Carnitine conjugation profiling in a selected cohort of patients with chronic fatigue syndrome

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ABSTRACT

Chronic fatigue syndrome (CFS) is classified by the World Health Organisation (WHO) as a non-communicable disease. Fatigue is a symptom commonly experienced by many individuals and is also a symptom associated with a wide variety of diseases, but once this fatigue becomes long lasting, persistent and debilitating, a case of CFS is considered. Research of CFS dates back to the nineteen hundreds, but unfortunately, no definite underlying cause or one single positive treatment has been identified. Diagnosis also poses a difficult task due to different criteria available, but also because of the lack in confidence of diagnosing doctors in making a positive diagnosis, because this disease is still poorly understood.

Recent studies and research found promising evidence that mitochondrial dysfunction may be considered as a possible underlying cause of CFS. Because mitochondria are responsible for the release of energy in cells, the connection between mitochondrial dysfunction and the underlying energy deficiency in CFS patients may indicate a good starting point for further investigation. L-carnitine plays an important role in energy metabolism and could possibly be used as potential biomarkers for energy related diseases such as CFS.

The first part of the study focused on method development and validation. A pre-existing high performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS) method coupled with electrospray ionisation (ESI) was further developed and validated to simultaneously quantify carnitine and acylcarnitines in human urine samples.

The second part of the study included application of the developed and validated method to urine samples of controls and possible CFS patients. All carnitines of interest could be detected and identified with this method, although the longer chain aclylcarnitines posed some difficulty. The aim of this study was to identify altered acylcarnitine profiles associated with possible CFS patients compared to control samples. At the end, principal component analysis (PCA) statistical analysis could not differentiate between the two groups, but two acylcarnitines were identified by the Mann Whitney test to have significant p-values, namely octanoylcarnitine (C8) and decanoylcarnitine (C10).

Although the method can be applied for acylcarnitine identification in urine samples, it is advised to pay attention to detecting the long chain acylcarnitines more efficiently in order to get the whole profile for comparison.

Key words: chronic fatigue syndrome; HPLC-MS/MS; carnitine; acylcarnitines; urine; mitochondria.

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"I can do all things through Christ who strengthens me"

Philippians 4:13

LIST OF ABBREVIATIONS, SYMBOLS AND UNITS

Symbols and units:

% – Percentage

°C – Degrees Celsius

µL – microliter

µmol/L – micromole per litre

µm – micrometre

g/mol – grams per mole

L/min – litre per minute

mL – millilitre

psi – Pound-force per square inch (pressure resulting from a force of one pound-force applied to an area of one square inch)

R^2 – correlation coefficient

V – volt

v/v – volume/volume (expressed as percentage)

Abbreviations:

ACN – Acetonitrile

AIDS – Acquired Immunodeficiency Syndrome

ATP – Adenosine Triphosphate

BOSS – Biotransformation and Oxidative Stress Status

C0 – free carnitine / L-carnitine

C2 – Acetylcarnitine

C3 – Propionylcarnitine

C4 – Butyrylcarnitine

C5 – Isovalerylcarnitine

C6 – Hexanoylcarnitne

C8 – Octanoylcarnitine

C10 – Decanoylcarnitine

C12 – Dodecanoylcarnitine

C14 – Tetradecanoylcarnitine

C16 – Palmitoylcarnitine

C18 – Octadecanoylcarnitine

CACT – Carnitine-acylcarnitine translocase

CAT – Carnitine acyltransferase

CBT – Cognitive behavioural therapy

CDC – Centre for Disease Control and Prevention

CE – Capillary electrophoresis

CE – Collision energy

CF – Chronic fatigue

CFS – Chronic fatigue syndrome

CFS/ME – Chronic fatigue syndrome / Myalgic encephalomyelitis

CNS – Central nervous system

CoA – Coenzyme A

CPT I – Carnitine palmitoyltransferase I

CPT II – Carnitine palmitoyltransferase II

CV – Coefficient of variation

ESI – Electrospray ionisation

FA – Formic acid

FDA – The USA Food and Drug Administration

FSS – Fatigue severity scale

GC-MS – Gas chromatography mass spectrometry

GET – Graded exercise therapy

GP – General practitioner

HCl – Hydrochloric acid

HPA – Hypothalamic-pituitary-adrenal

HPLC – High performance liquid chromatography

HPLC-MS/MS – High performance liquid chromatography tandem mass spectrometry

IS – Isotope stock solution

LC-MS/MS – Liquid chromatography tandem mass spectrometry

LLOQ – Lower limit of quantification

LOD – Limit of detection

Log-p – logarithm of partition coefficient

LOQ – Limit of quantification

m/z – Mass to charge

MCAD – Medium-chain Acyl-CoA dehydrogenase

ME – Myalgic encephalomyelitis

MRM – Multiple Reaction Monitoring

MS – Multiple sclerosis

MS/MS – Tandem mass spectrometry

MSQ – Medical symptoms questionnaire

NADH – Nicotinamide adenine dinucleotide

NCDs – Noncommunicable diseases

P – Partition coefficient

PCA – Principle Component Analysis

PCs – Principal components

PFS – Piper fatigue scale

POTS – Post orthostatic tachycardia syndrome

QC – Quality control

ROS – Reactive oxygen species

RSD – Residual standard deviation

TB – Tuberculosis

TIC – Total ion chromatogram

ULOQ – Upper limit of quantification

UPLC-MS/MS – Ultra high performance liquid chromatography tandem mass spectrometry

UV – Ultra violet

WHO – World Health Organisation

β-oxidation – Beta oxidation

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CHAPTER 1

1. INTRODUCTION

1.1 Introduction

Chronic, noncommunicable diseases (NCDs) are by definition non-transmissible and non-infectious medical conditions or diseases amongst people. According to the World Health Organization (WHO) (Alwan, 2011), NCDs are the leading cause of death worldwide, and ever increasing mortality from these diseases remain unacceptably high (Riley & Cowan, 2014). Present research is mostly focused on the diagnosis, etiology and treatment for these types of diseases. One disease known as chronic fatigue syndrome or myalgic encephalomyelitis (CFS/ME) is a vast topic of discussion in many articles. Fatigue is a common symptom experienced by many individuals, but once the fatigue becomes persistent and debilitating, a case of CFS/ME is considered (Afari & Buchwald, 2003). CFS/ME is a debilitating multisystem condition characterized by severe and incapacitating fatigue along with other symptoms including myalgia, muscle weakness and post-exertional malaise (Holmes *et al.*, 1988; Fukuda *et al.*, 1994; Smith *et al.*, 2015). The underlying etiology of CFS/ME is still unknown (Klonoff, 1992; Kumar & Kumar, 2006; Reuter & Evans, 2011; Castro-Marrero *et al.*, 2017) and the absence of diagnostic markers, as well as other factors such as similarities between symptoms of CFS/ME and other ill-defined diseases and the vague description of diagnostic criteria, makes diagnosing this disease much more problematic (Afari & Buchwald, 2003). Thus far diagnosis was based mainly on information obtained directly from patients by means of clinical interviews and questionnaires, resulting in incredulity of the reliability of this diagnostic method.

