Investigating the effect of anaesthesia on the metabolism of zebrafish (*D. rerio*)

M Burger

orcid.org/0000-0001-7559-5748

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Supervisor: Dr JZ Lindeque

Co-supervisor: Prof A Vosloo

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ABSTRACT

In recent years, zebrafish (*D. rerio*) have emerged as valuable research models, being widely used as models for aquaculture research, but also to model human metabolic diseases. The ethical guidelines stipulate that anaesthetics need to be applied before sampling of zebrafish. The effect of the anaesthetics on the metabolic profiles of zebrafish and the implications that this may have for zebrafish as research models, have mostly been overlooked. The primary hypothesis of this study was that anaesthetics will have an effect on zebrafish metabolism. The possibility for new hypotheses to be generated were not eliminated, since untargeted metabolomics are more hypothesis-generating than hypothesis-testing techniques. In this study, multi-platform metabolomics were used to investigate the metabolic alterations in zebrafish caused by three commonly used anaesthetics namely tricaine methanesulfonate (MS-222), eugenol and 2-phenoxyethanol (2-PE). The metabolomic analyses indicated that anaesthesia caused minimal metabolic alterations, with the concentration of only a small number of metabolites altered. It is hypothesized that these concentration changes are caused by ATP depletion and a stress response, which both lead to the upregulation of protein and lipid catabolism. Glycolysis and tricarboxylic acid cycle (TCA) intermediates remained relatively unaffected, but there are some indications that gluconeogenesis may be slightly upregulated. These effects are, however, limited by the short induction times of the anaesthetics. It was also determined that eugenol exhibited significantly less metabolic perturbations than the other anaesthetics with only three significantly altered metabolites, making it the preferred anaesthetic for metabolomic studies in zebrafish. General pathways impacted during anaesthesia include but are not limited to ß-oxidation, gluconeogenesis, urea cycle and amino acid transamination. In conclusion, anaesthetics used during sampling are not expected to have a significant effect on the suitability of zebrafish as research models. Further investigations are needed to confirm the hypotheses generated in this study.

Key terms: Zebrafish; anaesthesia; metabolomics; GC-MS; LC-MS; NMR; tricaine methanesulfonate; eugenol; 2-phenoxyethanol; aquaculture.

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ABBREVIATION	MEANING
1-mPMS	1-methoxy-5-methylphenazium methylsulfate
2-PE	2-Phenoxyethanol
AGAT	arginine:glycine amidinotransferase
ALP	Alkaline phosphatase
ALT	Alanine transaminase
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AMPK	AMP-activated protein kinase
AST	Aspartate transaminase
BCAA	Branched chain amino acids
CAT	Catalase
CK	Creatine kinase
CNS	Central nervous system
CPT	Carnitine palmitoyltransferase
CRAT	Carnitine O-acetyltransferase
CROT	Carnitine O-Octanoyltransferase
CV	Coefficient of variance
D_2O	Deuterated water
DTT	Dithiothreitol
EQM	Eugenol quinone methide
FDR	False discovery rate
GABA	Gamma aminobutyric acid
GAMT	guanidinoacetate *N*-methyltransferase
GCL	Glutamate cysteine ligase
GC-TOFMS	Gas chromatography-time-of-flight mass spectrometry
Glog	Generalised logarithm
GLUT	Glucose transporter
GPx	Glutathione peroxidase
GR	Glutathione reductase
GSH	Glutathione
GSSG	Glutathione disulfide
GST	Glutathione-S-transferase

KEGG	Kyoto Encyclopedia of Genes and Genomes
LC-MS	Liquid chromatography-mass-spectrometry
LC-MS/MS	Liquid chromatography-triple quadropole mass-spectrometry
LDH	Lactate dehydrogenase
MRM	Multiple reaction monitoring
MS-222	Tricaine methanesulfonate
mTOR	Mechanistic target of rapamycin
MVI	Missing value imputation
NAD	Nicotinamide adenine dinucleotide
NIST	National Institute of Standards and Technology
NMDA	N-methyl-D-aspartate
NMR	Nuclear magnetic resonance
PCA	Principal component analysis
PEPCK	Phosphoenolpyruvate carboxykinase
PLS-DA	Partial least squares-discriminant analysis
QC	Quality control
ROS	Reactive oxygen species
SOD	Superoxide dismutase
T3	Thyroxine
T4	Triiodothyronine
TBARS	Thiobarbituric acid reactive substances
TCA cycle	Tricarboxylic acid cycle
TR	Thyroid hormone receptor
TSP	Trimethylsilylpropionic acid
UDP	Uridine diphosphate

CHAPTER 1: INTRODUCTION

1.1 Background and motivation

Many researchers studying diseases, including metabolic defects, have started using zebrafish as disease models. There are several advantages when using zebrafish as models, such as accessible developmental stages, with embryo and larvae biology that make it possible to analyse developing pathologies using real-time imaging (Lieschke & Currie, 2007). Zebrafish present a fast and cost-effective model to study metabolic systems (Santoro, 2014), and nutritional requirements as well as modified diets in aquaculture because of their short development time and low cost compared to larger fish species (Ulloa, 2014). In fact, zebrafish are an important research tool in aquaculture, being the most widely used model for fish development (Rombough, 2007). They are used for various other aspects in aquaculture research like the use of anaesthesia (Rombough, 2007), growth, reproduction, pathology, stress and toxicology (Ribas & Piferrer, 2013).

Ethical guidelines stipulate that anaesthesia be used prior to handling or invasive sample collection, which includes terminal sample collection. However, a possible limitation overlooked so far is the effect of anaesthesia on their metabolism (and hence metabolic profiles). An ongoing study by the University of Kwazulu-Natal indicated that there are differences between the metabolism of fish anaesthetized with 2-phenoxyethanol (2-PE) and the control group (non-anaesthetized fish) (Sosibo *et al.*, in prep), but the extent of this effect on metabolism has yet to be fully elucidated (Velisek *et al*., 2007). This finding can potentially hamper metabolomics studies and interpretation of metabolic profiles; justifying the need to elucidate these effects. This study was thus aimed to determine whether compulsory anaesthetic use truly interfere with zebrafish metabolism, and to which extent pathways were perturbed. Three commonly used anaesthetics were tested in this study namely tricaine methanesulfonate (MS-222), 2-phenoxyethanol (2-PE) and eugenol (the active ingredient of clove oil).

1.2 Aim and objectives

1.2.1 Aim

The aim of this study was to investigate the effect of anaesthesia on the metabolism of zebrafish.

1.2.2 Objectives

To achieve the aim of the study, the objectives of the study were to:

1. Optimize the extraction procedures of the zebrafish samples.

2. Acquire and prepare zebrafish samples treated with MS-222, 2-PE and eugenol respectively (with appropriate controls) for metabolomic analysis.

3. Analyse treated and untreated zebrafish using a multiplatform metabolomics approach:
3.1 Untargeted gas chromatography time-of-flight mass spectrometry (GC-TOFMS),
3.2 Proton nuclear magnetic resonance (1H-NMR) spectroscopy with targeted data processing, and
3.3 Targeted liquid chromatography mass spectrometry (LC-MS) analysis of amino acids and acylcarnitines.

4. Process and compare the multiplatform data with standardised workflow.

1.3 Experimental design

The experimental design used to achieve the aim and objectives of this study is illustrated in **Figure 1.2.1.**.

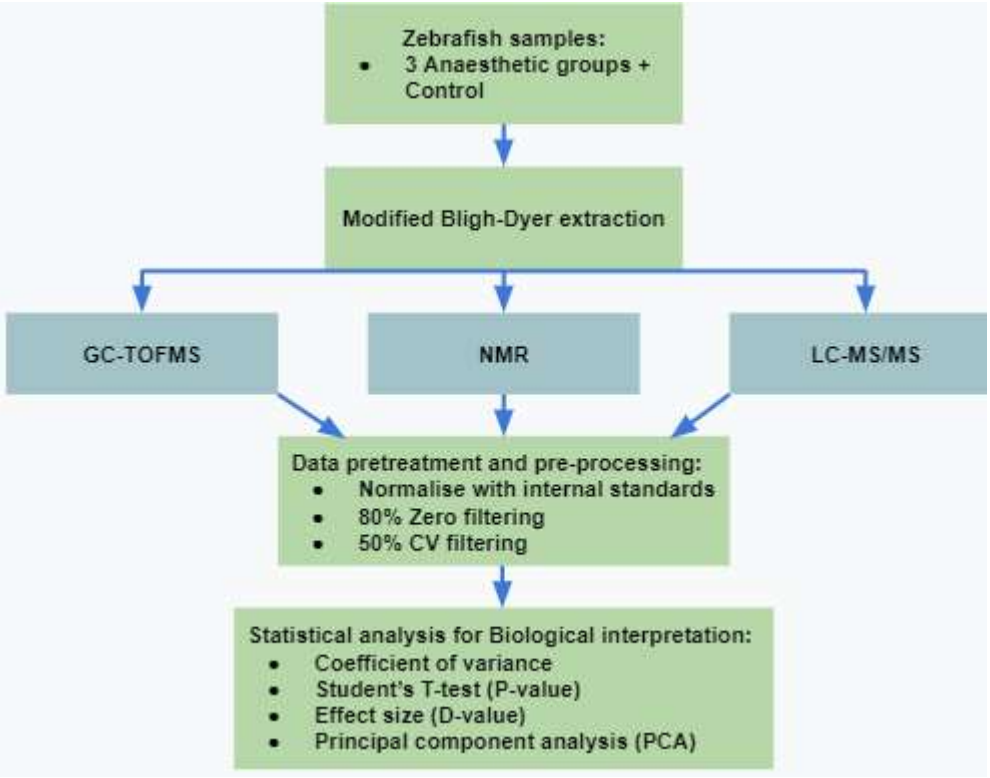

Figure 1.2.1: A diagram illustrating the experimental design used in the study.

1.4 Structure of dissertation and research outputs

This dissertation complies with the requirements prescribed by the North-West University (NWU; Potchefstroom Campus), South Africa, for the completion of the degree Magister Scientae (Biochemistry); and is structured in accordance with these guidelines, as shown below.

Chapter 1 provides a brief background and motivation for the study. It also provides the aim and objectives, along with the study design and the contributions made by co-authors, co-workers and collaborators.

Chapter 2 provides a literature review on the general concepts relevant to this investigation. It also investigates several factors that may play a role in zebrafish metabolism during anaesthesia, to better explain the reasons for the changes caused during anaesthesia.

Chapter 3 provides the methodology used in the study. The materials (reagents and chemicals) used are listed in this chapter, along with their origin (supplier). The experimental design and methods used are described in detail. Data processing, data validation and statistical analyses are also described.

Chapter 4 contains the results of all the methods that were used, and the results were compared to recent and relevant studies. A critical description of the results (accuracy, etc.) was also provided. The results are also discussed in this chapter.

Chapter 5 contains the final conclusions of the study considering the original aim of the study and the information obtained from the results as a whole. Future prospects are also provided in this chapter.

A bibliography of all references used in this dissertation is provided before annexures.
The findings of this investigation were presented orally at the annual metabolomics symposium that was hosted by Metabolomics South Africa (MSA) on the 20th-21st October 2021. Refer to Annexure D for proof of participation.

1.5 Author contributions

The primary author/investigator is Marcel Burger. All the co-authors, co-workers and collaborators, as well as their contributions made towards this dissertation are listed in **Table 1.1.** The following statement from the study promoters and primary author confirm their respective roles in this study and give permission that the data generated, and conclusions made may be included in this dissertation: “I declare that my role in the study, as indicated in **Table 1.1**, is a representation of my actual contribution, and I hereby give my consent that this work may be published as part of the M.Sc. dissertation of Marcel Burger.”

Table 1.2.1: The research team

Co-author/ co-worker/ collaborator	Contribution	Signature
Mr. Marcel Burger (B.Sc. Hons. Biochemistry)	Responsible for project planning, sample analyses, data analyses and writing of this dissertation, as well as all other documentation associated with this study.	
Prof. J. Zander Lindeque (Ph.D Biochemistry)	Served as supervisor and supervised all aspects of this study including project planning and design, sample analysis, data analyses, dissertation writing and documentation.	
Dr. Shayne Mason (Ph.D Biochemistry)	Assisted with the NMR analysis and interpretation of the NMR data.	
Prof. André Vosloo (Ph.D. Zoology)	Collaborator from UKZN who conceived the project and provided the zebrafish samples used in this study. He supervised sample collection, transport and final stages of the study.	

CHAPTER 2: LITERATURE STUDY

Before the commencement of any experiments, a literature study was done to get an overview of the topic from existing publications. In the literature review, information was gathered about the methods used in previous studies, the metabolism of zebrafish and the reported effects that anaesthesia has on zebrafish metabolism. The advantages and limitations of analytical instruments to be used in this study were also examined. In addition, any information that may assist in the final interpretation of the data was included in the literature study.\

2.1 Zebrafish as research models

2.1.1 Zebrafish as animal models for metabolism and biomedical research

Because *in vitro* studies are not able to accurately represent the complexity of metabolism in a multicellular context that exists *in vivo*, whole-animal approaches are needed to act as models for human metabolic conditions (Seth *et al*., 2013).

To better understand metabolic events and diseases, new experimental models which enable the enzyme–metabolite–phenotype association to be easily studied, are needed. To address this requirement, zebrafish are emerging as valuable model systems to elucidate the metabolic routes and regulation needed for cellular homeostasis (Santoro, 2014). Zebrafish can also be used to provide answers regarding central nervous system regulation of energy homeostasis (Schlegel & Stainier, 2007). In addition, zebrafish have also become one of the leading models for *in vivo* biomedical research (Collymore *et al*., 2013; Sánchez-Vázquez *et al*., 2011). Santoro (2014) and Lieschke & Currie (2007) reported that zebrafish are useful to not only study metabolism but also metabolic diseases and therapeutic treatment for these diseases. Zebrafish have also emerged as valuable tools for toxicology and target identification due to their potential in facilitating high-throughput drug discovery (Sánchez-Vázquez *et al*., 2011; Santoro, 2014). They have also been used to investigate the effect of microbiota on intestinal absorption and to measure autophagy and mitophagy *in vivo* (Santoro, 2014).

2.1.1.1 Advantages of zebrafish as animal models for metabolism and biomedical research

There are several advantages when using zebrafish as a research model for biomedical research. One of the major advantages is that genetic alterations can easily be introduced in zebrafish and that the genetic information and physiological processes used in humans are highly conserved in zebrafish. This makes zebrafish an excellent model to study existing human metabolic disorders (Santoro, 2014; Seth *et al*., 2013; Steele *et al*., 2014; Riché, 2018). Zebrafish also present a fast and cost-effective model to investigate cell metabolism (Santoro, 2014; Seth *et al*., 2013; Steele *et al*., 2014). Numerous metabolic pathways, especially the TCA (tricarboxylic acid) cycle and lipid metabolism, have been reported to be highly similar to those in humans (Santoro, 2014; Zuberi *et al*., 2019; Löhr & Hammerschmidt, 2011). Zebrafish embryos are also useful for metabolomic and fluxomic analyses and can even be analysed in multiwell plates due to their small size (Santoro, 2014; Löhr & Hammerschmidt, 2011). Other advantages include their high fecundity rate and the accessibility of their embryos (Löhr & Hammerschmidt, 2011). Zebrafish research facilities are also much easier, cheaper and faster to set up and maintain than that of rodents and mammals (Santoro, 2014; Seth *et al*., 2013). One advantage reported by Lieschke & Currie (2007), Steele *et al*. (2014) and Seth *et al*. (2013), is that the optical clarity of embryos and larvae enable real-time imaging of developing pathologies, and that all their developmental stages can be readily accessed. Seth *et al*. (2013), also reported that several organ systems in zebrafish are remarkably similar to human organs. Another advantage reported by Howe *et al*. (2013), is that the entire zebrafish genome is available, which is important for genetic analysis as well as interpretation of metabolic findings.

2.1.1.2 Disadvantages of zebrafish as animal models for metabolism and biomedical research

Using zebrafish does, however, have some limitations, like all animal models. One of the main limitations according to the literature is that zebrafish prefer to use lipids as an energy source rather than carbohydrates (Santoro, 2014). As ectotherms, zebrafish body temperatures are usually lower than mammals' depending on the temperature of their surroundings. This might have an effect on enzyme kinetics and metabolic fluxes when using it as model organism to study disease and other interventions (Santoro, 2014; Seth *et al*., 2013). Another limitation is that zebrafish live in environments where there are inevitably fluctuations in the oxygen concentration due to turbulence, depth, etc. (Santoro, 2014). In periods of low oxygen (hypoxia), zebrafish increase anaerobic respiration via glycolysis. Another limitation is the small size of zebrafish, which causes blood sampling to be a terminal procedure and thus limits repeated sampling (Seth

et al., 2013; Löhr & Hammerschmidt, 2011). This problem also limits the analysis of disease progression (Seth *et al*., 2013). The current techniques used to measure energy expenditure and food intake are also less sophisticated for zebrafish than that in rodents (Seth *et al*., 2013). Despite the mentioned limitations, the unique properties of zebrafish make it a powerful tool in the research of human metabolic diseases (Seth *et al*., 2013).

2.1.2 Zebrafish as models for aquaculture

Aside from biomedical research, zebrafish are also important research tools in aquaculture, being the most widely used model for fish development (Rombough, 2007). Conducting trials of different diets in aquaculture can be expensive and time-consuming for larger, slow-growing fish, while zebrafish have a fast development time and will significantly reduce the costs of studies. This makes zebrafish an attractive model animal to determine which interventions will optimise production in aquaculture (Ulloa, 2014). Ribas & Piferrer (2013) reviewed several studies conducted on zebrafish and reported that zebrafish can be used to investigate several different aspects of aquaculture research like stress, pathology, reproduction, nutrition, toxicology and growth.

2.2 Zebrafish metabolism

When using zebrafish as models for human metabolism, it is important to know the similarities and differences of zebrafish metabolism to human metabolism, to ensure appropriate interpretation.

According to Souza Anselmo *et al*. (2017) and Santoro (2014), the zebrafish is an emerging model to investigate human metabolism due to their genetic similarities with humans. It has also been reported that metabolic disease pathology in zebrafish was similar to that in humans and likewise many metabolic pathways (Schlegel & Stainier, 2007; Santoro, 2014). Li *et al*. (2010) conducted a comprehensive comparison between the genes present in humans and fish using several different established public databases like KEGG, BRENDA, etc. They made the important discovery that genes involved in metabolism are better conserved between humans and fish than any other genes; and reported on the most conserved and least conserved pathways (when comparing humans and zebrafish). The most conserved pathways consisted of 1- and 2-methylnaphthalene degradation, hyaluronan metabolism, sialic acid metabolism, hexose phosphorylation, the electron transport chain, limonene and pinene degradation, proteoglycan synthesis, glycosphingolipid biosynthesis, N-Glycan degradation, Di-unsaturated fatty acid beta oxidation, vitamin B1 (thiamine) metabolism, glycosphingolipid metabolism, glutamate

metabolism, the TCA cycle, vitamin B9 (folate) metabolism and linoleate metabolism. The least conserved pathways consisted of phytanic acid peroxisomal oxidation, glycosylphosphatidylinositol anchor biosynthesis, vitamin H (biotin), vitamin B12 (cyanocobalamin), glyoxylate and dicarboxylate metabolism, pentose and glucuronate interconversions, vitamin C (ascorbate) and aldarate metabolism.

Santoro (2014) also reported that the TCA cycle and lipid metabolism in zebrafish were particularly similar to humans. In support of this statement, Löhr & Hammerschmidt (2011) remarked that the cellular mechanisms that governs lipid metabolism are conserved between fish and mammals, while Schlegel & Stainier (2007) determined that zebrafish specifically possessed the mechanisms of lipid transport and synthesis used by humans. The main enzymes needed for *de novo* lipid synthesis were also reported to be present in zebrafish (Bou, 2016). Schlegel & Stainier (2017), also determined that genes known to be involved in regulation of lipid metabolism are conserved in zebrafish. Not only did Schlegel & Stainier (2007) report lipid metabolism to be similar, but also reported that the main enzymes of cellular energetics are conserved in zebrafish.

Similar to humans, lipid metabolism in zebrafish depends on carnitine conjugation for the transport of long chain fatty acids over the mitochondrial membrane so that mitochondrial β-oxidation can occur, yielding acetyl-CoA; which is in turn oxidized in the TCA cycle and converted to energy. Similar to that in humans, Bou (2016) reported that medium and long chain acyl-CoA's are converted to acylcarnitines in the outer mitochondrial membrane by CPT-1 (Carnitine palmitoyltransferase I). Like in mammals, CPT-1 is the rate-limiting enzyme for mitochondrial β-oxidation (Bou, 2016). The acylcarnitines are then transported over the inner mitochondrial membrane to the mitochondrial matrix, where CPT-2 (Carnitine palmitoyltransferase II) replaces the carnitine group with a CoA to yield acyl-CoA esters, which can then act as substrate for β-oxidation (Bou, 2016). Very long chain fatty acids undergo peroxisomal β-oxidation prior to mitochondrial β-oxidation (Bou, 2016). The products of peroxisomal β-oxidation (C8 and C6-CoA) are converted to acylcarnitines using the two enzymes CRAT (Carnitine O-acetyltransferase) and CROT (Carnitine O-Octanoyltransferase). The acylcarnitines are then transported to the mitochondria to undergo mitochondrial β-oxidation to yield acetyl-CoA (Bou, 2016).

Zuberi *et al*. (2019) conducted a study on the effect of aflatoxin B1 on the metabolic profiles of zebrafish. In their study, they determined that the results were consistent with those in mammals, and specifically mentioned carbohydrate, lipid and amino acid metabolism to be similarly affected in zebrafish and mammals. Perna *et al*. (2018) successfully used zebrafish as an animal model for sulphur amino acid metabolism, while Venturoni (2010), reported that zebrafish contain orthologs of all the enzymes used in the lysine oxidation pathway. Friedrich *et al*. (2011),

conducted a study where zebrafish were used as a model for maple syrup urine disease (MSUD), a branched chain amino acid (BCAA) defect. In the study by Friedrich *et al*. (2011), they determined that zebrafish are excellent models in the investigation of the mechanisms underlying MSUD. They mentioned that BCAA metabolism has not been fully elucidated in zebrafish, but that the same mechanisms used in the regulation of human BCAA metabolism are used in zebrafish. They proposed that branched chain ketoacid dehydrogenase regulate BCAAs in a similar way to mammalian systems. Mansfeld *et al*. (2015), also determined that the way branched chain amino acids (BCAA) regulate physical aging are conserved in zebrafish. By comparing the KEGG pathways of humans and zebrafish, it became evident that all the main amino acid biosynthesis pathways were similar between the two organisms. All the amino acids present in humans have been reported in studies conducted on zebrafish as well (Jia *et al*., 2019; Fu *et al*., 2018; Cofiel & Mattioli, 2009; Falco *et al*., 2016)

Seth *et al*. (2013), reported that zebrafish possess all the key organs required in humans for metabolic control. This has led to several metabolic diseases being successfully modelled in zebrafish. These diseases include obesity, non-alcoholic fatty liver disease, diabetes, atherosclerosis, diseases affecting β-cell function (Seth *et al*., 2013), glutaric acidemia type 1 (Venturoni, 2010), and glycine encephalopathy (Riché, 2018), which have all been modelled with great success in zebrafish. The success of these studies emphasizes the similarities of zebrafish and human metabolism.

