Development of a GC-MS/MS organic acid profiling method for abalone samples: challenges and future prospects

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PREFACE

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Lastly, to the Lord Almighty, thank you for carrying me through every challenge and for helping me rise even when I lost hope. Deep down, You knew how much completing this meant to me and Lord I thank you for making this possible.

ABSTRACT

Key words: **Abalone farming. Organic acids, Gas Chromatography, mass spectrometry, GC-MS/MS**

South African abalone (*H.midae*) locally known as Perlamoen in South Africa is a marine mollusc that is found on the South African shore-line. Due to overfishing and the high commercial value coupled to this marine mollusc, wild abalone stocks are on a decrease. Abalone farming plays and integral role in the aquaculture industry as it allows for additional resources to be produced, it also takes the pressure of wild abalone stocks.

Even though there are many research areas fulfilled in terms of the optimal farming and environmental factors for cultured abalone, there can still be additional factors on a biochemical level which could influence the period abalone takes to reach market size. Previous studies performed by our group, hypothesised that there is an absence of organic acids during hypoxia, therefore it can be hypothesised that abalone do not rely heavily on mitochondrial oxidative phosphorylation for energy production but on alternative energy pathways.

Metabolomics is an emerging field that allows for the use of multiple analytical platforms to detect and quantify small molecules such as organic acids. This study aimed to develop a GC-MS/MS organic acid profiling method for abalone samples. To test the best approach, two GC-MS/MS were developed, one used Chemical ionisation (CI) and the other Electron impact (EI). The CI MRM method provided reasonable linearity across the concentration range with most organic acids giving a $R^2 > 0.9$. However, certain organic acids such as Succinate, Malate, Lactate and Oxaloacetate show a decline in their curves, which could possibly be due to poor ionisation from CI, poor extraction, or compound degradation. The determined LOD and LOQs (from the curves) are relatively high, which highlight the issue of sensitivity with this method and CI in general. Overall, the EI method gave unsatisfactory response over the concentration range with less compounds having a $R^2 > 0.9$. Organic acids such as 3-Hydroxybutyric acid, Pyruvate and Lactate also showed low precision at the different concentrations. One of the reasons for this is the low specificity of the EI quantitative transitions which detect inferring compounds / peaks leading to inaccurate peak areas for each concentration.

It can be concluded that the method performance of the GC-MS/MS method fitted with CI is more satisfactory than EI, but day-to-day challenges with the CI source hinders further optimisation and use in the routine lab.

Future prospects include using alternative derivatisation methods that could improve EI MRM detection and linearity of organic acids and should be considered in future studies. The use of LC-MS/MS can also be explored for the detection and quantification of organic acids in abalone, especially as derivatives which could bridge the negative ionisation problem.

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CHAPTER 1 INTRODUCTION

1.1 Background and motivation

Abalone are a group of single-shelled marine molluscs that play an important role in the aquaculture industry. From a commercial point view, abalone can be cultured in man-made shore-based systems. This allows for tons of abalone to be cultured for export per year without depleting the country's natural resources. The aquaculture of abalone is one of the most developing industries that help sustain the South African economy. Since most of the farming dynamics are already well established, abalone farming can be considered the largest aquaculture sector in South Africa as it holds 76% share in the total value generated. It can be considered an important field to innovate, where the central focus of most research studies is based on increasing farming outputs and reducing animal losses. Unlike many other farming practices, the turnaround on investment in abalone aquaculture is considerably longer. It takes approximately 4 years for abalone to grow from seed to market size (80g). It is therefore mandatory to monitor health and growth, and to optimize it where possible.

Past studies allowed us to gain sufficient insight into abalone physiology as well as the numerous factors that influence the health and growth of farmed abalone. However, there are still substantial gaps in our basic knowledge regarding abalone biochemistry and metabolism. By investigating the metabolism of cultured abalone and understanding their metabolic responses to exposomal stimuli such as hypoxia, we can advance health- and growth-related practices in aquaculture. Moreover, biomarkers of stress and disease can be established and used to monitor farm animals on a routine basis.

Useful metabolic information can be acquired by profiling organic acids in animals – not only for the purpose of studying the metabolic responses in abalone but also to monitor their health. For this, an analytical method which quantifies selected organic acids is required. This study identified this need and aimed to develop a GC-MS/MS organic acid profiling method for abalone samples. To test the method's ability to profile organic acids in abalone, animals exposed to hypoxic conditions would be analyzed as hypoxia influences the concentration of organic acids severely. This was also seen in previous metabolomics-based studies performed by our group.

1.2 Aims and Objectives of study

The aim of this study is to develop a GC-MS/MS organic acid profiling method for abalone muscle samples. The following objectives were formulated to achieve this aim:

a) Optimize ionization and detection of selected derivatized organic acids on the GC-MS/MS instrument (i.e. optimize multiple reaction monitoring (MRM) transitions).
b) Optimize chromatographic separation of the derivatized organic acids.
c) Create matrix appropriate external calibrators to characterize new method and quantify organic acids
d) Establish method characteristics for each organic acid in terms of linearity, limit of detection (LOD), limit of quantification (LOQ), precision and accuracy.
e) Apply developed method to quantify and compare the organic acid profiles of abalone subjected hypoxia.

1.3 Experimental design of study

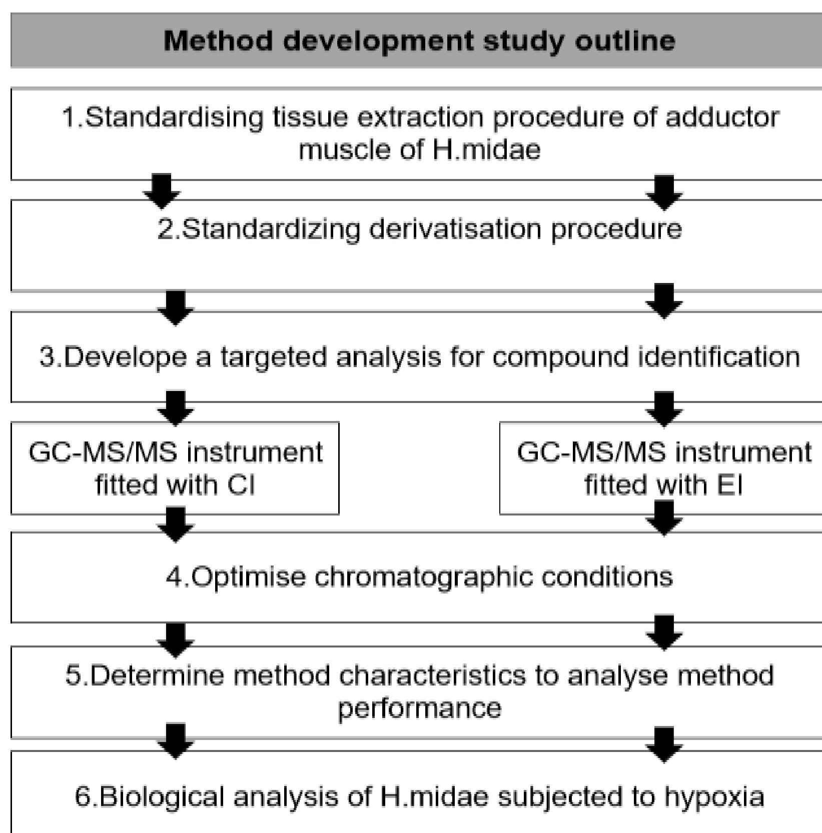


Figure 1-1: Schematic presentation of the experimental workflow.

As shown in Figure 1-1 above, this is method development study which includes standardization of tissue extraction procedures as well as developing a targeted analysis for compound identification using two popular ionization methods namely chemical ionization (CI) and electron impact (EI). Once the method is developed the next step would be to determine the method characteristics of in order to analyze method performance. This aims to explore options in order to prepare in-house machines to be compatible with abalone tissue samples. The last step of the study is the biological analysis of *H. midae* subjected to hypoxia.

1.4 Structure of this dissertation

This dissertation complies with the requirements of the NWU and are structured as follows:

Chapter 1 gives a brief background and motivation for this study, aims and objectives, structure of dissertation, and the contributions made by all collaborators. It also gives a comprehensive overview of what outcomes we aim to gain from this study.

Chapter 2 gives a descriptive literature overview of abalone biology as well as the aquaculture industry. It also gives insight on basic abalone metabolism and the influence of environmental hypoxia on it; and summarizes previous 'omics' research performed on abalone. Lastly, analytical techniques used for organic acid quantification are reviewed.

Chapter 3 highlights the methods and materials used for the study.

Chapter 4 showcases the results acquired during each step of method development and discussion thereof in relation to other methods.

Chapter 5 will discuss the conclusion of this study and will also link the information from previous studies and discuss future prospects.

1.5 Contributors and collaborations

The hypoxic exposure of abalone as well as the sample collection of the investigation has been conducted prior to the research study by HIK (Ltd) abalone farm in Hermanus and collaborators from the University of KwaZulu Natal.

CHAPTER 2 LITERATURE REVIEW

2.1 Introduction

Abalone, which is more commonly known as 'Perlemoen' (mother-of-pearl lining) to the locals of South Africa (Laas and Vosloo, 2010), is a group of single shelled marine invertebrates which belong to the phylum Mollusca, the class Gastropoda and the family Haliotidae (Venter *et al*., 2018a). Abalone has become a very popular seafood delicacy worldwide, where it is harvested on a mass scale in many countries (Venter *et al.,* 2018a). South Africa is considered one of the prime producers of wild abalone together with other countries such as Australia, Japan, New Zealand, Mexico and The United States (Mau & Jha, 2018). The high demand for abalone combined with the under-supply of natural abalone resources contributes to this marine animal's escalated monetary value. As the popularity for these highly sought-after marine molluscs continues to increase, natural abalone resources are under a high risk of depletion (Crookes, 2016). Negative factors such as overfishing, disease, ocean acidification and poaching cause a decline in natural abalone schools (Venter *et al*., 2018a). Fortunately, abalone farming plays an important role in the control of product supply and demand without putting pressure on the country's natural resources.

2.2 The basic biology of *H. midae*

As mentioned, abalone is single-shelled marine molluscs that belong to the phylum Mollusca (Venter *et al*., 2018a). Mollusca is considered one of the largest and most diverse animal phyla after the phyla Arthropoda (Hickman *et al*., 2001). The name Mollusca is derived from its distinctive soft body (Hickman *et al*., 2001). It is considered to be the most abundant and widespread group of molluscs that belong to the class Gastropoda as there are more than a 100 known species of these marine animals worldwide, all of which belong to the genus Haliotis (Hickman *et al*., 2001; Hahn, 1989). Six of these species are found in the South African coastal waters (Sales & Britz, 2001). *H. midae* is known as the largest of the five species found in South Africa and can be found in shallow reefs along the coast of the Western and Eastern Cape. *H. midae* is the only species of abalone cultivated in South Africa. The large size as

well as the abundance thereof makes this species commercially attractive (Genade *et al.*, 1988).

H. midae has a relatively simple biology (Figure 2-1), consisting of a shell that protects the muscular foot and soft body tissue. From an exterior angle abalone has a unique flattened shell where the mother-of-pearl lining and a row of respiratory pores extends from the left anterior margin of the shell. These pores close posteriorly as growth occurs (Venter *et al.*, 2016). Although Mollusca represents an incredibly diverse phylum, all molluscs share a common body plan which consists of a head-foot region and a visceral portion (Hickman *et al.*, 2001, Venter *et al.*, 2018a). The head-foot region is considered the more active area of the abalone and plays a role as the hydrostatic skeleton of the animal. It is composed of a muscular foot structure containing a hemolymph cavity (Hickman *et al.*, 2001, Venter *et al.*, 2018a). Abalone have some unique features which allows them to adapt to their environment. One such feature is its large muscular foot, which is used for locomotion and attachment (Venter *et al.*, 2018a).

The visceral portion can be characterized as the non-muscular metabolic section of the abalone which primarily consists of the digestive, circulatory, respiratory, and reproductive organs (Venter *et al.*, 2018a, Hickman *et al.*, 2001). Torsion is a term used for the rotation of the visceral organs in anti-clockwise direction through an angle of 180°. The digestive system consists of the mouth, buccal region, and esophagus. This area spreads posteriorly to the crop and terminates at the anus area which is situated dorsally to the gills due to torsion (Venter *et al.*, 2018a). The digestive system has many functions which include ingestion of food and absorption of nutrients (Maguire, 1998). The digestive gland also plays other unique roles such as energy storage in the form of lipids and glycogen, enzyme synthesis, gametogenesis, and antioxidant production (Venter *et al.*, 2018a).

The circulatory system contains the heart, arteries and all the numerous sinuses found through the abalone body, which permits hemolymph to circulate and fill the open spaces surrounding the internal organs (Venter *et al.*, 2018a). The respiratory system consists of a pair of bipectinate gills which are situated below the shell pores. They play an important role in gas exchange (Venter *et al.*, 2018a). During rest the right gill is continuously perfused, which depending on the demand allows for a continuous

supply of oxygen. When the oxygen demand increases, abalone diverts more hemolymph towards the left gill allowing for the oxygen uptake to gradually increase (Venter *et al.*, 2018a). It is interesting to note that hemocyanin has a reverse Bohr effect, where oxygen binds more firmly at a lower pH or higher carbon dioxide partial pressure. This gives abalone the unique ability to maintain oxygen saturation while clamping surfaces (Venter *et al.*, 2018a).

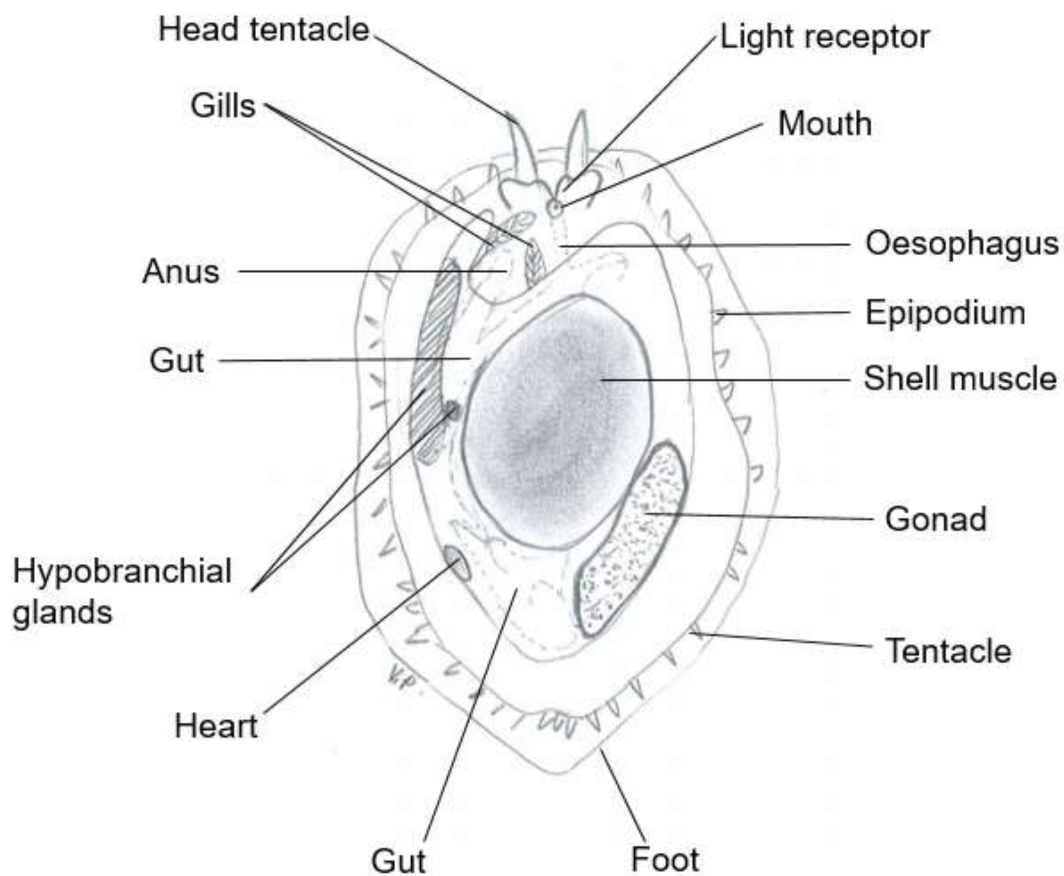


Figure 2-1: The basic biology of *Haliotis midae*

2.3 Abalone as a cultured species

Aquaculture in South Africa has existed from as early as the 1990s (Sales and Britz, 2008). Freezing, canning or live export to mostly far eastern countries are the prime destination for abalone which contribute millions to the South African economy (Venter

et al., 2018a). In the wild, abalone are vulnerable to capture as well as illicit trade which puts pressure on wild abalone stock production (Steinberg, 2005). Due to the continuous decline in wild abalone stocks a rapid development of abalone cultivation needed to take place (Troell *et al*., 2006). Abalone is cultured on man-made shore-based systems at a high density, where they are subjected to formulated artificial diets and a controlled farming environment to best aid their growth and development (Venter *et al*., 2018a). Abalone farms are mainly found in regions where coastal water temperature is comparable to the optimal temperature needed for fruitful growth. The success of intensive culture systems is vital, as abalone farming could serve the growing demand without depleting South Africa's natural abalone reserves (Venter *et al*., 2018a).

2.3.1 The economic impact

On a global scale the increase in the aquaculture of molluscs has become commercially important as annually the world's total production of molluscs is second to that of fin fish. Farmed abalone production exceeds that of fin-fish production value by over 900-fold (Mau and Jha, 2018); and hence many countries rely heavily on the molluscan production in the aquaculture sector (Mau and Jha, 2018).

Abalone can be considered one of South Africa's most successful exported aquaculture products as it contributes approximately 76% share of the total value generated by the aquaculture sector (Britz and Venter, 2016). Recent figures show that abalone have a high market value that is estimated to be approximately US$ 30-50 kg (Britz and Venter 2016). The commercial production in South Africa started in the 1980s with the annual production of farmed animals reaching to an impressive 934 tons by 2008 (Frachini 2011) Factors such as relatively cheap labor and adequate coastal water quality and infrastructure aided the fast-growing abalone industry in South Africa, making it one of the principal abalone producers outside Asia (Troell *et al*., 2006).