1.2 Problem Statement and Substantiation

Currently, there are no biological markers identified or diagnostic tests developed specifically for diagnosing CFS/ME (Afari & Buchwald, 2003). In a study conducted by Horton *et al.* (2010) they confirmed that the general practitioner (GP) not familiar with this condition find it difficult to diagnose CFS/ME. Problems causing this difficulty include the acceptance of CFS/ME as a real condition, thus causing a lack of confidence in making the diagnosis, the limitation of knowledge about CFS/ME as well as the lack of a diagnostic tests makes diagnosis even more uncertain.

This limitation opens the field to investigate and develop new methods to diagnose CFS/ME more efficiently.

A possible starting point for method development would be to identify L-carnitine and its derivatives (acylcarnitines) as potential diagnostic markers because of the critical role they play in energy production. L-carnitines' main responsibility is the transportation of long-chain fatty acids into the mitochondria for energy production by means of beta oxidation (β-oxidation) (McGarry & Brown, 1997; Jones *et al.*, 2005; Reuter & Evans, 2012). Mitochondrial β-oxidation can theoretically be divided into two steps/phases: 1) transporting acyl groups into the mitochondria and 2) chain shortening inside the mitochondria (Bartlett & Eaton, 2004). β-oxidation is the process in which L-carnitine is esterified to form short-, medium- and long-chain acylcarnitine derivatives. Not only does L-carnitine play a significant role in mitochondrial energy production by transporting long-chain fatty acids into the mitochondria, but also in the regulation of the intramitochondrial coenzyme A (CoA)/acyl-CoA ratio (Kuratsune *et al.*, 1994; Reuter & Evans, 2011). This means that an abnormality of energy metabolism and/or the increase of toxic acyl-CoA compounds inside the mitochondria can result from a deficiency in L-carnitine. It can be anticipated that carnitine and its esters can potentially serve as diagnostic markers in CFS/ME. According to a study conducted by Reuter and Evans (2011), chronic fatigue syndrome is not associated with alterations in total carnitine, acylcarnitine or free carnitine levels. They did however confirm significant differences in levels of certain carnitine species between healthy subjects and patients, especially long-chain acylcarnitines.

A wide variety of analytical methods have been developed for the detection, identification and quantification of carnitine and acylcarnitines in biological samples. Popular analytical methods for analysis of carnitines and acylcarnitines are based on chromatography, capillary electrophoresis, mass spectrometry and electrochemistry (Möder *et al.*, 2005; Dabrowska & Starek, 2014). More sophisticated methods apply chromatographic separation techniques such as high performance liquid chromatography (HPLC) with ultra violet (UV) detection, gas chromatography mass spectrometry (GC-MS) and capillary electrophoresis (CE) and electrospray ionisation (ESI) (Möder *et al.*, 2005). Carnitine and acylcarnitine butyl ester formation and tandem mass spectrometry (MS/MS) has become a popular method for detecting carnitines and acylcarnitines because of its sensitivity and rapidity.

The most widely used methods include high-performance liquid chromatography/electrospray ionization tandem mass spectrometry (HPLC-MS/MS) (Maeda *et al.*, 2007; Minkler *et al.*, 2008) or ultra-high performance liquid chromatography/electrospray ionization tandem mass spectrometry (UPLC-MS/MS). HPLC-MS/MS methods are increasingly becoming the more common method of choice for analysis of carnitine and acylcarnitines in urine because of its high selectivity and sensitivity. Acylcarnitines have a wide range of polarities (Vernez *et al.*, 2004) and because of this characteristic HPLC-MS/MS provides the advantage of simultaneous analysis of

different compounds, as well as the possibility for analysis of highly polar compounds with or without derivatization.

1.3 Research aims and objectives

1.3.1 Broad aim

The broad aim of this study is to identify altered acylcarnitine profiles that are associated with individuals diagnosed with chronic fatigue (CF), possible CFS/ME.

1.3.2 Study aim

The aim of this study is to investigate the urinary free carnitine and acylcarnitine profiles in patients diagnosed with CF, possible CFS/ME.

1.3.3. Objectives to accomplish this aim:

1. Standardization of acylcarnitine analysis
2. Optimization and validation of the HPLC-MS/MS method
3. Application to biological samples
4. Biostatistics analysis
5. Comparing urinary acylcarnitine profiles of healthy individuals with acylcarnitine profiles of individuals diagnosed with CFS/ME

1.4 Dissertation outline

1.4.1 Chapter 1: Introduction

The introduction gives an overview of chronic fatigue syndrome. The problem statement and substantiation is also discussed in the chapter and a brief overview is given regarding diagnostic limitations and methods used for identifying carnitine and its esters. Research aims and objectives are also stated here.

1.4.2 Chapter 2: Literature review

In this chapter, the available literature is given about chronic fatigue syndrome in general, an overview of speculated pathophysiology; as well as different diagnostic approaches and the accuracy of these approaches; and the possible treatment options including pharmacological and non-pharmacological approaches.

1.4.3 Chapter 3: Materials and methods

All chemicals and reagents used during this study are discussed in this chapter, including all methods followed in preparing stock solutions to be used. The HPLC-MS/MS method developed for simultaneous detection and quantification of urinary carnitine and acylcarnitines is described in this chapter, including validation parameters for method development as described by regulatory guidelines. Application of the developed and validated method to patient and control samples are also discussed in detail in this chapter.

1.4.4 Chapter 4: Results and discussion

Validation parameters results are given in this chapter and discussed in detail as well as patient and control sample results obtained. Furthermore, statistical analysis results are given and discussed in detail in this chapter.