According to Elo *et al*. (2007), many of the genes needed by humans for the regulation of glucose levels have been found in zebrafish, even though they rely more on lipids as an energy source (Santoro, 2014; Bou, 2016). Regulation of blood glucose levels by the hormones insulin and glucagon, as well as other proteins needed for glucose regulation like AMP-activated protein kinase (AMPK) and mechanistic target of rapamycin (mTOR) have been found to be conserved in zebrafish (Elo *et al*., 2007; Mansfeld *et al*., 2015). In addition to insulin and glucagon, the insulin/IGF-1 and sirtuin signalling pathways are also conserved in zebrafish (Mansfeld *et al*., 2015). Importantly, the expression of phosphoenolpyruvate carboxykinase (PEPCK), which is a rate-limiting enzyme in gluconeogenesis, is regulated by insulin and glucagon similarly to that in mammalian systems (Bou *et al*., 2016). Zebrafish also responded to anti-diabetic drugs by reducing blood glucose levels, which is similar to humans (which again highlight the usefulness of this model animal in drug discovery) (Bou *et al*., 2016). Despite having the insulin signalling pathways, zebrafish were reported to contain less insulin receptors and a lower capacity of glucose to stimulate insulin secretion possibly due to their greater reliance on lipids as energy source (Bou *et al*., 2016). According to Jensen *et al*. (2006), other labs demonstrated that the zebrafish counterparts of the human glucose transporters GLUT 1and GLUT 4 have been

identified in zebrafish, although GLUT 4 has been reported to have a lower affinity for glucose in fish than in mammals (Bou *et al*., 2016). This indicates that not only enzymes of glucose metabolism, but also key transporters are conserved in zebrafish which further strengthens zebrafish as a model to study central carbon metabolic pathways. These insights suggest that zebrafish may be an appropriate model to study glucose metabolism and interventions affecting it, although their greater reliance on lipids must be considered when using zebrafish as models for central carbon metabolism.

Ascorbic acid (vitamin C) plays an important role in energy metabolism because it regulates the activity of the perinucleolar compartment, especially AMP deaminase activity and thus affects AMP levels, which have a down-stream regulatory effect on various metabolic pathways (Kirkwood *et al*., 2011). According to Kirkwood *et al*. (2011), only a few species cannot synthesize ascorbic acid, including humans, guinea pigs and zebrafish. This similarity is important because ascorbic acid has such a widespread effect on metabolism. In the study by Kirkwood *et al*. (2011), they investigated the effect of an ascorbic acid shortage in zebrafish and determined that several metabolic pathways including purine metabolism, carnitine metabolism, glycerophospholipid metabolism and glutathione metabolism were at risk during an ascorbic acid shortage. They concluded that an ascorbic acid shortage caused a stress response similar to that in humans and that zebrafish is an appropriate model for investigating the effects of ascorbic acid on metabolism. Li *et al*. (2010), however, found that the enzymes involved in ascorbic acid metabolism were not highly conserved between humans and fish, which creates a contradiction in the literature. A possible explanation is that the enzymes involved in ascorbic acid catabolism may differ, while ascorbic acid's function as a cofactor may be conserved.

Although some pathways were reported to be poorly conserved in zebrafish or less utilized in the case of carbohydrate metabolism, overwhelming evidence indicated that the primary metabolism consisting of the TCA cycle, the electron transport chain, lipid metabolism, glycolysis and amino acid metabolism were highly conserved in zebrafish. Various regulation mechanisms, essential transporters and signalling pathways were also determined to be conserved in zebrafish.

2.3 Anaesthesia

2.3.1 Mechanism of different anaesthetics

When investigating the effect of anaesthetics on metabolism, it is important to have background knowledge on the mechanisms used by different anaesthetics. Knowledge of the mechanisms can give the researchers clues as to why some changes are observed after anaesthesia.

Virtually all mechanisms of anaesthetics are based on the blockage of action potentials in the nervous system. Action potentials are defined by a sequence of rapid changes in the membrane potential. In the event of an action potential, sodium channels across the plasma membrane open to allow the higher concentration of sodium ions on the outside of the cell to diffuse into the cell. This causes a depolarization of the plasma membrane, which in turn triggers neighbouring voltage-sensitive sodium channels to open and consequently propagating the action potential (Grider & Glaubensklee, 2019; Wann, 1993). After the initial depolarization, potassium ion channels open, causing potassium ions to stream out of the cell with their electrochemical potential, which causes a repolarization (Wann, 1993). When anaesthetics are applied, these action potentials are blocked by the inhibition of specific ion channels or receptors essential for the propagation of action potentials. A short explanation of the different channels and receptors involved, and the hypothesized mechanism of action of the three studied anaesthetics are discussed below.

Na/K-ATPases are important membrane proteins present in almost all animal cells and were reported to be highly conserved in zebrafish (Rajaroa, 2001). The function of Na/K-ATPases is to maintain the resting membrane potential by regulating the concentration of sodium and potassium ions across the plasma membrane (Rajaroa, 2001). This resting membrane potential is maintained by pumping three sodium ions out and 2 potassium ions into the cell for each ATP used (Xu, 2012). By maintaining the high sodium concentration on the outside of the cell, Na/K-ATPases also provide the driving force for various other active transport processes. These channels are involved in the transport of amino acid, glucose, citrate, succinate, calcium, phosphate and chloride over the cell membranes in most organs and tissues (Kreydiyyeh *et al*., 2000).

The mechanism whereby MS-222 inhibits action potential is to block voltage-sensitive sodium channels located in muscle membranes (Lynne, 2012; Ramlochansingh *et al*., 2014; Priborsky & Velisek, 2018) and to a lesser degree, potassium channels in the neuronal membranes (Priborsky

& Velisek, 2018). This mechanism is accompanied by various other biological changes because of the integral role of action potentials in the regulation of cellular functions.

Glutamate is the main excitatory neurotransmitter in the vertebrate central nervous system. It is involved in the activation of several synaptic receptors, including N-methyl-D-aspartate (NMDA) receptors, the non-NMDA kainate and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors (Vignes & Collingridge, 1997). When activated, these glutamate receptors enable fast propagation of action potentials between neurons and increase neural excitability by lowering the threshold of action potentials (Priborsky & Velisek, 2018). NMDA receptors accomplish this by initiating an influx of calcium and sodium and an efflux of potassium, while non-NMDA receptors only cause an influx of sodium and efflux of potassium (Priborsky & Velisek, 2018). The displacement of these ions causes a depolarization event for the propagation of action potentials (Ralph & Eduardo, 2007).

According to Lynne (2012), the exact mechanism of 2-phenoxyethanol in fish is unknown but is suspected to involve the suppression of central nervous system activity by expansion of neuronal cell membranes. Schmuck *et al*. (2000), Priborsky & Velisek (2018) did, however, find that 2-phenoxyethanol causes a reduction in n-Methyl-d-Aspartate (NMDA) and kainate receptor activity. This reduction in NMDA and kainic acid receptor activity induced a reduction of membrane currents.

Unlike the other anaesthetics that are reported to target specific receptors, eugenol is reported to have an effect on several different receptors (Sharma *et al*., 2012). It has been reported that eugenol has an agonistic effect on gamma aminobutyric acid (GABA), which is the main inhibitory neurotransmitter of the central nervous system (CNS) and is likely responsible for its anaesthetic effect (Guénette *et al*., 2007; Ramlochansingh *et al*., 2014). Eugenol also blocks vanilloid receptors and has an antagonistic effect on the NMDA receptors and Na/K-ATPase channels (Kreydiyyeh *et al*., 2000), which causes further suppression of action potentials and thus anaesthetic effect (Guénette *et al*., 2007). Vanilloid receptors are excitatory ion channels present on pain receptors, which contribute to the detection of chemical and thermal stimuli that causes a pain sensation. These stimuli usually lead to the opening of vanilloid channels, an influx of Ca^{2+} ions and subsequent depolarization, which initiates an action potential (Caterina & Julius, 2001).

After investigating the mechanisms of the different anaesthetics, it became evident that the mechanisms mostly involve disruption of sodium, potassium, calcium and glutamate receptors and/or ion channels. The concentrations of these compounds were found to be strictly regulated, especially in neuronal cells. This regulation often involves active transport, which may cause a

change in these channels or compounds to have an effect on ATP concentrations, which may in turn, have adverse effects on total metabolism.

2.3.2 Breakdown products of anaesthetics

The catabolism (or biotransformation) of the different anaesthetics is very convoluted and include a vast number of derivatives and products. To accurately interpret metabolomic results, it is important to know the pathways involved in the origin of some of the intermediates and products that could be included in the untargeted metabolomic analyses.

When MS-222 (ethyl 3-aminobenzoate) enters the body, it is rapidly metabolized in the liver and to a small degree in the gills via two main biotransformation pathways (Rombough, 2007; Wayson *et al*., 1976). In one pathway, the ethyl groups of MS-222 are hydrolysed to form m-aminobenzoic acid. This step is followed by the addition of an acetyl group to produce m-acetylaminobenzoic acid. In the other pathway, the amine group is acetylated first to yield ethyl-m-acetyl aminobenzoate. After acetylation, the acetyl group is hydrolysed to yield m-acetylaminobenzoic acid, similar to the first pathway (Rombough, 2007; Priborsky & Velisek, 2018). The main products of MS-222 biotransformation are thus ethyl-n-acetyl aminobenzoate, m-aminobenzoic acid and m-acetylaminobenzoic acid. The polar m-aminobenzoic acid and its acetyl derivatives, like m-acetylaminobenzoic acid, are excreted through the kidneys while the remaining MS-222 and non-polar products ethyl m-aminobenzoate and its acetyl conjugates like ethyl-m-acetylaminobenzoic are excreted through the gills (Hunn, 1970; Rombough, 2007; Priborsky & Velisek, 2018).

The main biotransformation pathway of 2-PE is a two-step oxidation process where 2-PE is firstly converted to 2-phenoxyacetaldehyde by alcohol dehydrogenase and then to phenoxyacetic acid by aldehyde dehydrogenase (Lilienblum, 2016; Dréno *et al*., 2019). Phenoxyacetic acid is the main product of 2-PE biotransformation (Lilienblum, 2016; Dréno *et al*., 2019; Kim *et al*., 2015). There are two other pathways of biotransformation of 2-PE namely ring sulfonation after hydroxylation, and conjugation with glucuronic acid. The products of these three pathways can be hydroxylated at the ring and in one case, oxidised to a carboxylic acid at the terminal hydroxyl group (Lilienblum, 2016). The structures of these products are provided in the article by Lilienbulum (2016).

The biotransformation of eugenol (3-hydroxy-4-methoxyphenyl-3′-propene) is complex because eugenol and its two common derivatives methyl eugenol and isoeugenol (Zhao *et al*., 2017; Khan *et al*., 2019) participate in numerous conjugation reactions to form phenolic conjugates, which make up 99% of the products according to Fischer *et al*. (1990). The main pathway of eugenol

biotransformation firstly involves conjugation with glucuronic acid to form eugenol-4-O-glucuronide, which is the main product of eugenol biotransformation (>50%) (Fischer *et al*., 1990; Nohmi & Fukushima, 2016). In the next step, a phenoxy radical (1-hydroxy eugenol) is formed after hydrogen peroxide oxidises the eugenol molecule. Glutathione can reduce this compound back to eugenol. 1-Hydroxy eugenol is converted to the eugenol quinone methide (EQM) in the following step, whereafter the EQM binds to glutathione to form eugenol-glutathione conjugates (Nohmi & Fukushima, 2016; Thompson *et al*., 1989).

There are numerous other derivatives that may form during eugenol biotransformation. These are 1′-hydroxy-methyleugenol, 3′-hydroxy-methylisoeugenol, 3′-oxo-methylisoeugenol, 2′,3′-dihydroxy-2′,3′-dihydromethyleugenol (Nohmi & Fukushima, 2016), 2-methoxy-4-propylphenol, 4-ethylguaiacol (Khan *et al*., 2019), 4-hydroxy-3-methoxyphenyl-propane, 3-(4-hydroxy-3-methoxyphenyl)-propionic acid, 3-(4-hydroxy-3-methoxyphenyl)-propane-1,2-diol, 3-(4-hydroxy-3-methoxyphenyl)-propylene-1,2-oxide etc. (Fischer *et al*., 1990). The pathways involved in the formation of these products are the epoxide diol pathway, thiophenol synthesis, substitution by propionic acid, allylic oxidation, and migration of the double bond (Fischer *et al*., 1990).

When xenobiotics enter the body, they are often detoxified by conjugation reactions with glutathione (GSH). This reaction is catalysed by GST and represents the first step of the mercapturic acid pathway (Townsend & Tew, 2003). After conjugation with GSH, most of the conjugates are excreted from the cells or excreted into bile in the case of hepatocytes. The γ-glutamyl moiety of GSH conjugates are cleaved, yielding a cysteinyl-glycine conjugate. The cysteinyl-glycine moiety is in turn cleaved by a dipeptidase, which leads to the formation of a cysteinyl conjugate. The cysteinyl conjugate lastly undergoes N-acetylation, leading to the production of mercapturic acid, which can be excreted in the urine (Lu, 2009). Occasionally, the product itself may be toxic, but the mercapturic acid pathway mostly leads to detoxification of the xenobiotic involved (Lu, 2009).

Evidently, the pathways involved are complex and the products and by-products are numerous. The long list of reactions and products may, however, give clues as to the origin of some compounds that may be detected in an untargeted metabolomic analysis.

2.3.3 Stress response

A common consequence of all anaesthetics is the initial activation of a stress response (Lynne, 2012). Although this stress response is less than that witnessed in fish handled without anaesthesia (Topic Popovic *et al*., 2012), it could nonetheless have a marked effect on metabolism. Hence, this effect should also be known in order to interpret the metabolic findings appropriately.

The stress response is elicited due to the potential taste and smell of the anaesthesic, as well as possible skin irritation and loss of balance before the anaesthesia has its intended effect (Topic Popovic *et al*., 2012). Hypoxia caused by the cessation of respiration also activates a stress response (Congleton, 2006; Iwama *et al*., 1989; Velisek *et al*., 2011; Lepic *et al*., 2014).

The stress response results in the activation of neuroendocrine systems, which causes catecholamines and corticosteroids to be released, especially epinephrine (adrenaline) and cortisol (Gingerich & Drottar, 1989; Löhr & Hammerschmidt, 2011; Lynne, 2012). According to Topic Popovic *et al*. (2012), catecholamines are released rapidly but their concentration decreases rapidly as well, whereas corticosteroids are released more slowly but remain in the blood for a longer time. Löhr & Hammerschmidt (2011) also conducted a study where they compared the functions of the different endocrine systems between humans and zebrafish. In this study, the authors determined that all the major processes that are regulated by the (neuro)endocrine systems e.g. energy homeostasis, feeding circuits, osmoregulation, calcium homeostasis etc. were conserved in zebrafish.

Elevated levels of stress hormones cause ventilation rate, cardiac output and brachial blood flow to increase. Plasma glucose also increased initially while chloride decreased as a response to stressors (Topic Popovic *et al*., 2012; Lynne, 2012). The mechanisms whereby catecholamines increase plasma glucose levels rely on the up regulation of glycogen phosphorylase, the rate-limiting enzyme of glycogenolysis (Brás *et al*., 2018) and the inhibition of pyruvate kinase, which causes inhibition of glycolysis and activation of gluconeogenesis (Wright *et al*., 1989; Kuo *et al*., 2015). The increased glucose is expected to have a profound effect on metabolism due to its central role in metabolism. Fu *et al*. (2018) reported that stress caused a higher energy demand in zebrafish, which led to the upregulation of protein catabolism to release amino acids for energy production. Fu *et al*. (2018), also reported a disruption in nitrogen metabolism, which is linked to several other compounds including amino acids. This disruption in nitrogen metabolism caused a general increase in amino acids, especially glycine and glutamine. The increase in the synthesis of stress hormones have also been reported to cause precursor metabolites to be altered (Fu *et*

al., 2018). Phenylalanine is a precursor for catecholamines, dopamine, norepinephrine, epinephrine and other neurotransmitters, while it is also needed for tyrosine and tryptophan synthesis and the formation of phosphoenolpyruvate during glycolysis (Fu *et al*., 2018). Fu *et al*. (2018) reported a significant increase in phenylalanine during oxidative stress. This increase was caused by elevated protein catabolism since phenylalanine is an essential amino acid. The change in phenylalanine concentrations may affect several pathways like tyrosine biosynthesis, glycolysis etc. (Fu *et al*., 2018).

After reviewing the stress responses that may result from the use of anaesthetics, the question arises whether it can be prevented altogether. According to Topic Popovic *et al*. (2012), pre-anaesthesia sedation has been used for MS-222 and has successfully reduced the stress response. Conversely, Congleton (2006), reported that low levels of anaesthesia elicited a stress response while higher levels brought about anaesthesia quickly enough to prevent changes in cortisol levels. In the study by Congleton (2006), it was however reported that an early rise in glucose could not be prevented, even when high concentrations of anaesthetics were used. A more in-depth discussion of the metabolic alterations induced by anaesthetics follows.

2.4 Effect of anaesthetics on metabolism

2.4.1 Commonly altered metabolites

The literature up to date did not provide a comprehensive scope of the metabolic changes caused in zebrafish particularly after the use of the different anaesthetics – hence the aim of this study. There are, however, numerous studies conducted on other types of fish which may give some valuable insights into the effects of these anaesthetics.

Conflicting results are reported in many of the reports, probably due to the wide variety of fish species and experimental conditions used. To resolve this problem, a composite profile was constructed for the effect of each anaesthetic on the metabolism by combining the results that were reported by the majority of studies in order to deduce a generalized effect of the anaesthetics on fish. The information provided below may thus be influenced by the type of fish used and may differ slightly from zebrafish but could highlight metabolic pathways worth studying.
The most complete review of the effects of MS-222, 2-phenoxyethanol and eugenol on fish metabolism was published by Priborsky & Velisek (2018). The authors summarized the metabolic effect of each of these anaesthetics according to the species of fish used. Their findings are summarized in **Table 2.1 - 2.3**, with the information of all of the fish species combined to highlight common perturbations and dominant trends. In this summary of metabolic effects, it is important

to note that the experimental conditions of the studies reviewed differed greatly. Some of the metabolites or enzyme concentrations/activities were only measured in specific organs. The authors also did not specify in all cases if enzyme activities or concentrations were affected. Nonetheless, their findings are included to assist with the biological interpretation in this study.

Table 2.1: A summary of the effect of tricaine methanesulfonate (MS-222) on the metabolism of different fish species according to literature (Priborsky & Velisek, 2018).

Compound/Enzyme	Studies reporting an increase*	Studies reporting a decrease*	No difference reported	Consensus
Alanine transaminase (ALT)	3	0	0	Increase
Alkaline phosphatase (ALP)	1	2	0	No trend
Ammonia	1	3	0	Decrease
Aspartate transaminase (AST)	2	0	0	Increase
Catalase (CAT)	3	0	0	Increase
Cholesterol	-	-	-	-
Glucose	15	0	5	Increase
Glutathione peroxidase (GPx)	0	2	0	Decrease
Glutathione reductase (GR)	1	2	0	No trend
Inorganic phosphate	0	3	0	Decrease
Lactate	8	3	2	Increase
Lactate dehydrogenase (LDH)	2	2	0	No trend
Lysozyme activity	1	0	1	No trend
pH	0	2	0	Decrease
Superoxide dismutase (SOD)	0	4	0	Decrease

Table 2.1. (continued)

Thiobarbituric acid reactive substances (TBARS)	2	1	0	No trend
Total protein	4	0	1	Increase
Triglycerides	3	1	0	Increase

* increased/decreased metabolite concentration or enzyme activity

According to several studies conducted on different fish species and especially the review article published by Priborsky & Velisek (2018) (as summarized in **Table 2.1**), the effects of MS-222 include an increase in the following metabolites or biochemical indices: glucose, triglycerides, lactic acid, serum urea and total protein levels in blood. MS-222 also caused an increase in some enzyme activities or concentrations namely catalase (concentration in different organs) and transaminase activity (AST and ALT) (Priborsky & Velisek, 2018; Velisek *et al*., 2011; Lynne, 2012; Congleton, 2006; Chen, 2019). MS-222 caused a decrease of the following metabolites or biochemical indices: ammonia, pH and inorganic phosphate. The enzyme activities or concentrations decreased by MS-222 anaesthesia were SOD and glutathione peroxidase (not specified whether it was enzyme activities or concentrations) (Priborsky & Velisek, 2018; Velisek *et al*., 2011; Lynne, 2012; Congleton, 2006). Sanchez-Vasquez *et al*. (2011) also confirmed that MS-222 causes hypoxia (due to the cessation of respiration), respiratory acidosis and hyperglycaemia specifically in zebrafish, which supports the findings by Priborsky & Velisek (2018). These changes suggest a reliance on anaerobic metabolism during anaesthesia (Lynne, 2012). Topic Popovic *et al*. (2012), also reported that hypoxia was the cause of increased glucose, potassium, magnesium, sodium, lactate, and lysozyme activity in fish anaesthetized with MS-222. Congleton (2006) and Lepic *et al*. (2014), reported that the rapid increase in blood glucose was due to the release of catecholamines that were triggered during the stress response.

Increased enzyme activity has been reported as a consequence of the stress experienced during anaesthesia. During stress, an energy crisis often follows, which causes increased activity of transaminases (ALT, AST) in an attempt to replenish the TCA cycle with transamination products in order to alleviate the energy crisis (Velisek *et al*., 2011; Lepic *et al*., 2014). ALT catalyses the conversion of alanine and α-ketoglutarate to pyruvate and glutamate, respectively (Watford, 2000). An increase in ALT activity may thus contribute to a decrease in alanine. Glutamate is then converted to glutamine by the addition of ammonia. This reaction is catalysed by glutamine synthetase (Watford, 2000). Alanine and glutamine usually transport ammonia from extrahepatic tissue to the liver where the ammonia can be converted to urea, by the urea cycle. Glutamine is converted back to glutamate (and ammonia) which in turn, is converted to aspartate in a

transamination reaction by AST. Both glutamate and aspartate donate amino groups to the urea cycle to produce urea as a means to eliminate toxic ammonia. This may explain the increased urea and decreased ammonia associated with increased ALT and AST activity (Watford, 2000). Gomulka *et al*. (2008) reported that the decrease in ammonia may also be due to reduced metabolic activity. The increase in transaminase activity is expected to influence the amino acid concentrations as well.

Other enzymes which may be increased due to tissue damage is alkaline phosphatase (ALP), lactate dehydrogenase (LDH) and creatine kinase (CK) (Congleton, 2006; Velisek *et al*., 2011), although there was no consensus in the literature to confirm this. Increased blood ALP levels would indicate liver or kidney damage while increased CK in blood indicates muscle damage (Velisek *et al*., 2011). Increased blood lactate is an indication of increased anaerobic metabolism. Elevated total protein in blood may be caused by increased haemolysis (Roman *et al*., 2009).

MS-222 also affected internal organs, which may cause reactive oxygen species to increase. Respiration rate was reported to decrease, which accompanied alterations in blood carbon dioxide and oxygen levels. It was also reported that MS-222 interfered with cytochrome P-450, which is a broad range of heme-dependent monooxygenase enzymes present in membranes including the inner membrane of the mitochondria (Williams *et al*., 2000; Topic Popovic *et al*., 2012). Cytochrome P-450 enzymes are involved in a wide variety of reactions and substrates throughout primary and secondary metabolic pathways (Munro *et al*., 2007; Pandey *et al*., 2019; Williams *et al*., 2000; Danielson, 2002; Topic Popovic *et al*., 2012). These enzymes are widely reported to be important for drug degradation as well as the degradation of other compounds like retinoic acid, fatty acids, carcinogens, steroids etc. and may affect different metabolic systems (Pandey *et al*., 2019; Williams *et al*., 2000; Danielson, 2002). Not only are they involved in degradation pathways, but also in the synthesis of bile acids, cholesterol, steroid hormones etc. (Williams *et al*., 2000). According to Munro *et al*. (2007), the majority of Cytochrome P-450s are used to catalyse the reductive scission of oxygen. For this reaction, they use electrons from NADH, NADPH and redox partner proteins. The involvement of NADH and NADPH may prove to be an important factor because of these cofactors' wide role in metabolic pathways. Conversely, Kolanczyk *et al*. (2003) found no effect of MS-222 anaesthesia on cytochrome P-450 enzymes in rainbow trout.

Table 2.2: A summary of the effect of 2-phenoxyethanol (2-PE) on the metabolism of different fish species according to literature (Priborsky & Velisek, 2018).