As with most farms, the central focus of research is on increasing the output and reducing animal loss. However, unlike many other farming practices, the turnaround

on investment in abalone aquaculture is considerably longer as it can take approximately four years for abalone to grow from a seed to a market sized animal of approximately 80 g / 90 mm under intensive culture conditions, as opposed to the 6 to 8 years it takes for wild abalone to reach a mature size. Considering this, it is priority to maximize the growth of these animals to increase revenue. Monitoring farmed abalone health during the four years, is also crucial for farmers to ensure minimum losses and sufficient return on investment.

2.3.2 The goal of optimum growth for farmers

In farming the concept 'growth' could be shortly described as the process by which ingested food is converted to body mass (Venter *et al*., 2018a). Growth can therefore be described as the relationship between anabolism (biosynthesis) and catabolism (biodegrading) (Venter *et al*., 2018a). Optimizing growth has always been the main priority for farmers, in terms of shell length and live weight (Venter *et al*., 2018a).

Farms have both hatcheries and grow-out facilities. The hatchery is usually divided into four highly specialized divisions, each one representing a life cycle phase, namely the brood stock, larvae, settlement, and the weaning phase (Venter *et al*., 2018a). Once these developmental phases are complete, abalone move to the grow-out phase where the conditions provided allow for optimal growth until market size is reached (Venter *et al*., 2018a). This latter phase is known to be the most expensive phase for an abalone farmer as it is the longest of the developmental stages (Venter *et al*., 2018a).

Abalone are considered a model farming animal as it possesses many traits that can contribute to its success as a farmed animal. Firstly, abalone require a small group of adults as a brood stock. They have a non-feeding planktonic larval stage whereafter they only consume plant-based food as juveniles - this is a fairly inexpensive phase for the farmer. Abalone also have high survival rates in congested surroundings (Venter *et al*., 2018a). Abalone are relatively sedentary animals; therefore, they use minimal energy for movement which allows surplus energy (from a metabolic point of view) to be directed towards anabolic processes, allowing growth to take place (Venter *et al*., 2018a). However even after implementing optimal growth parameters, variance

in growth rates and slow growers can still be present in the brood stock. Slow growers can be defined as abalone which fail to grow to market size in the required period even after optimizing all factors that may influence their environment (Venter *et al.*, 2018a). To ensure that growth is always favored factors such as the temperature of water, spawning as well as the type of diet is optimized.

Understanding the prime culture conditions can be a very useful tool in the management of industrialized aquaculture systems. For years this has been the most researched area, with most farms following the same principles and strict farming conditions to allow optimal growth (Venter *et al.*, 2018a). However, stressors such as hypoxia can have a negative influence on anabolic processes and overall growth. Insight in these metabolic processes can be beneficial for farmers, as it could shed some light one all factors that negatively affect the growth process. In an unstressed environment where sufficient oxygen is available, anabolic processes and the synthesis of macromolecules are favored, leading to growth but in a stressed environment, cell homeostasis is disturbed, and alternative energy pathways are activated which can in-turn have a negative effect on growth.

2.4 Feeding, growth and metabolism

In all walks of life, living organisms maintain themselves by obtaining nutrients from the environment they inhabit. Abalone can be classified as an opportunistic herbivore as they typically feed on plant-based foods (Venter *et al.*, 2018a). In a farming environment, a farmer’s choice of feed is very important as it can have a direct influence on growth rates of the farm grown animals. To achieve an optimum yield a farmer needs to choose a feed that complements the abalone’s digestive system and that is fairly inexpensive. Farmed abalone are mostly fed pelletized food which is formulated from proteins, carbohydrates, lipids, vitamins, and minerals which are put together by an alginate binder (Venter *et al.*, 2018a).

2.4.1 Basic metabolism

Basic metabolism can be briefly defined as the sum of all chemical changes that convert nutrients to energy and chemical end products of cells. Two important purposes of metabolism are to synthesize biological molecules and to generate energy to drive vital functions that allow cells to grow, reproduce and repair (Venter *et al.*, 2018b). Metabolites are simple organic compounds within a cell, tissue, organ, and biological fluid which constitute the metabolome (Venter *et al.*, 2018b). They are intermediates in cellular energy transformation and through the process of biosynthesis they form various sets of building blocks such as amino acids, sugars, nucleotides, and fatty acids (Garrett and Grisham, 2001).

Like most other organisms, metabolism in abalone can be divided into three important pathways namely, catabolism, anabolism and amphibolism. Catabolism is the oxidation of complex nutrient molecules through exergonic reactions resulting in the production of energy in the form of ATP. Through these processes carbohydrates, lipids and proteins are metabolized to a common intermediate molecule known as acetyl-CoA which serves later as a substrate in the tricarboxylic acid (TCA) cycle. In the TCA cycle it generates reduced electron carriers for ATP production via the oxidative phosphorylation system (OXPHOS) and end products such as water, carbon dioxide and ammonia (Garrett and Grisham, 2001, Venter *et al.*, 2018a).

Anabolism on the other hand can be described as the synthesis of biomolecules such as proteins, polysaccharides, lipids and nucleic acids from simple precursors such amino acids, sugars, fatty acids, and nitrogenous bases. Reactions involve the formation of new covalent bonds and an input of chemical energy to drive endogenic processes. The ATP generated from catabolism is used to drive these reactions, together with NADPH for the reductive reactions required during this process (Garrett and Grisham, 2001).

Amphibolism is the process where pathways can act both as catabolic and anabolic pathways where they form intermediates which play a role in both (Garrett and Grisham, 2001, Venter *et al.*, 2018a). The concept growth can be vaguely summarized as the process by which ingested food is converted into body tissue (Venter *et al.*, 2018a).

In an ideal farming environment, sufficient energy should be produced to drive anabolic processes. Catabolism of dietary carbohydrates through glycolysis produces adequate substrate for energy production (via TCA cycle and OXPHOS) and anabolic products such as glycogen and lipids (Venter *et al.*, 2018b, Venter *et al.*, 2018c). Lipid reserves are used for energy production during times of fasting or stress. Consequently, amino acids acquired from the diet (essential) or synthesized (non-essential) are mainly used for protein synthesis and less to replenish TCA intermediates and energy levels (Venter *et al.*, 2018b, Venter *et al.*, 2018c). Since OXPHOS produces the bulk of energy in most organisms (through aerobic metabolism) it is understandable that anaerobic situations should be absent for an ideal growth profile to be created. Stressors such as hypoxia have a negative effect on these animals and cause abalone to re-direct their energy production with the induction of alternative catabolic pathways that often result in wasting - the breakdown of muscle mass and stored fat (Venter *et al.*, 2018b, Venter *et al.*, 2018c).

2.4.2 The effect of stress and hypoxia on metabolism

Hypoxia can be defined as a deficiency in the amount of oxygen reaching the tissue or muscle of an organism (Venter *et al.*, 2018b). In the aquaculture industry hypoxia is a very common factor studied among researchers concerned about marine health as it is commonly associated with significant losses of animals as well as the significant decrease in the growth of surviving animals. Abalone experience hypoxia when they are taken out of tanks for activities such as general size grading or to conduct cleaning procedures on tanks. In South Africa, factors such as interrupted power supply, which affect water flow in tanks, or an increase in temperature contribute to hypoxic conditions (Venter *et al.*, 2018b).

As mentioned above, one of the most important roles of metabolism is to sustain and maintain adenosine triphosphate (ATP) levels. Oxygen is essential for this process and if absent, marine invertebrates such as abalone are forced to activate alternative energy pathways. A low ATP:ADP ratio stimulates catabolic processes to replenish ATP levels, which can be detrimental to growth (Venter *et al.*, 2018b). Moreover, previous results also showed that functional and environmental hypoxia create an

imbalance in homeostasis (Venter *et al.*, 2018b, Venter *et al.*, 2018c), which affect growth further.

a) Initial response to hypoxia

i. ***Restoration of redox imbalance and ATP production during anaerobic metabolism***

Alternative energy pathways such as substrate-level phosphorylation are activated in order to restore instant energy levels when mitochondrial respiration becomes inadequate (Venter et al., 2018a, Venter *et al.*, 2018b). Substrate level phosphorylation can be broken down into two main metabolic categories, namely phosphagen breakdown where abalone makes use of phosphoarginine for instant energy liberation. Previous studies which tested the effect of functional hypoxia confirmed this as results showed large pools of arginine detected in most tissue samples where it also showed creatine was present in the foot muscle which suggested that energy can also be maintained by phosphocreatine metabolism (Venter *et al.*, 2018b). Anaerobic glycolysis also aids with ATP replenishment as shown by the increase in lactate, alanine and several opines. Previous studies which discussed the effect of environmental hypoxia on abalone showed an adverse effect as results by showing a decrease in arginine and creatine levels which indicates that further breakdown pathways are up regulated. It can also be due to metabolic shuttles between tissues (Venter *et al.*, 2018b). Initial energy replenishment is often short-lived and therefore needs to further be assisted by anaerobic respiration which helps compensate for the loss of energy by the breakdown of glycogen, proteins, and lipids (Venter *et al.*, 2018b).

Anaerobic glycolysis is also activated to restore the redox (NADH: NAD^+) imbalance when oxygen levels are reduced, and the mitochondrial respiration is slowed down. Previous studies reported elevated levels of lactate, several opines, succinate and alanine (Venter *et al.*, 2018b). During prolonged hypoxia some ATP is provided by the phosphoglycerate kinase and pyruvate kinase reactions in the glycolysis pathway. These reactions are only able to take place if NAD^+ levels are adequate. Therefore,

other complementary glycolysis pathways are employed for NAD^+ recovery namely the glucose-lactate pathway where pyruvate is converted to lactate by oxidizing NADH to NAD^+ to avoid metabolic shutdown (Venter *et al.*, 2018b). The excessive amounts of lactate produced influences the intracellular pH, so to avoid acidotic shock *H. midae* produces alanine and opines via glucose opine pathway. Opines are anaerobic end products produced by pyruvate and several amino acids which convert NADH to NAD^+. The glucose/aspartate-succinate pathway is also activated to sustain NAD^+ levels. In previous studies this is supported by the decrease in aspartate levels and the increase in alanine- and succinate levels in the TCA cycle (Venter *et al.*, 2018b).

ii. ***The role of Carbohydrate metabolism during hypoxia.***

In the event where ATP is required rapidly, carbohydrates play a vital role in the ATP production. Previous studies report an increase in several sugar metabolites such as galactose and mannose accumulation during hypoxia (Venter *et al.*, 2018b). When anaerobic metabolism is activated a release of carbohydrates from glycogen stores, glycoproteins as well as the upregulation of gluconeogenesis takes place. This leads to elevated TCA cycle intermediates such as citrate, aconitate, isocitrate and succinate. The accumulation of these intermediates could be due to possible impaired mitochondrial respiration as well as the down regulation of the TCA cycle via isocitrate dehydrogenase. In vertebrates this is subsequently done to prevent the animal from metabolic energy waste (Venter *et al.*, 2018b).

b) Intermediate response to hypoxia

iii. ***The role of protein metabolism during hypoxia***

During hypoxia protein catabolism is activated. This can be due to the altered AMP to ATP ratio which usually occurs in low energy states. Previous studies reported an increase of AMP detected in the right gill and adductor muscle of *H. midae* as well as an increase in amino acid concentrations in those and other tissues (Venter *et al.*,2018a). When the ratio increases AMP activated protein kinase (AMPK) is activated

by means of a conformational chnge (Venter *et al.*, 2018a). AMPK then plays a vital role in signaling the catabolism of proteins which subsequently inhibits anabolism and cell growth (Venter *et al.*, 2018a). In a stressed environment the amino acid alanine is a key survival response as it aids in the buffering of H^+ ions and the regulation of osmotic pressure (Venter *et al.*,2018a). When glucogenic amino acids are elevated during hypoxia they are converted to alanine via the transamination of pyruvate. Previous studies have reported the increase of alanine and pyruvate in tissues that support this. They also report an increase in serine concentrations which plays a vital role as an energy substrate for gluconeogenesis and the formation of pyruvate (Venter *et al.*, 2018b). Aspartate and asparagine were seen to decrease during environmental hypoxia while glutamate increased. This could be due to its role in the replenishment of oxaloacetate during hypoxia, while glutamate plays a role as an intermediate in amino acid metabolism it is also associated with the TCA cycle as the amination of alpha-ketoglutarate to produce glutamate (Venter *et al.*,2018a).

Previous studies also showed the levels of the ketogenic amino acids such as leucine and lysine were significantly decreased in the adductor muscle and left gill after the exposure to environmental hypoxia (Venter *et al.*, 2018a). Leucine and other branched chain amino acids are substrates for gluconeogenesis under conditions of increased ATP demand. In addition to their role in energy production certain amino acids such as opines, alanine, arginine, asparagine, aspartate, cystine, glutamate, and others play a role as osmolytes during hypoxia as they help maintain intracellular osmolarity (Venter *et al.*, 2018a).

c) Final response to hypoxia

iv. *The role of lipid metabolism during hypoxia*

Previous studies showed that a few lipids were altered after exposure to environmental hypoxia. This suggests that cellular production via lipid metabolism was limited, and that the role of lipid metabolism is the final response against hypoxia (Venter *et al.*, 2018a). In unchallenged circumstances Haliotids cannot synthesize all the fatty acids required for growth and strongly rely on dietary intake for these stores. If they have a

restricted intake of lipids this could also explain the decrease effect in altered fatty acids (Venter *et al.*, 2018a). The fatty acylcarnitines that were reported elevated in *H. midae* were free carnitines, acetylcarnitine, butyryl-carnitine and palmitoylcarnitine. This could be due to the inhibition of the β-oxidation pathway and the redox imbalance which can result in increased levels of fatty acids (Venter *et al.*, 2018a). Previous studies showed elevated palmitoleate during functional hypoxia. which could be associated with the increase in palmitoylcarnitine, carnitine and acetylcarnitine (Venter *et al.* 2018b). These metabolites show that β-oxidation is compromised in both functional and environmental hypoxic episodes.

2.5 Investigating abalone metabolism

2.5.1 Untargeted metabolomics

Metabolomics can be considered an empowering research tool that is used to investigate metabolic alterations associated with chemical, biological and physiological processes (Wishart *et al.*, 2022). Metabolomics is defined as the non-biased identification and quantification of all metabolites (i.e. metabolome) in a biological system, using highly sensitive and selective analytical techniques. These techniques will be discussed in more detail in the upcoming sections (Wishart et al., 2022). The metabolome consists of thousands of metabolites, defined as the building blocks for cellular function (Schrimpe-Rutledge *et al.*, 2016); and is considered the final downstream product of the genome, transcriptome and proteome which contains a wealth of knowledge. It serves as a direct representative of the functional state or phenotype of a cell (Markus M. Rinschen *et al.*, 2019) since metabolites are products of both inherited biological factors and environmental factors. Hence, studying it can assist researchers in bridging the gap of knowledge between the genotype and phenotype (Schrimpe-Rutledge *et al.*, 2016).

Metabolomics can be divided into two main categories namely untargeted and targeted analysis (Schrimpe-Rutledge *et al.*, 2016; Griffiths *et al.*, 2010). Untargeted metabolomics aims to give a comprehensive or global view of all low weight molecules within an organism, resulting in large datasets of hundreds to thousands of detected

small molecules (molecular weight less than 1500 Daltons) that can be mapped into networks and pathways (Griffiths *et al.*, 2010). There is no single analytical technique that can identify and quantify all metabolites of interest in a biological sample (Griffiths *et al.*, 2010), hence many untargeted metabolomics studies make use of a multiplatform approach. One of the main challenges which untargeted analysis face is the identification of detected metabolites. Available databases are often used to identify detected signals, but many remain unidentified. Moreover, metabolites identified solely on mass spectra or retention time (one physical attribute at a time) are often miss-identified leading to incorrect interpretations (Schrimpe-Rutledge *et al.*, 2016). Because of these limitations, untargeted metabolomics is considered a hypothesis generating approach which requires followed-up with more targeted methods to confirm findings (metabolite identities) or to implement a method for biomarker screening in a biological sample.

Figure 2-2 represents the flow of information from untargeted studies to more targeted study. Both types of approaches play a valuable role in obtaining information in order to fill gaps in biological information. Since untargeted metabolomics produces large data sets, it is not commonly utilized in a routine set-up. Targeted methods are popular in routine laboratory settings where they are optimized to quantify a limited number of metabolites with acceptable accuracy and precision.

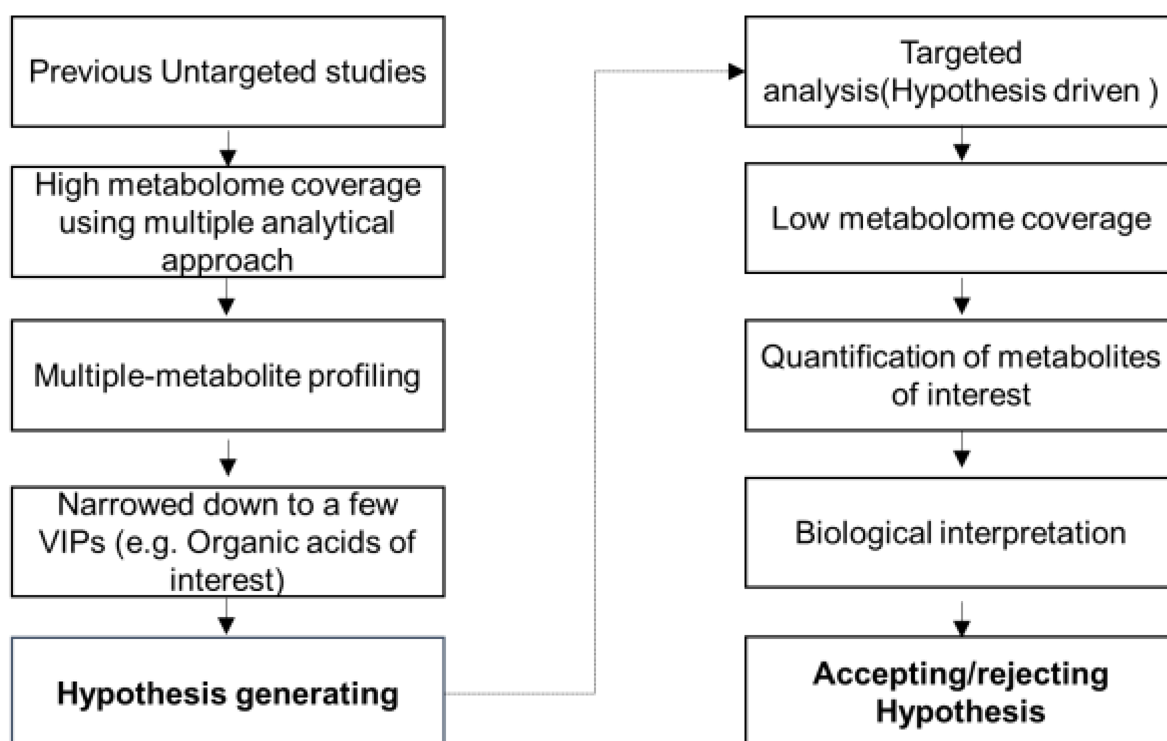


Figure 2-2: Figure representing the flow of information from previous untargeted studies to a targeted study.