1.4.5 Chapter 5: Conclusion and future prospects

In this chapter a conclusion based on the results obtained are made and discussed and based on this, recommendations for future research are discussed.

1.4.6 Chapter 6: References

References used during this study is listed in this chapter according to the guidelines as stipulated in the North West University's referencing guide. Part of this chapter is the Appendix list of tables used for this study.

CHAPTER 2

2. LITERATURE REVIEW

2.1 Chronic Fatigue Syndrome

Fatigue is a symptom commonly experienced by many individuals and has both physical and mental aspects. Prolonged fatigue individuals are experiencing is normally defined as self-reported, temporary fatigue lasting for one month or more, has an underlying cause such as disease and has a major impact on day to day functioning and quality of life (Fukuda *et al.*, 1994; Afari & Buchwald, 2003). When an individual suffers from severe incapacitating fatigue, which cannot be explained by any known medical condition, it may indicate chronic fatigue syndrome, also known as myalgic encephalomyelitis (CFS/ME).

CFS/ME was earlier referred to as the chronic Epstein-Barr virus syndrome and was also known as chronic mononucleosis or chronic mononucleosis-like syndrome. The United States Centre for Disease Control and Prevention (CDC) proposed a new name for this illness, namely chronic fatigue syndrome and also developed a case definition as a guideline for research (Holmes *et al.*, 1988; Fukuda *et al.*, 1994). According to the CDC, chronic fatigue is defined as self-reported, prolonged and persistent fatigue lasting for 6 or more consecutive months. The case definition includes major and minor criteria that must be fulfilled, as well as symptom criteria before a case of CFS/ME can be considered.

Because of the lack of knowledge, an International Consensus Panel consisting of researchers, clinicians, teaching faculty and an independent advocate came together with the aim of developing a universally usable criteria based on the current existing knowledge of CFS/ME. The Canadian Consensus Criteria (Carruthers *et al.*, 2003) is a clinically usable consensus criteria and encourage diagnosis based on symptom clusters with regard to specific pathogenesis. Carruthers *et al.* (2011) emphasizes the concern regarding the misunderstanding of CFS/ME as well as the problem in classifying the illness as psychological instead of a physical illness. The development of the International Consensus Criteria was established by using the Canadian Consensus Criteria as starting point with significant changes, including the elimination of the six-month waiting period before a diagnosis can be made.

2.2 Pathophysiology

CFS/ME is classified as a neurological disorder by the WHO (WHO, 2016). There has been many proposals regarding the origin of CFS/ME, from earlier theories focusing on symptom occurrences due to acute viral infections – the Epstein-Barr virus (Holmes *et al.*, 1988) to psychiatric disorders, central nervous system (CNS) involvement and environmental factors (like organophosphates and pollution, including stressful environments and being exposed to toxic chemicals) that could play a role (Ax *et al.*, 2001; Ferrero *et al.*, 2017). A genetic study done by Kerr *et al.* (2007), identified seven clinical phenotypes, but three distinct clusters seems to be prevalent amongst CFS/ME: (1) Vascular system abnormalities (blood flow – decreased pressure), (2) CNS sensitization (widespread pain, increased sensitivity) and (3) impaired energy production (fatigue and exhaustion). Myhill *et al.* (2009) also suggested that mitochondrial dysfunction can cause the abnormalities mentioned in clusters (1) and (2) as the mitochondria is responsible for adenosine triphosphate (ATP) generation for all body processes. Yet, despite all the research, CFS/ME is still referred to as an illness of unknown pathophysiology (Ax *et al.*, 2001; Afari & Buchwald, 2003; Kumar & Kumar, 2006; Castro-Marrero *et al.*, 2017).

2.2.1 Neurological

Research point towards the involvement of the CNS as the onset point for CFS/ME (Demitrack, 1994). Impaired cognition is a key diagnostic feature for CFS/ME and is observed in as many as 85% of patients (Tiersky *et al.*, 1997). Depression often co-exists with CFS/ME and has been found to affect cognitive functioning (Tiersky *et al.*, 1997).

Because the CNS plays an important role in cognitive actions, any structural or functional impairment of the brain and/or spinal cord can cause dysfunction of CNS control. Subjective cognitive complaints, including distractibility / decreased concentration, forgetfulness and impaired reasonability are common and well documented amongst CFS/ME patients (Afari & Buchwald, 2003). Neurocognitive studies reveals that patients suffer from memory, learning as well as information processing impairment (Evengård & Klimas, 2002). According to Evengård and Klimas (2002) magnetic resonance imaging described changes in the white matter of the brain, but is still to be confirmed, where as other results remain inconclusive (Shepherd, 2006). Other studies of brain metabolism found that acetylcarnitine uptake is decreased (Kuratsune *et al.*, 2002), choline uptake is increased (Puri *et al.*, 2002; Chaudhuri *et al.*, 2003) and that serotonin fluctuates (Badawy *et al.*, 2005).

2.2.2 Neuroendocrine and immunological

There is evidence supporting abnormalities in T and B lymphocytes in CFS/ME (James *et al.*, 1992), as well as cytokine abnormalities, but inconsistent results have been reported.

Many patients acknowledge stress as possible factor for onset of some symptoms. According to Evengård and Klimas (2002), stress impairs the functioning of the immune system, it is thus possible that neuroendocrine and immunological abnormalities found in CFS/ME patients may be due to cytokine imbalances. Parker *et al.* (2001) furthermore reported abnormalities in the hypothalamic-pituitary-adrenal (HPA) axis and also abnormalities of the serotonin pathways in CFS/ME subjects. This can cause an altered physiological response to stress and can explain some of the reported symptoms experienced by patients with CFS/ME (Afari & Buchwald, 2003). Neuroendocrine hypo-activity of the HPA-axis has also been reported by other research groups (Shepherd, 2006), predominantly a reduced output of cortisol has been observed.

Unfortunately, contradicting results about the dysfunction of the immune system has been reported. The most likely argument remains that following a precipitating infection, an ongoing change in the immune system's functioning occurs which indicates that cytokine activation may take place, causing flu-like symptoms (James *et al.*, 1992; Evengård *et al.*, 1999; Shepherd, 2006).

2.2.3 Environmental

Environmental stressors such as pollution or organophosphates can explain allergic reactions reported by patients, but so far, no scientific evidence has been reported that supports this statement (Ax *et al.*, 2001).