Compound/Enzyme	Studies reporting an increase*	Studies reporting a decrease*	No difference reported	Consensus
ALP	3	0	3	No trend
ALT	1	1	3	No change
Ammonia	1	2	0	No trend
AST	3	1	3	No trend
Catalase	1	0	0	No trend
Cholesterol	2	0	0	Increase
Glucose	13	0	3	Increase
Glutathione peroxidase (GPx)	0	2	0	Decrease
Glutathione reductase (GR)	1	2	0	No trend
Inorganic phosphate	1	0	0	No trend
Lactate	1	2	1	No trend
LDH	-	-	-	-
Lysozyme activity	-	-	-	-
pH	-	-	-	-
SOD	0	4	0	Decrease
TBARS	1	0	0	No trend
Total protein	2	0	2	No trend
Triglycerides	0	1	1	No trend

* increased/decreased metabolite concentration or enzyme activity

When the summarized results of Priborsky & Velisek (2018) were considered for 2-PE as displayed in **Table 2.2**, along with other articles, the inconsistency in the literature became apparent. A general trend could however be found for several indices. 2-PE caused an increase in the following metabolites or biochemical indices: glucose, cholesterol, lactate and blood carbon dioxide (Lynne, 2012; Pucéat *et al*., 1989). Some enzyme activities were also increased namely AST and ALT (Priborsky & Velisek, 2018; Velisek *et al*., 2011; Lepic *et al*., 2014; Javadi Moosavie *et al*., 2014). 2-PE caused a decrease in some indices namely blood oxygen and pH (Lynne, 2012), as well as some enzymes namely SOD and glutathione peroxidase (not specified whether it was enzyme activities or concentrations) (Priborsky & Velisek, 2018). Lepic *et al*. (2014),

Velisek & Priborsky, Velisek & Svobodova (2004a, b) and Velisek *et al*. (2007) determined that 2-phenoxyethanol did not have an effect on LDH concentration after experiments on several fish species. The increase in AST and ALT can be attributed to amplified transamination processes similar to MS-222 anaesthesia (Javadi e *et al*., 2014). In terms of ammonia concentration, there were a lot of discrepancies in the literature, with Lepic *et al*. (2014) reporting an increase in ammonia while several studies found no effect of 2-PE on ammonia concentration (Velisek & Svobodova *et al*., 2004a, 2005b, 2007, 2009 [as cited by Lepic *et al*., 2014]) and several studies reporting a decrease in ammonia concentration (Priborsky & Velisek, 2018). The widely differing results concerning ammonia makes the effect of 2-PE on ammonia unclear, although the effect is expected to be roughly similar to that in MS-222. Javadi Moosavie *et al*. (2014) and Bolasina (2006) reported that the increase in cholesterol levels was associated with an upregulation of cortisol synthesis and may also indicate increased hepatic activity.

Pucéat *et al*. (1989) compared the effects of MS-222 and 2-phenoxyethanol on rainbow trout (*Salmo gairdneri*) hepatocyte metabolism. In this study they found no difference in the hepatic concentration of glucose, lactate, glycogen ATP, ADP and AMP between the two treatments. They did find that 2-PE treatment caused less glucose production in hepatocytes than MS-222 and attributed it to an inhibition of glycogenolysis due to a lower amount of stress caused by 2-PE. The reason for increased glucose and lactate after 2-PE anaesthesia is that the anaesthesia caused a stress response and consequently a release of catecholamines, leading to an increase in glucose (Congleton, 2006; Lepic *et al*., 2014). Hypoxia also caused a shift to anaerobic respiration, which explains the elevated lactate (Velisek *et al*., 2011; Topic Popovic *et al*., 2012).

Table 2.3: A summary of the effect of eugenol on the metabolism of different fish species according to literature (Priborsky & Velisek, 2018).

Compound/Enzyme	Studies reporting an increase*	Studies reporting a decrease*	No difference reported	Consensus
ALP	0	3	0	Decrease
ALT	3	0	1	Increase
Ammonia	4	2	0	Increase
AST	3	2	1	No trend
Catalase	1	0	0	No trend
Cholesterol	-	-	-	-
Glucose	22	2	8	Increase

Table 2.3. (continued)

Glutathione peroxidase (GPx)	0	2	0	Decrease
Glutathione reductase (GR)	0	2	0	Decrease
Inorganic phosphate	2	2	0	No trend
Lactate	3	2	2	No trend
LDH	0	1	1	No trend
Lysozyme activity	2	0	0	Increase
pH	0	1	0	No trend
SOD	0	4	0	Decrease
TBARS	2	0	0	Increase
Total protein	4	1	2	Increase
Triglycerides	5	0	1	Increase

* increased/decreased metabolite concentration or enzyme activity

When the results of Priborsky & Velisek (2018) (as summarized in **Table 2.3**) and several other studies were considered for eugenol, the metabolites or biochemical indices commonly found to increase were, glucose, ammonia, triglycerides, TBARS and total protein in blood (Priborsky & Velisek, 2018). The following enzyme activities or concentrations were also increased: lysozyme activity, ALT activity, T3 and T4 (not specified whether it was enzyme activities or concentrations) (Holloway *et al*., 2004 [as cited by Sanchez-Vasquez *et al*., 2011]). Priborsky & Velisek (2018) found several enzyme activities or concentrations to decrease namely ALP activity, SOD, glutathione peroxidase and glutathione reductase. Eugenol also inhibited several proinflammatory enzymes like nitric oxide synthase, lipoxygenase and cyclooxygenase (Pramod *et al*., 2010) and importantly inhibited NADH oxidase, which is integral to ATP synthesis and may disturb the redox balance and ATP levels (Usta *et al*., 2002).

The increase in glucose is caused by the increase in catecholamines as part of the stress response (Congleton, 2006; Lepic *et al*., 2014). Although most studies indicated increased lactate as expected during hypoxia, there were several studies reporting a decrease or no change in lactate after eugenol anaesthesia (Priborsky & Velisek, 2018). This inconsistency may be caused by the stress response, which may reduce the reliance of fish on anaerobic metabolism during anaesthesia (Velisek *et al*., 2011). The increase in ALT is expected to be an attempt to alleviate the energy crisis by increasing the transamination products, especially pyruvate, which could in turn be used in energy pathways (Velisek *et al*., 2011; Lepic *et al*., 2014). The effect of eugenol on the urea cycle seems to differ from that in MS-222 and 2-PE, with no clear trend of increased

AST or decreased ammonia. Lepic *et al*. (2014) and Gomulka *et al*. (2008), reported that protein metabolism may be altered during anaesthesia. Total protein levels in blood may be an indication of increased haemolysis (Roman *et al*., 2009). Because eugenol inhibits Na/K-ATPase channels, which provide the driving force for the transport of many compounds from the intestines, the absorption of many compounds is impaired, including glucose and amino acids (Kreydiyyeh *et al*., 2000). Kreydiyyeh *et al*. (2000) specifically reported that the absorption of alanine was significantly lowered after eugenol anaesthesia. Kumaravelu *et al*. (1996) reported that eugenol inhibits glucose-6-phosphate dehydrogenase in the pentose phosphate pathway and subsequently reduces glutathione reductase as well. These changes indicate that the GSH/GSSG (glutathione/glutathione disulfide) ratio cannot be maintained during a NADPH deficiency.

Some studies reported that eugenol caused an increase in the thyroid hormones thyroxine (T3) and triiodothyronine (T4) for a short time (10 min) after the application of anaesthesia (Holloway *et al*., 2004 [as cited by Sanchez-Vasquez *et al*., 2011]). The concentrations of T3 and T4 were measured after MS-222 treatment as well, but it did not show a significant difference between the MS-222 treated and the control group. T3 is secreted into the bloodstream, which transports it to the organs. In the organs, it is converted to its active form T4. T4 binds to the nuclear thyroid hormone receptor (TR) which regulates carbohydrate and cholesterol metabolism by direct actions on gene-expression and by crosstalk with other nuclear receptors. T3 also causes increased transcription of acetyl CoA carboxylase, which converts acetyl CoA to malonyl CoA. Malonyl CoA in turn, promotes lipogenesis and inhibits palmitoyl transferase, which is responsible for converting long-chain fatty acyl-CoAs to acylcarnitines to enable their translocation into the mitochondrial matrix for β-oxidation. The increased T3 and T4 shortly after the application of eugenol, is thus expected to have an influence on the concentration of long-chain fatty acyl-CoAs and acylcarnitines (Muller *et al*., 2014).

The main conclusions that were made after summarizing the work of Priborsky & Velisek (2018) and numerous other studies were that all the anaesthetics caused an increase in blood glucose and a decrease in the antioxidant enzymes SOD and glutathione peroxidase, which indicates reactive oxygen species (ROS) production. Several other enzymes and compounds involved in antioxidant activity (catalase, TBARS, GR) were also affected for some of the anaesthetics. It was also determined that the majority of anaesthetics caused increased transaminase activity. Lastly, it was widely reported that the hypoxia caused by cessation of respiration in turn caused a shift to anaerobic glycolysis for all the anaesthetics. This shift to anaerobic metabolism may, however, be counteracted by the stress response, as reported for eugenol anaesthesia by Velisek *et al*.

(2011). However, it is also worth noting that fish can experience some degree of hypoxia when anaesthetized which could favour the shift to anaerobic glycolysis.

2.4.2 Effect of anaesthetics on redox state

Along with ATP/ADP ratio, the cell redox potential (NAD/NADH ratio) is one of the most important regulators of main cellular processes like glycolysis (Tilton *et al*., 1991; Lin & Guarente, 2003) and the TCA cycle (Lin & Guarente, 2003; Berg *et al*., 2002). The redox state is also often considered as a reflection of the total metabolic state (Lin & Guarente, 2003). Some of the enzymes regulated by the NAD/NADH ratio are glyceraldehyde 3-phosphate dehydrogenase, pyruvate dehydrogenase (Lin & Guarente, 2003), lactate dehydrogenase, glutamate dehydrogenase, malate dehydrogenase, ß-hydroxybutyrate dehydrogenase (White & Schenk, 2012), isocitrate dehydrogenase (Igamberdiev & Eprintsev, 2016) and α-ketoglutarate dehydrogenase (Zdzisińska *et al*., 2016). Each of these enzymes represent a metabolic shuttle between two metabolites, with the involvement of NAD/NADH in the reactions (White & Schenk, 2012).

To measure the redox state directly can be very challenging due to the constant flux between protonated and de-protonated forms of NAD. White & Schenk (2012) did, however, report that the redox state can be indirectly measured by determining the ratios of the metabolites involved in the NAD-dependant metabolite shuttles. This method of determining the NAD/NADH ratio is called the metabolite indicator method (MIM).

Although some anaesthetics like eugenol are reported to disturb the redox potential (by inhibiting NADH oxidase) (Usta *et al*., 2002), the effect of anaesthetics on the cellular redox state is still extremely contradictory in the literature (Papurica *et al*., 2015) and information on specific anaesthetics, especially MS-222 and 2-PE is lacking. The contradictory nature of present literature on the effect of anaesthetics on the cellular redox state supports the need for further investigation into the matter.

2.4.3 Effect of ATP/ADP ratio on metabolism

After investigating the mechanisms of the different anaesthetics, it became evident that most of the anaesthetics used influence depolarization and repolarization of neurons by affecting ion channels. When the resting membrane potential is lost, ATP is rapidly used by these ion channels to attempt correcting the membrane potential. This may deplete cellular ATP levels (Santos *et al*., 1996), exacerbated by the functional hypoxia associated with respiratory inhibition. In some

cases, the exact mechanism affecting these ion channels are still debated (Kishikawa *et al*., 2018). Kishikawa *et al*. (2018) proposed an alternative theory to help explain the anaesthetic effect, hypothesizing that anaesthetics may inhibit proteins in the electron transport chain, which causes loss of mitochondrial membrane potential and thus decreased ATP synthesis. Because ATPase ion channels rely on ATP to maintain membrane potential, a depletion of ATP will lead to a loss of membrane potential, causing the anaesthetic effect. In either case, ATP is decreased, which was confirmed in the study by Kishikawa *et al*. (2018).

The decreased ATP experienced during anaesthesia naturally leads to a lower ATP/ADP ratio. The ATP/ADP (and ATP/AMP) ratio is an important regulator of main cellular processes, being one of the main factors that determine whether anabolic or catabolic pathways are favoured (Kim *et al*., 2016; Towler & Hardie, 2007). In conditions of a low ATP/ADP ratio, several enzymes are activated to alleviate the energy crisis. A notable enzyme activated by a low ATP/AMP ratio is the AMP-activated protein kinase (AMPK) system. This system acts as a central regulator of energy homeostasis by maintaining a favourable balance between anabolic and catabolic processes, depending on the energy need. In the event of depleted ATP, the AMPK system is activated, which in turn activates ATP-producing catabolic pathways like glycolysis, fatty acid oxidation and autophagy. When activated, the AMPK system also inhibits anabolic processes that consume ATP like lipogenesis, protein synthesis and glycogenesis (Kim *et al*., 2016; Towler & Hardie, 2007; Lehner & Quiroga, 2016). In addition to the AMPK system, several more pathway-specific enzymes are regulated by the ATP levels, especially enzymes that are part of glycolysis and the TCA cycle (Berg *et al*., 2002; Sussman *et al*., 1980). The enzymes activated by a low ATP/ADP ratio includes phosphofructokinase (which is the rate-limiting enzyme of glycolysis) (Passonneau & Lowry, 1964), pyruvate dehydrogenase, citrate synthase, isocitrate dehydrogenase and alpha-ketoglutarate dehydrogenase (Berg *et al*., 2002). Protein catabolism is also reported to be upregulated during an energy crisis to provide amino acids for energy production (Fu *et al*., 2018).

It is evident that altered ATP levels have a widespread effect on cellular metabolism. Advanced analytical techniques are thus needed to generate comprehensive metabolomic data sets and to assess whether pathways regulated by ATP levels are altered in this study.

2.5 Metabolomics

2.5.1 Analytical approaches

Metabolomics is one of the new additions to the “omics” revolution. The addition of metabolomics proves invaluable in extending “omics” from the genetic level (genotype) to small molecule level

(phenotype), which helps researchers to answer questions that could not be fully answered by other omics methods (Du Preez *et al*., 2017; Idle & Gonzalez, 2007). Metabolomics can be defined as the systematic non-biased identification and quantification of all the metabolites in a specific biological system, using various analytical techniques supported by statistical, mathematical and computational analysis (Du Preez *et al*., 2017; Idle & Gonzalez, 2007). The metabolome is the downstream product of the genome, transcriptome and proteome and influenced by the environment. Moreover, the metabolome is dynamic, unlike the static genome, and provides insight into the phenotype of the host (Du Preez *et al*., 2017).

The rapid development of metabolomics as a research field can be attributed to the remarkable developments in analytical chemistry including nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS) platforms, along with the development of multivariate data analysis software, which makes it possible to deconvolute the large data matrices generated from metabolomic analyses (Idle & Gonzalez, 2007). The main analytical platforms used for metabolomic analyses are gas chromatography (GC), liquid chromatography (LC), usually coupled to different MS techniques, and NMR spectroscopy. Each of the platforms have different advantages and disadvantages, which determine their applications depending on the aim of the investigation (De Villiers & Loots, 2013). The complexity of the metabolome and the different strengths and weaknesses of the different analytical platforms often creates the need for a multi-platform approach to identify and quantify as many metabolites as possible (Scalbert *et al*., 2009; Xiao *et al.,* 2012; Garcia & Barbas, 2011). GC-MS has been widely considered as the golden standard for the detection and quantification of many compounds related to toxicology, forensic science, environmental contaminants, metabolism and pharmaceuticals (Garcia & Barbas, 2011).

The GC component of the instrument achieves initial separation of the molecules on the basis of volatility, polarity and interaction with the capillary column. After initial separation has been achieved, the molecules are captured by the MS. The MS ionises the molecules, which causes them to fragment into specific fragmentation patterns. The ionised fragments are then accelerated toward the detector, which detects them based on time-of-flight or mass-to-charge ratio, depending on the type of MS (Sneddon *et al*., 2007). GC-MS has the advantages that it is highly sensitive, efficient and quantitative (Garcia & Barbas, 2011; Scalbert *et al*., 2009). The fragmentation spectra are not instrument dependent, making it reproducible (Garcia & Barbas, 2011). The reproducibility in turn, allows the creation of public databases for the identification of detected species (Garcia & Barbas, 2011). GC-MS allows identification of detected species based not only on fragmentation patterns (mass spectrum), but on retention time as well (Garcia & Barbas, 2011; Scalbert *et al*., 2009). Moreover, deconvolution software makes it possible to identify more than 300 metabolites using appropriate data processing methods (Fiehn, 2016).

The disadvantages of GC-MS are that only volatile compounds or compounds that are volatile after derivatization can be analysed (Garcia & Barbas, 2011). Due to the need for derivatization, demanding sample treatment may be necessary (Garcia & Barbas, 2011).
LC-MS was developed in the mid-1990s to compensate for some of the limitations of GC-MS. Since then, the use of LC-MS in clinical laboratories has seen enormous growth (Grebe & Singh, 2011). An LC-MS system makes use of a liquid mobile phase to separate compounds based on polarity, charge and/or size (depending on stationary phase). The polarity of the mobile phase is gradually changed, which causes compounds to separate from the sample mixture and elute when the mobile phase is at a specific polarity. As compounds elute, they are detected by the MS system (Grebe & Singh, 2011). Unlike GC-MS, LC-MS has the advantage that it can detect non-volatile compounds, thermally unstable compounds and compounds having a wide range of polarity, which removes the need for demanding derivatization steps in the pre-treatment of samples (Xiao *et al.,* 2012). LC-MS also has the advantages that it allows for faster analytic turnaround times, higher throughput and simpler workflows than GC-MS, as well as higher sensitivity and specificity (Grebe & Singh, 2011). This platform does have some limitations, which includes ion suppression (Scalbert *et al*., 2009), inconsistent retention times, highly manual workflows, complex instrumentation, higher running costs, and isotope-based identification (Grebe & Singh, 2011; De Villiers & Loots, 2013).

NMR spectroscopy is a very important platform for metabolomics because it addresses the limitations experienced by MS techniques and vice versa. NMR resides on the basic principle that nuclei are electrically charged and usually have an inherent spin. When the nuclei are exposed to an external magnetic field, they can be elevated to a higher energy state. When the nuclei are allowed to return to their original energy state, they submit a specific radio frequency which can be measured as a signal intensity (Keeler, 2010). The signal intensity is directly proportional to the number of nuclei submitting that specific frequency (resonance) (Bharti & Roy, 2012). The main advantages of NMR are that it is highly reproducible, it can be used to determine molecular structure, it can quantify metabolites without the use of a reference standard, it has a short measuring time, it is non-destructive, it has high precision and accuracy, it allows the identification of metabolites with identical masses and it can be used to detect compounds that are difficult to ionise or derivatize (Bharti & Roy, 2012; Markley *et al*., 2017). When NMR data are integrated with two-dimensional (2D) NMR metabolomics databases, they increase the accuracy of identification for metabolites that are recorded in these databases (Bingol, 2018). This improved identification is due to 2D NMR's ability to differentiate between overlapping signals that may exist in large molecules (Idström *et al*., 2016). The major limitation of NMR is that it has a lower sensitivity than MS methods (Garcia & Barbas, 2011; Markley *et al*., 2017).

The main advantages and disadvantages of the three analytical instruments used in this study are summarized in **Table 2.4.**

Table 2.4: Summary of the advantages and disadvantages of the analytical instruments used in this study.

Instrument	Advantages	Disadvantages
GC-TOFMS	Highly sensitive	Only volatile compounds or compounds that are volatile after derivatization can be analysed
	Fragmentation spectra are not instrument dependent, making it reproducible	Demanding sample treatment may be necessary
	Extensive public databases available for identification of features	
	Allows identification based on retention index	
	Deconvolution software makes it possible to identify a large amount of more than 300 metabolites	
LC-MS/MS	Compounds that are non-volatile or thermally unstable can be detected	Ion suppression may occur
	Faster analytic turnaround times	Inconsistent retention times
	Higher throughput	Highly manual workflows
	Simple sample preparation	Complex instrumentation
	High sensitivity	High running costs

Table 2.4 continued:

	High specificity	Isotope-based identification
NMR	Highly reproducible	Lower sensitivity than MS methods
	Can be used to determine molecular structure	
	Quantification possible without the use of a reference standard	
	Has a short measuring time	
	Non-destructive	
	High precision and accuracy	
	Allows the identification of metabolites with identical masses	
	Can detect compounds that are difficult to ionise or derivatize	

Metabolomic approaches can be classified as either “targeted” or “untargeted”. The different approaches are often used in collaboration with each other because the strengths and weaknesses of the different approaches tend to compensate for each other (Ribbenstedt *et al*., 2018). Untargeted metabolomics aim to detect as many metabolites as possible in a single analysis, in an effort to determine the global metabolic response to diseases, environmental factors or genetic disorders (Xiao *et al.,* 2012; Ribbenstedt *et al*., 2018). To identify significant metabolites from the hundreds of variables detected, untargeted methods need to be combined with advanced multivariate statistics and chemometric software. Untargeted metabolomics have the advantages that it is a comprehensive approach that can be used to detect novel biomarkers and that it provides a basis for targeted analyses (Ribbenstedt *et al*., 2018; Roberts *et al*., 2012). The disadvantages of untargeted metabolomics are firstly that they are never truly unbiased because the sample preparation, stationary phase, ionization mode etc. may cause a bias towards some substances (Ribbenstedt *et al*., 2018). Other disadvantages include that the large datasets require a lot of time to process and that further validation procedures are often needed after biomarkers were identified (Roberts *et al*., 2012). Targeted approaches are used to analyse a specific group of metabolites related to specific metabolic pathways, enzymes or end-products

(Ribbenstedt *et al*., 2018; Roberts *et al*., 2012). Targeted metabolomics are especially useful for diagnostics and have the advantages that it has a higher precision than untargeted methods and it can be optimised for specific classes of metabolites to remove the possible dominance of high abundance molecules (Roberts *et al*., 2012). This may in turn, increase the sensitivity of targeted approaches (Roberts *et al*., 2012). Targeted approaches do, however, have the limitation that it provides a limited coverage of the metabolome, which may cause important metabolic perturbations to be overlooked (Ribbenstedt *et al*., 2018).

In the investigation regarding the effect of anaesthetics on zebrafish metabolism, it is important to get a complete overview of the metabolic perturbations caused by the anaesthesia. Due to the need for a comprehensive metabolic picture, it was decided to use all the most common metabolomic instruments described above (GC-MS, LC-MS and NMR) in order to harness all of the advantages and compensate for all of the different disadvantages of the instruments. These instruments were applied according to their strengths in both untargeted and targeted analyses. This ensured that the full potential of not only the instruments but also of the different metabolomic approaches could be utilized in this investigation to provide a global view of zebrafish metabolism.

Before the commencement of a metabolomic analysis as explained above, the metabolites must be chemically extracted from the tissue. The nature of metabolomics, which ambitiously attempts to provide an overview of total metabolism, creates the need for an extraction method that extracts the largest possible number of metabolites. The extraction methods used for this study are explained in more detail below.

2.5.2 Extraction methods

There are numerous different methods available, of which variations of the Bligh and Dyer method are the most popular to extract metabolites from tissue homogenate (Jensen, 2008). During the Bligh and Dyer extraction method, samples are prepared in biphasic conditions. The final extract thus contains a top polar phase consisting of water, methanol and hydrophilic metabolites while the bottom apolar phase consists of chloroform and contains hydrophobic metabolites like lipids (Raja *et al*., 2020). There are also modified versions of the Bligh and Dyer extraction where the final extract is monophasic (Wu *et al*., 2008). The biphasic and monophasic extraction methods each have their own advantages and disadvantages. The monophasic method has the advantage that it reduces the complexity and time needed for the procedure while the biphasic method may have a higher probability of contamination due to the retrieval of the bottom apolar phase (Gil *et al*., 2018). According to Salem *et al*. (2016) the two-phase method has the advantage that it

extracts compounds with a wide range of polarities and in diverse compound classes. This may be an important determining factor when choosing between the two methods.