2.5.2 Targeted analysis of organic acids

Targeted approaches are aimed at identifying and quantifying a limited number of known metabolites. This approach is hypothesis driven and in many cases the identification of compounds is already known. Predefined and optimized metabolite signals are used to obtain the relative abundances and concentrations of the *a priori* known metabolites (Griffiths *et al.*, 2010). One of the main advantages of targeted analysis is that absolute quantification of metabolites can be obtained (Schrimpe-Rutledge *et al.*, 2016).

In this study the main aim is to develop a targeted method to analyze specific organic acids in abalone tissue. Previous metabolomics investigations on abalone were unable to detect several important organic acids. This is likely due to their lower concentrations in abalone samples which warrant more specific methods for analysis (Venter *et al.*, 2018b). Organic acids play an integral role and can be considered the

center of energy metabolism. Carbohydrates, lipids and even amino acids are converted to these intermediates. Targeted analysis of organic acids can therefore reveal a lot about the metabolism of an organism. The different analytical technologies used in targeted metabolic studies will be discussed in the following sections, with special focus on analytical platforms suitable for organic acid analysis.

2.5.3 Analytical technologies

Most analytic technologies consist of two main components namely, separation and detection technologies commonly referred to as hyphenated techniques. These two components work together to improve selectivity (or specificity) of a technique when detecting metabolites in complex biological samples. Popular separation techniques available are liquid chromatography (LC) and gas chromatography (GC) while specific detection technologies popular in metabolomics include mass spectrometry (MS) and nuclear magnetic resonance (NMR) (Schrimpe-Rutledge *et al.*, 2016). Each technique has a certain bias when it comes to chemical classes and concentration ranges, hence a combination of these techniques is often used in untargeted metabolomics to cover as much of the metabolome as possible; with appropriate selectivity to distinguish between thousands of chemical signals (Schrimpe-Rutledge *et al.*, 2016). Some of the popular analytical platforms mostly used are discussed below.

2.5.3.1 H^1-NMR

NMR (specifically H^1-NMR) is a non-destructive, relatively unbiased analytical method that allows accurate measurement of many different analytes in a sample (especially biofluids). One of the main advantages is that NMR requires little to no sample preparation and there is no need for chemical derivatization. NMR methods are often automated and easily reproducible (Wishart *et al.*, 2022). A major weakness, however, is the poor dynamic range that limits detection to abundant metabolites (Want, Cravatt and Siuzdak, 2005). Moreover, although sample preparation and analysis can be automated, data analysis and interpretation cannot (presently). Spectral interpretation

and quantification of target metabolites required a dedicated NMR specialist which limits its use in routine analysis.

2.5.3.2 GC-MS

GC-MS is one of the pioneering methods in metabolomics with some older methods dating back as far as the 1960's (Griffiths *et al.*, 2010). GC-MS has several advantages over other analytical methods (like LC-MS) such as higher chromatographic resolution and reproducible electron impact (EI) spectra that can aid identification of compounds. This makes compound identification much easier as prior knowledge and databases can be used. In metabolomics, GC-MS is often used to profile the primary metabolism which includes fatty acids, amino acids and organic acids (Want, Cravatt and Siuzdak, 2005). Because of the nature of GC, larger, involatile compounds such as those associated with secondary metabolism (like steroids and heme structures) are usually better analyzed by LC-MS. Also, many primary metabolites are also involatile at the temperature range of the instrument and require derivatization to increase volatility (Griffiths *et al.*, 2010). A major drawback of GC-MS with EI as ion source, is that the molecular ion of detected compounds is low in abundance or completely absent. This is not only problematic when attempting to identify unknown signals but also when applying the technique in a routine setting with the use of selected ion monitor (SIM). With the SIM functionality, compounds are targeted by monitoring a single, 'specific' mass at a specific retention time. However, with no intact molecular ion, less-specific fragment ions are found when using EI which can lead to problems if chromatography is poor. This limitation can be overcome with chemical ionization CI where $[M+H]^+$ or $[M+NH_4]^+$ ions can be used which improves specificity (Griffiths *et al.*, 2010).

2.5.3.3 LC-MS

LC-MS has become a leading technology for both polar and apolar compound analysis (Wishart *et al.*, 2022). Some of the advantages that LC-MS offers is the electrospray ionization (ESI) approach, which gives the ability to analyze low and high mass compounds. Other advantages include good quantification capabilities and

reproducibility, high sensitivity, minimal sample preparation and automation (Want, Cravatt and Siuzdak, 2005). This is especially true for LC triple quadrupole (LC-MS/MS) instruments, which is one of the workhorse instruments in clinical labs. The power of this tandem mass spectrometry is seen when used in a targeted functionality like single reaction monitoring (SRM), which is also referred to as multiple reaction monitoring (MRM) when more than one compound is detected (Griffiths *et al.*, 2010). As seen in Figure 2-3, with this setup, the first mass analyzer (Q1) is parked on a m/z value of interest while the second analyzer (Q3) monitors a specific fragment ion. The second quadrupole (Q2) functions as a collision cell, by introducing a collision gas like argon or nitrogen into the flight path of the selected ion. Hence only a compound with the correct precursor – and product ion combination are detected which makes this setup very specific.

A main drawback in LC-MS approaches is that analysis can be time consuming when used in untargeted mode or when baseline separation of all peaks of interest is required. This is because LC has lower chromatographic resolution than GC (Wishart *et al.*, 2022). Also, ESI is very susceptible to matrix effects (like salts) leading to ionization suppression or enhancement. Another drawback specifically with organic acids is that these compounds are best detected as negative ions ($R\text{-}COO^{-}$). Negative ionization is known to give less sensitivity than positive ionization (Want, Cravatt and Siuzdak, 2005). Because of this, GC-MS is still the preferred approach when it comes to the measurement of organic acids (Bergou et al.,2017).

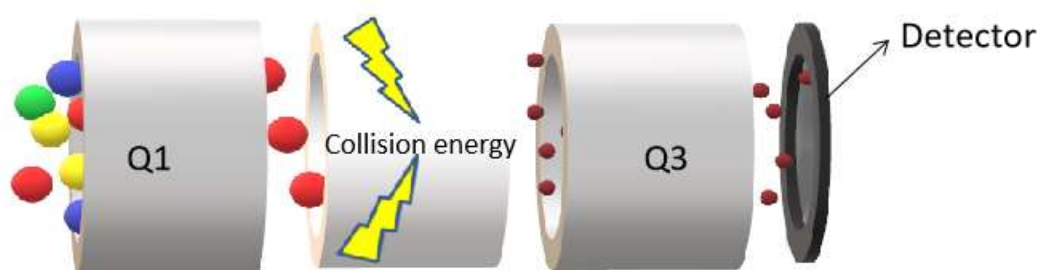


Figure 2-3: Figure representing the function of multiple reaction monitoring (MRM)

2.5.3.4 GC-MS/MS

The GC-QQQ (triple quadrupole) combines the best of two worlds – the superior chromatographic resolution of GC and the selective and sensitive detection of tandem mass spectrometry (MS/MS). This combination seems to be ideal for analyzing organic acids in abalone samples.

There are two types of ion sources that can be used in a GC-QQQ instrument namely electron impact (EI) and chemical ionization (CI). One of the main shortcomings of the use of electron ionization is the absence of the molecular ion of the compounds analyzed. This hard ionization technique fragments compounds into small ions not suitable for tandem MS. For example, silylated metabolites produce a common and dominant 73 m/z ion. Further fragmentation of this ion before Q3 would lead to even smaller fragments that are also unspecific. Ideally, the molecular ion should be preserved and used as it is more specific and can also produce specific fragments when fragmented in the collision induced dissociation (CID), or Q2. To allow for the preservation of the intact molecular ion and other high molecular weight fragments, a softer ionization technique such as chemical ionization (CI) can be used (Kvitvang, Andreassen *et al.*, 2011). In a nutshell, CI uses an ionization gas such as methane to transfer a proton onto the metabolite allowing the formation of positive ions. Negative ions may also be produced by changing the ionization gas respectively (De Hoffmann, 2000).

CHAPTER 3 METHODS AND MATERIALS

3.1 Introduction

This chapter discusses the details regarding the reagents, standards and solutions purchased for this study. The consumables and equipment used in the analytical experiment is also highlighted. The analytical parameters used during instrument analysis and optimization will also be discussed in detail.

3.2 Chemicals and reagents

3.2.1 Organic acid standards

Organic acid standards of high purity were purchased from Sigma-Aldrich: oxaloacetic acid (04126), citric acid (C83155), 2-ketoglutaric acid (75892), succinic acid (293075), fumaric acid (47910), malic acid (M9138), aconitic acid (122750), sodium pyruvate (P2256), lactic acid (L6402), 3-hydroxybutyric acid (166898), isovalerylglycine (04478), 3,3-dimethylacrylic acid (D138606), 2-oxoisovaleric acid (198994), 3-hydroxyisobutyric acid (16842), 3-methylglutaconic acid (44108), 3-methylglutaric acid (M47604), 3-hydroxy-3-methylglutaric acid (H4392), 4-methyl-2-oxovaleric acid (68255), sodium phenylpyruvic acid (P8001), 3-phenyllactic acid (P7251), 4-hydroxyphenyllactic acid (H3253) and 4-hydroxyphenylpyruvic acid (114286). 3-Phenybutryic acid (116807) was purchased as the internal standard from Sigma-Aldrich.

3.2.2 Extraction and derivatization reagents

Acetonitrile (BJ015CS), chloroform (BJ049CS) and methanol (BJ230CS) were purchased from Honeywell (Burdick & Jackson). Sodium chloride (7647-14-5) used for making the saline solution was purchased from Merck and MilliQ water used was obtained from a Millipore Milli-Q water purifying system. Derivatization agents purchased for the preparation of organic acids were methoxyamine hydrochloride (226904), o-bis (trimethylsilyl) trifluoroacetamide (BSTFA) with trimethylchlorosilane

(TMCS) (33155). Oximation mix was prepared in an amber reagent bottle by dissolving 200 mg methoxyamine in 10 mL pyridine.

3.2.3 Preparation of organic acid stocks and internal standards

A 15 mL stock solution of all standards was prepared to a concentration of 100 ppm (µg/ml) and stored in the fridge at 4 ˚C. Distilled water was added to each stock to obtain a final volume of 15 mL. Enough of each standard was derivatized and analyzed during the optimization process.

3.2.4 External calibrators

External calibrators in the correct cellular matrix were to be used to ensure reproducible quantification of target organic acids. This part of the method was done by stripping spare abalone tissue homogenates from metabolites by means of dialysis. Firstly, a saline solution was prepared by weighing 9 g of sodium chloride in a weighing boat. The salt was then added to 1000 mL distilled water to prepare the saline solution. Using a Potter-Elvehjem homogenizer, 3.5 g of the dissected tissue was homogenized in 12 mL of saline solution. The homogenized tissue was transferred to SnakeSkin™ Dialysis tubing (68035) purchased from Thermo Fisher, with a 3.5 kDa cut-off. The homogenate was dialyzed in 1 L of saline solution for 48 hours. Saline was replaced after 24 hours with fresh saline solution.

The dialyzed tissue was prepared and analyzed with a standard metabolomics procedure commonly used at our institution to confirm that metabolites were absent. The process briefly: 100 µL of dialyzed sample was transferred to a 2 mL Eppendorf microtube.150 µL of acetonitrile and 150 µl methanol was added to the sample for sample extraction. The sample was centrifuged for 10 minutes at 2000 rpm at 4 ˚C. The supernatant was transferred to a GC vial and dried under a gentle stream of nitrogen at 37 ˚C until all the solvent has been evaporated. The sample was derivatized (Section 3.6) and analyzed on the GC-MS/MS (in scan mode) to evaluate if the sample was stripped correctly.

The stripped tissue samples were used to create external calibrators. Aliquots of 500 µL of the stripped tissue was spiked with organic acid standards to create a concentration range: 0, 0.5, 1, 5, 10, 15, 25, and 50 ppm. During method characterization, these external calibrators are treated exactly as biological, experimental samples.

3.3 Consumables and equipment

The consumables used for the duration of this study were the following: Eppendorf safe lock 5 mL tubes (0030119401); safe lock micro 2 mL tubes (00300120094); Eppendorf 2-200 µL tips (EP0030000870) and 50-1000 µL tips (EP0030000919) purchased from Sigma-Aldrich. Pasteur glass pipettes [(150 mm) (612-1701) and (230 mm) (612-1702)] were purchased from Monitoring & Control Laboratories (Pty) Ltd. For the GC-MS analysis of samples Agilent blue screw caps (5182-0717), 250 µL pulled point glass inserts (5183-0717) and 2 mL screw top clear vial (5182-6106) were purchased from Chemetrix. The 100 µL Hamilton syringe (HAM204052-1EA) used was purchased from Merck. A Dissection Kit was purchased from Kampus Pharmacy (Potchefstroom, SA) for the dissection of the tissue samples.

The general laboratory and analytical equipment used for this study is situated in the Biochemistry department of the North-West University, Potchefstroom campus. The equipment used included the following: A Potter-Elvehjem homogenizer (P7859-1EA) purchased from Sigma Aldrich, a AdventurerTM AX224 Microbalance (3100602) purchased from Ohaus Corp.NJ, US, a fixed angle microcentrifuge (60130084) purchased from Hermle, DE, A DB3 heating block (FDB03OD) purchased form Techne,UK, and lastly a Agilent 780A GC-MS purchased from Chemetrix.

3.4 Animals and biological samples

Dissected adductor muscle samples from abalone exposed to hypoxia were obtained from Prof Andre Vosloo (School of Life Sciences, University of KwaZulu-Natal, Durban) who is collaborating with HIK Abalone Farm Pty (Ltd) in Hermanus. All research in the past has been carried out under Department of Agriculture, Forestry and Fisheries (DAFF) Aquaculture Research Permit (DAFF Permit No.1304476). Ethical approval for this study was acquired from AnimCare (NWU-00172-18-A5). Housing and experiment conditions are described by Venter *et al.,* (2018).

At the time of sampling the chosen animals were removed from their respective holding systems and gently stroked dry with paper towels. A gender assessment was done for each individual by observing the color of the gonad. The abalone were dissected, and samples snap frozen for a standardized time period (approximately 10 minutes). During dissection, the abalone were shucked separately using a scalpel, working rapidly from the anterior to posterior axis, cutting longitudinally through the foot between the mantle and the distal surface of the foot. Abalone samples were transported on dry ice to the NWU's Centre for Human Metabolomics where it remains stored at -80 ˚C.

3.5 Tissue homogenization and metabolite extraction

Sample preparation plays an important role in the discovery of biomarkers as it prepares the sample for the detection of low abundance molecules and it prevents analytical artefacts from entering the subsequent analysis (Griffiths *et al.*, 2010). Using a Potter-Elvehjem homogenizer, the tube with 3.5 g of dissected tissue was topped up with 7.5ml of saline solution. The dial was turned to 200 and the abalone sample was homogenized for 10 minutes till it resembled a milky liquid. Once homogenized it was dialyzed to prepare external calibrators as stated in Section 3.2.4

A single phase, modified Bligh-Dyer extraction was used to extract organic acids as described by Venter *et al.* (2016). Briefly, 100 µL of the sample along with 560 µL methanol, 200 µL water, 50 µl internal standard (3-phenylbutyric acid) and 180 µL of chloroform were added to a 2 mL Eppendorf microtube. The samples were vortexed

for 1 minute and incubated on ice for 10 minutes. Thereafter, the samples were centrifuged at 25 000xg for 10 minutes at 4 ˚C after which 500 µL of each supernatant was transferred to a clean GC vial and dried under a gentle stream of nitrogen at 37 ºC. The external calibrators and samples were injected in triplicates to obtain data.

3.6 Oximation and silylation

After the drying process 50 µL of the oximation reagent was added to the sample. The sample was then vortexed for 1 minute and incubated for 60 minutes at 60 ˚C. Silylation was followed by adding 50 µL BSTFA (containing 1% TMCS) to the samples. The samples were vortexed again for 1 minute and incubated for 60 minutes at 60 ˚C. It should be noted that the organic acid standards used to set up the MRM transitions did not undergo the extraction process but were merely dried and derivatized.

3.7 Optimizing MRM transitions with GC-MS/MS instrument fitted with chemical ionization (CI)

To set up an MRM method, each individual standard was injected twice. During the first injection, the instrument scanned / detected all the masses in the range of 50 - 800 m/z. The oven was set to ramp from 70 – 300 °C in 8 minutes for this process. The mass spectrum of the peak(s) belonging to the respective standard was inspected to confirm the compound (corresponding the recorded molecular ion with that of the compound) and to select precursor ions for the next round. One or two ions of interest were selected for the product ion scan. The intact molecular ion (if present) and the most abundant/dominant ion for each compound was selected for further fragmentation and product ion scan.

During the second injection, a product ion scan was performed. The compounds underwent selective fragmentation using different collision energy (CE) voltages in the collision induced dissociation (CID) chamber and their product ions (fragments) recorded. The masses and intensities of the various product ions formed at the different voltages were compared. As a rule of thumb, the ions with the highest intensity and mass were selected to get sensitive but also specific detection for each compound of interest. A table of the MRM transitions (precursor - and product ion

pairs) were constructed in an MRM method with the selected CE voltages. Two transitions per compound was selected: the qualitative transition, which is more specific, to confirm the identity of the compound; and the quantitative transition, which gives the best sensitivity, to use for quantification.