Brown *et al.* (2013) reported that environmental toxicity increases the burden on the body (caused by pollutants), toxins include pesticides, insecticides, mercury, lead and nickel. Unfortunately with the limitations of research reports and variable exposure, no concluding evidence can be confirmed. One report identified disturbances in hypothalamic function after toxic exposure, together with more severe immune system dysfunction (Racciatti *et al.*, 2001; Devanur & Kerr, 2006; Brown *et al.*, 2013). Devanur and Kerr (2006) also stated that toxin exposure plays a role in the development of fatigue symptoms because of the influence on the immune system. This statement has also been confirmed (Devanur & Kerr, 2006; Kerr *et al.*, 2007). Organophosphate concentrations was found higher in CFS/ME patients than in control subjects with known toxin exposure during a study conducted by Dunstan *et al.* (1995). In research conducted by Stephens

et al. (1996), they demonstrated that exposure to organic phosphates can cause abnormalities in the nervous system.

2.2.4 Energy production / transport impairment and mitochondrial dysfunction

Mitochondria play an important role in cellular respiration and generating metabolic energy (ATP) which is used during daily activities and exercise, this means if less mitochondria are active, a build-up of lactic acid may occur even with a low level of exercise. This limits muscle performance and can contribute to the post-exertional malaise and fatigue reported by CFS/ME sufferers (Burns *et al.*, 2012). According to Myhill *et al.* (2009), there is a lot of evidence suggesting and supporting mitochondrial dysfunction in CFS/ME patients. Mitochondrial dysfunction is a physiological factor considered to be one of the contributing factors of CFS/ME (Brown, 2014). Some reports go as far as to say that mitochondrial dysfunction may be fundamental to the pathophysiology of CFS/ME (Pieczenik & Neustadt, 2007; Bains, 2008; Maes, 2011; Brown, 2014).

The main energy producing pathway, for especially muscle and cardiac cells, are the fatty acid oxidation pathway, which takes place inside the mitochondrial matrix. Long chain fatty acids are transported into the mitochondrial matrix with the aid of L-carnitine, where it is oxidised to release energy. Smits *et al.* (2011) reported a decreased number of mitochondria in muscle biopsy samples from CFS/ME patients when compared to control subjects. While mitochondrial function remained unaffected, they also found that the rate of ATP production was within normal range in patients when compared to subjects with mitochondrial disorders. They actually stated that they can reliably differentiate between CFS/ME sufferers and people with mitochondrial disorders. Other muscle biopsy studies also indicated fewer active mitochondria in CFS/ME patients in comparison to healthy controls (Myhill *et al.*, 2009; Burns *et al.*, 2012), as well as abnormal mitochondria being observed during research done by James *et al.* (1992) and Behan *et al.* (1991). During a study conducted by Lengert and Drossel (2015), they found reduced mitochondrial activity in patients with CFS/ME. They also reported that the ATP levels of CFS/ME patients reaches critically low concentrations during high intensity exercise.

The decreased capacity of mitochondrial ATP energy production in CFS/ME pathophysiology observed during exercise, may be one of the foremost contributors to exercise intolerance found in these patients and depleted ATP and fatigue-like symptoms can possibly be due to mitochondrial dysfunction (Myhill *et al.*, 2009; Booth *et al.*, 2012).

As L-carnitine plays an essential role in energy production in the mitochondria, some studies indicate that L-carnitine and acetyl carnitine compounds were decreased in serum samples. This can possibly be due to the high demand of fatty acid transportation into the mitochondria for energy production. Armstrong *et al.* (2015) suggests that there is a connection between mitochondrial function and a decreased use of aerobic respiration because of the decreased use of oxygen, contribution to reactive oxygen species (ROS) found in CFS/ME patients. This again points to the possible involvement of mitochondria in CFS/ME patients.

2.3 Diagnosis

Diagnosis of CFS/ME is a difficult task because there are not yet any form of laboratory diagnostic test as well as no diagnostic markers for accurate diagnosis (Klonoff, 1992; Kumar & Kumar, 2006; Fernández *et al.*, 2009; Castro-Marrero *et al.*, 2017). Diagnosis is based on the occurrence of a number of signs and symptoms which are poorly understood (Reuter & Evans, 2011). Furthermore, diagnosis is more difficult due to different diagnostic criteria being used; and physicians' limited knowledge and understanding of this illness often leads to it being considered as a psychological illness instead of a physical one.

CFS/ME is firstly defined by CDC as clinically evaluated, unexplained persistent or relapsing chronic fatigue of new or definite onset (not lifelong) and is not improved by rest. Second, the simultaneous presence of four or more of the following symptoms; i) self-reported impairment in short term memory or concentration, ii) Sore throat, iii) tender cervical or axillary lymph nodes, iv) muscle pain and headaches of a new type, pattern or severity, v) unrefreshing sleep, vi) post-exertional malaise lasting more than 24 hours (Fukuda *et al.*, 1994; Kumar & Kumar, 2006). It is critical to exclude physical and psychiatric diseases which may cause fatigue. The criteria, according to the CDC are summarized in **Table 2.1**.

What makes diagnosis even more difficult is the different diagnostic criteria being used across the world. Other criteria include the Australian definition (Lloyd *et al.*, 1990), the Oxford definition (Sharpe *et al.*, 1991), the Canadian Consensus Criteria (Carruthers *et al.*, 2003) and the International Consensus Criteria (Carruthers *et al.*, 2011). Of these mentioned, the International Consensus Criteria is more widely used and was derived from the Canadian Consensus Criteria. In **Table 2.2** a summary of the Canadian Consensus Criteria is given. The starting point to gather medical information from patients is to do physical and mental clinical evaluations to identify symptoms and experiences of individuals as well as making use of medical symptom questionnaires like the Piper Fatigue Scale (PFS) and the Medical Symptoms Questionnaire (MSQ). With help from these questionnaires, medical history and symptom severity can be

obtained more easily, but this leads to a diagnosis made more commonly based on exclusion rather than a diagnostic criteria. During the physical and mental clinical examination, any and all other possible treatable or diagnosable illnesses should be excluded, and are usually confirmed with laboratory screening tests (Afari & Buchwald, 2003; Carruthers *et al.*, 2003). For a diagnosis to be made according to the CDC, major criteria 1 and 2 must be fulfilled, and of the minor criteria, 8 or more of the 11 symptom criteria; or 6 or more of the 11 symptom criteria and 2 or more of the 3 physical criteria must be fulfilled.