When conducting a metabolite extraction, different methods can also be used to add the extraction solvents, which include a two-step, stepwise and an all-in-one tube approach. Wu *et al.* (2008), compared the different approaches on the basis of extraction yield, sample throughput and reproducibility. The authors concluded that the two-step method where water and methanol are added initially, followed by additional water and chloroform at a later stage, produced the highest reproducibility, throughput and extraction yield. The results that were generated by Wu *et al.* (2008), informed the decision to use the two-step method in the current study as well.

After completion of the literature study, the experimental part of the study could commence. Information from the literature study was used throughout the experimental stage and during data interpretation to ensure that the methods used were up-to-date and that any conclusions or hypotheses generated were sufficiently supported by the literature.

CHAPTER 3: METHODOLOGY

3.1 Materials

Methanol (Honeywell, cat # 67561), water (Honeywell, cat # 7732185) and chloroform (Honeywell, cat # 67663) were used during the metabolite extraction.

During the GC-TOFMS analysis, BSTFA (O-bis[trimethylsilyl]trifluoro acetamide) +1% TMCS (trimethyl-chlorosilane) (Sigma Aldrich, cat # 15238-25mL), Methoxyamine (Sigma Aldrich, cat # 226904-1G) and Pyridine (Sigma Aldrich, cat # 270407-1L) were used to derivatize the samples while nonadecanoic acid (Sigma Aldrich, cat # N5252-1G) were added as internal standard.

For the LC-MS analysis, the following amino acid and acylcarnitine isotopes were used for quantification: Citrulline, Arginine, Glycine, Glutamic acid, Phenylalanine, Methionine, Isoleucine, Valine, octadecanoylcarnitine (C18), hexadecanoylcarnitine (C16), tetradecanoylcarnitine (C14), dodecanoylcarnitine (C12), decanoylcarnitine (C10), octanoylcarnitine (C8), valerylcarnitine (C5), butyrylcarnitine (C4), propionylcarnitine (C3), acetylcarnitine (C2), free carnitine (C0). To derivatize the samples for LC-MS analysis, butanolic hydrogen chloride was used consisting of 80% 1-Butanol (Sigma Aldrich, cat # 281549-100mL) and 20% acetylchloride (Fluka, cat # 00990-100mL). The solvents used as mobile phases consisted of water (Honeywell, cat # 7732185) and 0,1% formic acid (Fluka, cat # 94318-50mL-F) (solvent A) and acetonitrile (Honeywell, cat # 017-4) (solvent B).

During the NMR analysis, dried extracts were resuspended in water (Honeywell, cat # 7732185). A deuterated buffer was added consisting of potassium phosphate salt (Sigma-Aldrich, cat # 60218-500G), deuterium oxide (Merck, cat # S5635266420) and an internal standard namely trimethylsilylpropionic acid (TSP) (Merck, cat # S5633352419).

3.2 Experimental design

The experimental design of this study (as summarized in **Figure 1.2.1.**), involved the treatment and acquisition of zebrafish samples. After sample acquisition, the extraction methods were optimized (refer to Annexure A). The optimized extraction procedure was used to extract the metabolites from the zebrafish samples. The next step was to do metabolomic analysis of the samples. The sample extracts were divided in three for the three platforms namely GC-TOFMS, LC-MS/MS and NMR that were used to conduct the metabolomic analyses. After completion of the metabolomic analyses, data pre-treatment and pre-processing were done, which included

normalisation with internal standard, zero filtering and coefficient of variance (CV) filtering. The final step of the experimental workflow was to identify significant compounds and compare the data of the different experimental groups using statistical analyses. The statistical analyses used for final interpretation of the data were student's t-tests (p-values), effect size (d-values) and principal component analyses (PCA). The different steps listed in the experimental design are described in detail below.

3.3 Zebrafish treatment and sample acquisition

Each of the 3 treatments and the control group required 7 fish (28 fish in total). A further 10 fish were available for optimization of the pharmacokinetics and another 10 for the optimization of the sample preparation methods.

To apply the anaesthesia, fish were moved from the holding tank to a container with clean aerated water, where they remained for an hour for handling recovery before anaesthesia was applied. To apply the anaesthesia, bath treatment was used where stock solutions of anaesthetics were dissolved in the water to achieve the desired concentration. The concentrations of the different anaesthetics that were needed to reach stage 4 anaesthesia in a specific time interval (60-80 seconds) were determined previously (Sosibo *et al.*, in prep) (Grush *et al*., 2004; Khosravanizadeh *et al*., 2020). The scale used in this study defines stage 4 anaesthesia as anaesthesia that results in a total loss of equilibrium (Scherelk & Moyle, 1990). For each group, 7 fish were sampled at the time interval when stage 4 anaesthesia was reached. The same time intervals and vehicle controls were used for the control group, but without any anaesthesia (Velisek *et al*., 2011).

The concentrations of the anaesthetics required to achieve stage 4 anaesthesia in 60-80 s were as follows:
MS-222: 150 ppm (buffered with $NaHCO_3$)
2-PE: 700 ppm
Eugenol: 90 ppm (in EtOH)

After the zebrafish were exposed to the above-mentioned doses and time intervals of anaesthesia, they were euthanized by removal and direct decapitation. Control fish underwent the same euthanasia process but without the use of anaesthetics. The samples were stored at -80°C and the frozen samples were transported to the NWU Mitochondrial Laboratory on dry ice.

For this study, whole fish samples were used for the analysis due to the small size of the fish, which makes blood sampling impractical. Whole fish samples also ensure a more complete picture of the fish's total metabolism (Seth *et al*., 2013; Löhr & Hammerschmidt, 2011).

3.4 Metabolite extraction

Following method optimization (see Annexure A), experimental samples were weighed to perform pre-analysis normalisation. The tissue (whole fish) was crudely minced using a razor blade, where after 4 µL methanol and 2 µL water were added for each mg of tissue. A 5mm and a 7 mm stainless steel bead was added to each tube. A Retch M400 vibration mill was then used to homogenize the mixture for 10 min at 30 Hz. The homogenate was separated from the beads, whereafter the contents were transferred to clean tubes. The supernatant of each homogenate was divided into 3 microcentrifuge tubes, one for GC-MS, one for LC-MS and one for NMR. A two-phase extraction protocol previously described (Lindeque *et al*., 2013), albeit with slight modification, was used to extract metabolites from the samples. During the extraction protocol, additional water, methanol, chloroform, and internal standard (nonadecanoic acid, at a concentration of 50 ppm) were added to each of the tubes in the ratios described in the established, optimized two-phase extraction protocol (Annexure A). The tubes were vortexed for 30 seconds and then incubated on ice for 10 min. To achieve phase-separation, the samples were centrifuged at 2000×g for 5 min at 4°C. After extraction, 300 µL of the polar phase and 150 µL of the apolar phase were transferred to 2mL microcentrifuge tubes for NMR and screw top vials for GC-MS and LC-MS analysis. The extracts were dried under a gentle stream of nitrogen at 37°C and stored in a -80°C fridge until platform specific preparation procedures could be conducted (Venter *et al*., 2018a).

3.5 Quality control samples

After the samples were homogenized, 70uL of each sample were combined in a new 5mL microcentrifuge tube to form a single pooled quality control (QC) sample. The samples were vortexed and 100uL were aliquoted into fifteen 2mL microcentrifuge tubes. The aliquoted samples were extracted and analysed in the same way as the experimental samples. 7 QCs were prepared for GC-MS and LC-MS respectively, to be injected after every 5 experimental samples. The NMR QC samples were injected at the beginning, middle and end of the analytical batch. The QC samples' purpose was to ensure that the data obtained are reliable and to correct for any analytical drift caused by within-batch effects. For GC-MS and LC-MS, the first QC injection was repeated five times to equilibrate the analytical instrument.

3.6 Metabolomic analysis

Because of the complementary nature of the different analytical platforms, three metabolomic techniques namely GC-TOFMS, NMR and LC-MS were used to get a comprehensive profile of the treated and control groups' metabolism.

3.6.1 Sample preparation

After extraction and phase-separation, the GC-TOFMS aliquots were removed from the freezer and dried under a nitrogen stream at 40 °C for 30 min to ensure there was no residual moisture that could interfere with the derivatization. After the extracts were dried, the samples were oximated and silylated. To oximate the samples, 50µL of the oximation reagent, which consisted of 200 mg methoxyamine dissolved in 10mL pyridine, was added to the samples. The vials were capped, vortexed for 1 min and incubated for 60 min at 60 °C using a heat block. After the samples were oximated and left on the bench to reach room temperature, 50µL BSTFA, containing 1% TMCS, was added to the samples. The samples were vortexed for 1 min and incubated at 40 °C for 60 minutes. After derivatization was completed, the samples were transferred to glass GC vials with inserts and capped before conducting an untargeted GC-TOFMS analysis (Venter *et al*., 2016).

The aliquots designated for LC-MS analysis were derivatized by adding 300µL 3N butanolic hydrogen chloride (HCl) to the dried samples and incubating it at 60°C for 30min. The Butanolic HCl solution used was freshly prepared by adding acetyl chloride to a 1-butanol solution in a 1:4 ratio. After butylation, the samples were dried again for 60min. using a nitrogen sample evaporator at 40 °C. The dried samples were re-dissolved in 50uL of water and 50uL acetonitrile and transferred to vial inserts before LC-MS/MS analysis.

Before the NMR analysis, dried sample extracts were re-dissolved in 100µl ultra-pure water. The samples were centrifuged at 12,000×g for 5min to remove any remaining particles and macromolecules. After centrifugation, 54uL of the re-dissolved samples were mixed with 6uL of a 1.5M potassium phosphate monobasic deuterated buffer solution (90% H_2O:10% D_2O; pH 7.4) containing TSP and transferred to 2mm micro NMR glass tubes (Mason *et al*., 2018).

3.6.2 Instrumentation

3.6.2.1 GC-TOFMS

The GC-TOFMS analysis was done with an Agilent 7890A GC coupled to a LECO Pegasus HT mass analyser (TOFMS). An Agilent DB1 column (20m × 0.180mm × 0.18µm) was used for chromatographic separation. In the Autosampler method used for every run, a sample volume of 1 µL was injected with a 1:5 split ratio. The front inlet temperature was kept constant at 250 °C. The oven temperature was initially maintained at 50 °C for 1.0 min, whereafter it was increased as follows: 5 °C/min to 100 °C, 10 °C/min to 160 °C, 13 °C/min to 230 °C, 20 °C/min to 300°C where it was maintained for 2 min. This added up to a total run time of ~30 min per sample. Helium was used as carrier gas at a constant flow of 1.40 mL/min. The ion source temperature was maintained at 200 °C while the transfer line temperature was kept at 225 °C for the entire duration of the run. An acquisition delay was applied for the first 350 sec which served as a solvent delay. Data were captured with an acquisition rate of 20 spectra (40–950 m/z) per second, with a detector voltage of 50 V over the daily tune voltage and electron energy of −70 V (Venter *et al*., 2018b).

3.6.2.2 LC-MS/MS

After the samples were prepared, they were subjected to reverse phase liquid chromatography using an Agilent 6410 LC system. A sample injection volume of 1 µL was used, with an autosampler temperature of 4 °C. The column used for separation was an Agilent C18 SB-Aq column (2.1 × 100 mm, 1.8 µm), fitted with a guard pre-column. The column temperature was set to 45 °C. The mobile phase solvents consisted of water (A) and acetonitrile (B) respectively, each with 0.1% formic acid (Venter *et al*., 2017).

The gradient used to perform the separation was as follows: 0 min 5% (B); 1 min 5% (B); 6 min 25% (B); 10 min 25% (B);15 min 100% (B); 20 min 100% (B) and 21 min 5% (B). A flow rate of 0.2 mL/min was used for the entire run except for the time slot between 15 min and 20 min where a flow rate of 0.3 mL/min was used. The remaining gradient was maintained for 4 min using a flow rate of 0.2 mL/min. The post-run was used to ensure that all the analytes have eluted and to ensure column equilibration. The gradients used and the post run time resulted in a total runtime of 28 minutes per sample. Mass spectrometric detection of the target compounds was performed using an Agilent 6410 Triple Quadrupole equipped with positive electrospray ionisation. A drying gas temperature of 300 °C was used with a drying gas flow of 7.5 L/min and nebuliser pressure of 30 psi. The target metabolites namely amino acids and acylcarnitines were detected using

multiple reaction monitoring (MRM). The optimized MRM transitions and source conditions are summarized in Supplementary **Table C1 (Annexure C)**.

3.6.2.3 1H-NMR

To conduct the 1-H NMR analysis, a Bruker Advance III HD NMR spectrometer operating at a frequency of 500 MHz was used. This instrument was equipped with a triple-resonance inverse (TXI) (1H, 15N, 13C) probe head and x, y, z gradient coils. The NMR instrument was calibrated per sample by automated tuning, matching, locking and shimming using the standard Bruker routines ATMA, LOCK, and TopShim. The NMR spectra had a spectral width of 6000 Hz and consisted of 256 transients in 32,000 data points. The sample temperature was kept at 27°C and the water resonance was pre-saturated by single-frequency irradiation during a 4 second relaxation delay, with a 90° excitation pulse of 8 µs, acquiring spectra at 256 scans per sample (runtime of 32 min per sample). The deuterium signal was used for the automated shimming of the sample. The resonance line widths for TSP and metabolites were <1 Hz at half the height of the peak. Fourier transformation and phase and baseline correction were done automatically. Bruker Topspin (Version 3.5) software was used to process spectral data while Bruker AMIX (Version 3.9.12) software was used to distinguish and identify metabolites (Ellinger *et al*., 2013; Irwin *et al*., 2016; Venter *et al*., 2018b).

3.6.3 Data analysis

After metabolomic analysis, vast amounts of raw data were generated in the form of NMR and mass spectra, which is extremely difficult and time-consuming to process manually. To process the raw data sets into a meaningful and understandable format, established data mining and extraction procedures were used (Dettmer *et al*., 2007). The data mining process is displayed in **Figure 3.1** below and described in the following sections:

Figure 3.1: Data mining process

3.6.4 Data extraction

In this study, the GC-TOFMS data were extracted using the Leco-ChromaTOF GC software package. ChromaTOF was used to perform peak detection, baseline subtraction and deconvolution in this study as described in Venter *et al*. (2018b). The baseline tracking mode was set to have an offset of 1. The software was allowed to automatically select smoothing parameters. An expected peak width of 3 s and signal-to-noise ratio of >20 was used to detect peaks. Additionally, the detector was set to only use masses between 100 and 950 m/z as model ions, and any true peak had to contain five apexing masses. Two spectral libraries namely the National Institute of Standards and Technology (NIST) 2011 mass spectral library and an inhouse created library (Reinecke *et al*., 2012) were used to identify features. A feature had to reach a spectral match similarity of 80% before it could be given a tentative identity. To eliminate spectral matching of incorrect derivatives or non-derivatized compounds, only identities which contained at least one silicon (Si) element in its formula were allowed to be identified as a metabolite (Venter *et al*., 2018b). The identity (spectral match) of the important compounds were confirmed by comparing retention time or retention indices to that of the libraries and previously analysed standards (Section 3.6.10).

LC-MS data were firstly examined for retention time drifts and the presence of metabolites and isotopes using Agilent's MassHunter Qualitative software. After the qualitative evaluation of the data, Agilent's MassHunter Quantitative software was used to convert the raw data to a useful data matrix by performing supervised peak integration. The data matrix containing the integrated peak areas and retention times was then downloaded for data pre-processing and normalisation.

During the NMR analysis, Bruker Topspin (V3.5) software was used to perform automatic shimming of the sample based on the deuterium signal. The resonance line width of TSP was determined to be <1HZ, which indicated that the shimming had been successful. The Bruker Topspin software was also used to perform automatic Fourier transformation and phase and baseline correction were done. Bruker AMIX (V3.9.12) software was used to further process the NMR spectra. The NMR spectra was divided into 0.02 ppm sized spectral bins for the interval of 0.5-10 ppm, excluding the region of the water peak (4.69-5.21ppm). After the pre-processing steps using Bruker AMIX software, a data matrix was created consisting of 473 bins (Lindeque *et al*., 2018). The data matrix was exported to Microsoft Excel for further processing.

3.6.5 Data pre-processing and normalisation

Pre-processing can be described as all the editing that is needed to prepare the data for statistical analysis (Liland, 2011), which willreveal relevant information (Nielsen *et al*., 2010). An important part of data pre-processing is normalisation with an internal standard to correct for any non-biological variation in the data (Steinfath *et al*., 2008). The established pre-processing and normalisation steps included zero filtering, coefficient of variation (CV) filtering, missing value imputation (MVI), normalisation with nonadecanoic acid and batch effect verification.

3.6.5.1 Zero Filtering

Zero filtering is described as removing features with extensive missing values to create a more complete data set (Venter *et al*., 2015). The application of a zero filter makes the information generated after statistical analysis more meaningful. In this study, a supervised 80% zero filter was applied. This means that there needed to be a value for at least 80% of samples in at least one group for the compound to be retained in the data set. The features that did not meet this criterion were removed because they did not contribute any valuable biological or statistical information to this experiment.

3.6.5.2 Coefficient of variation filter

In a metabolomic analysis and especially a GC-TOFMS analysis, it is likely that some of the features will not be reliably measured due a number of reasons including degradation, the formation of oxidation products etc. Irrespective of the reason, these features need to be removed from the data set, because they will introduce unwanted variation and give a false reflection of the actual sample, leading to complications in the statistical analysis and incorrect interpretation. To address this problem, a 50% QC CV value filter was applied to further reduce this non-biological variation in the data (Doerfler *et al*., 2012). The CV values of the QC's were used to determine precision because it was the same sample that was analysed throughout the entire run, making it a good indicator of features displaying unwanted variation. All features with a QC CV > 50% were removed.

3.6.5.3 Normalisation

Normalisation is a technique used to correct for any non-biological variation that may occur during an experiment and to determine the relative concentration of the metabolites. Different methods can be used to normalise metabolomics data, each with its own advantages and disadvantages.

In the GC-MS experiment an internal standard namely nonadecanoic acid was used for normalisation. The data was normalised by dividing all the peak areas of a sample with the peak area of nonadecanoic acid whereafter it is multiplied by the original concentration of the internal standard (50ng per mg tissue). The LC-MS data was normalised similarly albeit by using the stable isotopes. The peak area of each compound detected during the analysis was divided by the peak area of the closest eluting stable isotope of the same class (amino acids or acylcarnitines) and multiplied by the concentration of these isotopes (5.3ng per mg tissue). During the NMR analysis, all the compounds detected were scaled and normalised relative to the TSP peak for quantification (Dona *et al*., 2014).

3.6.6 Batch effect verification

In metabolomics studies, it is important that the measurements remain consistent and within the minimum amount of between- and within-batch variation. If any batch effect is observed, there are batch correction methods that must be applied. During this study, all the samples were analysed in one batch, ruling out any between-batch effects. To confirm that there was no within-batch drift, a principal component analysis (PCA) plot was used. No visible effect could be observed for any of the instruments, thus no correction methods had to be applied.

3.6.7 Missing value replacement

MS datasets usually have numerous missing values originating from biological and/or technical sources and are often due to the concentration of compounds simply being below the detection limit of the analytical platform. Hence, to assume a missing value equals zero is biologically incorrect since normal metabolites are usually present in a sample, but some only at low concentrations. Zero values in the data also causes complications in statistical analyses. In this study, the missing value problem was overcome by replacing the missing values with 1/5th the detection limit (Hrydziuszko & Viant, 2012; Xia *et al*., 2009) using MetaboAnalyst 5.0 software.

3.6.8 Data pre-treatment

Data pre-treatment was done using MetaboAnalyst 5.0 software before statistical analysis (Xia *et al*., 2015). The combined data matrix was transformed using the generalised logarithm (glog) transformation function before statistical analysis (Lindeque *et al*., 2015). Generalized log transformation is used to scale the data so that the intensity of the weaker signals is not overwhelmed by the stronger signals in multivariate statistics. Moreover, the distribution of variables is also normalized so that parametric statistical tests can be used across the board for

all variables. The software does this by calculating a transform parameter that minimises the technical variance of the data (Parsons *et al*., 2007).

3.6.9 Statistical analysis

After the raw data was processed to a suitable data matrix by cleaning, normalisation, pre-treatment etc., statistical analysis could be conducted using MetaboAnalyst. Before the experimental samples were subjected to statistical analyses, the QC samples were assessed using CV value percentages and visual inspection (principal component analysis) to determine whether any within-batch variation occurred (Wehrens *et al*., 2016; Xia *et al*., 2012) - hence verify data quality. The statistical tools used for this study are displayed in **Figure 3.2**.

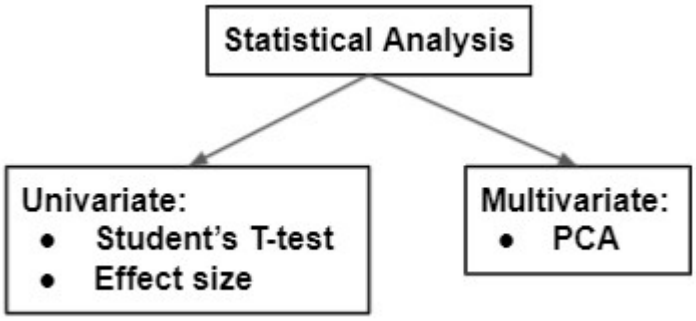

Figure 3.2: Statistical approach used for feature selection and verification of data quality

3.6.9.1 Data overview (multivariate)

After metabolomic analysis, it is useful to conduct statistical tests that can present a qualitative overview of the data to indicate the separation (if any) between different experimental groups. In this study, PCA was used to get an overview of the data before quantitative analysis. PCA is an unsupervised multivariate statistical test which ignores any previous information of group labels. PCA determines the directions of maximum variance in a data set (X) while ignoring the class labels (Y). PCA projects the data onto a lower dimensional space while recording the maximum possible amount of data from the previous data set. The unsupervised nature of a PCA test ensures that it displays a clear view of the natural separation (or grouping) of the data. PCA scores were plotted with 95% confidence ellipses to indicate grouping of the data more effectively.

3.6.9.2 Feature selection (univariate)

Feature selection is the process where metabolites (features) that differ significantly between experimental groups are identified. Two univariate statistical methods were used to construct a

list of significant features: Student's T-tests (hypothesis testing) were used to identify metabolites that differed significantly between the groups and Cohen's d-value (effect size) was used as a magnitude measure of the differences. Volcano plots were used to display the relationship between d-values and p-values and to identify significant features with $p < 0.05$ and $d > 0.8$ (as detailed below).

3.6.9.2.1 Student's T-test

As part of the univariate statistical analysis, a student's T-test was performed to find metabolites that differed significantly in concentration between the control and respective anaesthetic groups. The three anaesthetic groups were not compared with each other as this was not within the scope of this study. Initially, false discovery rate (FDR) corrected p-values were used to determine whether zebrafish metabolism was significantly affected. Due to the low amount of significant metabolites, metabolites with a non-FDR corrected p-value <0.05 were deemed significant for the purpose of hypothesis generation (Lindeque *et al.*, 2018).

3.6.9.2.2 Effect size

The concentration of all the features were used to determine effect size (d-value), which is very similar to fold change but is scaled to the highest standard deviation instead of the control group mean. By calculating effect size instead of fold change, practical significance is ensured. The effect size was calculated by taking the absolute difference between the means of the groups and dividing it by the maximum standard deviation of the two groups. Only features with a d-value >0.8 were labelled as important (Ellis & Steyn, 2003; Lindeque *et al.*, 2018).

3.6.9.2.3 Volcano plots

After conducting the univariate analysis, volcano plots were created from the acquired p- and d-values to identify metabolites that meet the abovementioned requirements (cut-offs) (Terburgh *et al.*, 2019). It also allows one to see whether metabolite concentrations were markedly elevated or decreased in the anaesthetic groups compared to the control – which is not obvious from the p and d values alone. In the volcano plots, the x-axis consisted of the effect size raised to the third power (d^3), while the y-axis consisted of the negative log transformed p-value (Terburgh *et al.*, 2019). A feature was only regarded as significant if it was located above the visualized threshold of significance for both p- and- d-values.