A dynamic MRM method was finally constructed after chromatographic conditions were optimized. MRM transitions (with the respective collision energy for each transition) are accompanied by the respective retention time of the compound of interest. With dynamic MRM, the respective transitions are only scanned at specific time points in the run and not cycled throughout the entire run which gives arguably better sensitivity and cycles per second.

3.8 Optimizing MRM transitions with GC-MS/MS instrument fitted with Electron impact (EI)

The same process described in the previous section was followed to set up the MRM table for electron impact ionization. Online databases such as the Human metabolome database were used to confirm the spectra of each compound of interest and the number of TMS-groups attached. It must be noted that when using EI, the impact can cause the molecular ion to be low in abundance or completely absent from the spectra, making it more difficult to select ions for further fragmentation. In fact, EI fragmentation is harsh and results in numerous low m/z ions in high abundance. Again, the largest mass (m/z) with the largest intensity was selected for further fragmentation as mentioned above. A dynamic MRM method was finally constructed after chromatographic conditions were optimized.

3.9 Optimizing chromatographic conditions

Chromatographic conditions should be optimized to allow maximum specificity (chromatographic resolution) in the shortest amount of time (for routine methods) without compromising sensitivity. Since MRM detection already add a degree of specificity to the method, a compromise can be made between chromatographic resolution and time. However, since many compounds produce identical fragment ions

(especially with EI), baseline separation of the target compounds was still required. It was reasoned that baseline separation of all standards allows the for the MRMs to be time-programmed (dynamic MRMs) by adding the time of elution of the component of interest to the MRM table.

The oven temperature gradient was adapted by lowering the rate at which the temperature increased at certain points in the run. Table 3-1 shows the temperature gradient values used during separation and Figure 3-1 shows a visual representation of the temperature gradient. The metabolite stocks were analyzed with different temperature gradients to optimize the separation of the compounds. The flow rate was also adjusted to aid chromatographic resolution and detection. additionally different flow rates were tested to assess cinematographic resolution.

Table 3-1: Optimized temperature gradient used for analysis of target organic acids.

	Rate ***(˚C/min)***	***Target temperature*** ***(˚C)***	***Hold time*** ***(min)***
Initial		70	2
Ramp1	15	150	0
Ramp2	10	220	0
Ramp3	15	300	1

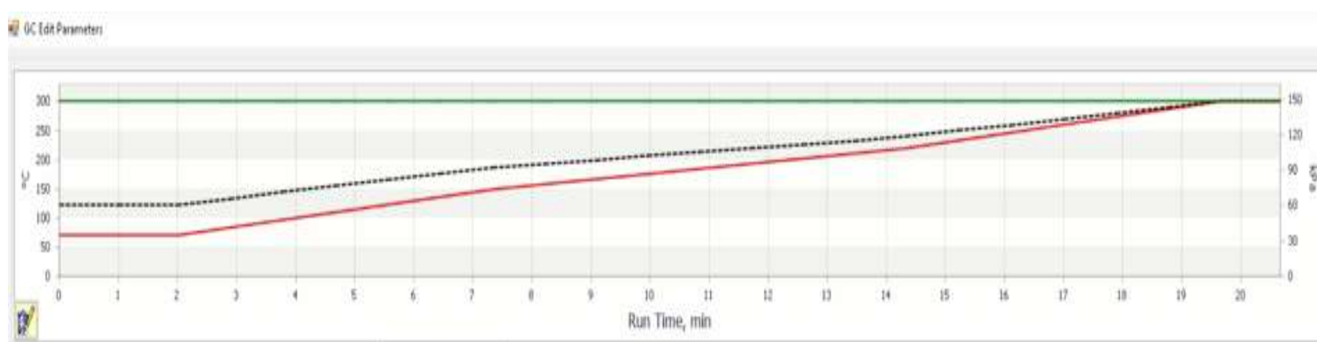


Figure 3-2: Optimized temperature gradient used for analysis of target organic acids where the green line represents the temperature of the thermal Aux (˚C) and the red line the temperature gradient of the oven (˚C)

3.10 Characterizing the performance characteristics of the standardized EI and CI methods

In order to characterize the performance of the CI and EI methods, external calibrators were prepared as stated in Section 3.3 and analyzed. Three replicates of each concentration were prepared and analyzed to construct calibration curves for each compound of interest. From this the following was determined: precision at different concentration levels, limit of detection (LOD), limit of quantification (LOQ), and the linearity R^2 of each compound. These performance characteristics were calculated according to the ISO17025 guidelines. The LOD was calculated to represent the average integrated noise signal in the blank sample plus three times the standard deviation of the integrated noise signal. The intensity was converted to concentration using the internal standard and calibration curve. The LOQ was calculated the same way except that the blank average was calculated and added to 10 times the standard deviation. Note that this was done for both EI and CI methods. For accuracy, an additional sample of 5 ppm was injected and quantified with the calibration curves created. The closer the determined concentration to the actual concentration (5 ppm), the higher the accuracy (expressed as a percentage). When the determined concentration is below 5 ppm, a percentage below 100% are provided and when the determined concentration is above 5 ppm, a percentage above 100% is provided.

3.11 GC-MS/MS setup and conditions.

The Agilent GC-MS/MS system consisted of a model 7890B gas chromatograph fitted with an Agilent HP-5 column (30 m x 0.25 mm 0.25 μm) which separated the compounds using the temperature gradient shown in Table 3-1. For sample analysis, 0.5 μL was injected in splitless mode (purge delay of 30 sec) with the help of an Agilent 7693 auto sampler. The inlet temperature was kept at 250 ˚C throughout the entire run. Helium was used as carrier gas at a flow rate of 1 mL/min. Chemical ionization took place at tune voltage (~90 V at time of use) while the standard -70 V was used for electron impact ionization. The source chamber and transfer line were 250 ˚C and 280 ˚C, respectively. The target compounds were detected, after a 3.5 min solvent delay. The CI and EI sources were changed at the time of analyses to assess each.

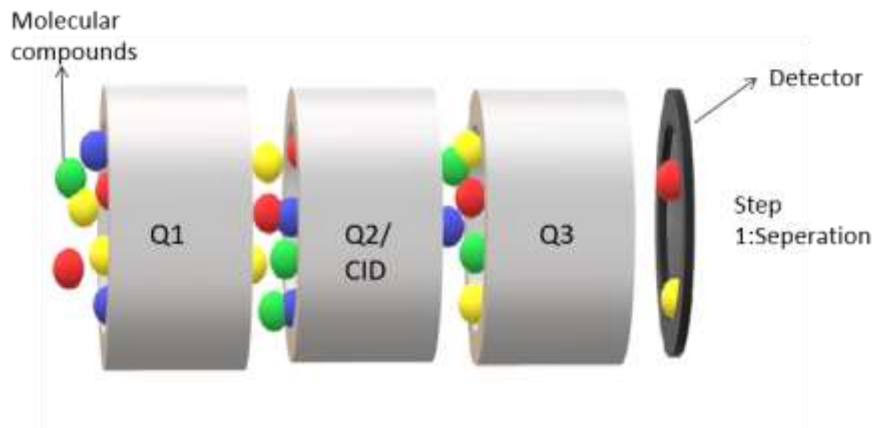
3.12 GC-MS/MS data processing

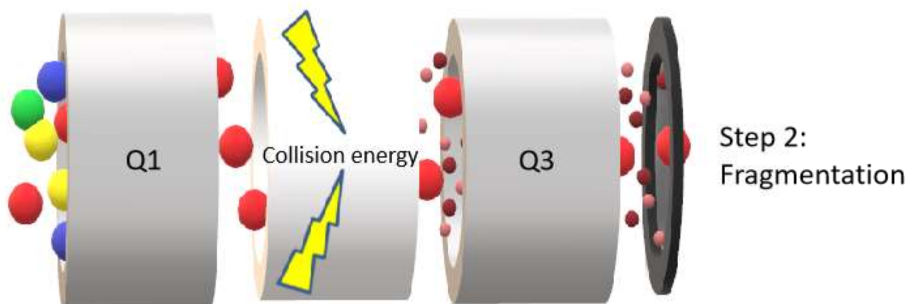
Peak detection and integration were done with Agilent's MassHunter Quant software. Extracted data was exported to MS Excel where calibration curves and abovementioned calculations were done.

CHAPTER 4 RESULTS AND DISCUSSION

4.1 dMRM transitions for chemical ionization (CI)

The process of setting up MRM transitions for selective detection of target compounds is shown in Figure 4-1 (Step 1-3). At each of these steps, a pure, derivatized organic acid was injected. With Step 1, the mass analyzer was allowed to scan for all masses (50 – 600 m/z) and the mass spectra of the detected peak(s) investigated to confirm the standard, see whether the organic acid of interest give one or more derivatized peaks, and to select a precursor ion for further fragmentation (example shown in Figure 4-2). The highest mass (for better specificity) with the highest intensity (for better sensitivity) was selected if the molecular ion $[M+H]^+$ was absent. During Step 2, only the selected precursor ions were guided to the collision cell and fragmented at different collision energies (CE volts) to produce product ion spectra. These spectra were compared to find suitable fragments (product ions) for the MRM. An example of this can be seen in Figure 4-7 and 4-8 below. Again, selection was based on the highest mass at the highest intensity (example shown in Figure 4-3). Two product ions with their corresponding collision energy were selected - one as quantifier and the other as qualifier. The final step (Step 3) entailed setting up the MRM table and confirming specific detection of the standard when analyzed in a mixture with other standards, with the optimized conditions (Figure 4-4, Table 4-1).





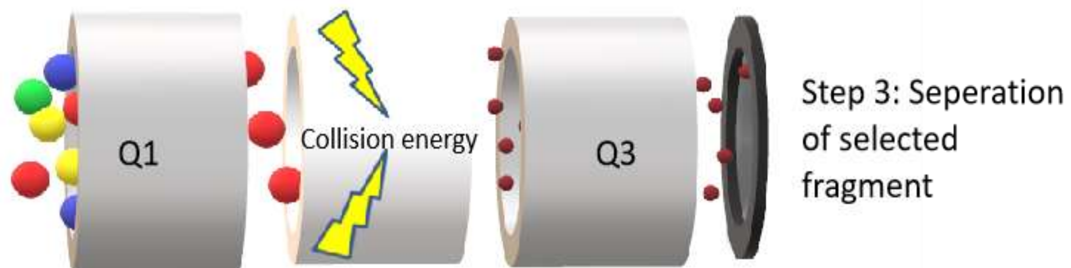


Figure 4-1: Schematic presentation showing the process of setting up multiple reaction monitoring (MRM) transitions.

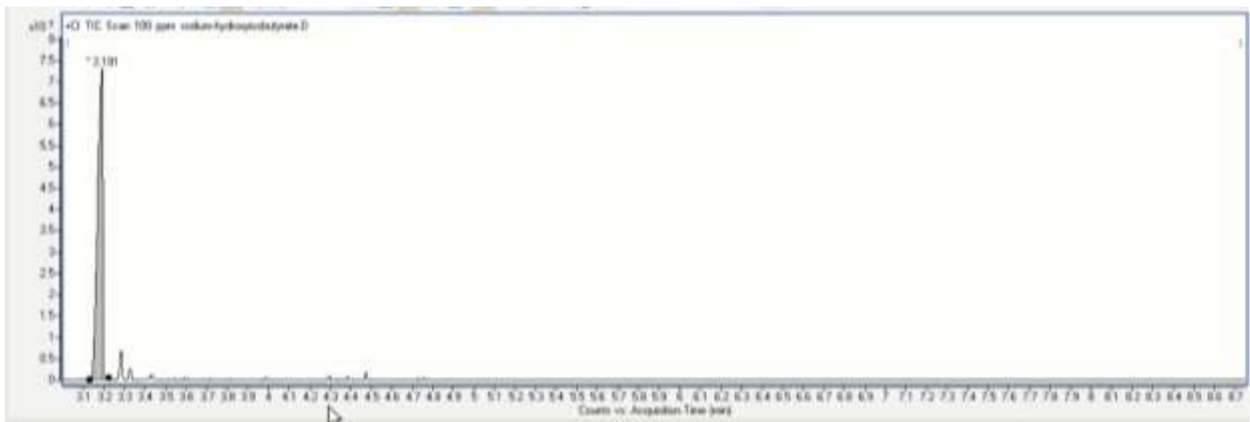


Figure 4-2: Chromatogram showing a scan of derivatized hydroxyisobutyrate standard

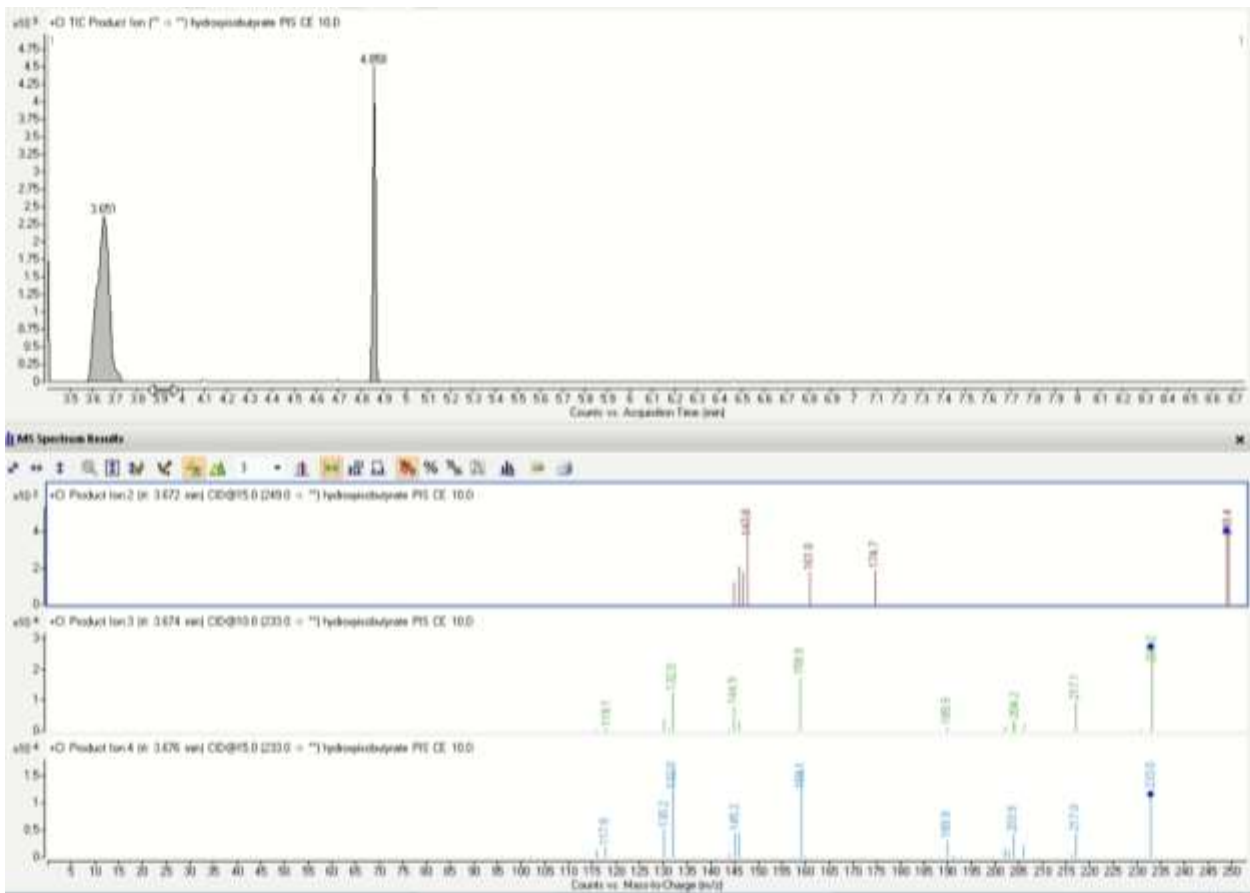


Figure 4-3: Chromatogram and mass spectra of product ion scan of hydroxyisobutyrate at different collision energies.

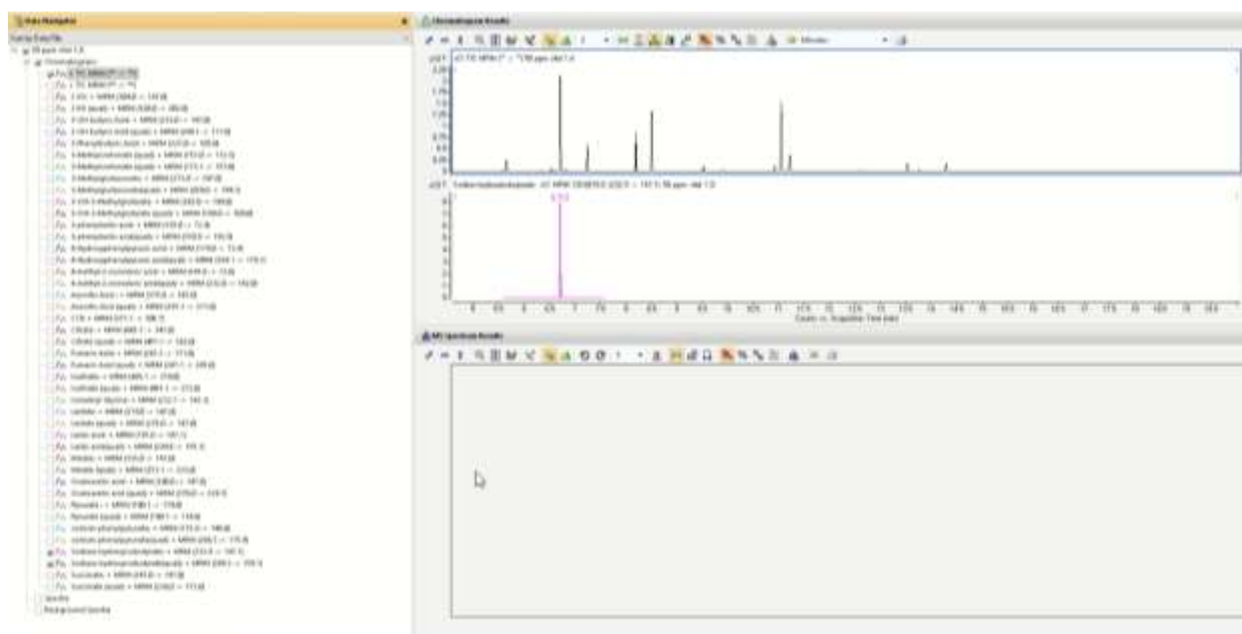


Figure 4-4: Chromatogram showing the specific detection of hydroxyisobutyrate with optimized MRM conditions.