Table 2.1: Proposed diagnostic criteria for CFS/ME according to the CDC, as defined by Holmes et al. (1988) and Fukuda et al. (1994).

Major criteria:	
1. A new onset of persistent, incapacitating fatigue in a person who has no previous history of similar symptoms. The fatigue does not improve with bed rest and causes impairment of a patients' normal daily activity level for a period lasting at least six months.	
2. Other medical conditions that may produce similar symptoms must be excluded.	
Minor criteria:	
Symptom criteria: symptoms begun with or after onset of fatigue and lasted for a period of 6 months or more.	
1. Mild fever (oral temperature of 37.5°C-38.6°C)	2. Sleep disturbance
3. Sore throat	4. Neuropsychological complaints
5. Painful anterior and posterior cervical or axillary lymph nodes	6. Migratory arthralgia without joint swelling or redness
7. Muscle discomfort or myalgia	8. Unexpected generalized muscle weakness
9. Prolonged generalized fatigue lasting > 24 hours after previously tolerated exercise	10. Generalized headaches of new pattern or severity
11. Development of main symptom complex over a few hours or days	
Physical criteria – assessed by a physician on at least two occasions two months apart.	
1. Non-exudative pharyngitis	
2. Low grade fever (oral temperature of 37.5°C-38.6°C)	
3. Palpable or tender anterior or posterior cervical or axillary lymph nodes	

An expert Medical Consensus Panel came together in 2001 to establish a working case definition, diagnostic and treatment protocols for CFS/ME. Carruthers and colleagues (2003) presented a systematic clinical working case definition which encourages a diagnosis based on characteristic patterns of symptom clusters regarding specific pathogenesis areas. Different symptom clusters are used because of the unlikeliness of all CFS/ME cases sharing a single disease model.

According to the Canadian Consensus Criteria, for a patient to be diagnosed with CFS/ME, the criteria for fatigue, post-exertional malaise and/or fatigue, pain and sleep dysfunction will be met. Two or more cognitive/neurological manifestations should be present, at least one symptom from two of the autonomic, immune and neuroendocrine manifestations and should also adhere to item seven (7) as described in **Table 2.2**.

Table 2.2: Canadian Consensus Criteria as a clinical working case definition of CFS/ME proposed by Carruthers et al. (2003).

1.	**Fatigue** Significant degree of fatigue of new onset, persistent or unexplained Recurrent mental and/or physical fatigue that reduces level of activity significantly.
2.	**Post-exertional malaise and/or fatigue** Loss of physical and mental stamina Rapid cognitive and muscle fatigue Post-exertional malaise and/or fatigue and/or muscle pain The tendency of other associated cluster symptoms to aggravate Pathological slow recovery period, 24 hours or longer
3.	**Sleep dysfunction** Unrefreshed sleep Sleep quantity rhythm disturbances (like reversed sleep rhythms)
4.	**Pain** Significant degree of myalgia Pain in joints and/or muscles (can spread in a migratory nature) Significant headaches of new onset, form or severity
5.	**Neurological / Cognitive manifestations** Concentration impairment and short-term memory consolidation; confusion; information processing and word retrieval difficulty; disorientation; sensory and perceptual disturbances; ataxia, muscle weakness and fasciculation. * Cognitive or sensory overload (e.g. hypersensitivity) could lead to 'çrash' periods and/or anxiety

Table 2.2 continued.

6. (A) <u>Autonomic Manifestations</u>

Postural orthostatic tachycardia syndrome (POTS); Orthostatic intolerance-neurally mediated hypotension; Delayed postural hypotension; Extreme paleness; Nausea and irritable bowel syndrome; Light-headedness; Urinary frequency and bladder dysfunction; Exertional dyspnoea; Palpitations with or without cardiac arrhythmias

(B) <u>Neuroendocrine manifestations</u>

Sweating episodes; Recurrent feelings of feverishness and cold extremities; Loss of thermostatic stability; Extreme heat and cold intolerance; Marked weight change (anorexia) or abnormal appetite; Worsening of symptoms with stress; Loss of adaptability

(C) <u>Immune manifestations</u>

Recurrent sore throat; Tender lymph nodes; General malaise; New food, medication and/or chemical sensitivities; Recurrent flu-like symptoms

7. <u>Persistence of at least six months</u>

Usually have a distinct onset, but may be gradual. Three months is applicable to children

For diagnosis, symptoms must fall within the time range of the onset of the illness. It is highly unlikely for an individual to suffer from all the symptoms mentioned in criteria 5 and 6. Symptom clusters present may fluctuate and change over time.

<u>Exclusion criteria (confirmation with laboratory testing and imaging):</u>

- Active disease processes explaining the majority of the prominent symptoms of pain, fatigue, sleep disturbances and cognitive dysfunction.
- Certain diseases including Addison's disease Cushing's syndrome, hypothyroidism, hyperthyroidism, Diabetes Mellitus, cancer, iron deficiency, iron overload syndrome and other treatable forms of anaemia.
- Treatable sleep disorders like upper airway resistance syndrome and obstructive or central sleep apnoea.
- Rheumatological disorders including rheumatoid arthritis, polymyositis, lupus and polymyalgia rheumatic.
- Immune disorders including acquired immunodeficiency syndrome (AIDS)
- Neurological diseases such as multiple sclerosis (MS), Parkinsonism, myasthenia gravis and B12 deficiency
- Infectious diseases like Tuberculosis (TB), Chronic hepatitis and Lyme disease
- Primary psychiatric disorders and substance abuse

<u>Co-morbid entities associated with CFS/ME include:</u>

Fibromyalgia syndrome, Myofascial Pain syndrome, Temporomandibular Joint syndrome, Irritable Bowel syndrome, Interstitial cystitis, Raynaud's Phenomenon, Prolapsed Mitral valve, Depression, Migraine, Allergies, Multiple chemical sensitivities, Hashimoto's thyroiditis and Sicca syndrome.