3.6.10 Feature Identification

To identify features found to be significant in the GC-TOFMS analysis, the mass spectra were compared to the National Institute of Standards and Technology (NIST) mass spectral library and an in-house mass spectral library created by Reinecke *et al*. (2012). Features were required to contain at least one silicon (Si) element in its formula to be allowed. This was to ensure that no non-derivatized compounds or incorrect derivatives could be identified (Venter *et al*., 2018b). To ensure reliable identification, features were not only compared based on mass spectra, but the retention times or retention indexes were also compared to that of previously analysed standards and reference retention indexes acquired from the NIST20/2020/EPA/NIH RI database and assessed based on chromatographic principles (Zenkevich, 2013; Wei *et al*., 2014). Finally, the features that could be identified with a high confidence level (level one identity) were awarded with metabolite identities and were further discussed in the results (Schymanski *et al*. 2014).

For the LC-MS analysis, a level one identity could be awarded to all metabolites due to the targeted nature of multiple reaction monitoring (MRM) (Schymanski *et al*. 2014).

After the NMR spectral bin data were processed statistically, important bins were identified. These bins were, in turn, used to identify important metabolites using Bruker pH 7.0 spectral libraries of pure compounds. The metabolite identities were confirmed using both one-dimensional and two-dimensional information to achieve a level one identification (Schymanski *et al*., 2014).

CHAPTER 4: RESULTS AND DISCUSSION

4.1 Data quality and batch effect verification

Before the statistical analysis of the metabolomics data was done, the quality of the data was assessed for the different instruments. This was done to ensure that no unwanted experimental variance or within-batch effect was present that could affect the biological variance and interpretation (Xia *et al*., 2012). The QC samples (GC-TOFMS: QC1-7; LC-MS: QC1-5; NMR: QC1-3) and the experimental samples were compared using principal component analysis (PCA) to determine whether any within-batch drift took place and to detect any possible natural grouping in the data (Lindeque *et al*., 2015). The data used were already cleaned using an 80% zero filter and a 50% CV filter. The PCA plots are displayed in **Figure 4.1**.

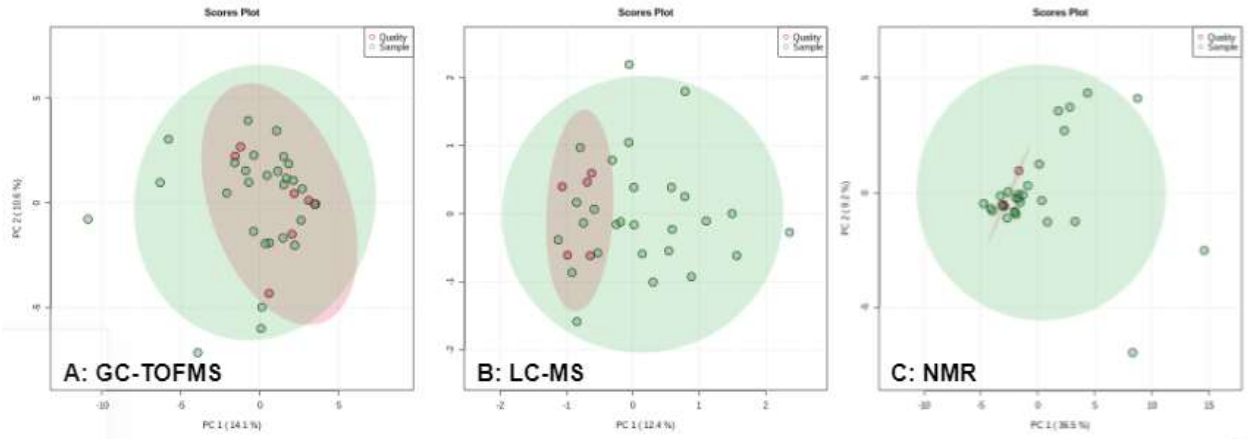

Figure 4.1: PCA score plots where the natural grouping of the QC samples and the experimental samples are qualitatively analysed for the three instruments that were used during this study.

The PCA plots indicate that the quality control samples (red), and the experimental samples (green) overlapped in the three datasets. Moreover, no obvious trend related to run order was detected which confirms that no significant within-batch drift occurred. The quality control samples did show closer grouping than the experimental samples but were still not tightly grouped in GC-TOFMS and LC-MS data. This can be attributed to the fact that the grouping of QC samples in PCA plots are relative to the amount of metabolome (total) variation in the experimental samples. If the experimental samples display low variation (as it was determined in later statistical methods), the QC samples will not group tightly even though it has acceptably low variance. Since no within-batch drift was detected in any of the platform-specific datasets, no correction methods had to be applied. Hence, the data were of appropriate quality for further analysis.

4.2 Overview of data before feature selection

After it was confirmed that the data from the different platforms were of acceptable quality, it was re-analysed with principal component analyses (PCA) but without the QC samples. PCA was done to qualitatively compare the control and different anaesthetics groups for any natural separation, and hence conspicuous metabolic differences. The three different anaesthetic groups and the control group were analysed and plotted together (displayed in **Figures 4.2-4.5**).

4.2.1 GC-TOFMS analysis

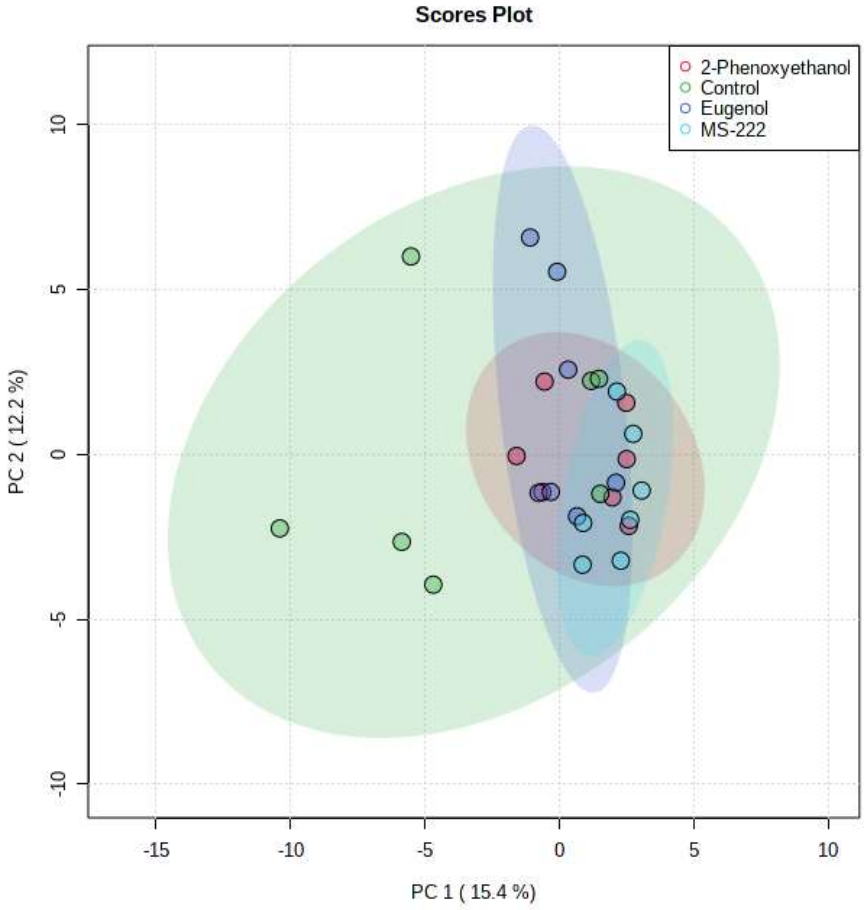

Figure 4.2: PCA score plot displaying the summarized variance of the GC-TOFMS data from the control samples and the three anaesthetic groups. The clustering of the different experimental groups give an indication of the natural grouping of the data.

The PCA score plot in **Figure 4.2** displays close clustering of the anaesthetic groups, which indicate that the studied metabolomes were relatively similar. The anaesthetic groups also had significant overlap with the control group which hint that the primary metabolism was not significantly perturbed. The control group had more within-group variation than the anaesthetic groups but there was still no definite separation between the control and anaesthetic groups.

Assessment of the PCA score plot thus did not indicate major variation between control and anaesthetic groups when evaluated visually.

4.2.2 LC-MS/MS analysis

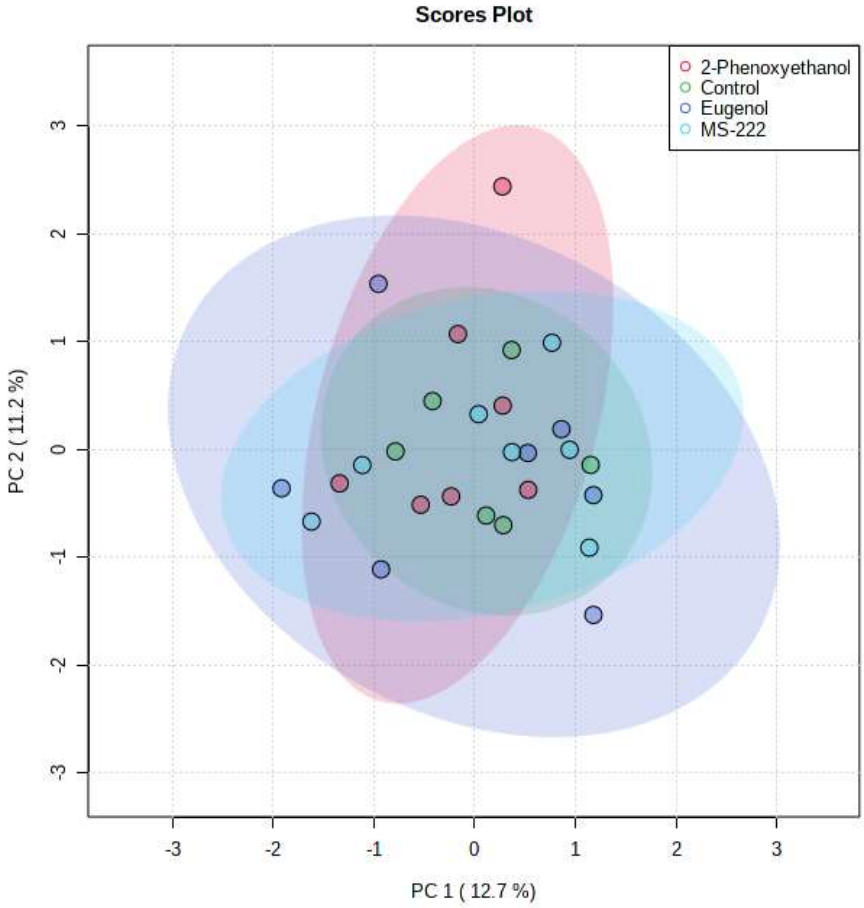

Figure 4.3: PCA score plot displaying the summarized variance of the LC-MS/MS data from the control samples and the three anaesthetic groups. The clustering of the different experimental groups give an indication of the natural grouping of the data.

The PCA score plot in **Figure 4.3** displayed no separation between the control and any of the anaesthetic groups, similar to the PCA score plot of the GC data. This visual examination thus did not indicate any significant variation in the concentrations of the targeted amino acids and acylcarnitines. Interestingly, the variance of the control group is smaller compared to variance seen in the GC-TOFMS and NMR bin data.

4.2.3 NMR analysis

Identification and proper integration of NMR spectral data is a cumbersome exercise. It is therefore common practice in metabolomics to simply align and bin the spectra to screen the data for peaks (bins) that significantly differ between the experimental groups; after which only the peaks of interest are properly integrated and quantified. The statistical significance of the re-integrated/ quantified peaks is then verified. The NMR data is thus analysed twice. The PCA score plot of the NMR spectral bin data is displayed in **Figure 4.4**.

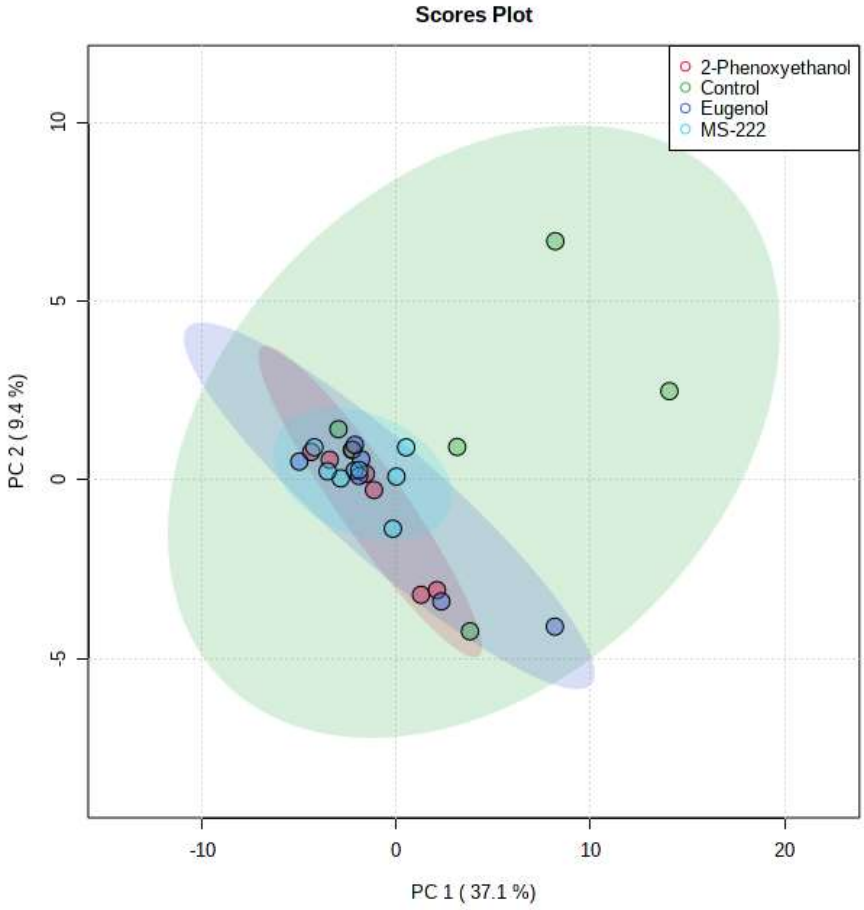

Figure 4.4: PCA score plot displaying the summarized variance of the NMR spectral bin data from the control samples and the three anaesthetic groups. The clustering of the different experimental groups gives an indication of the natural grouping of the data.

The PCA score plot of NMR spectral bin data displayed closer grouping between the anaesthetic samples than the control samples. This correlates with the observation from the GC-TOFMS and LC-MS/MS analyses. The NMR spectral bin data also displayed no separation between control and anaesthetic groups, which indicates that the anaesthetics did not have a widespread effect

on zebrafish metabolism, especially the highly abundant metabolites commonly screened with this platform.

After VIP bins were identified and quantified properly, it was subjected to the same statistical analysis. A PCA score plot (**Figure 4.5**) was generated to get an overview of the processed NMR data.

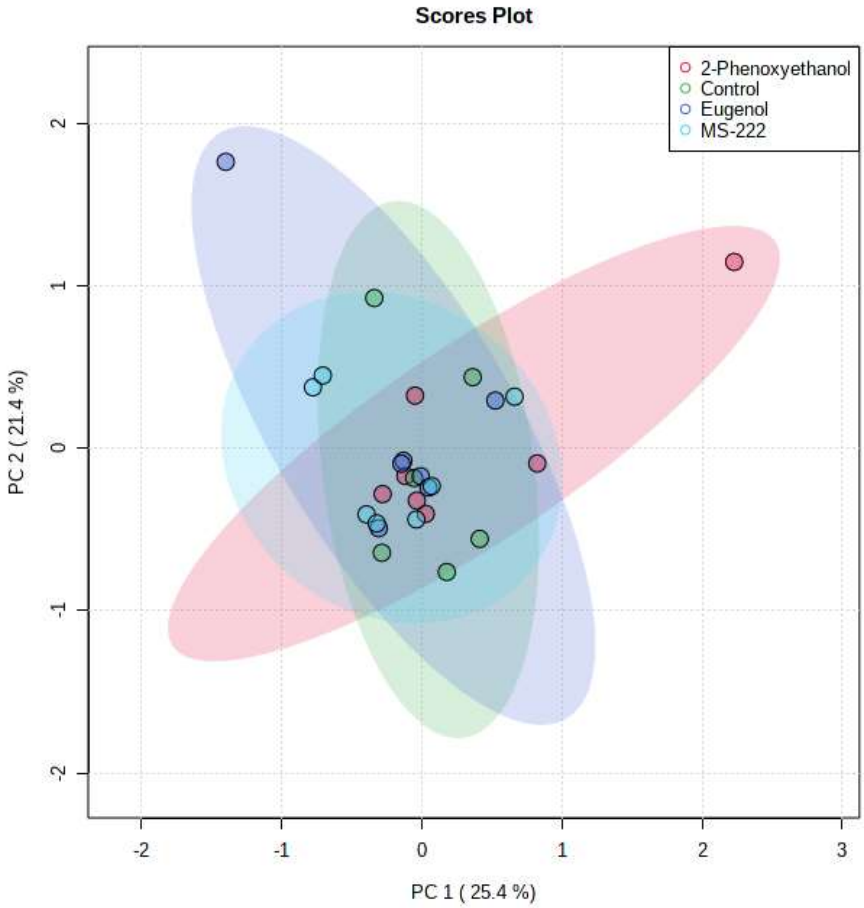

Figure 4.5: PCA score plot displaying the summarized variance of the quantified NMR data from the control samples and the three anaesthetic groups. The clustering of the different experimental groups give an indication of the natural grouping of the data.

The quantified NMR data consisting of the concentrations of important metabolites did not display natural grouping when analysed using a PCA score plot (**Figure 4.5**). The PCA score plot thus indicated that there was no momentous shift in zebrafish metabolism due to the application of anaesthetics.

Since none of the PCA plots displayed separation between experimental groups, it was decided to not use PLS-DA for feature selection due to its tendency to overfit data that has no natural separation (Kelly *et al*., 2018).

4.3 Feature selection (Univariate statistical analysis)

An important step in metabolomic workflows is to identify biologically important features that display significant variation between experimental groups. Initially, FDR-corrected p-values were used to assess whether anaesthetics had a significant effect on zebrafish metabolism. The FDR-corrected p-values yielded only three significant features over three platforms (see Annexure B), which was an additional indication that anaesthetics did not have a significant effect on zebrafish metabolism. Although minimal metabolic changes were recorded using FDR-corrected p-values, it is expected that the short induction times of the anaesthetics may limit any effect of anaesthesia on metabolism. Because of the nature of this study, which was more hypothesis-generating than hypothesis-testing, it was thus decided to make the feature selection process slightly less stringent by using non-FDR corrected p-values. This was done to identify subtle metabolic alterations, which could, in turn, assist in generating hypotheses regarding the aim of this study.

After the initial univariate analyses, the data from all three of the instruments were combined. Student's t-tests (non-FDR corrected p-values) and effect sizes (d-values) were then utilized in the form of volcano plots to identify significant features. The relationship between d-values and p-values are displayed in separate volcano plots for the different anaesthetics (**Figure 4.6 [A-C]**). Positive values on the x-axis indicated that the treatment groups had elevated metabolite concentrations. Conversely, negative d-values meant that the treatment group had decreased metabolite concentrations (Terburgh *et al*., 2019). The threshold for significance was +/- 0,512 for the x-axis and 1,301 for the y-axis. These values were derived from the significance threshold for the original p-and-d values namely $p < 0,05$ and $d > 0,8$ respectively. Only metabolites that were located above the threshold values of both axes were selected as significant features (Lindeque *et al*., 2018).

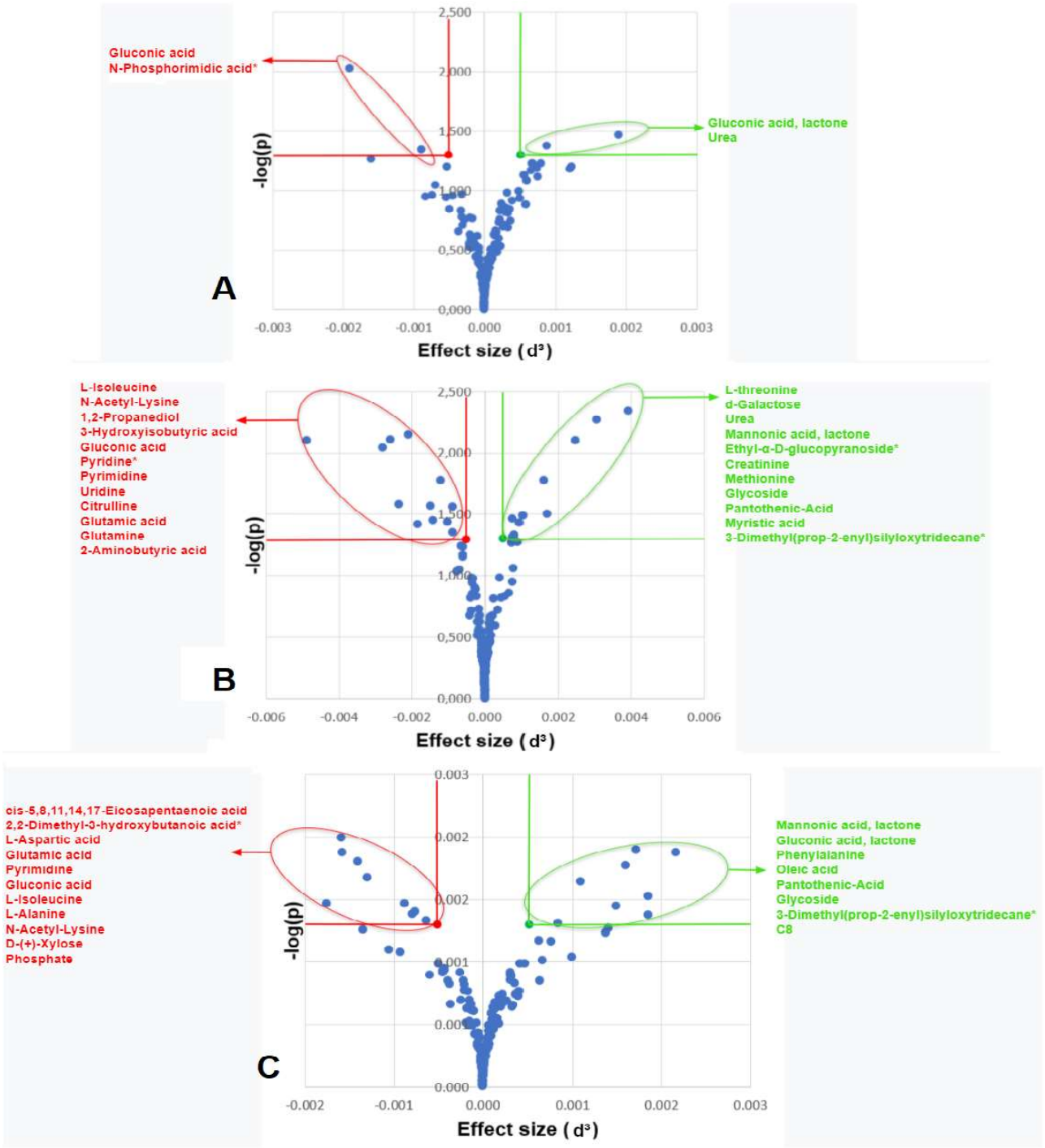

Figure 4.6: Volcano plots displaying multi-platform metabolomics results of A: Eugenol, B: MS-222 and C: 2-Phenoxyethanol treatments. The x-axis represents the effect size raised to the third power (d^3) and the y-axis represents the negative logarithm of the T-test p-values. The green and red lines indicate the threshold of significance for both axes while the green and red ellipses indicate metabolites that were significantly increased and decreased respectively. The features are listed in descending order of significance. Features marked with a (*) were not awarded a level one identity and were thus omitted from further discussions.