Table 4-1 shows the precursor-product ion pairs (MRM transitions) optimized for each of the target organic acids. As mentioned, two transitions per compound were selected. The purpose of the qualifier is to confirm the identity of the compound as this transition is more specific (but not always as sensitive in detecting the compound of interest). The quantitative transition gives the best sensitivity and is used for quantifying the target compound. In Figure 4-5 a chromatogram for Aconitic acid can be seen, here is a good example of qualifier transition as the molecular ion $[M+H]^+$ of 391.2 is present, this allows for us to confirm the identity of the compound. The retention time for each compound was recorded after chromatographic optimization (Section 4.3) and included in the table to allow dynamic MRM (dMRM) detection. During dMRM the respective transitions are only scanned at specific time points in the run and not cycled throughout the entire run which gives arguably better sensitivity and cycles per second.

It can be noted that 4-methyloxovaleric acid and isovalerylglycine use the same transition and elute at the same retention time which makes specific detection and quantification difficult. Ideally, chromatographic conditions should be optimized further

to resolve these compounds. Fortunately, the second transition is more specific and can aid the MS in distinguishing between the two compounds.

It should be noted that all single or triple quadrupole mass spectrometers must continuously scan through each m/z or MRM transition listed in the method. The more masses or transitions there are, the longer the cycle time (the time it takes to record all transitions / masses once – measured as mass spectra per second). The longer the cycle time the lower the dwell time (the time the mass analyzer “stands still” at a mass or transition) and thus sensitivity of the method. Consequently, there is a compromising relationship between cycle time and dwell time. For example, if you have 5 transitions in your method and you specify a dwell time of 50 milliseconds (ms) for each transition, then it will take ~250 ms to cycle through all the transitions, which means that one could get 4 cycles (or mass spectra) per second. For a peak that is 3 seconds wide, at least 12 data points can be captured over the peak. Note that in a practical environment this can be slightly higher as it takes the MS a few milliseconds to switch through transitions.

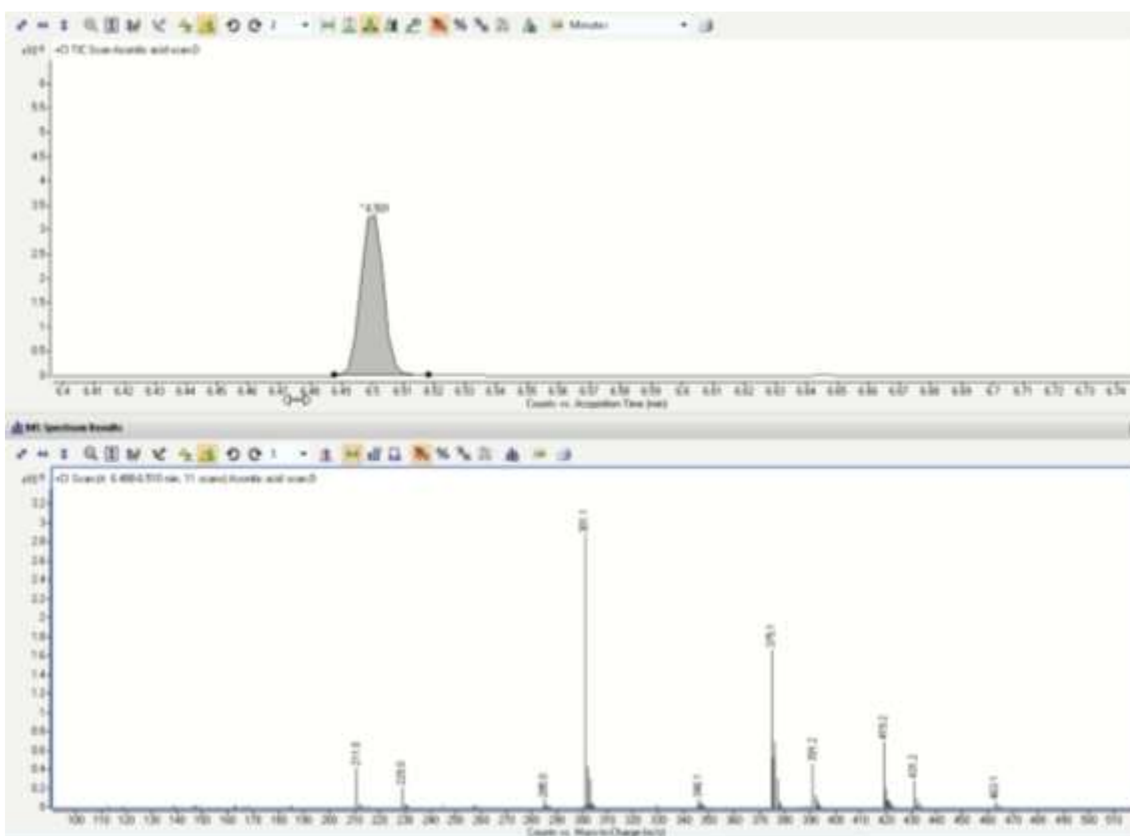


Figure 4-5: Chromatogram and mass spectra of derivatized aconitic acid.

One of the main drawbacks with using a standard MRM method for so many transitions (Table 4-1), is the low dwell time. Agilent’s default dwell time is 200 ms and other methods at our institution successfully use a dwell time as low as 40 ms. For this reason, it was decided to optimize a dMRM method instead of conventional MRM as dMRM limits the number of transitions per time window. However, despite this the dwell time in the optimized dMRM method remained low (Table 4-1) which has caused a decrease in the sensitivity of the method. An additional method was tested with a shorter list of MRM transitions (qualifier transitions removed) but the improvement in sensitivity was negligible (even though more data points across the peaks were acquired). This is thus one instance where chromatographic resolution and a narrow peak width did not favor the detection technique. We can see an example of a narrow peak in Figure 4-6. More on this is shown in Section 4.4.

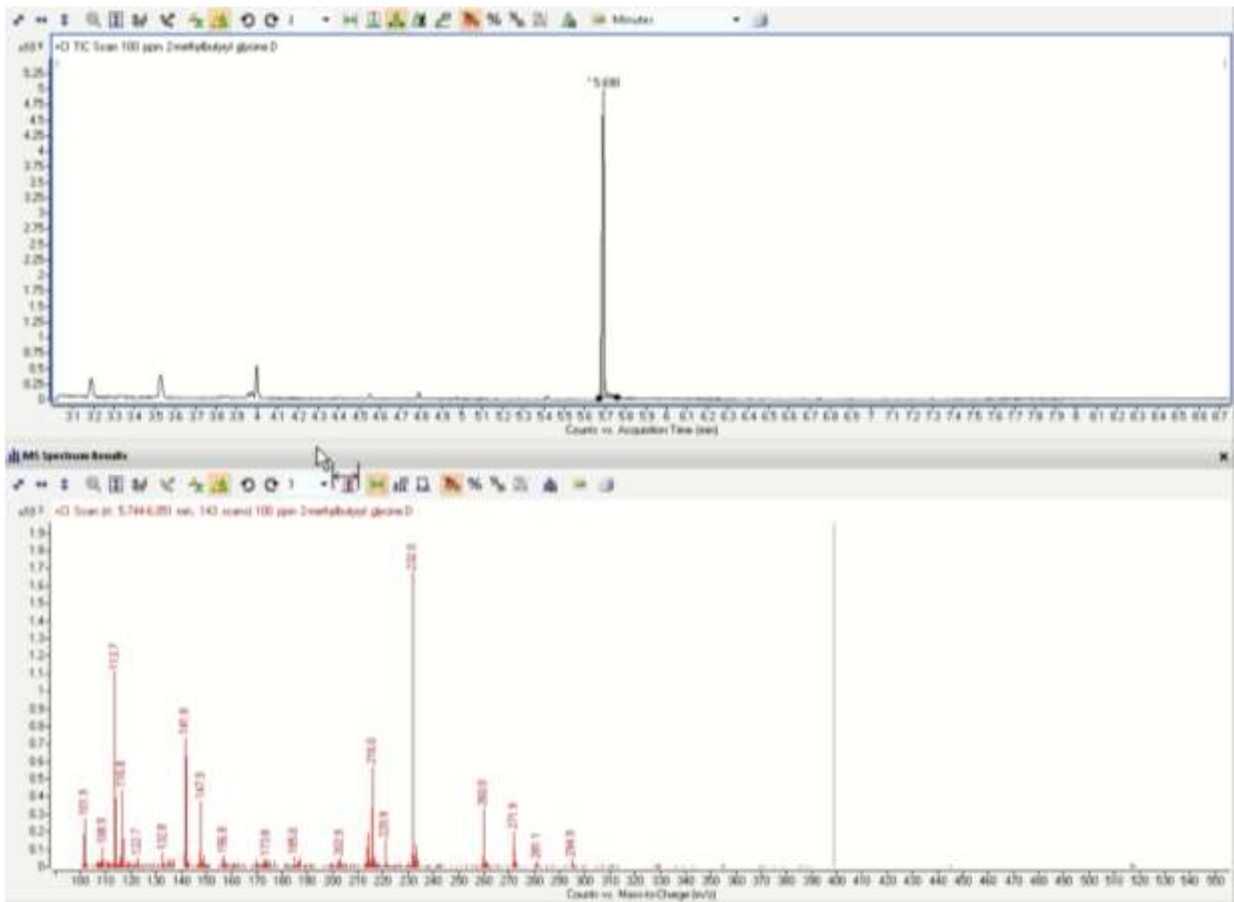


Figure 4-6: Chromatogram and mass spectrum of 2-methylbutyryl glycine

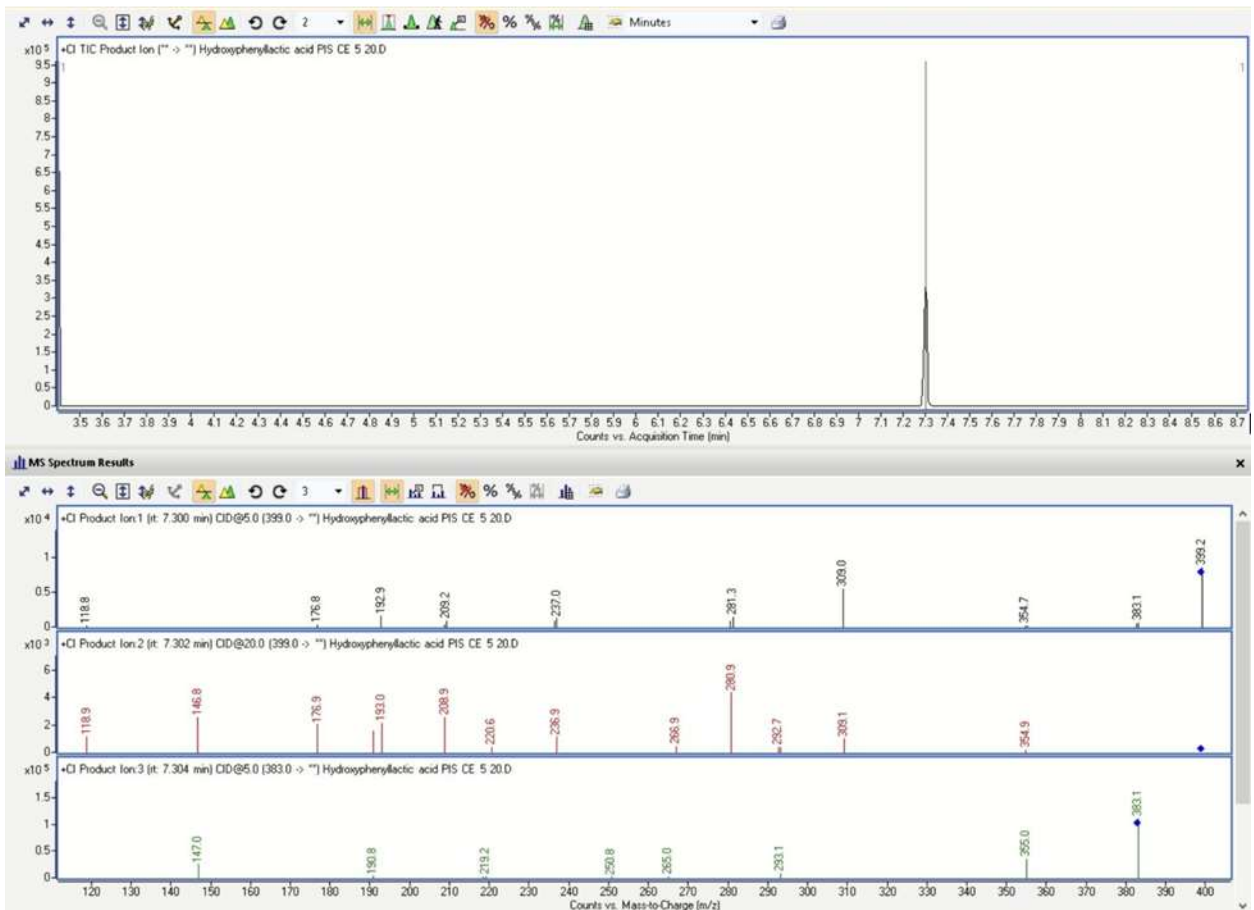


Figure 4-7: Chromatogram and product ion mass spectra of 4-hydroxyphenylpyruvate at different collision energies and while using CI.

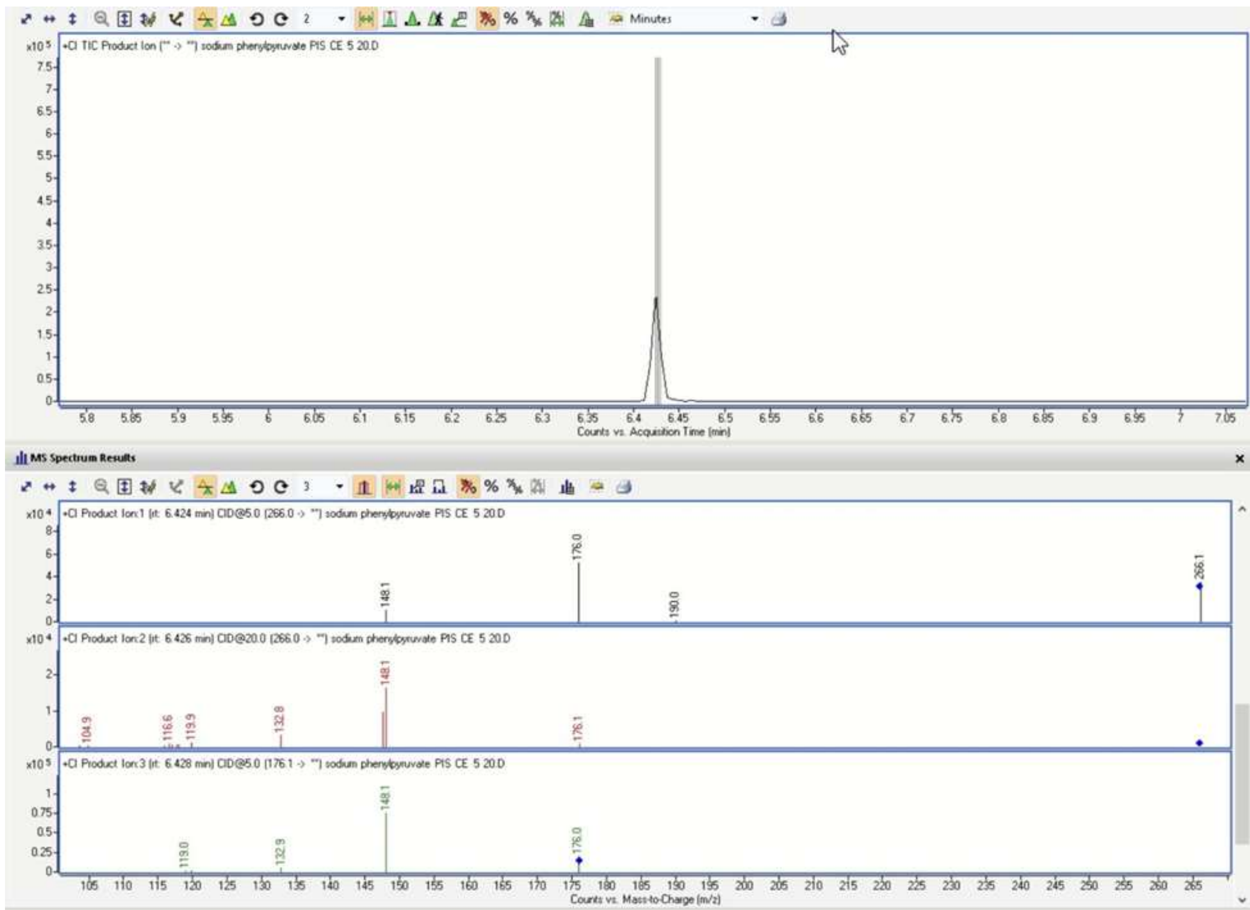


Figure 4-8: Chromatogram and product ion mass spectrum of phenylpyruvate at different collision energies and while using CI

Table 4-1: Optimized dMRM transitions and retention times when using CI.