If an individual suffers from prolonged unexplained fatigue, but do not meet other symptom criteria for CFS/ME, a diagnosis of Idiopathic Chronic fatigue should be considered

2.3.1 Guidelines for consideration when applying the Clinical case definition

A patient's total illness has to be assessed and can be done by obtaining a complete symptom description from the individual and by observation. Variability of symptoms from one individual to the next will occur, but a coherence of symptoms will be shown by according to what applies to the individual and when there is a case where coherent symptoms are absent, a diagnosis of CFS/ME is doubted. Severity of symptoms are judged to have a dramatic negative impact of more or less 50% on an individual's life. Symptom severity ranking should be part of the ongoing clinical evaluation and it should be kept in mind that this will vary from one individual to the next. It is important to try and separate primary symptoms from secondary symptoms and other factors that can intensify primary symptoms (Carruthers *et al.*, 2003).

2.3.2 Fatigue questionnaires used and their reliability

Fatigue is a completely subjective experience and is defined by persistent weakness, tiredness or physical and/or mental exhaustion (Dittner *et al.*, 2004). Different scales are available and during a study conducted by Dittner *et al.* in 2004, they assessed a total of 30 different scales and reported the purpose, structure and evidence of psychometric properties of each and classified them as either unidimensional or multidimensional. Furthermore, they advise clinicians to choose fatigue scales based on the specific needs that has to be fulfilled. The fatigue severity scale (FSS) being one of the most common scales used is classified as unidimensional as it measures only the impact of fatigue and does not include measurement of the severity and intensity of fatigue related symptoms.

2.3.2.1 Piper fatigue scale

The Piper fatigue scale (PFS) is classified as a multidimensional scale measuring phenomenology and severity of symptoms (Piper *et al.*, 1998). The PFS has received a lot of criticism from clinicians and patients, as it takes a long time to complete and patients state that questions are difficult to understand.

Internal consistency was found to be very high, but also found that the original PFS had limitations in terms of psychometric qualities, and therefore a revised PFS was developed and validated in 1998 (Dittner *et al.*, 2004). With the revised PFS internal consistency remained high and it also proved easy to score.

2.3.2.2 Medical symptoms questionnaire

The Medical symptoms questionnaire (MSQ) is a clinical tool used for the evaluation of physical signs and symptoms (Mallar, 2008). It consists of a total of 71 questions with an easy scoring point system and measures various mental, physical and emotional symptoms. Scores above 75 are usually associated with significant symptomology.

The MSQ displays high clinical ability and reasonable face validity as a subjective measure of physical symptoms and can be considered reliable when administered on two consecutive days as reported by Mallar (2008) based on research conducted.

In conclusion, fatigue scales and symptoms questionnaires are only reliable when answered truthfully by patients and can thus differ from day to day or week to week, depending on the individual's daily experiences.

2.3.3 Other approaches for diagnosing CFS/ME

According to Bains (2008) there is no obvious metabolic problems that could lead to CFS/ME, although a common finding is a reduced level of oxidative metabolism (McCully *et al.*, 1996; Bains, 2008) and also an increase in lactate production (Lane *et al.*, 1998).

Kuratsune *et al.* (1994) measured carnitine and acylcarnitines in CFS/ME patients with an enzymatic cycling method, and reported that during this study they found acylcarnitines to be deficient in CFS/ME patients compared to controls. Jones *et al.* (2005) conducted a radio-enzymatic assay study in 2005 to asses plasma and urinary carnitine and acylcarnitines in patients with CFS/ME based on the role carnitine plays in mitochondrial energy production, but they found no significant differences in urinary or plasma total, free or acylcarnitnes. In another study conducted by Casado *et al.* (2005), capillary electrophoresis (CE) was used to determine urinary electrophoretic profiles of CFS/ME patients and reported peak differences when compared to a control group that may be of significance as biomarkers. According to Myhill *et al.* (2009), they observed strong implications that mitochondrial dysfunction is the immediate cause of CFS symptoms through ATP profiling tests done. Smits *et al.* (2011) conducted a study to determine the extent of mitochondrial involvement in CFS/ME and found that mitochondrial content was decreased in CFS/ME in comparison to healthy controls although it did not discriminate between CFS/ME and individuals with mitochondrial disorders.

Previous studies as mentioned above have investigated the endogenous plasma carnitine levels in patients with CFS/ME, but contrasting results were obtained (Kuratsune *et al.*, 1994; Plioplys

& Plioplys, 1995; Jones *et al.*, 2005). One study used a radiochemical assay to determine carnitine and acylcarnitine levels in serum, but did not report any significant findings (Soetekouw *et al.*, 2000). Reuter and Evans (2011) still suggested that CFS/ME may be associated with carnitine homeostasis being altered and that a study needs to be conducted in order to confirm this hypothesis. When considering previously conducted studies, the majority of the research were done on serum samples and very little studies on urine samples.

2.4 Treatment

There still remains no universally successful treatment option for CFS/ME. The prevalence of CFS/ME in the community is roughly 0.2 – 0.7% and 0.5 - 2.5% in primary care (Reuter and Evans, 2011). Treatment approaches have mainly been focused on symptoms and the relief thereof in order to improve daily functioning of patients. These approaches include non-pharmacological, which aims to improve general wellbeing of patients with the focus on exercise and psychological aspects whereas pharmacological treatment aims to improve symptoms through pharmaceutical drugs.

Different therapeutic approaches for a possible treatment for CFS/ME have been examined in the last decade, but only one seem to produce significant results, namely cognitive behaviour therapy along with gradual physical exercise (Fernández *et al.*, 2009).

2.4.1 Non-pharmacological

2.4.1.1 Graded exercise therapy

This approach is used due to the symptoms of muscle fatigue and pain. There have been reports of improvement of symptoms in CFS/ME patients from numerous studies, especially treatment focusing on individuals (Fulcher & White, 1997; Wearden *et al.*, 1998; Afari & Buchwald, 2003; Shan, 2007). These studies indicate that exercise therapy needs to be sustained over a continued period of time, to see improvements in general fitness levels and to help cope with post exertional malaise (Afari & Buchwald, 2003). This can be achieved by finding a balance between physical and mental activity.

Even though graded exercise therapy (GET) shows promising results, contradicting results have been reported about the effect GET has on patients, particularly on group focused GET (Fulcher & White, 1997; Wearden *et al.*, 1998; Shepherd, 2006), and it is for this reason that it is encouraged to plan such programs with great care, based on individual needs and progression based on their symptom severity and exercise recovery (Revelas & Baltaretsou, 2013). Some reports show up to a 50% improvement in symptoms (Luyten *et al.*, 2008; Brown, 2014).