The features found to be significant according to the volcano plots are displayed in **Figures 4.6 (A-C)**. The concentration of only 4 features differed markedly in the zebrafish treated with eugenol, as shown in the volcano plot (A). In contrast, MS-222 and 2-phenoxyethanol affected the concentration of 23 and 17 features respectively as shown in the volcano plots (B & C). The significant features that could be identified with high confidence levels were awarded metabolite identities and added to **Table 4.1** for further discussion.

Table 4.1: List of significant metabolites

			Eugenol			MS-222			2-PE			
Class	**Metabolite**	**ID level**	**P-values**	**Abs. D-values**	**Direction**	**P-values**	**Abs. D-values**	**Direction**	**P-values**	**Abs. D-values**	**Direction**	**Instrument**
Acylcarnitine metabolism	Octanoylcarnitine	1	0,184	0,53		0,25	0,53		0,049	0,94	↑	LC
Amino acid metabolism	L-Alanine	1	0,666	0,22		0,97	0,02		0,034	0,96	↓	GC
	L-Aspartic acid	1	0,054	1,17		0,37	0,45		0,013	1,16	↓	GC
	Creatinine	1	0,107	0,69		0,03	1,02	↑	0,127	0,84		GC
	2-Aminobutyric acid	1	0,111	0,77		0,04	0,96	↓	0,642	0,21		GC
	Methionine	1	0,059	0,87		0,04	0,98	↑	0,238	0,51		GC
	Glutamic acid	1	0,299	0,56		0,04	1,01	↓	0,016	1,12	↓	GC
	Glutamine	1	0,197	0,67		0,03	0,96	↓	0,504	0,35		LC
	L-threonine	1	0,151	0,67		0,00	1,58	↑	0,205	0,58		GC
	N-Acetyl-Lysine	1	0,149	0,69		0,01	1,41	↓	0,041	0,93	↓	GC
	Pantothenic acid	1	0,237	0,53		0,05	0,93	↑	0,017	1,17	↑	GC
	Phenylalanine	1	0,254	0,60		0,15	0,77		0,042	1,23	↑	LC
	Citrulline	1	0,090	0,89		0,02	1,06	↓	0,218	0,71		LC
	Urea	1	0,042	0,96	↑	0,01	1,35	↑	0,103	0,75		GC
	3-Hydroxyisobutyric acid	1	0,546	0,29		0,03	1,33	↓	0,820	0,11		GC
	L-Isoleucine	1	0,511	0,32		0,01	1,70	↓	0,083	0,98		GC
Carbohydrate metabolism and derivatives	d-Galactose	1	0,140	0,67		0,01	1,45	↑	0,103	0,78		GC
	D-(+)-Xylose	1	0,144	0,79		0,06	0,86		0,039	0,91	↓	GC
	Mannonic acid, lactone	1	0,065	1,06		0,03	1,19	↑	0,013	1,29	↑	GC
	Gluconic acid	1	0,010	1,24	↓	0,01	1,28	↓	0,021	1,09	↓	GC
	Gluconic acid, lactone	1	0,034	1,24	↑	0,19	0,71		0,030	1,23	↑	GC
	1,2-Propanediol	1	0,276	0,53		0,01	1,37	↓	0,301	0,53		GC

Table 4.1. (continued)

Energy metabolism	Phosphate	1	0,063	0,81	0,29	0,57		0,047	0,86	↓	GC
Lipid metabolism	Eicosapentaenoic acid	1	0,252	0,58	0,31	0,50		0,034	1,21	↓	GC
	Myristic acid	1	0,873	0,08	0,05	0,91	↑	0,570	0,33		GC
	Oleic acid	1	0,328	0,56	0,71	0,19		0,013	1,20	↑	GC
Other reactions	Glycoside	1	0,729	0,18	0,05	0,93	↑	0,035	1,14	↑	GC
Pyrimidine metabolism	Pyrimidine	1	0,643	0,23	0,03	1,14	↓	0,055	1,11		GC
	Uridine	1	0,999	0,00	0,04	1,12	↓	0,150	0,72		GC

*Only metabolites with significant p- and- d-values were awarded with a direction (↓↑).

The confidently identified metabolites that differed significantly between the control and respective anaesthesia groups (**Table 4.1**) were compared using a Venn diagram (**Figure 4.7**) to illustrate the metabolites mutually significant for two or more of the anaesthetic treatments. The goal of the Venn diagram was to display the relationship between the different anaesthetic treatment groups (Venter, 2013). All the metabolites that are displayed in the Venn diagram had to meet the requirement of having level one identities as well as significant p-values (<0,05) and d-values (>0,8). Those features that could not be confidently identified were excluded, hence the number of metabolites shown in **Figure 4.7** differs from the number detected in the volcano plot (e.g., four features were markedly affected by eugenol but only three were successfully identified and further discussed).

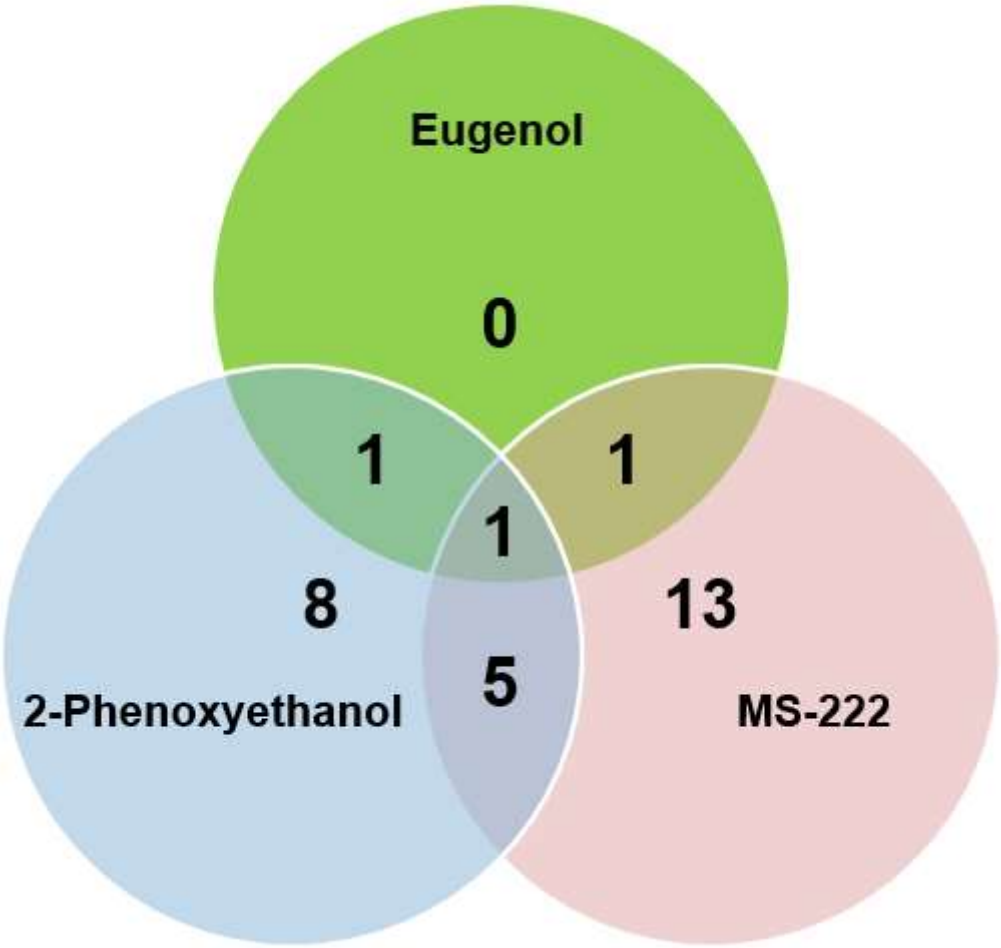

Figure 4.7: Venn diagrams of significant metabolites

The Venn diagram in **Figure 4.7** indicates the relationship between the different anaesthetics concerning the number of significant metabolites. MS-222 affected the concentration of twenty metabolites while 2-phenoxyethanol affected fifteen and eugenol only three. From this Venn

diagram it can be clearly determined that eugenol caused the least amount of disturbance to zebrafish metabolism.

Furthermore, the only metabolite that was significantly affected by all three anaesthetics was gluconic acid. Gluconic acid-lactone was the only metabolite that was shared between eugenol and MS-222 exclusively, while urea was the only metabolite shared between eugenol and 2-phenoxyethanol exclusively. The following metabolites were shared by MS-222 and 2-phenoxyethanol: glutamic acid, n-acetyl-lysine, pantothenic acid, mannonic acid-lactone and glycoside.

No metabolites were exclusive for eugenol. The following metabolites were significant only for MS-222: creatinine, 2-aminobutyric acid, methionine, glutamine, threonine, citrulline, 3-hydroxyisobutyric acid, isoleucine, galactose, 1,2-propanediol, myristic acid, pyrimidine, uridine. The following metabolites were significant for 2-phenoxyethanol exclusively: octanoylcarnitine, l-alanine, aspartic acid, phenylalanine, d-(+)-xylose, phosphate, eicosapentaenoic acid and oleic acid.

It is important to note that the concentrations of the metabolites that were significantly influenced by only one or two of the anaesthetics, could also have been altered by the remaining anaesthetics; however, these were simply not seen as such due to it not reaching the threshold of significance. The effect direction (increased/decreased) was mostly consistent over all three anaesthetics, with some exceptions (refer to **Table 4.1**).

4.4 Biological interpretation

The pathways potentially affected by the anaesthetics were assessed using the Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathway database. The relevant pathways were then added manually to a custom pathway map (**Figure 4.8**) that displays all the significant metabolites in an interconnected and holistic manner. The effect of the anaesthetics was mainly interpreted and discussed as one intervention (one group) especially as the PCA plots did not indicate marked differences in the total metabolism of the different anaesthetic groups. The basic mechanisms of the anaesthetics are also similar namely that ion channels are inhibited to diminish the cellular membrane potential and thus block action potentials. Consequently, an increase/decrease caused by any of the three anaesthetics are indicated as an arrow in **Figure 4.8** in an attempt to elucidate the general metabolic effect of anaesthetics. The pathways that contained significantly altered metabolite concentrations included: (A) glycolysis / gluconeogenesis; (B) alanine, aspartate and glutamate metabolism; (C) fatty acid ß-oxidation;

(D) cysteine and methionine metabolism; (E) D-glutamine and D-glutamate metabolism; (F) galactose metabolism; (G) glycine, serine and threonine metabolism; (H) lysine degradation; (I) pantothenate and CoA biosynthesis; (J) pentose and glucuronate interconversions; (K) pentose phosphate pathway; (L) phenylalanine metabolism; (M) propanoate metabolism; (N) pyrimidine metabolism; (O) urea cycle (P) branched chain amino acid metabolism; (Q) electron transport chain.

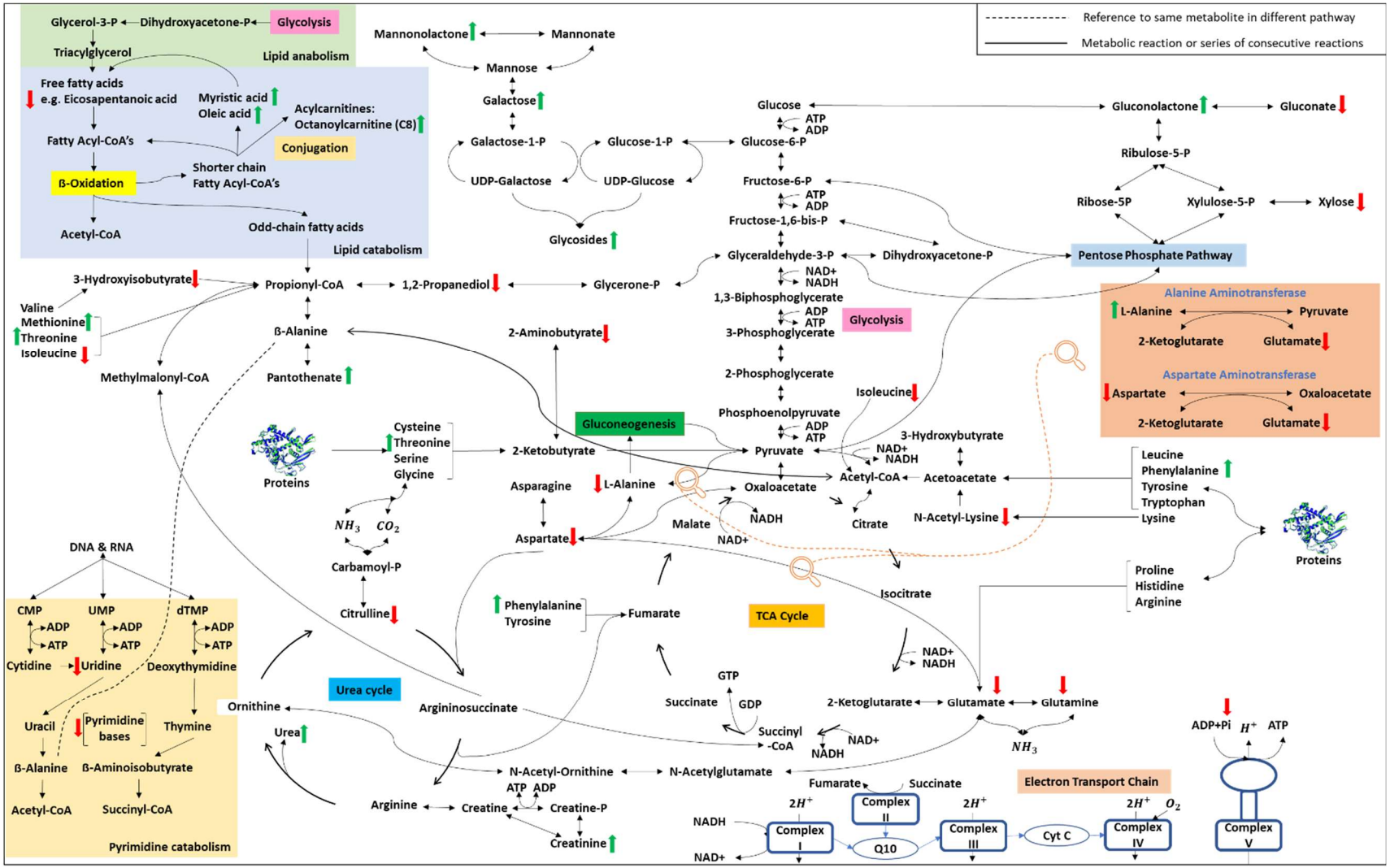

Figure 4.8: Metabolic map of *D. rerio* metabolic response during anaesthesia

Elevated metabolites found as a result of the three different anaesthetics (eugenol, MS-222 and 2-phenoxythanol) are indicated with green arrows and decreased metabolites with red arrows.

After a preliminary assessment of the metabolic pathways depicted in **Figure 4.8**, it was determined that zebrafish metabolism did not exhibit extensive pathway upregulation or downregulation due to a single anaesthetic. This supported the observations made from the PCA plots in **Figures 4.2-4.5**. Because only some metabolites were altered in most pathways, it was difficult to identify clear patterns and draw irrefutable conclusions on the general effect of the anaesthesia on metabolism. To generate a hypothesis in terms of the reason that some metabolites were elevated or decreased, information on the mechanisms of anaesthetics, regulation mechanisms, ion channels and an integrated manual pathway analysis were used.

4.4.1 Catabolic and anabolic pathways

When the metabolic response to anaesthesia in **Figure 4.8** is considered, it is important to note that catabolic processes appear to be more active than anabolic processes after the application of anaesthesia. This observation can be made due to the elevation of some ß-oxidation products (myristic acid, oleic acid and octanoylcarnitine) and the elevation of some essential amino acids (methionine, threonine, phenylalanine) and urea, which indicates active protein catabolism; albeit mainly in the fish that received MS-222 and 2-phenoxyethanol. None of the intermediates of glycolysis or the TCA cycle were significantly altered, indicating that these pathways remained relatively unaffected during anaesthesia. Alanine levels were, however, decreased in the 2-PE group, which may indicate increased gluconeogenesis (Kuo *et al*., 2015) resulting in elevated glucose levels as found in the literature and supported by this study (Priborsky & Velisek, 2018).

There are two possible explanations for the upregulated catabolism of lipids and proteins. The first is that ATP is depleted in an attempt to correct the lost membrane potential caused by the anaesthesia (Kishikawa *et al*., 2018). In summary, the suspected lower ATP/ADP or ATP/AMP ratio will upregulate catabolic pathways to alleviate the ATP shortage (Fu *et al*., 2018; Venter *et al.,* 2018a; Lehner & Quiroga, 2016; Towler & Hardie, 2007). The second explanation is that a stress response caused by the anaesthetics before the anaesthetic effect sets in, causes catabolic pathways to be upregulated (to increase energy levels in times of fight-or-flight) (Lynne, 2012; Topic Popovic *et al*., 2012; Gingerich & Drottar, 1989; Löhr & Hammerschmidt, 2011). These two possible explanations are closely related since the aim of the stress response is also to increase ATP production in times of stress (Fu *et al*., 2018). The effect that ATP levels and stress has on the upregulation of metabolism are explained in more detail below.

4.4.2 Effect of ATP levels on the upregulation of catabolism

Energy in the form of ATP plays a central role in metabolism, being a key factor needed to drive chemical reactions, transport molecules, activate transcription, provide energy for mechanical work etc. Because of the reliance of cells on ATP, the metabolic pathways leading to ATP production are tightly regulated (Berg *et al*., 2002; Sussman *et al*., 1980). To maintain a suitable ATP concentration, several key enzymes are regulated by the ATP concentration. In the case that ATP is depleted during anaesthesia, as proposed by Kishikawa *et al*. (2018), proteins, amino acids (Lindeque *et al*., 2018) and lipids are rapidly broken down to provide substrates for ATP production (Venter *et al*., 2018a; Lehner & Quiroga, 2016; Reinecke *et al*., 2012). To achieve this, several catabolic enzymes are activated in an attempt to accelerate ATP production. An important enzyme activated by a low ATP/AMP ratio is the AMP-activated protein kinase (AMPK) system, which in turn activates ATP-producing catabolic pathways like glycolysis, fatty acid oxidation and autophagy (Towler & Hardie, 2007). When activated, the AMPK system also inhibits anabolic processes that consume ATP like lipogenesis, protein synthesis etc. (Kim *et al*., 2016; Towler & Hardie, 2007; Lehner & Quiroga, 2016). The acceleration of fatty acid oxidation by AMPK is suspected to be the main reason that ß-oxidation products (myristic acid, oleic acid and octanoylcarnitine) are elevated while a substrate of ß-oxidation namely eicosapentaenoic acid is decreased. This effect was especially prevalent due to 2-PE anaesthesia and to a lesser degree MS-222. Eugenol did not display this effect.

AMPK also plays a key role in the regulation of creatine kinase (Palmer *et al*., 2008). When AMPK is activated, it up regulates creatine kinase activity, leading to the conversion of creatine phosphate to creatine and ATP (Palmer *et al*., 2008). Since creatine is spontaneously converted to creatinine, the elevated creatinine levels measured after MS-222 may be explained by the up regulation of creatine kinase (Feher, 2017). MS-222 appears to be the only anaesthetic causing this effect. In addition to the AMPK system, several more pathway-specific enzymes are regulated by the ATP levels, especially enzymes that are part of glycolysis and the TCA cycle (Berg *et al*., 2002; Sussman *et al*., 1980). Some of the enzymes reportedly activated by a low ATP/ADP ratio is phosphofructokinase (Passonneau & Lowry, 1964), pyruvate dehydrogenase, citrate synthase, isocitrate dehydrogenase, alpha-ketoglutarate dehydrogenase (Berg *et al*., 2002) and glutamate dehydrogenase (Xiao *et al*., 2016). The catabolic pathways and enzymes that were activated due to low ATP levels may help explain the increase or decrease of several significant metabolites. More details are provided below.

Firstly, glutamate dehydrogenase is responsible for the conversion of glutamate to alpha-ketoglutarate and is activated under low-ATP conditions to feed glutamate into the TCA cycle for energy production. The up regulation of glutamate dehydrogenase may explain why glutamate

and its precursor glutamine are decreased during anaesthesia, albeit not in the eugenol group (Xiao *et al*., 2016). Glutamine is also involved in the *de novo* synthesis of Arginine in the gut and kidneys, with citrulline as an intermediate of this pathway (Curis *et al*., 2005; Rabier & Kamoun, 1995). When glutamine levels decrease due to conversion to TCA cycle intermediates for energy, less glutamine is available as substrate for *de novo* arginine synthesis. This may cause a decrease in citrulline in the gut and kidneys, the gut being the main source of circulating citrulline (Curis *et al*., 2005). Whole-body citrulline concentrations are also influenced by the urea cycle (liver) and the nitric oxide cycle, but to a lesser degree (Curis *et al*., 2005; Wu, 1998). The reason that an accelerated urea cycle (as was the case in this study) did not significantly cause an increase in whole-body citrulline levels is because almost all hepatic citrulline is converted to argininosuccinate immediately and no citrulline is released into general circulation from hepatocytes (Curis *et al*., 2005).

As previously mentioned, the amino acid concentrations were expected to be elevated in the anaesthetic group due to increased protein catabolism (Reinecke *et al*., 2012). However, the amino acid profile gave mixed results with some amino acids elevated (methionine, threonine, phenylalanine) and other decreased (isoleucine, citrulline, aspartate, glutamate, glutamine, alanine). Most of the alterations were confined to glucogenic amino acids and amino acids that are both glucogenic and ketogenic. The purely ketogenic amino acids namely lycine and leucine were not significantly altered. Some products or derivatives of amino acids were also altered namely 3-hydroxyisobutyrate (an intermediate of valine catabolism) and N-acetyl-lysine (a lysine derivative released from proteins) were both decreased. Although amino acids and their derivatives are involved in numerous different reactions, which complicates the biological interpretation, it is proposed that the mixed results are due to a complex equilibrium between amino acid release by protein catabolism and accelerated amino acid breakdown for energy production (Lindeque *et al*., 2018). Glucogenic amino acids may also be involved in gluconeogenesis (refer to section 4.4.3). Alterations in amino acid concentrations are limited to the MS-222 and 2-PE groups.

The metabolomic analysis performed in this study determined that different sugar derivatives were elevated in the anaesthetic group. These metabolites include mannonolactone (a derivative of mannose) and gluconolactone (a derivative of glucose). There are various possible factors which may help explain an increase in these metabolites, one being that elevated glucose can cause an increase in these derivatives (see Section 4.4.3). A possible explanation is linked to the effect of ATP depletion on hexokinase activity. Hexokinase is an ATP-dependant enzyme responsible for the phosphorylation of both mannose and glucose (Heise & Abel, 2005). If ATP levels are

severely diminished, as suspected in this case, this reaction may be impaired. Excessive glucose and mannose may then be converted to gluconolactone and mannonolactone respectively.

In this study, uridine and a metabolite identified as “pyrimidine” (suspected to represent a pyrimidine base namely uracil or thymine) were decreased in the MS-222 group. The reason for this decrease is difficult to determine because uridine and pyrimidines are involved in various pathways which includes pyrimidine synthesis, nucleoside salvage, pyrimidine catabolism etc. (Zhang *et al*., 2020). Nucleoside salvage and *de novo* nucleic acid synthesis are energy intensive pathways, making use of ATP in several reactions within the pathways. Lower ATP levels can hinder nucleoside salvage and nucleic acid synthesis and cause pyrimidine and uridine to be catabolised instead. Catabolism of these metabolites can also produce acetyl-CoA and succinyl-CoA, a process which may be up regulated to alleviate the ATP shortage experienced during anaesthesia (Zhang *et al*., 2020). Additionally, uridine and uracil catabolism (and propionyl-CoA from fatty acid oxidation) leads to the formation of ß-alanine, which is converted to pantothenate, a metabolite significantly increased in this study. The increase in pantothenate can thus be explained by a combination of uridine/uracil catabolism and increased fatty acid oxidation as explained earlier. A decrease in uridine may also cause a decrease in uridine diphosphate (UDP) because uridine is a precursor for UDP (Zhang *et al*., 2020). Because UDP is needed for glycogen synthesis and galactose breakdown, this may contribute to the elevated galactose and its derivative mannonolactone as was determined in this study (Thoden *et al*., 1997).