Compound name	Precursor Ion (m/z)	Product ion (m/z)	Retention time (min)	Average dwell time (ms)	CE Voltage (eV)
3-methylcrotonate (quan)	173.1	157	5.09	2.8	6
3-methylcrotonate (qual)	157	112.5	5.09	2.8	12
Pyruvate (quan)	190.1	174	5.6	3.1	15
Pyruvate (qual)	190.1	114.8	5.6	2.3	20
Oxaloacetic acid (quan)	290	147	6.3	2.3	19
Oxaloacetic acid (qual)	378	229.7	6.3	2.3	15
3-Hydroxybutyric acid (quan)	233	147	6.6	2.7	20
3-Hydroxybutyric acid (qual)	249.1	117	6.6	2.7	17
Hydroxisobutyrate (quan)	232.9	147.1	6.6	2.1	15
Hydroxisobutyrate (qual)	249.3	159.1	6.6	2.1	10
Lactic acid (qual)	235	147	7.03	3.6	10
Lactic acid (quan)	191	147.1	7.03	3.6	5
4-methyloxovaleric acid (quan)	141.8	72.8	7.2	2	5
4-methyloxovaleric acid (qual)	232	142	7.2	2	10
Isovaleryl glycine	232.1	142.1	7.2	2	5
Succinate (quan)	247	147	8.1	2.4	17
Succinate (qual)	236	173	8.1	2.4	10
Fumaric acid (quan)	261.1	171	8.5	2.4	12
Fumaric acid(qual)	261.1	245	8.5	2.4	12
3-Methylglutaconate (quan)	273	147	9.4	4	20
3-Methylglutaconate (qual)	289	199.1	9.4	4	5
3-Phenylbutyric acid	237	105	9.5	2.5	10
2-Ketoglutaric acid (quan)	304	147	10	3.2	20

2-Ketoglutaric acid (qual)	320	202	10	3.2	12
Malate (quan)	335	147	10	3.2	15
Malate (qual)	351.1	233	10	3.2	10
3-Phenyllactic acid (quan)	193	72.9	11.05	5.3	10
3-Phenyllactic acid (qual)	310.9	192.9	11.05	5.3	5
Phenylpyruvate (quan)	175.9	148	11.05	5.3	5
Phenylpyruvate (qual)	266.3	175.9	11.05	5.3	5
3-Hydroxymethylglutarate (quan)	363	199	11.2	6.4	10
3-Hydroxymethylglutarate (qual)	199	109	11.2	6.4	10
Aconitic acid (quan)	375	147	12	5.2	23
Aconitic acid (qual)	391.1	211	12	5.2	15
Citrate (quan)	465.1	347	13.53	8.8	18
Citrate (qual)	481.1	363	13.53	8.8	12
4-Hydroxyphenylpyruvate (quan)	179	72.9	13.7	13.5	10
4-Hydroxyphenylpyruvate (qual)	354.1	179.1	13.7	13.5	5

4.2 MRM transitions for electron impact (EI) ionization

Table 4-2 show the precursor-product ion pairs (MRM transitions) optimized for each of the target organic acids when using EI. As mentioned, two transitions per compound were selected. Due to inherent limitations of using EI-MS/MS, only a conventional MRM method was set up instead of a dMRM method. EI is considered a hard ionization technique. Consequently, the small, common occurring fragment ions (masses) were abundant with larger ions at very low intensity. The low intensity, high mass precursor ions were selected as qualifiers while the high intensity, low mass precursors were used as quantifiers as seen in Figure 4-9. Unfortunately, the low specificity of the quantifiers hindered quantification as many other organic acids produced the same precursor-product ions and were thus detected, including organic acids not part of our target list.

Table 4-2: Optimized MRM transitions and retention times when using EI.

Compound name	**Precursor Ion (m/z)**	**Product ion (m/z)**	**Dwell time (ms)**	**CE Voltage (eV)**
3-3dimethylacrylic acid (Qual)	171.9	156.1	20	5
3-3dimethylacrylic acid (Quan)	156.1	82	20	5
3-Hydroxy-3-methylglutaric acid	247.2	115.1	20	10
Pyruvate (quan)	131.2	73.1	20	6
Pyruvate (qual)	217	131.5	20	8
3-Hydroxybutyric acid (qual)	191	147	20	8
3-Hydroxybutyric acid (quan)	147	72.8	20	5
Hydroxisobutyrate (quan)	147.7	73	20	10
Hydroxisobutyrate (qual)	233.2	147.7	20	6
Lactic acid (qual)	219	75.1	20	10
Lactic acid (quan)	75.1	72	20	10

4-methyloxovaleric acid (quan)	82	74	20	7
Fumaric acid	245	147.2	20	10
3-Methylglutaconate (quan)	170.1	127.1	20	6
3-Methylglutaconate (qual)	288.2	170.1	20	5
Malate (quan)	147.1	731.	20	8
Malate (qual)	335	147.1	20	10
3-Phenyllactic acid (quan)	149	85	20	10
3-Phenyllactic acid (qual)	222	149	20	10
3-methyl-2-oxobutyrte (Quan)	157.8	72.9	20	8
3-methyl-2-oxobutyrte (Quan)	185.8	157.8	20	10
3-Hydroxymethylglutarate (quan)	115.1	72	20	8
Aconitic acid (quan)	211	147	20	10
Aconitic acid (qual)	391.1	211	20	10
Citrate (quan)	273.2	183.1	20	5
Citrate (qual)	347.1	273.2	20	7
4-Hydroxyphenylpyruvate (qual)	267.2	192.8	20	10
4-Hydroxyphenylpyruvate (quan)	192.8	73	20	7
4-Hydroxyphenyllactic acid	307.5	178.8	20	10
Isovaleryl glycine (Qual)	216.2	85	20	10
Isovaleryl glycine (Quan)	85	53	20	10
4-methyloxovaleric acid	202	82	20	7
Hydroxyphenyllactic acid (Quan)	178.8	73.1	20	10

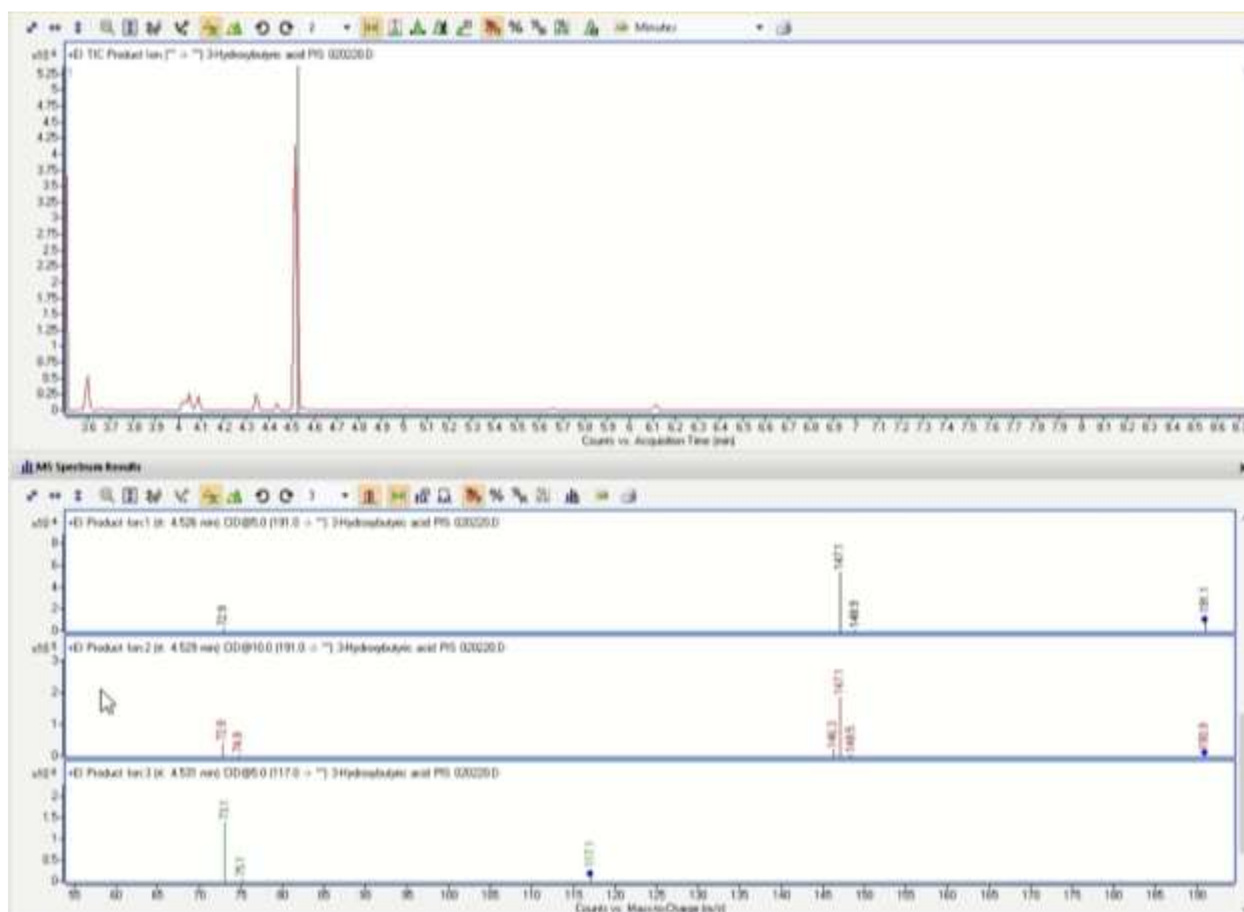


Figure 4-9: Chromatogram and product ion mass spectrum of 3-hydroxybutyric acid at different collision energies and while using EI.

4.3 Optimizing chromatographic separation

The oven temperature gradient was adapted to allow baseline separation of most organic acids (Figure 4-10e). The rate at which the oven temperature raised from 70 – 300 ˚C were adapted to allow better separation of most organic acids but without increasing the run time of this routine, targeted method too much (which would defeat the purpose of high-throughput analysis). The optimized gradient is shown in Figure 3-1.

Moreover, as seen in Figure 4-10, many peaks were detected despite the targeted nature of the optimized EI method. This clearly indicates the unspecific detection of compounds with the transitions set up in EI mode. Hence, despite using a targeted approach, the data generated were similar to untargeted (scan) data which meant that

one had to identify and quantify target organic acids with data analysis software. Again, this defeats the purpose of high-throughput, routine quantitation).

4.4 Optimizing flow rate

As mentioned, one of the problems identified while testing the MRM detection methods was the limited number of data points over a peak. Very thin peaks often contained only three data points which created a distorted triangle shaped peak. Integration of such a peak area gave inaccurate quantitation. To circumvent this problem, less MRM transitions were tested as well as slower chromatographic flow rates (shown in Figure 4-10). Slower flow rates give broader peaks which could provide more data points across the peak. As a rule of thumb an optimal number of data points would be 12 - 20.

As shown in Figure 4-10, peak separation was not severely influenced when the flow rate of the carrier gas (helium) was lowered from 1.8 ml/min to 1 ml/min. However, the lower flow did not provide much broader peaks and this not much improvement in quantitation. This will be shown and discussed in Section 4.6. Another way to broaden peaks which however was not explored, is the lower column length. Instead of using a 30 m column, one can use a 5 – 10 min column.

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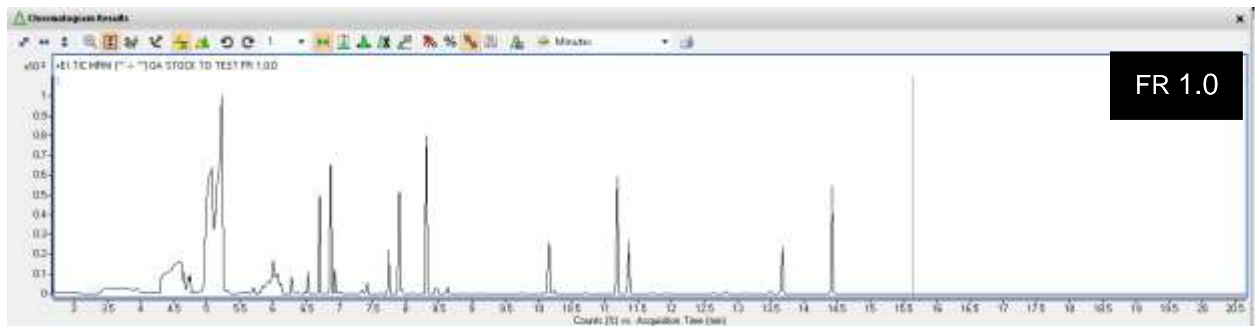
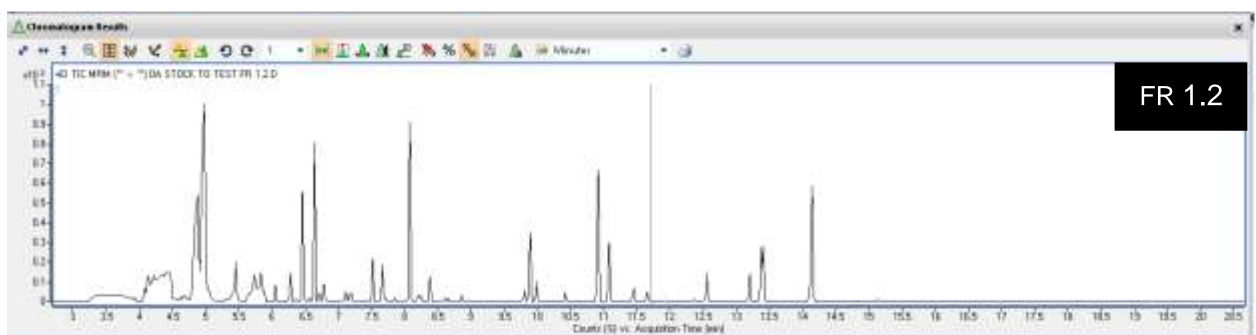
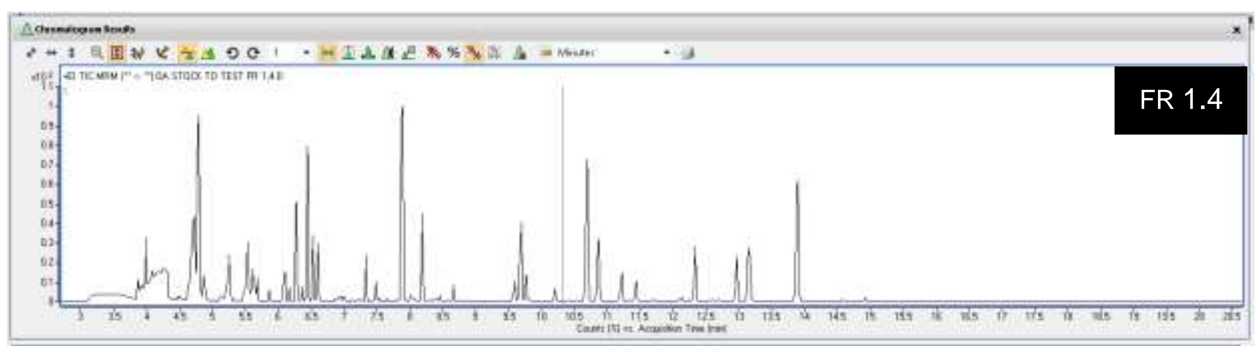


b)



c)



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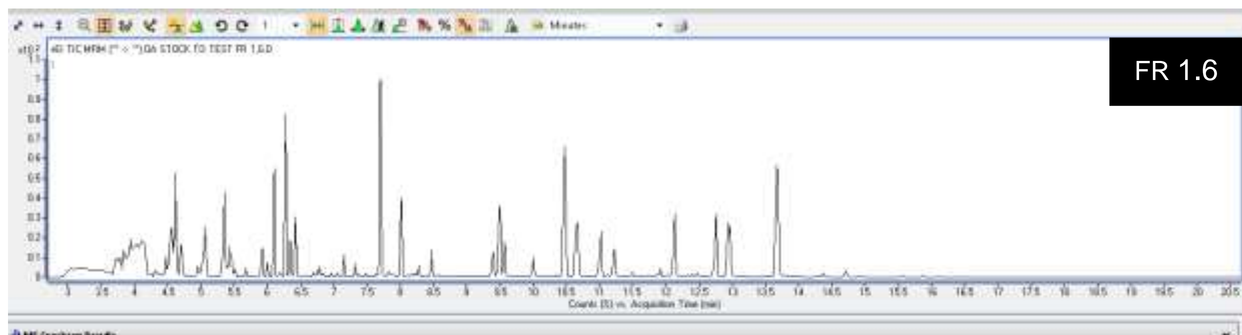

e)

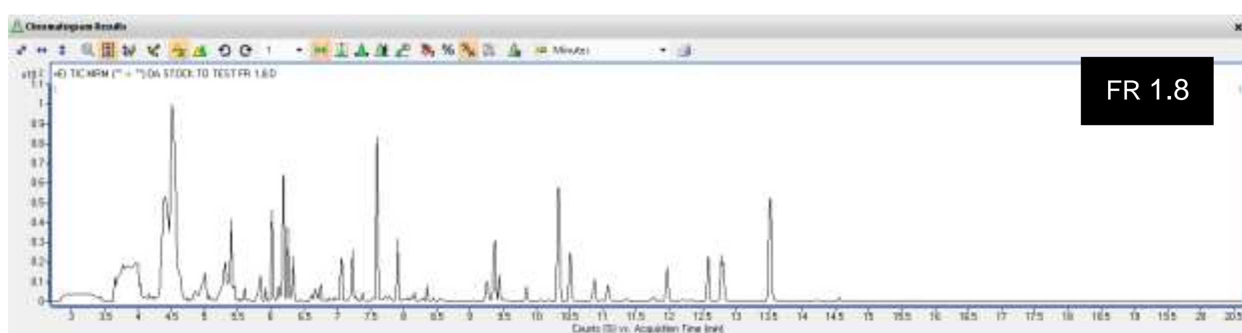

Figure 4-10: Chromatograms at varying flow rates.