Another approach used together with GET is pacing, this is where an individual finds a balance between activity and rest, by accepting the limitations of CFS/ME and avoiding any activities that can exceed these limitations to prevent intensifying the symptoms (Burns *et al.*, 2012). GET shows promise as a treatment option as results obtained in studies indicate improvement of fatigue after twelve continuous weeks compared to control groups (McBride & McCluskey, 1991), one exception being patients suffering from depression, where pharmacological intervention is needed, but only shows a short term result (Revelas & Baltaretsou, 2013).

2.4.1.2 Cognitive behavioural therapy

GET is usually combined with cognitive behavioural therapy (CBT) as an approach for CFS/ME treatment.

Cognitive therapy involves a series of techniques which is based on the principles of behaviour modifications and the cognitive theory, aimed at the strengthening of the modification of thoughts and behaviour related to the patients' symptoms and distress (Sharpe *et al.*, 1991; Fernández *et al.*, 2009). Protocols developed for this treatment modality is mostly based on three key factors namely 1) control and coping with disease-associated stress, 2) programmed physical exercise and 3) cognitive restructuring (Deale *et al.*, 1997; Fernández *et al.*, 2009). CBT is a form of psychological therapy and focuses on improving the behavioural and thinking patterns of patients to conclusively change the way a person feels. It helps patients to cope with CFS/ME more effectively (Brown, 2014).

Roberts *et al.* (2009) reported an increase in cortisol levels after only six months of CBT therapy, which makes it one of a few treatment options to have this effect on CFS/ME patients. There is however also reports indicating that some individuals feel worse after treatment, but this can be due to the combination with GET, as stated earlier (White *et al.*, 2007).

In 2017, Castro-Marerro *et al*. suggested that CFS/ME is a physical illness, and not a psychological one, which means that CBT cannot cure the illness. Although CBT shows promising results in improvement of an individual's functioning, it did not show the re-establishment in their ability to work (Chambers *et al.*, 2006) and it is therefore suggested to continue GET and CBT intervention as it shows promise towards the improvement of symptoms. It cannot on the other hand, be considered as a primary intervention for CFS/ME, as no study thus far could prove that GET and CBT can reverse the illness (Castro-Marrero *et al.*, 2017).

2.4.2 Pharmacological

No confirmed pharmacological treatment recommendations with conventional medicine has been proposed and no USA Food and Drug Administration (FDA) approved drugs for the treatment of CFS/ME is confirmed. The absence of diagnostic markers makes treating CFS/ME much more difficult (Evengård *et al.*, 1999). In general the studies done until now provides insufficient data for effective and conclusive treatment (Evengård & Klimas, 2002), however, suggestions for treatment of symptoms have been made with a fair amount of positive results reported.

Pharmacological treatment is based on symptoms portrayed by individuals diagnosed with CFS/ME and is specific to each individual as symptom severity and prevalence differ from one patient to the next. The aim of symptomatic treatment has been described to effectively relief symptoms but not to cure CFS/ME, as no certain treatment have yet been established (Shepherd, 2006; Shan, 2007; Castro-Marrero *et al.*, 2017).

The proposed strategies for the pharmacological treatment for CFS/ME is summarised in **Figure 2.1**, which include the most common approaches based on symptoms shown by patients.

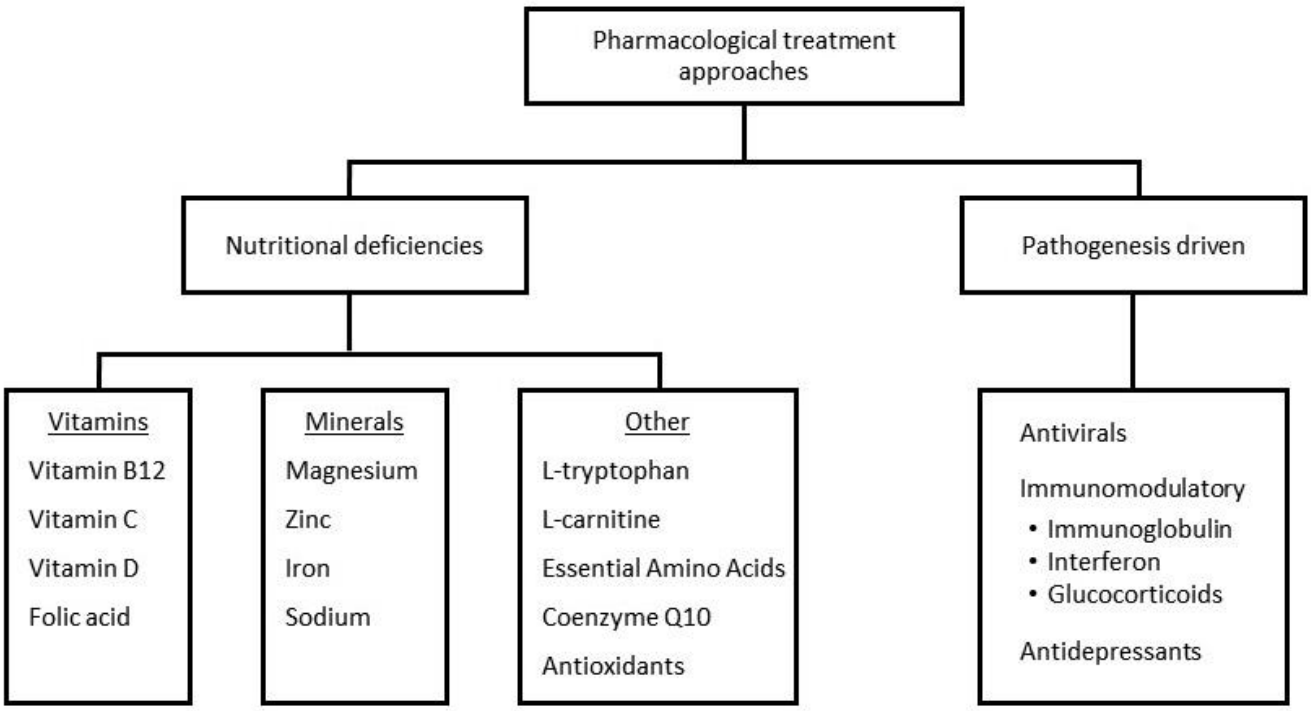


Figure 2.1: Summary of pharmacological treatment strategies for CFS/ME

As shown in the figure, treatment approaches have been based on what is believed to be causes of the symptoms portrayed by individuals diagnosed with CFS/ME.