One seemingly unexpected aspect of the metabolic profile in **Figure 4.8** is that glycolysis and TCA cycle intermediates are unaffected, although glycolysis is reported to be up regulated by low ATP levels. Up regulated glycolysis is expected to result in lower glucose which was not observed. An explanation for this lies in the stress response, which up regulates glucose production (Topic Popovic *et al*., 2012; Lynne, 2012). Since the systemic metabolome were studied and not organ specific metabolism, detected glucose levels likely reflected gluconeogenesis. The extra glucose that results from the stress response can thus be used for glycolysis and prevent the glucose depletion associated with increased glycolysis. The stress response experienced by the zebrafish during anaesthesia application is described in Section 4.4.3.

4.4.3 Effect of the stress response on upregulation of catabolism

As described earlier, zebrafish experience a stress response after the application of anaesthetics and before stage four anaesthesia is reached (Lynne, 2012). This stress response causes the rapid release of stress hormones named catecholamines, which includes adrenaline and

noradrenaline (Topic Popovic *et al*., 2012). Elevated levels of catecholamines causes several physiological changes like increased blood flow, ventilation rate etc., but importantly for this study it is reported to also cause an increase in plasma glucose (Topic Popovic *et al*., 2012; Lynne, 2012). The increase in plasma glucose is a result of catecholamines that up regulate glycogenolysis and gluconeogenesis (Section 2.5) (Wright *et al*., 1989; Kuo *et al*., 2015). An up regulation of gluconeogenesis may also help explain the decreased alanine concentrations since alanine is a major precursor of gluconeogenesis (Kuo *et al*., 2015). Although glucose did not reach the threshold of significance in the statistical analysis, it appeared to be slightly increased for all three anaesthetics in both the GC-MS and NMR analysis. An increase in plasma glucose may have occurred, but the use of whole-fish samples may have obscured the data to some extent due to differential tissue-specific responses. Other sugars and their derivatives were, however, significantly elevated, which may be partly explained by an increase in plasma glucose that shunt into the respective carbohydrate pathways (Bierenstiel & Schlaf, 2004). The sugars and their derivatives that were elevated are gluconolactone (a glucose derivative), galactose and mannonolactone (which is a derivative of galactose and mannose).

Another common effect of stress reported by Fu *et al*. (2018), is that protein catabolism is up regulated to release amino acids for energy production. During stressed conditions and the energy crisis associated with it, the activity of transaminases and especially alanine aminotransferase (ALT) are increased in an attempt to replenish the TCA cycle with transamination products (Watford, 2000). This causes increased conversion of alanine to pyruvate, which may further explain why alanine is decreased in this study (Watford, 2000). Aspartate aminotransferase also catalyses the conversion of glucogenic amino acids to alpha-ketoglutarate by transamination, which may help explain the decrease in glutamate and glutamine that was experienced in the MS-222 and 2-PE groups in this study (Venter *et al.,* 2018a; Velisek *et al*., 2011; Lepic *et al*., 2014).

4.4.4 Effect of anaesthetics on other pathways and reactions

When the metabolic pathways in **Figure 4.8** were analysed to generate a hypothesis as to why the involved metabolites were increased or decreased, most metabolites could be explained by either the effect of low ATP levels or the stress response. There were, however, significantly elevated or decreased compounds that are the result of other effects and reactions that are not fully elucidated. One example of such a metabolite is glycoside, which was elevated in the anaesthetic groups of 2-phenoxyethanol and MS-222. “Glycoside” is a very general term which refers to many combinations of sugars and aglycones. They are reported to be formed from UDP-

sugars like UDP-glucose and UDP-galactose via various reactions (De Bruyn *et al*., 2015). It can also be regarded as breakdown products of glycosylated proteins and peptides (like transferrin).

Other significant metabolites that could not be explained by the up regulation of catabolism are 1,2-propanediol (elevated in MS-222 group), xylose (decreased in 2-PE group), gluconate (decreased in all three groups) and inorganic phosphate (decreased in 2-PE group). In the case of gluconate, it is known that it exists in a complex equilibrium with gluconolactone (which was elevated in this study). It is possible that the anaesthetics directly or indirectly causes a shift in the equilibrium in the direction of gluconolactone production, but the mechanism is unknown. Xylose may be converted to xylulose-5-phosphate and metabolised in the pentose phosphate pathway, but the mechanism of regulation due to anaesthesia is also unknown for this reaction. Although anaesthesia was expected to cause a decrease in inorganic phosphate as reported in the review article by Priborsky & Velisek (2018), and determined in this study, the exact reason for this effect could not be explained due to the vast number of reactions it is involved in. All the metabolites listed above, which are involved in unknown mechanisms, are part of secondary metabolic pathways and are not expected to have an effect on the suitability of zebrafish as research models.

In addition to the metabolic pathways that were studied, the redox state (NADH:NAD ratio) was also investigated in the form of the lactate:pyruvate (LP) ratio, which is a ratio that is commonly used as an indicator of the redox state (White & Schenk, 2012) (refer to Section 2.7). The LP ratios of the control and anaesthetic groups were compared using the existing data matrix where the three analytical instrument's data were combined. (**Figure 4.9.**).

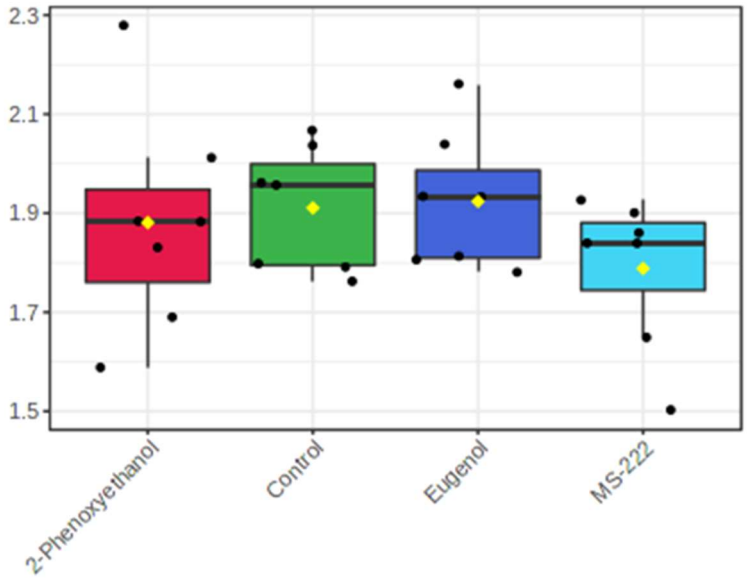

Figure 4.9: Boxplot of the lactate:pyruvate ratios as determined for the control group and anaesthetic groups

No statistical difference was found between the experimental groups. Although not significant, MS-222 treatment resulted in a slightly lower LP ratio than the other anaesthetics.

CHAPTER 5: FINAL CONCLUSIONS AND FUTURE PROSPECTS

5.1 Final conclusions

The effect of anaesthetics on zebrafish metabolism was successfully elucidated in this study. In summary, the treatment of zebrafish with anaesthetics had minimal effects on their metabolism. Only a small number of metabolites were significantly altered, and no large-scale perturbations were detected in primary metabolic pathways. The anaesthetics are thus not expected to have a significant effect on the suitability of zebrafish as research models. The effect on zebrafish metabolism is, however, expected to be limited by the short induction times of the anaesthetics before sampling (60-80 seconds). The metabolites that were altered are hypothesized to be a result of ATP depletion and a stress response. Both an ATP depletion and the stress response appear to up regulate protein and lipid catabolism. Glycolysis is, however, differently affected by these factors, being up regulated by ATP depletion and down regulated by the stress response. Except for some glycolysis derivatives, the opposing effects of these factors on glycolysis seem to neutralise each other as indicated by the relatively unaffected glycolysis intermediates. It was determined that MS-222 had the most significant effect with twenty significantly altered metabolites. 2-Phenoxyethanol had the second largest effect with fifteen significantly altered metabolites. Eugenol had a very small effect with only three altered metabolites. This leads to the recommendation that eugenol should be used as an alternative anaesthetic in studies where zebrafish are used to study metabolism. The final choice of anaesthetic should, however, consider the availability, regulations, cost and safety aspects of the anaesthetic. Follow-up studies are needed to test the hypotheses considering the suspected ATP depletion, stress response and the direct and indirect metabolic consequences associated with it.

5.2 Critical evaluation and future prospects

Some limitations were experienced in this study, which must be addressed in follow-up studies. The first limitation is that this study was mainly untargeted, making it a hypothesis generating study. This approach has the limitation that it is not well suited to test specific effects and hypotheses like the redox state, possible ATP depletion, stress response etc. Future studies have the advantage that it can target specific metabolites to confirm or disprove existing hypotheses. Although not a limitation, it would also be favourable for follow-up studies to perform tissue-specific instead of systemic screening. Lastly, it remains difficult to separate the effect of anaesthesia and the stress response which will always be a confounder in metabolic studies. Despite these limitations, the study was still successful because it managed to generate hypotheses as to the effect of anaesthetics on the metabolism of zebrafish. The possible effects identified also correlated strongly with effects described in the literature, albeit with different research aims and objectives.

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ANNEXURE A

Extraction methods optimization: methods

Before starting the analysis, various steps in the extraction protocols had to be optimized to identify the methods that provided the best possible metabolome coverage and the lowest amount of inter-sample variation. The extraction steps that were optimized includes the homogenization methods, the ratio of extraction solvents added before homogenisation and the extraction methods. For the homogenization methods, a Potter-Elvehjem homogenization and a vibration mill homogenization method was compared. For ratio of extraction solvents, the most suitable ratio of methanol and water added to the sample before homogenization had to be selected. The ratios that were compared were 1mg sample:4uL methanol:2uL water vs. 1mg sample:2uL methanol:1uL water. Additionally, three possible extraction methods namely single-phase, two-phase and Fiehn extractions had to be compared to choose the most suitable option. The main factors that played a role in the choice of extraction method was reproducibility and amount of analytes extracted.

To conduct the above-mentioned optimization steps, three consecutive experiments were done, each with a different variable. Firstly, two different ratios of sample:methanol:water were added to tissue samples, followed by vibration mill homogenization, a standard two-phase extraction method, derivatization and a GC-TOFMS analysis. In the second experiment, the best-performing solvent ratio of the previous experiment was used, followed by the two different homogenization methods respectively, a two-phase extraction method, derivatization and GC-TOFMS analysis. In the third experiment, the best performing solvent ratio and homogenization methods were used, followed by the three possible extraction methods, derivatization and a GC-TOFMS analysis.

For the first experiment, three whole-fish samples were divided in two equal halves (from head to tailfin) and weighed. Thus, the experiment consisted of two groups, each containing three halve zebrafish. For the first group, the halves were crudely minced using a razor blade, where after 4uL methanol and 2uL water were added for each mg of tissue. For the second group, 2uL methanol and 1uL water were added for each mg of tissue after crudely mincing the tissue. Along with these solvents, both a 5- and 7 mm stainless steel bead was added to each tube. A Retch M400 vibration mill was then used to homogenize the mixture for 10 min at 30 Hz. The homogenate was separated from the beads, where after the contents were transferred to a clean tube and centrifuged at 3000 ×g for 10 min at 4 °C. 100uL of the supernatant from the homogenate was then transferred to microcentrifuge tubes and subjected to a Two-Phase Bligh-Dyer extraction. An established protocol was used for the extraction. Additional water, methanol,

chloroform and internal standard (nonadecanoic acid, at a concentration of 50ppm) was added to each of the tubes in the ratios described in the established two-phase extraction protocol. The tubes were vortexed for 30 seconds and then incubated on ice for 10 min. To achieve phase-separation, the samples were centrifuged at 2000 ×g for 5 min at 4 °C. Consistent volumes of the polar and apolar phases were transferred to GC-vials, derivatized (BSTFA + % TMCS) and analysed on a GC-TOFMS. After data cleaning and processing, coefficient of variation (CV) values were determined for each analyte. When all the results were considered, it was decided that a 1:4:2 ratio of sample:methanol:water would be used due to the importance of reproducibility and the number of analytes extracted.

For the second experiment, the homogenization methods served as the variable. To commence the experiment, three whole body adult zebrafish were thawed to 2-4 °C and then split in halves. Each halve was weighed to calculate the volume of extraction solvents to be used. The halves from each sample were transferred to 15 mL centrifuge tubes and labelled as S1-3A and S1-3B respectively. Methanol and water were added to both samples in a ratio of 1mg sample:4uL methanol:1uL water. The first group (S1A-S3A) was then homogenized using a Potter Elvehjem homogenizer while the second group (S1B-S3B) was homogenized in the presence of 5 mm and 7 mm stainless steel beads using a vibration mill. The vibration mill operated at a frequency of 30Hz for 3 minutes. After homogenization the samples were centrifuged for 5 minutes at 3000 g and 4 °C and subjected to a standard two-phase extraction. Consistent volumes of the polar and apolar phases were transferred to GC-vials, derivatized (BSTFA + % TMCS) and analysed using GC-TOFMS

For the third experiment, two whole body adult zebrafish were thawed to 2-4 °C, crudely minced and pooled. The pooled samples were transferred to one 15 mL centrifuge tube where after methanol and water was added in a ratio of 1:4:2. The pooled sample was homogenized in the presence of a 5 mm and 7 mm stainless steel bead using the vibration mill for 3 minutes at 30 Hz. After homogenization, 100 µL of the homogenate were transferred to 9 separate microcentrifuge tubes. Of these 9 tubes, 3 where subjected to single-phase extraction, 3 were extracted using two-phase extraction and 3 where extracted using the Fiehn extraction method (acetonitrile:isopropanol:water). Standard protocols and solvent ratios were used for all the extraction methods. The extracts we transferred to GC-vials, derivatized (BSTFA + % TMCS) and analysed using GC-TOFMS.

Extraction method optimization: results

CV values were calculated for the two experimental groups in the first experiment where the ratio of extraction solvents were used as the variable. The CV values from the GC-TOFMS analysis are displayed in **Figure A1(a)** where the CV values were plotted against the percentage of metabolites. For each percentage value displayed on the graph, that specific percentage of metabolites detected have a smaller CV value than the corresponding CV value on the X-axis. A PCA plot was also generated to assist the comparison of the two solvent ratios (**Figure A1(b)**).

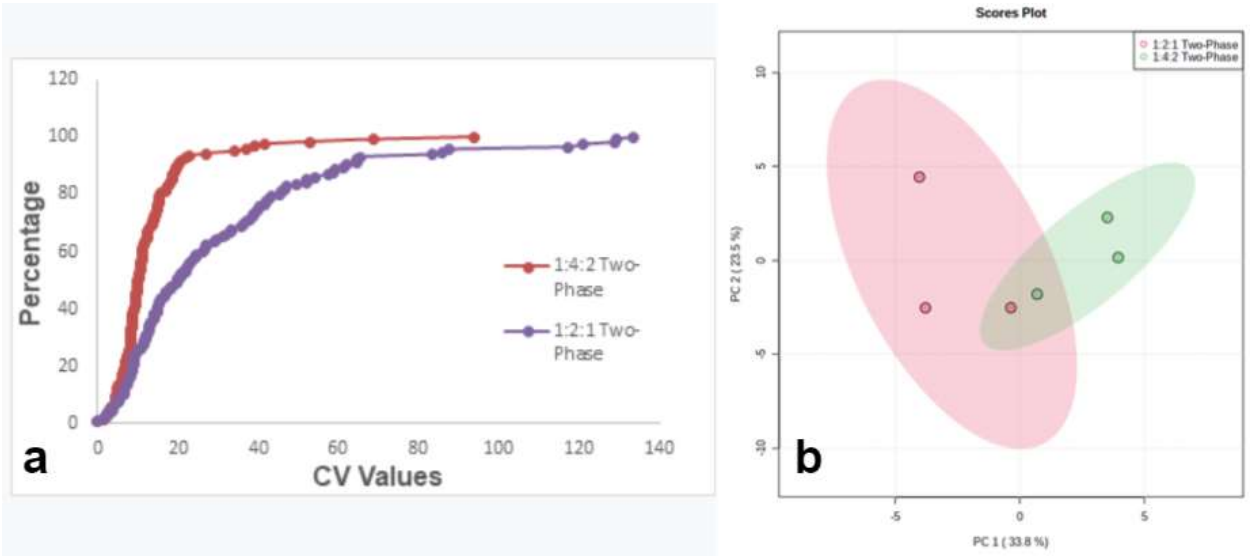

Figure A1: (a) CV value graph and (b) PCA plot where the two groups of different extraction solvent ratios are compared.

In this representation of the CV values, the curve of 1:4:2 extraction is shifted significantly to the left, indicating that a higher percentage of metabolites had a lower CV value for this solvent ratio. The lower CV values of this method indicates that it had the lowest amount of variance between the two extraction solvent ratios. The PCA plot supported this finding due to the closer grouping of the 1:4:2 group (displayed in green). In the 1:4:2 group, 122 analytes were detected in at least 2 of the 3 samples while only 115 analytes were detected in the 1:2:1 two-phase method. The higher number of analytes detected in the 1:4:2 methods may be due to the 1:4:2 ratio providing better metabolome coverage, but the exact reason is unknown. The lower CV values and high number of analytes detected using the 1:4:2 two-phase extraction makes it the preferred ratio for this study.

The same statistical analysis was conducted for the second experiment where the homogenization methods were compared. The CV values derived from this experiment's GC-TOFMS data was also displayed as a CV graph (**Figure A2(a)**). A PCA plot was also generated

to help compare the two homogenization methods (**Figure A2(b)**). In the PCA plot, the vibration mill group is displayed in red while the Potter Elvehjem group is displayed in green.

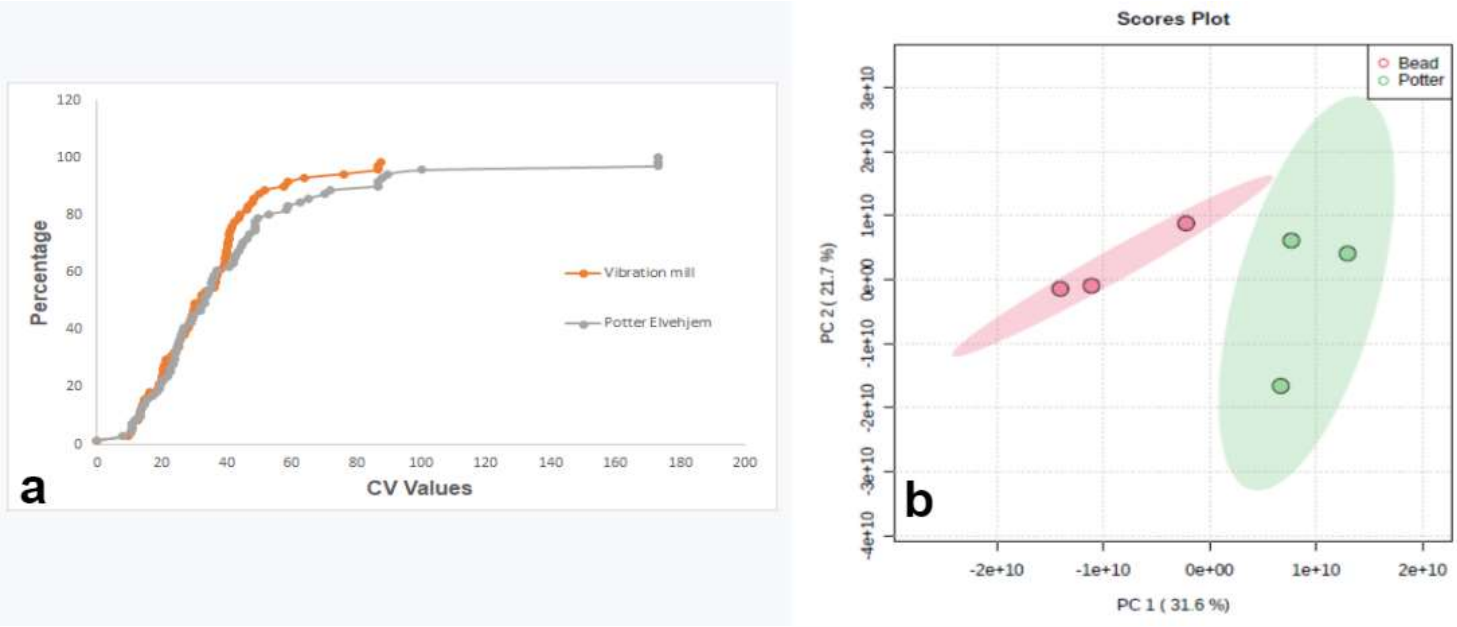

Figure A2: (a) CV value graph and (b) PCA plot where the two groups of different homogenization methods are compared.

In the CV graph displayed above, the curve of the vibration mill homogenization method is shifted slightly to the left, especially concerning features with CV values between 40% and 50%. This indicates that the vibration mill homogenization method yielded lower CV values for a higher percentage of metabolites. The lower CV values of this method indicates that it had the lowest amount of variance between the two homogenization methods. The PCA plot supported this finding due to the closer grouping of the vibration mill (bead beating) group (displayed in red). The amount of metabolites detected was roughly similar for the two groups. The choice was made to use the vibration mill method for the rest of the study due to the lower variance exhibited by the data from the vibration mill group.

For the third experiment, the data was treated in a similar fashion. Firstly, CV values were calculated, and a CV graph was constructed. Secondly, a PCA plot (**Figure A3(b)**) was generated for further comparison between the three extraction methods. The CV graph is displayed in **Figure A3(a)** where the grey data points represent the two-phase method, the green data points the single-phase method and the blue data points the Fiehn method. In the PCA plot, the blue dots represent the two-phase method, the green dots the single-phase method and the red dots the Fiehn method.

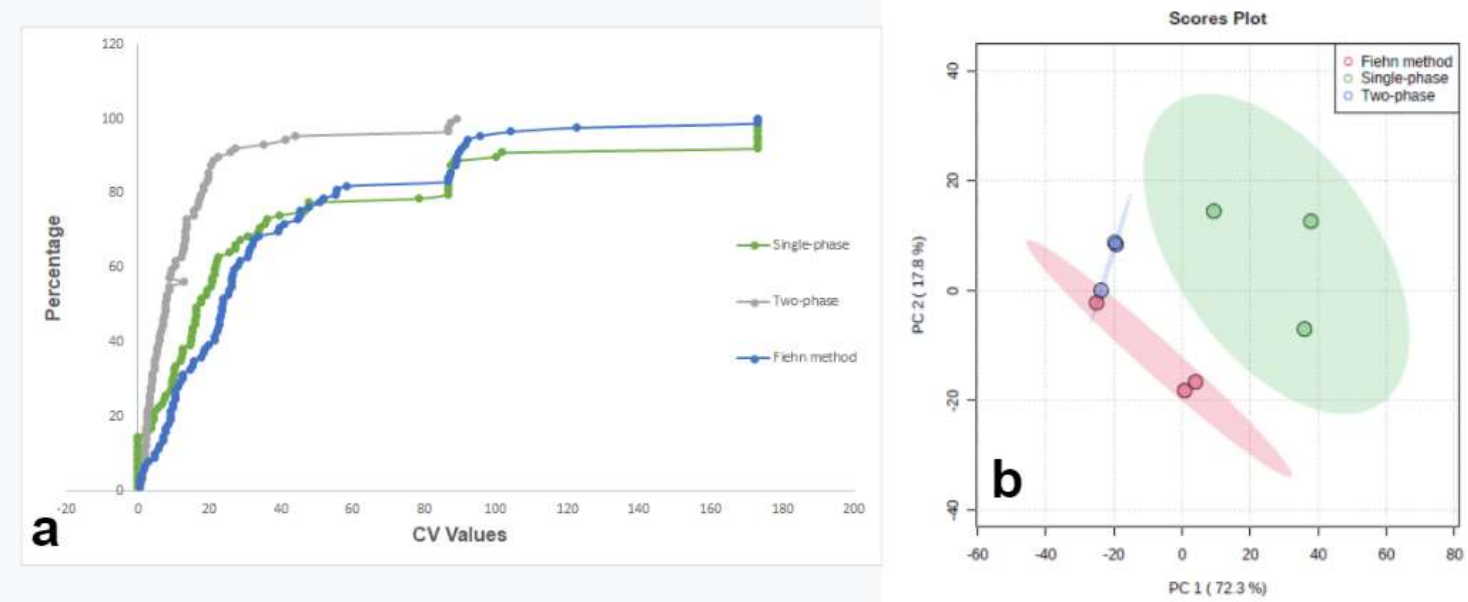

Figure A3: (a) CV value graph and (b) PCA plot where the three groups of different extraction methods are compared.