4.5 Optimizing extractions

The abundance of the organic acids extracted with the standard method (single phase, modified Bligh-Dyer) commonly used at our institution were higher (Figure 4-12) compared to those extracted with the acidic method (Figure 4-11). From the above results it was concluded that the standard method would be used in conjunction with the optimized MRMs to characterize and implement the method in biological samples

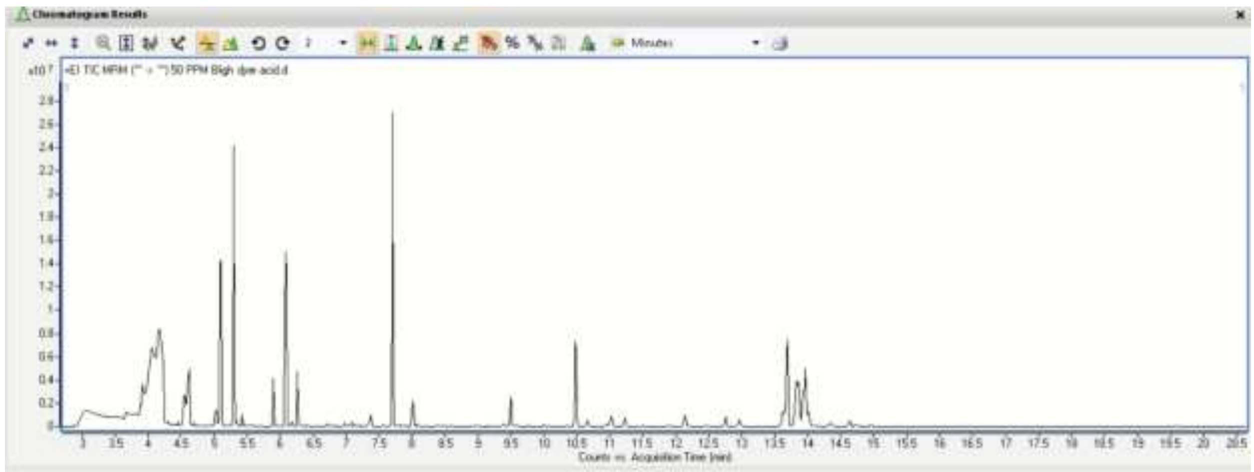

Figure 4-11: Chromatogram of organic acids extracted with acidic method.

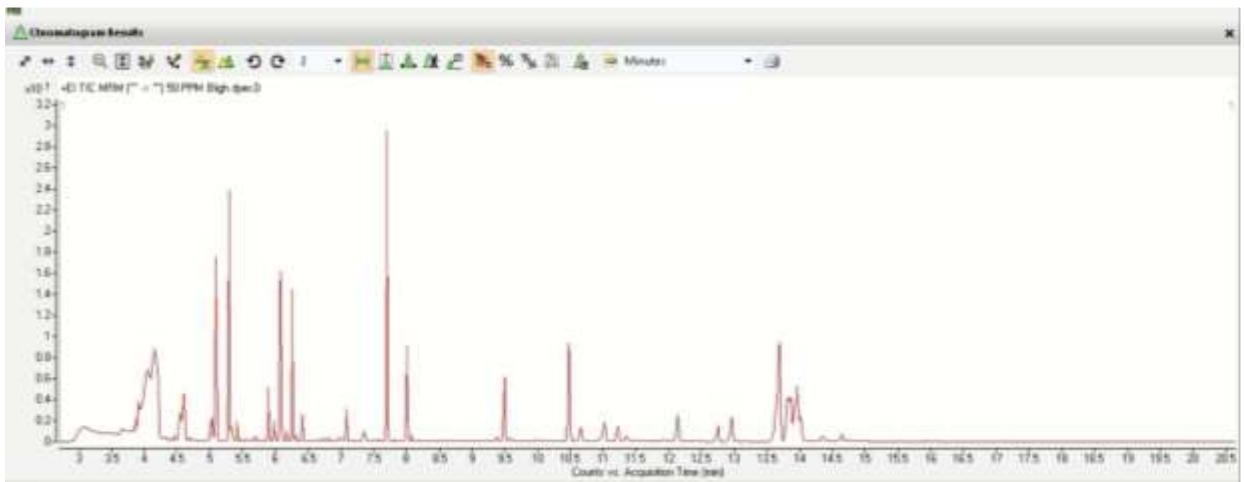

Figure 4-12: Chromatogram of organic acids extracted with standard method.

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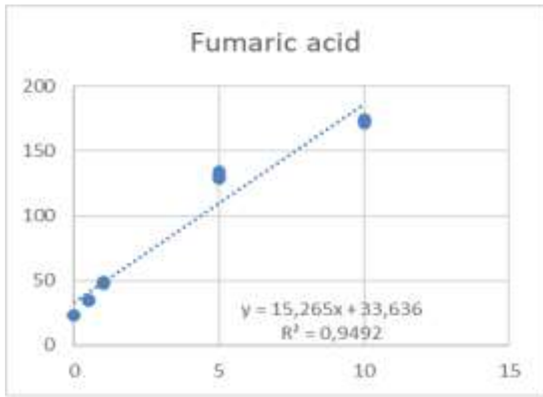
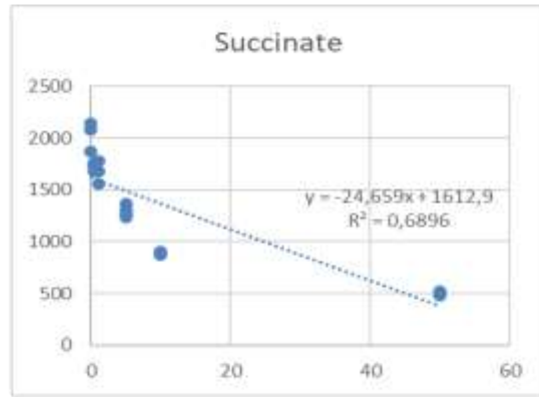
4.6 Performance characteristics of the standardized CI method

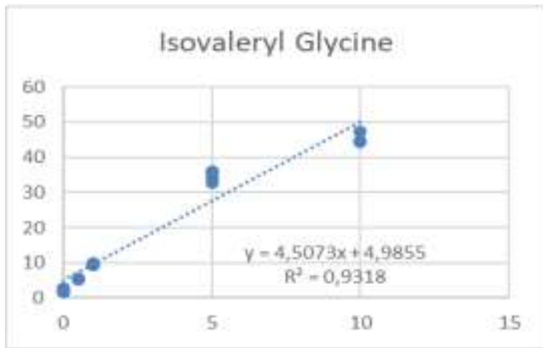
The calibration curve of each target organic acid is shown in Figure 4-13, with method performance characteristics listed in Table 4-3. The characteristics of the CI and EI methods will be discussed together in the following section

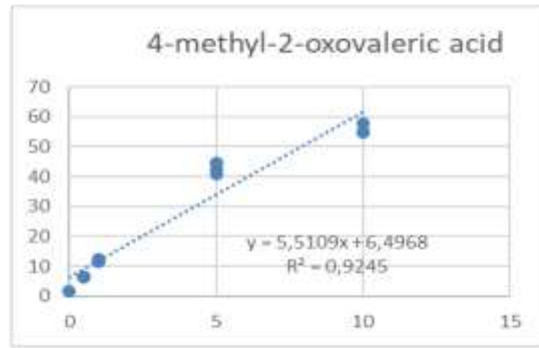
Table 4-3: Analytical performance parameters for GC-MS/MS method using CI.

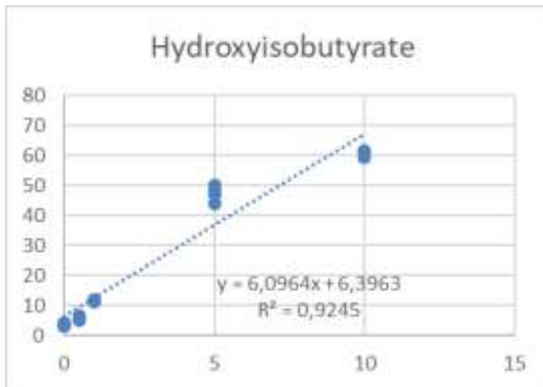
Compounds	R^2	Linearity (ppm)	LOD (ppm)	LOQ (ppm)
2-Ketoglutaric acid	0,964	0-50	2,45	36,14
3-Hydroxy-3-methylglutaric acid	0,919	0 - 50	8,27	12,68
3-Hydroxybutryric acid	0,924	0 - 50	6,18	30,54
3-Methylglutaconic acid	0,983	0 - 50	12,12	170,24
3-Phenyllactic acid	0,939	0 - 50	5,30	17,38
3-Methylcrotonate	0,386	0-50	21,89	661,58
4-Methyl-2-valeric acid	0,925	0 - 50	6,81	16,35
4-Hyrdroxyphenylpyruvic acid	0,822	0 - 50	5,02	38,02
Aconitic acid	0,970	0 - 50	2,29	17,73
Citric acid	0,970	0 - 50	1,90	29,36
Fumarate	0,939	0-50	3,14	3,14
Isovaleryl glycine	0,931	0-50	6,19	6,19
Lactic acid	0,260	0 - 50	205,56	2592,87
Malic acid	0,652	0 - 50	26,71	836,16
Oxaloacetic acid	0,637	0-50	51,89	1007,10
Pyruvic acid	0,850	0 - 50	3,66	144,60
Phenylpyrute	0,956	0-50	3,14	25,95
Succinate	0,670	0-50	34,41	938,54

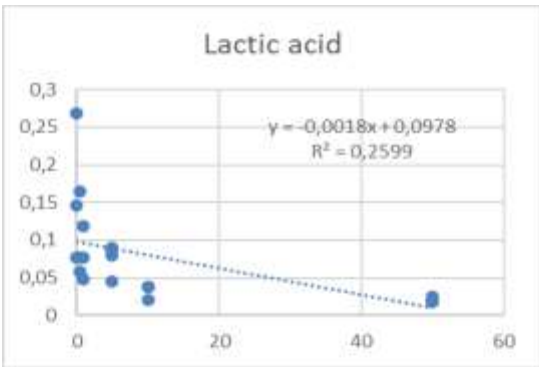


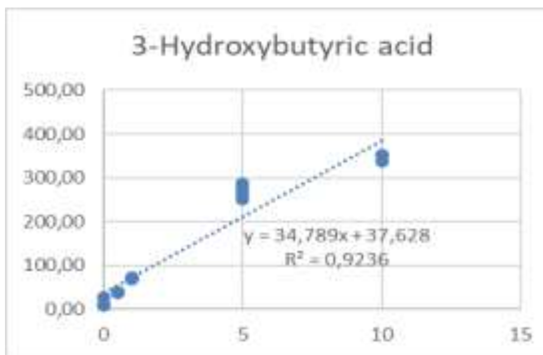


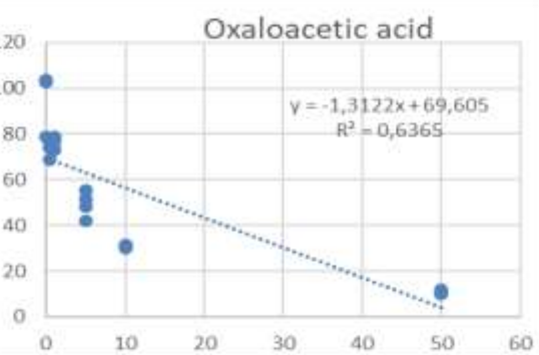








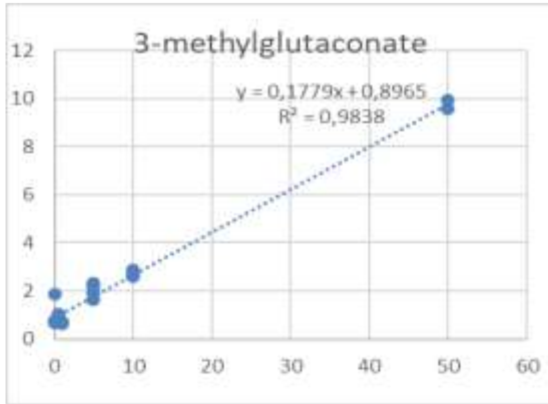


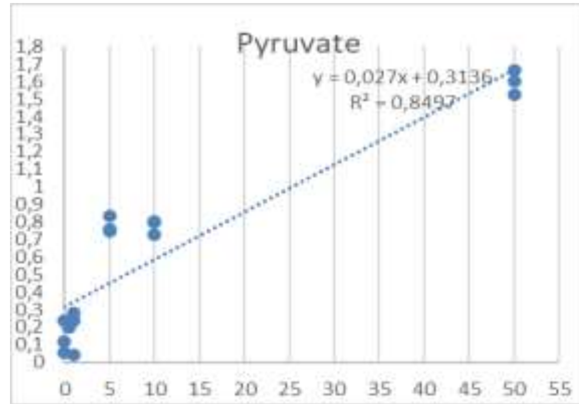


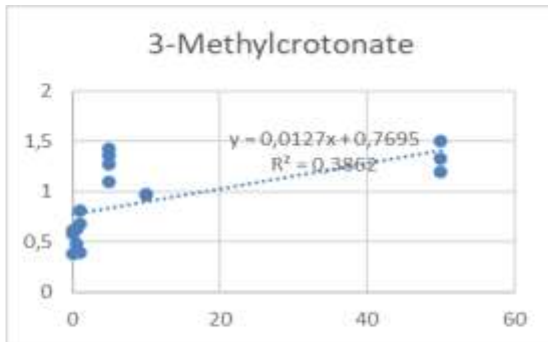
Y-axis is Normalized peak area

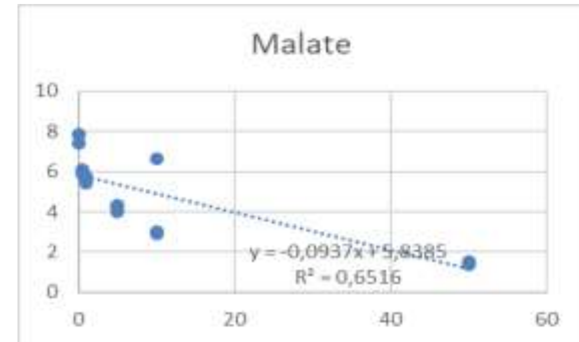
X-axis is Concentration (µg/ml)

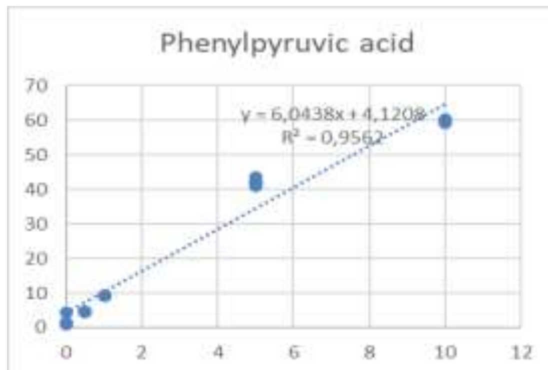
Y-axis is Normalized peak area

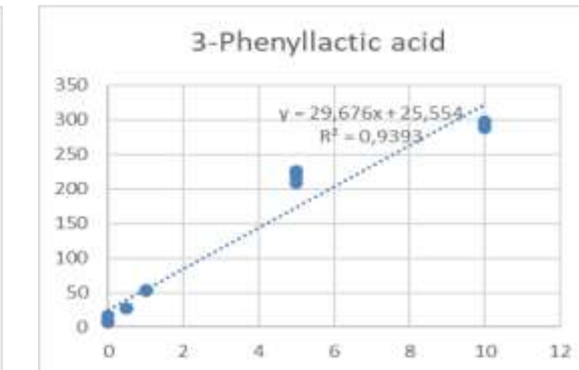


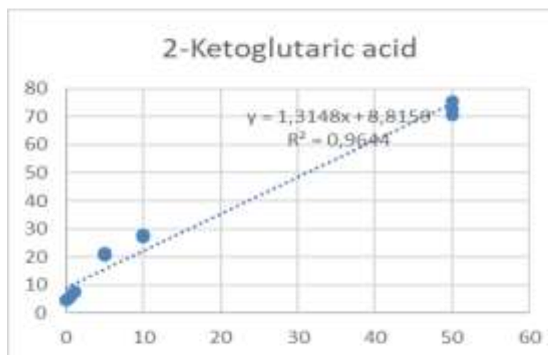


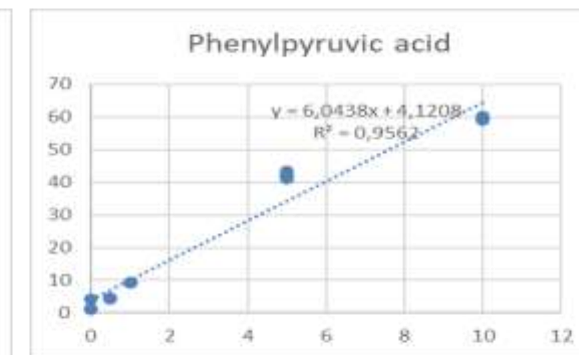






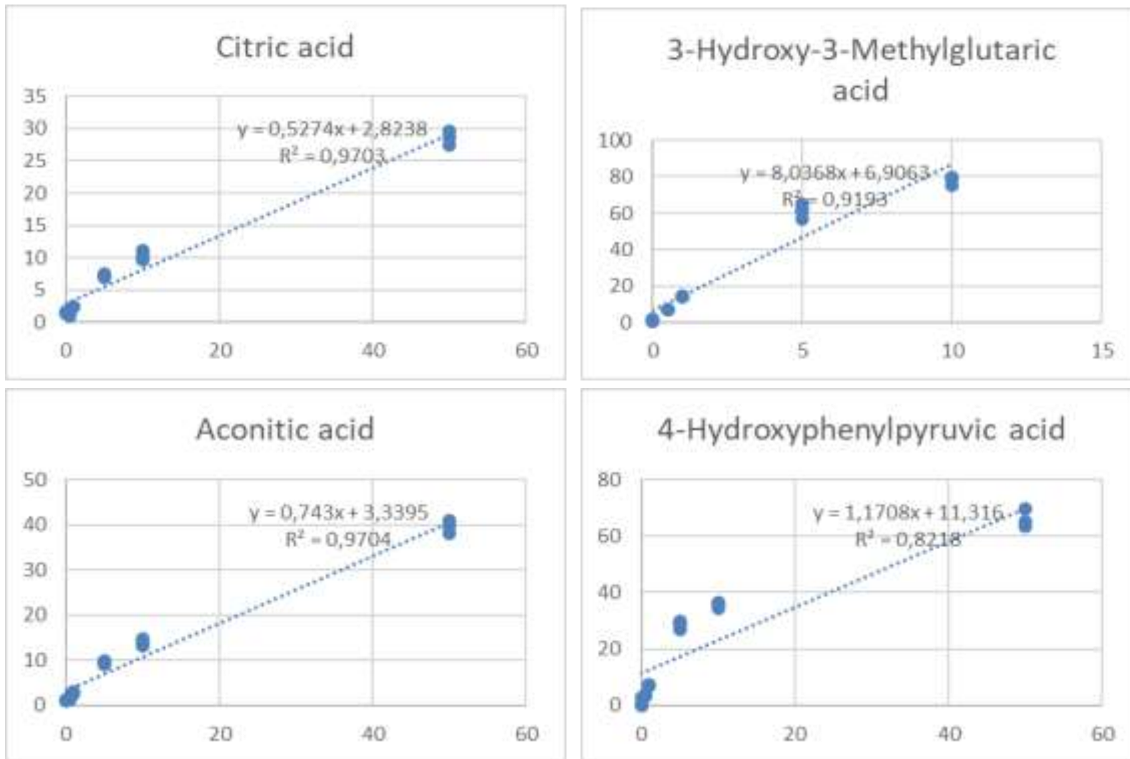






X-axis is Concentration (µg/ml)

Y-axis is Normalized peak area



X-axis is Concentration (µg/ml)

Figure 4-13: Calibration curves for target organic acids detected with GC-MS/MS using CI

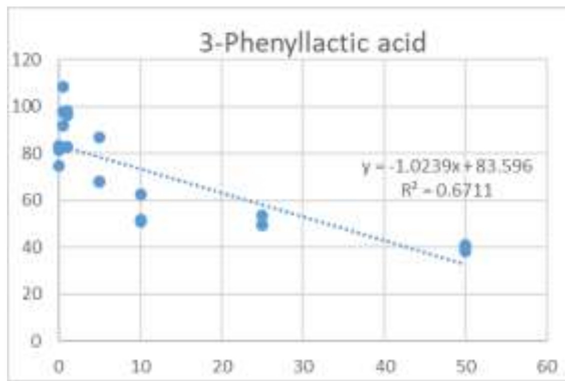
4.7 Performance characteristics of the standardized EI method

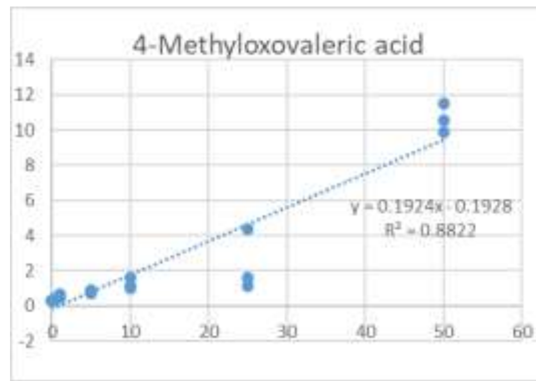
The calibration curve of each target organic acid is shown in Figure 4-14, with method performance characteristics listed in Table 4-4. The characteristics of the CI and EI methods will be discussed together in the following section.