In the CV graph displayed in **Figure A3 (a)**, the curve of the two-phase extraction method is shifted significantly to the left of the other two extraction methods (displayed in grey). This indicates that a higher percentage of metabolites had a lower CV value for this extraction method. The two-phase method is followed by the single-phase method (green) and finally the Fiehn method (blue). The lower CV values of the two-phase method indicates that it had the lowest amount of variance between the three extraction methods. The PCA plot supported this finding due to the closer grouping of the two-phase group (displayed in blue) than the other two groups. In the two-phase group, an average of 86 analytes were detected in at least 2 of the 3 samples while only 66 analytes were detected in the single-phase group and 82 in the Fiehn group. The lower CV values and higher number of analytes extracted using the two-phase extraction makes it the preferred extraction method for this study.

After completion of the extraction method optimization experiments, it was decided that a solvent ratio of 1mg sample:4uL methanol:2uL water would be added before doing a vibration mill homogenization. The homogenate would be extracted using a two-phase method.

ANNEXURE B

Data overview: T-tests

As part of the univariate statistical analysis, Student's t-tests were performed for each platform to find metabolites that displayed significant variation between the control and anaesthetic groups. Metabolites with a false discovery rate (FDR) corrected p-value <0.05 were deemed to differ significantly between groups and were therefore labelled as important while metabolites with a p-value >0.05 were excluded from the data set since they did not contain statistical significance (Lindeque *et al*., 2018). The student's t-test displayed in **Figure B1-B4** indicated all the features that had an FDR corrected p-value lower than 0,05 and were thus deemed significant according to the GC-TOFMS, LC-MS and NMR analysis respectively.

GC-TOFMS analysis

The results of the student's t-test generated from the GC-TOFMS data is displayed in **Figure B1**.

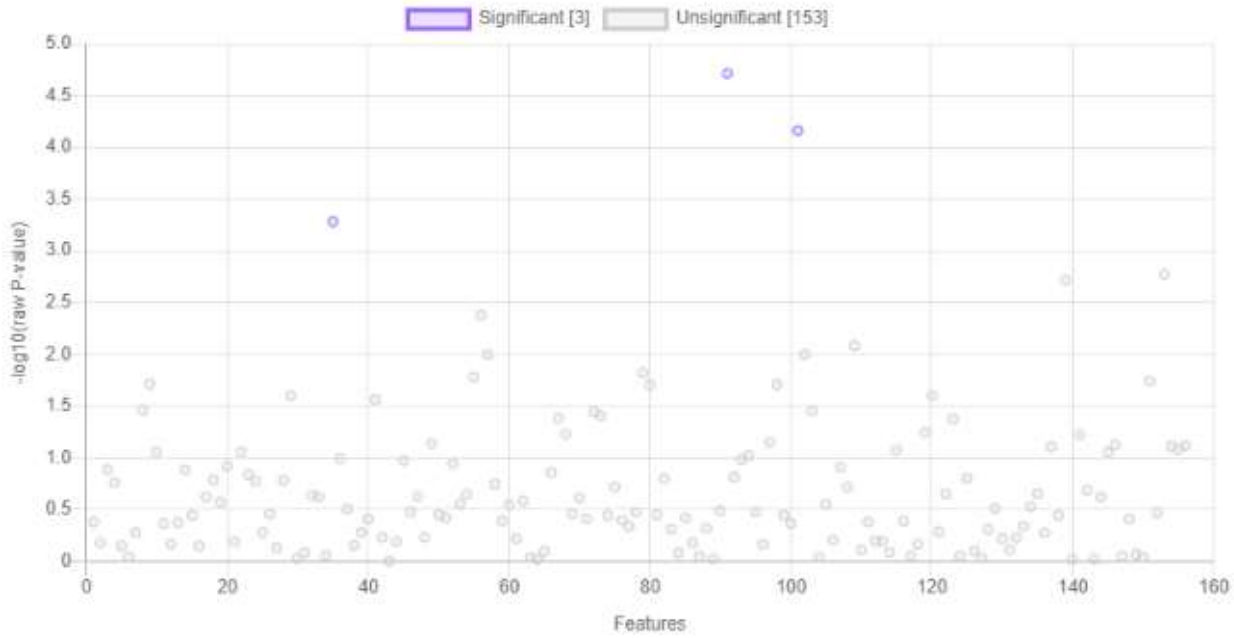

Figure B1: Student's t-test of the GC-TOFMS data (Control vs Treatment group).

Only three features were found to be significant by the T-test. The features found to be significant were added to the feature selection table (**Table 4.1**).

LC-MS analysis

A student's t-test was also done for the LC-MS analysis to give an overview of the data. The results of the Student's t-test are displayed in **Figure B2**.

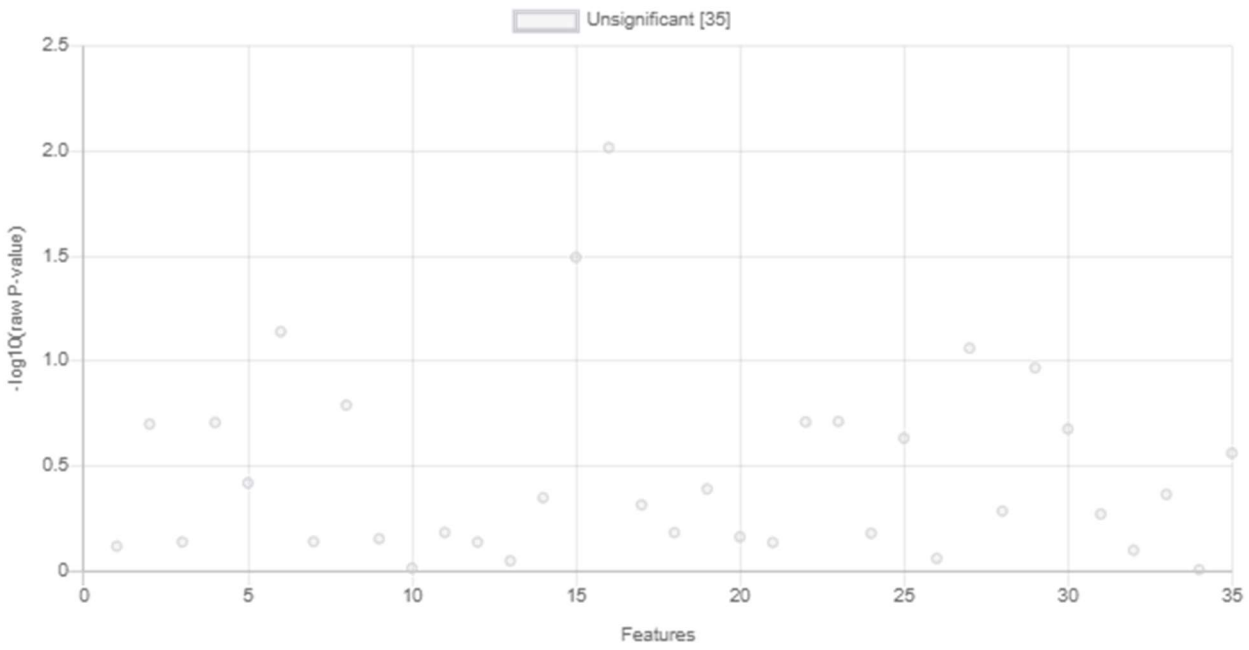

Figure B2: Student's t-test of the LC-MS data (Control vs Treatment group).

No features were found to be significant according to the student's t-test, which did the assessment based on FDR-corrected p-values.

NMR analysis

The NMR data underwent an extra round of statistical analysis because NMR data is generated as spectral bins first. These spectral bins had to be subjected to a statistical analysis to find VIP bins. The VIP bins were then identified, and the metabolites identified were subjected to a second statistical analysis. After the spectral bins were subjected to statistical analysis, a student's t-test was generated and is displayed in **Figure B3**. In this case, the spectral bin data was, however, not FDR corrected since this was only a preliminary statistical analysis.

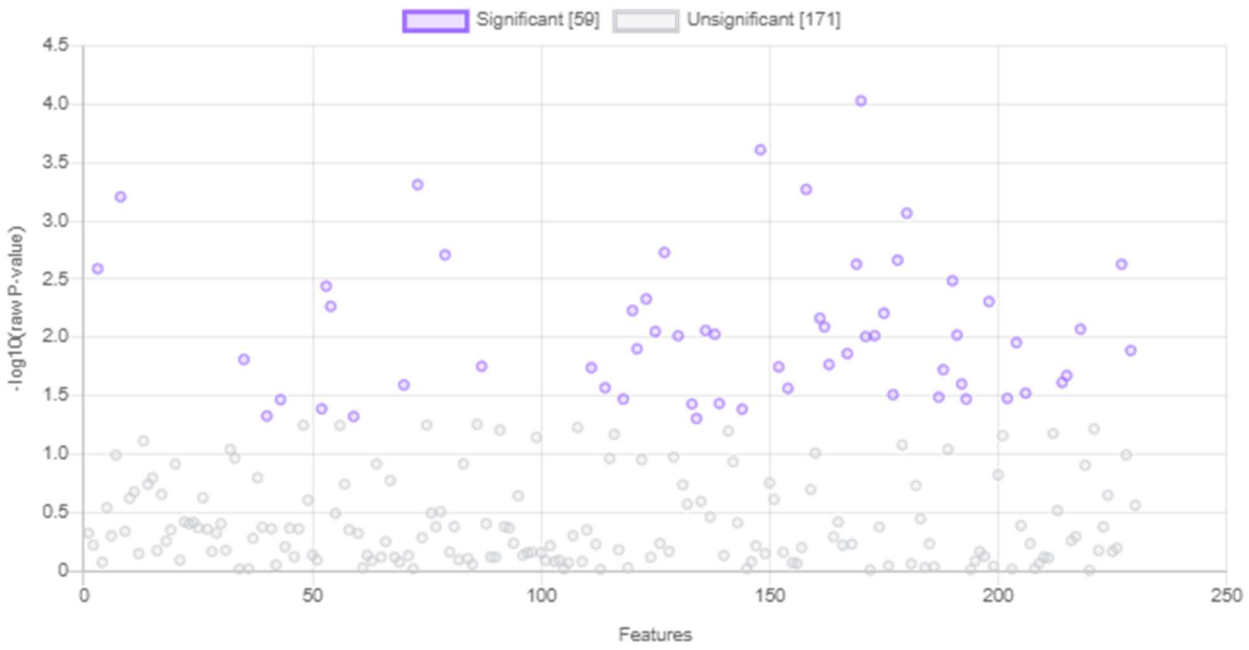

Figure B3: Student's t-test of the NMR spectral bin data (Control vs Treatment group).

The student's t-test of NMR spectral bins found 59 bins to be significant (non-FDR corrected). These bins were added to a list of VIP bins and were re-integrated, identified and quantified using Bruker pH 7.0 spectral libraries. The identified metabolites were subjected to another round of statistical analysis and the student's t-test generated from that analysis are displayed in **Figure B4**.

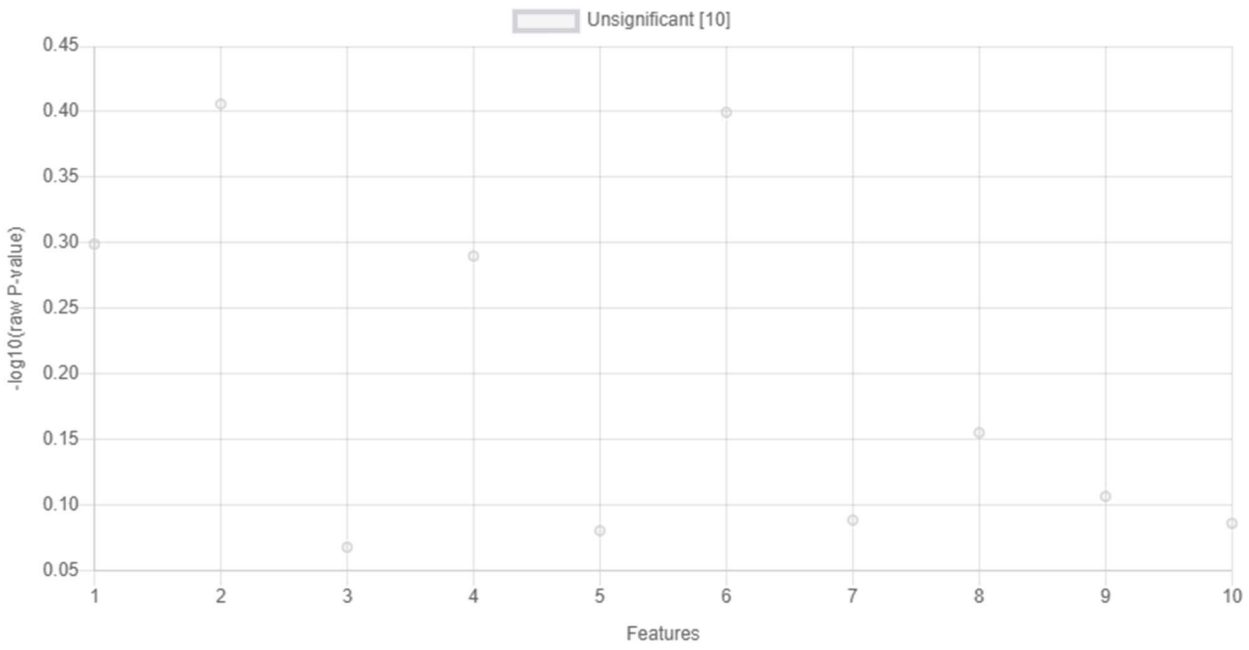

Figure B4: Student's t-test of the quantified NMR data (Control vs Treatment group).

As displayed in **Figure B4**, a t-test of the quantified NMR data found no metabolites that differed significantly between control and anaesthetic groups when FDR-correctd p-values were used to determine significance.

In the first round of univariate statistics, FDR-corrected p-values were used. The FDR-corrected p-values yielded only three significant features over three platforms, which was an additional indication that anaesthetics did not have a significant effect on zebrafish metabolism. Although minimal metabolic changes were recorded using FDR-corrected p-values, it is expected that the short induction times of the anaesthetics may limit any effect of anaesthesia on metabolism. Because of the nature of this study, which was more hypothesis-generating than hypothesis-testing, it was thus decided to make the feature selection process slightly less stringent by using non-FDR corrected p-values. This was done to identify very subtle metabolic alterations, which could, in turn, assist in generating hypotheses regarding the aim of this study.

ANNEXURE C

Table C.1: MRM transitions of butylated amino acids and acylcarnitine's and their respective isotopes.

	Compound Name	Precursor Ion	Product Ion	Dwell	Fragmentor	Collision Energy
Window 1	Cystine	297	151.9	60	89	12
	Citrulline IS	236.2	219.1	50	93	8
	Citrulline IS	236.2	105.1	60	85	16
	Arginine IS	235.2	74.3	60	100	40
	Citrulline	232.2	70.1	60	94	28
	Arginine	231.2	70.1	60	93	28
	C0 IS	221.2	103.1	60	110	20
	C0	218	103	60	110	16
	Histidine	212.1	110.1	60	99	16
	Lysine	203.2	84.1	60	94	0
	Glutamine	203.1	84.1	60	84	24
	Homocysteine	192.1	90.1	60	155	12
	Asparagine	189.1	70.1	60	89	20
	Cysteine	178.1	76	60	180	20
	Threonine	176	74.2	60	84	12
	Proline	172.1	70.2	60	80	20
	Serine	162	60.1	60	69	12
	Alanine	146.1	90.1	60	60	4
	Glycine IS	134.1	78.2	60	60	4
	Glycine IS	132.1	76.1	60	55	4
Window 2	C18 IS	487.5	85.1	45	160	36
	C18	484	85.1	45	155	36
	C16 IS	459.4	85.1	45	120	28
	C16	456.4	85.1	45	160	36
	C14 IS	431.4	84.9	45	150	44
	C14	428.4	85.1	45	150	28
	C12 IS	403.3	85.1	45	145	28
	C12	400.3	85	45	155	28

Table C1 (continued)

C10 IS	375.3	85.1	45	145	24
C10	372.3	85	45	135	28
C8 IS	347.2	85.2	45	105	24
C8	344.3	85.1	45	115	28
Cystathionine 2BE	335.2	190.1	45	97	13
C6	316	85.1	45	120	24
C5 IS	311.2	85	45	140	20
C5	302.2	85.1	45	105	24
Cystine	297	151.9	45	89	12
C4 IS	291.2	160.9	45	80	16
C4	288.2	85.1	45	110	20
C3 IS	277.2	148	45	100	12
Glutamic acid IS	265	248.1	45	100	8
C2 IS	263.2	246.1	45	95	4
Tryptophane	261.2	84.1	45	94	28
Glutamic acid 2BE	260.2	158	45	100	9
C2	260.2	85	45	100	20
Aspartic acid 2BE	246.2	144	45	100	9
Tyrosine	238.2	136.1	45	89	12
Phenylalanine IS	227.2	125.1	45	90	12
Phenylalanine	222.2	120.1	45	80	12
Methionine IS	209.1	107.2	45	80	8
Methionine	206.1	104.1	45	60	8
Isoleucine IS	198.2	96.2	45	80	12
Homocysteine	192.1	90.1	45	155	12
Isoleucine	188.2	86.2	45	80	8
Leucine	188.2	86.1	45	65	8
Valine IS	182.2	80.2	45	60	12
Cysteine	178.1	76	45	180	20
Valine	174.2	72.2	45	80	12
Proline	172.1	70.2	45	80	20

ANNEXURE D

The findings of this investigation were presented virtually at the 2nd Metabolomics South Africa Symposium; of which parts of the symposium brochure is shown as evidence of participation and scientific contribution.

2ND METABOLOMICS SOUTH AFRICA (MSA) SYMPOSIUM & ANNUAL GENERAL MEETING

Virtual Sessions

20-21 October 2021

MSA

METABOLOMICS SOUTH AFRICA

ACGT

AFRICAN CENTRE FOR GENE TECHNOLOGIES

BACKGROUND

Metabolomics South Africa (MSA) is a non-profit organization, affiliated to the International Metabolomics Society, that aims to promote and improve the profile of metabolomics research and technology in South Africa (and by extension in Africa), foster networking, training, capacity building, information sharing, mentoring, career opportunities, leadership training and professional development. The symposium will continue to build capacity for principal investigators, postgraduate and PhD students currently performing or planning to work in the metabolomics field. Additionally, the event is intended to foster further collaborations amongst metabolomics researchers within SA and across the globe by providing a platform for researchers to network and to identify synergies.

MEETING DETAILS AND LINKS

Day 1: Zoom Meeting ID: 953 2230 2020
Click Here

Day 2: Zoom Meeting ID: 915 8348 2299
Click Here

ORGANIZERS AND LEADERSHIP STRUCTURES

Committee of directors (CoD)

Dr Fidele Tugizimana (UJ & Omnia) – Chairperson – ftugizimana@uj.ac.za
Dr Aurelia Williams (NWU) – Deputy Chairperson - Aurelia.Williams@nwu.ac.za
Prof Duncan Cromarty (UP) – Deputy Chairperson - duncan.cromarty@up.ac.za
Prof Du Toit Loots (NWU) – Secretary - Dutoit.Loots@nwu.ac.za
Dr Wilma Augustyn (TUT) – Member - augustynw@tut.ac.za
Prof Gerhard Prinsloo (UNISA) – Member - prinsg@unisa.ac.za
Dr Gabriel Mashabela (SU) – Member - gabrielm@sun.ac.za
Ms Tracy Snyman (Wits) – Member - tracy.snyman@wits.ac.za
Mr. Daniel Mutithu (UCT) – Member - mtidan008@myuct.ac.za
Mr. Molati Nonyane (ACGT) – Member - molati.nonyane@up.ac.za

Communications subcommittee

Ms Kekeletso Chele (UJ) - Ckekeletso@gmail.com
Ms Anza-Tshilidzi Ramabulana (UJ) - ramabulanaanza@gmail.com
Mr Molati Nonyane (ACGT) - molati.nonyane@up.ac.za

Training and conferences subcommittee

Dr Aurelia Williams (NWU) - aurelia.williams@nwu.ac.za
Dr Lungile Sitole (UJ) - lsitole@uj.ac.za
Prof Gerhard Prinsloo (UNISA) - prinsg@unisa.ac.za
Dr Olayemi ELUFIOYE Taiwo (University of Ibadan, Nigeria) - toonitaiwo@yahoo.com
Dr Shayne Mason (NWU) - 21487855@nwu.ac.za
Dr Fidele Tugizimana (UJ & Omnia) - ftugizimana@uj.ac.za

PROGRAMME: DAY 2 - 21 OCTOBER 2021

	MORNING SESSION (DAY 2) CHAIR: DR AURELIA WILLIAMS	
09:00-09:05	Welcome	Dr Aurelia Williams North-West University Deputy Chairperson of MSA
09:05-10:05	Metabolomics – an indispensable tool in medicinal plant research	Prof A Viljoen Tshwane University of Technology
10:05-10:25	Asparagine content as potassium deficiency biomarker in soybean (Glycine max) leaves and pods	Gustavo dos Santos Cotrim São Paulo State University, Brazil
10:25-10:45	Investigating the effect of anaesthesia on the metabolism of zebrafish (D. rerio).	Marcél Burger North-West University
10:45-11:05	Mass spectral molecular networking to decode the chemical space of Bacillus strains	Ms. Lerato Nephali University of Johannesburg
11:05-11:20	TEA/COFFEE BREAK	
11:20-12:20	Molecular Networking	Dr F Tugizimana University of Johannesburg & Dr E Madala University of Venda
12:20-12:40	Metabolites levels correlate to tumour stage and survival in Pancreatic Ductal Adenocarcinoma patients	Nnenna Elebo University of Witwatersrand
12:40-13:00	Application of agricultural biotechnology as mean to attenuate the impact of synthetic pesticides – A mini-review	Nurmahomed, W University of South Africa
13:00-13:20	Metabolomic Analysis of Oat (Avena sativa L.) Plants: A Strategy for Cultivar Identification and Differentiation	Mrs Chanel Pretorius University of Johannesburg
13:20-14:00	LUNCH	

Investigating the effect of anaesthesia on the metabolism of zebrafish (D. rerio)

Authors: Marcél Burger, Zander Lindeque, Prof André Vosloo
Email: marcelburger1102@gmail.com

In recent years, zebrafish (D. rerio), have emerged as valuable research models, being widely used as models for aquaculture research, but also to model human metabolic diseases. The ethical guidelines, however, stipulate that anaesthetics needs to be applied before sampling of zebrafish. A possible limitation overlooked so far is the effect of the anaesthesia on their metabolic profiles and the implications that this may have for zebrafish as research models. In this study, multi-platform metabolomics were used to investigate the metabolic alterations that three commonly used anaesthetics namely MS-222, eugenol and 2-phenoxyethanol caused in zebrafish. The metabolomic analyses indicated that anaesthesia caused minimal metabolic alterations, with only a small number of altered metabolites. The relatively few metabolites that were altered are hypothesized to be caused by ATP depletion and a stress response, which both lead to the upregulation of catabolic processes. This effect may, however, be limited by the short induction times of the anaesthetics. It was also determined that eugenol exhibited significantly less metabolic perturbations than the other anaesthetics with only three significantly altered metabolites, making it the suggested anaesthetic for metabolomic studies using zebrafish. In conclusion, anaesthetics used during sampling are not expected to have a significant effect on the suitability of zebrafish as research models. Further investigations are needed to confirm the hypothesis related to the pathway-specific effects of the anaesthetics.