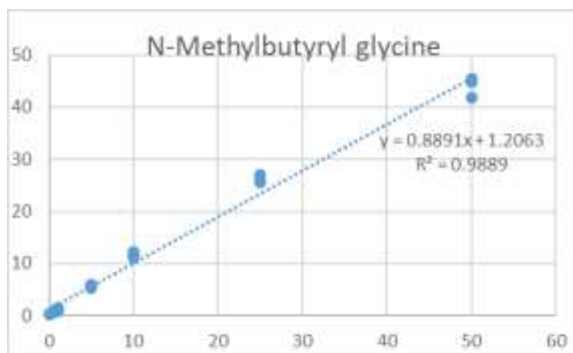
Table 4-4: Analytical performance parameters for GC-MS/MS method using EI.

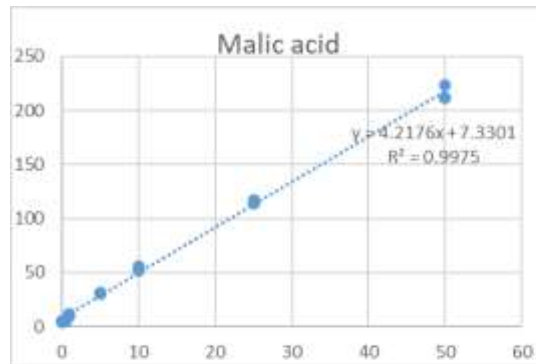
Compounds detected by EI	R^2	Linearity (ppm)	LOD (ppm)	LOQ (ppm)
3,3-Dimethylacrylic acid	0,987	0 - 50	0,30	13,35
3-Hydroxy-3-methylglutaric acid	0,987	0 - 50	1,21	18,11
3-Hydroxybutryric acid	0,184	0 - 50	76,89	1356,13
3-Methylglutaconic acid	0,627	0 - 50	26,47	846,00
3-Phenyllactic acid	0,671	0 - 50	9,03	829,34
4-Methyloxovaleric acid	0,882	0 - 50	0,49	13,92
4-Hyrdroxyphenylpyruvic acid	0,948	0 - 50	2,70	16,73
Aconitic acid	0,990	0 - 50	0,11	2,63
Citric acid	0,935	0 - 50	1,25	5,45
4-Hydroxyphenyllactic acid	0,941	0 - 50	3,42	4,07
Lactic acid	0,435	0 - 50	14,63	27,22
Hydroxyisobutyric acid	0,321	0 - 50	9,32	494,45
Isovaleryl glycine	0,989	0 - 50	1,10	1,20
Malic acid	0,998	0 - 50	0,21	0,63
Pyruvic acid	0,278	0 - 50	21,66	170,17

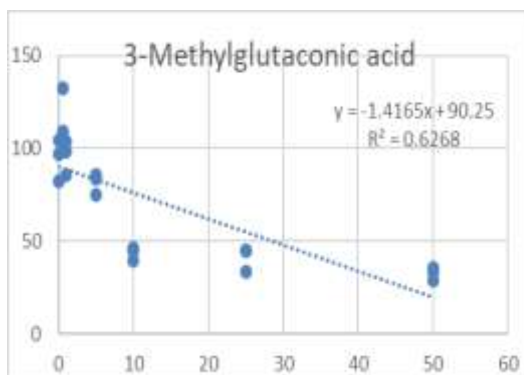
Y-axis is Normalized peak area

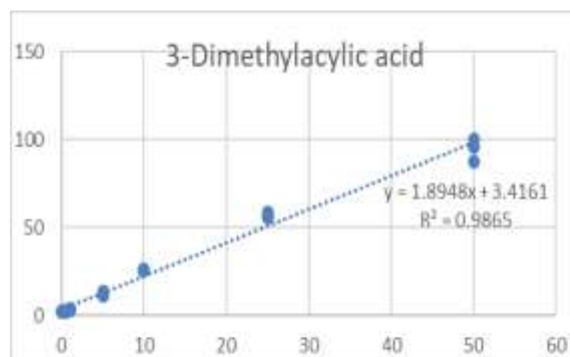


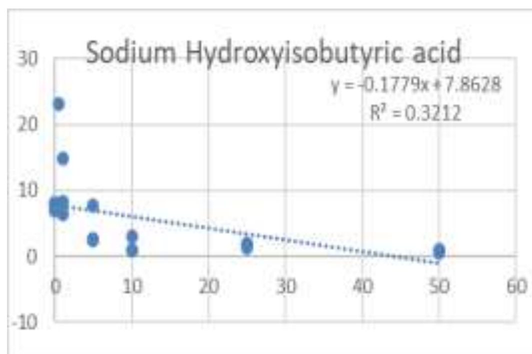


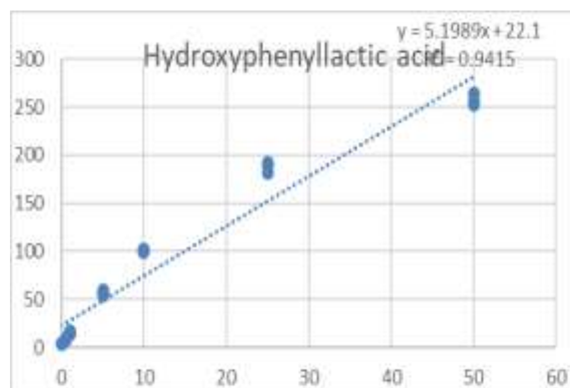




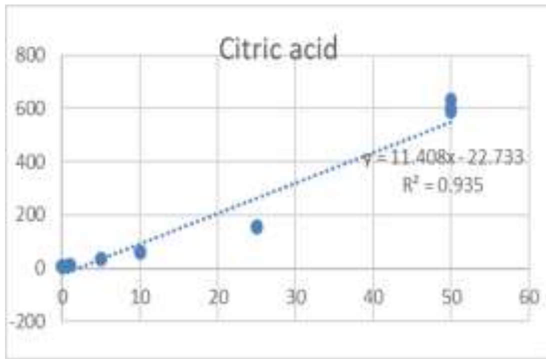


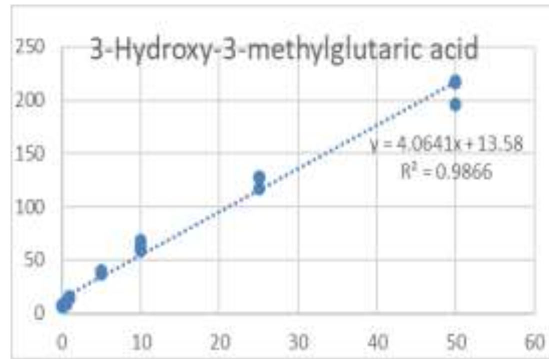




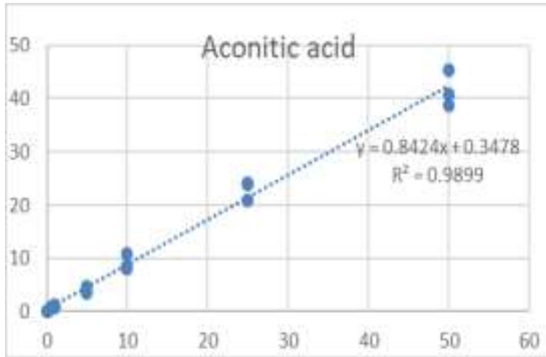


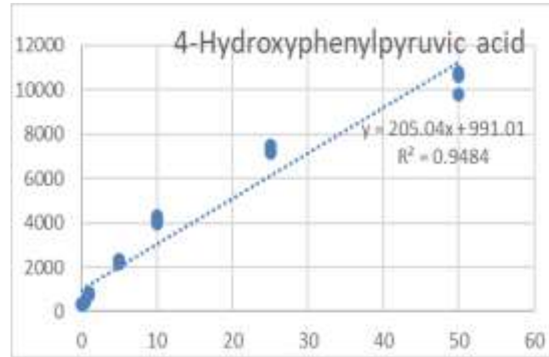
X-axis is Concentration (µg/ml)

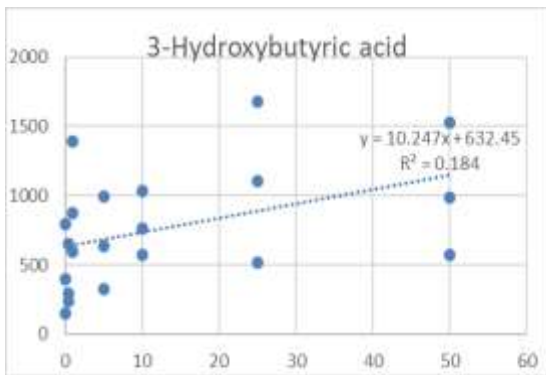


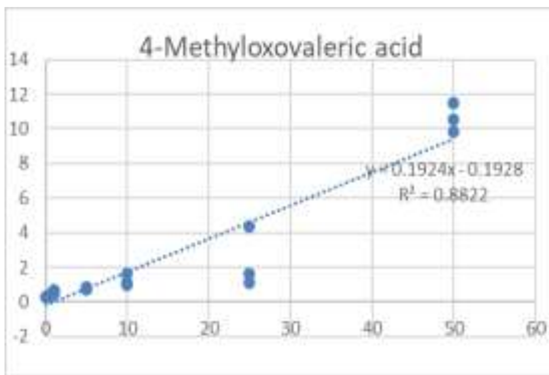


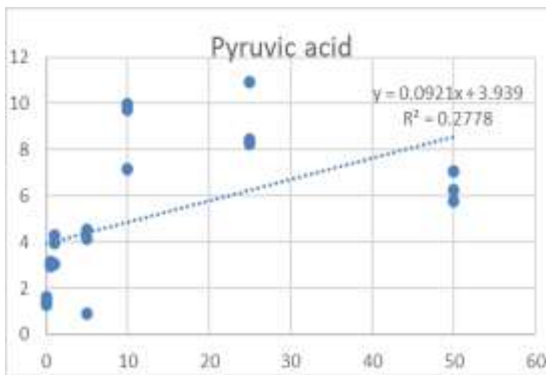
Y-axis is Normalized peak area

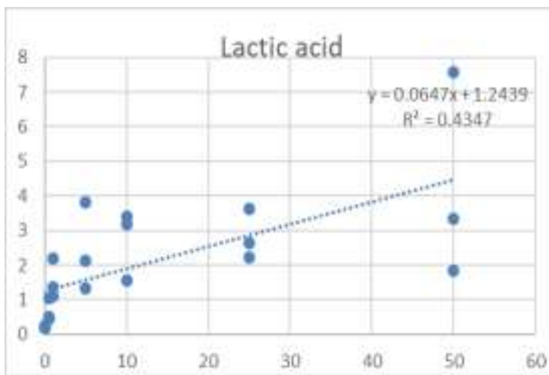












X-axis is Concentration (µg/ml)

Figure 4-14: Calibration curves for target organic acid detected with GC-MS/MS using EI

The CI MRM method provided reasonable linearity across the concentration range with most organic acids giving a R^2 > 0.9, as seen in Figure 4-13 and Table 4-3. However certain organic acids such as succinate, malate, lactic acid and oxaloacetate show a decline in their curves, which could possibly be due to poor ionization from CI, poor extraction or compound degradation. For example, it is known that oxaloacetate can spontaneously decarboxylate within 7 hours after sample collection / preparation. However, these remain speculations that require further testing. The LOD and LOQs (determined from the curves) are relatively high which emphasizes the issue of sensitivity with this method and CI in general. Since organic acids are found in low concentrations in abalone, it is unlikely that the standardized CI method would be able to detect and quantify selected organic acids sufficiently.

Overall, the EI method gave an unsatisfactory response over the concentration range with only a few compounds having a R^2 > 0.9. Organic acids such as 3-hydroxybutyric acid, pyruvic acid and lactic acid also showed low precision at the different concentrations. One of the reasons for this is the low specificity of the EI quantitative transitions which detect inferring compounds/ peaks leading to inaccurate peak areas for each concentration. Moreover, certain organic acids could not be detected accurately and are therefore missing in Table 4-4 and Figure 4-14. These are 2-ketoglutartate, 3-methylcrotonate, 4-methyl-2-valeric acid, fumarate, succinate and oxaloacetic acid. Whether metabolite extraction could be blamed for this poor response is open for investigation albeit poor results were also found when extraction is omitted, and "clean" standards are analyzed.

One of the true differences between the CI and EI method is seen in the detection and quantification of malic acid. With the CI method, malic acid did not show a proper linear response with the concentration range while the EI method produced good results (decent linearity, and low LOD and LOQ). However, specificity of the EI method remains poor as malic acid, 3-hydroxy-butyrate and hydroxy-isobutyrate are detected with the same transition (147 → 73 m/z) which makes automation difficult if one must confirm peak integration and identity after every analysis. Therefore, because organic acids often share similar structural properties, it can be noted that CI are a more selective method with the production of intact molecular ions (used for 3-methylcrotonate, 4-methyloxovaleric acid, hydroxyisobutyrate, isovaleryl glycine, 3-

phenyllactic acid, phenylpyruvate, 4-hydroxyphenylpyruvate, lactic acid, hydroxyphenylactic acid, 3-phenyllactic acid and 4-methyl-2-oxovaleric acid).

4.8 Challenges

Evidently the optimized detection methods did not satisfy ISO17025 performance criteria and would not provide accurate quantification of organic acids in abalone tissue. Apart from the abovementioned results, we also experienced many other challenges with the CI source and implementation thereof worth noting. The sensitivity of the chemical ionization dropped significantly over a short period of time due to the source chamber getting dirty. This meant that the source had to be cleaned frequently, which is clearly not ideal for high-throughput analysis. Moreover, the sensitivity drop-off after a cleaning session was also severe which often affects the detection in a single batch. This contributed to the high LOD and LOQ reported above.

As mentioned, the main limitation of EI is the production of small fragments that are not suitable for MS/MS (especially w.r.t. specificity). CID of EI fragments often result in even smaller fragments below our mass detection window of 50 m/z. Since EI MRMs did not show improved specificity compared to conventional SIM methods, its use as detection method is questionable.

Traditionally, tandem MS has been associated with LC and not GC. Since peaks are usually broader in LC analysis compared to GC, one hardly struggles with finding enough data points over a peak (or detection cycles per compound). The hyphenation of tandem MS with GC thus introduced another challenge which was the low number of data points over a chromatographic peak, which complicated accurate peak integration and ultimately quantification. This means that either broader peaks are needed, or multiple methods should be set up which detect only a small number of compounds to limit the number of MRMs.

Finally, one of the main challenges as frontier users of this technology is the lack of support from vendors and peers, especially since this technology is relatively new. A very limited number of papers are published where this technology has been used;

and when it is used, it is rather used as an exploratory system which is able to fragment unknown compounds further for structural elucidation.

CHAPTER 5 CONCLUSIONS

The aim of this study was to develop a GC-MS/MS organic acid profiling method for abalone muscle samples. MRM transitions for the detection of selected derivatized organic acids were successfully optimized for both CI and EI. The chromatographic separation of the target organic acids was also successfully optimized to allow baseline separation of most compounds. Matrix appropriate external calibrators were successfully created to characterize the new method and quantify organic acids. However, the performance characteristics of the CI and EI methods were unsatisfactory and could not be implemented to study the organic acid levels in abalone exposed to hypoxia. The method performance of the GC-MS/MS method fitted with CI is more satisfactory than EI, but day-to-day challenges with the CI source hinders further optimization and use in the routine lab.

5.1 Future Prospects

Derivatization is necessary for the analysis of polar, non-volatile metabolites (organic acids) with GC-MS. Alternative derivatization methods could improve EI MRM detection and linearity of organic acids and should be considered in future studies. For example, tert-Butyldimethylsilyl (tBDMS)derivatisation provides larger derivatives with a more intense fragment ion of [M-57] which has the same level of specificity than the molecular ion due to the higher masses of tBDMS derivatives.

The use of LC-MS/MS can also be explored for the detection and quantification of organic acids in abalone, especially as derivatives which could bridge the sensitivity problem of negative ionization.

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