In the paragraphs to follow, a brief overview of the general findings regarding the treatment approaches will be given.

Brown (2014) states that B vitamins (pyridoxine, riboflavin and thiamine) is essential for mitochondrial function and that vitamin B supplementation could improve overall energy and feelings of weakness. Vitamin D could help with the improvement of general fatigue and weakness, depression and muscle pain. Nicotinamide adenine dinucleotide (NADH), the active form of niacin (vitamin B3) showed improvement of symptoms in patients (Forsyth *et al.*, 1999; Santaella *et al.*, 2004). Werbach (2000) reports on several studies conducted where patients reported increased stamina, energy or well-being within two to three weeks of treatment with vitamin B12, with a substantial amount of vitamin B12 administered to obtain symptomatic relief. Vitamin C has been shown to enhance immune function and increased immunoglobulin levels in CFS/ME individuals and vitamin C also contains antiviral activity (Brown, 2014).

Furthermore, Brown (2014) reported a case control study where energy levels and emotional state improved with treatment of intravenous magnesium as well as the improvement of overall health with weekly magnesium injections as reported by Evengård and Klimas (2002).

Reduced zinc levels have been associated with the increase in severity of symptoms. There is evidence suggesting that zinc supplementation can influence fatigue, mood, oxidative stress and

immune function positively, but there is no clinical trials regarding CFS/ME yet to confirm this expectation.

L-carnitine shows significant symptom improvement of pain fatigue and cognitive function within four to eight weeks of supplementation (Brown, 2014). According to Castro-Marrero *et al.* (2017), during a study conducted in 2008, patients reported a significant difference in physical and mental fatigue compared to control subjects. With L-acetylcarnitine supplementation similar results have been obtained (Werbach, 2000).

Behan *et al.* (1990) reported a significant improvement in fatigue, myalgia, dizziness, depression and concentration with treatment of essential fatty acids. Gamma linolenic acid, eicosapentaenoic acid and docosahexaenoic acid have been proved to improve the CFS/ME symptoms mentioned above, as reported by Brown (2014).

Antioxidants may be a safe and effective way for improving symptoms of CFS/ME sufferers and offers an improved quality of life (Maric *et al.*, 2014). A combination of natural antidepressants including coenzyme Q10 and NADH could prove beneficial in alleviating fatigue and providing insight into the pathogenesis of CFS/ME (Castro-Marrero *et al.*, 2017).

Some antiviral treatment approaches documented thus far shows promise in improvement and even recovery in some individuals (See & Tilles, 1996) compared to other studies where no significant improvement in depression or quality of life were noted (Vollmer-Conna *et al.*, 1997; Afari & Buchwald, 2003; Castro-Marrero *et al.*, 2017). Glucocorticoids delivered positive results with placebo-controlled trials, with improvement of fatigue reported (McKenzie *et al.*, 1998; Cleare *et al.*, 1999), whereas hydro-cortisol intervention proves promising but has not yet been recommended for clinical use (Castro-Marrero *et al.*, 2017).

Antidepressants provided relief of symptoms with the improvement in quality of life and health perception with reduced fatigue (Evengård & Klimas, 2002; Solomon *et al.*, 2003; Revelas & Baltaretsou, 2013). Cleare *et al.* (1999) stated that most antidepressants interact with other drugs and that some of these interactions can be very serious. Tricyclic antidepressants are known to relief symptoms like sleeplessness and low energy levels in CFS/ME and only requires low dosage compared to patients suffering from depression (Evengård *et al.*, 1999; Castro-Marrero *et al.*, 2017). Despite of these findings reported, the use of antidepressants remains controversial.

2.5 Carnitine

L-carnitine occurs naturally in all mammalian species and is synthesized mainly from the amino acids lysine and methionine in the liver, kidneys and the brain, (Kelly, 1998; Vaz & Wanders, 2002; Reuter & Evans, 2012) but is also primarily obtained through the diet. L-carnitine is an essential metabolite and has a significant role in especially energy metabolism, where it is responsible for the transport of long chain fatty acids into the mitochondria for beta-oxidation (β-oxidation).

L-carnitine also helps in regulating the acyl-Coenzyme A/Coenzyme A (acyl-CoA/CoA) ratio (McGarry & Brown, 1997) and stores energy in the form of acetylcarnitine (Vaz & Wanders, 2002).

2.5.1 Role in energy metabolism

As mentioned earlier, the role of carnitine in mitochondrial energy metabolism is crucial, as long chain fatty acids cannot cross the mitochondrial membranes by themselves. Carnitine acts as a carrier molecule for these fatty acids, and transports them into the mitochondria where they can be oxidized to release energy. The structure of L-carnitine is given in **Figure 2.2**.

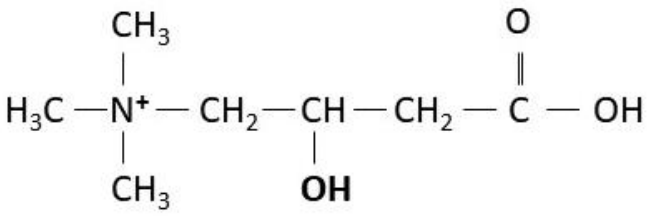


Figure 2.2: Structure of L-carnitine

Mitochondrial fatty acid oxidation is a process that happens inside the mitochondrial matrix, where long chain fatty acids are broken down to release energy. The whole process starts when activation of long chain fatty acids happens through acyl-CoA synthase forming a long chain acyl-CoA. Carnitine palmitoyltransferase I (CPT I) located in the outer membrane, trans-esterifies long chain Acyl-CoA to L-carnitine, where the acyl moiety is transferred from the long chain fatty acid to the hydroxyl group of the carnitine, forming a long chain acylcarnitine which can then be transported across the inner mitochondrial membrane through the carnitine-acylcarnitine translocase (CACT) carrier.

Inside the matrix, transesterification of long chain fatty acids to intramitochondrial CoA takes place through carnitine palmitoyltransferase II (CPT II) and as a result, carnitine is released which can leave the mitochondria through CACT. Carnitine acetyltransferase (CACT) located in the mitochondrial matrix can convert short- and medium-chain acyl-CoAs into acylcarnitines by using intramitochondrial carnitine and can then also leave the mitochondria via CACT. This whole process is visually explained by **Figure 2.3**.

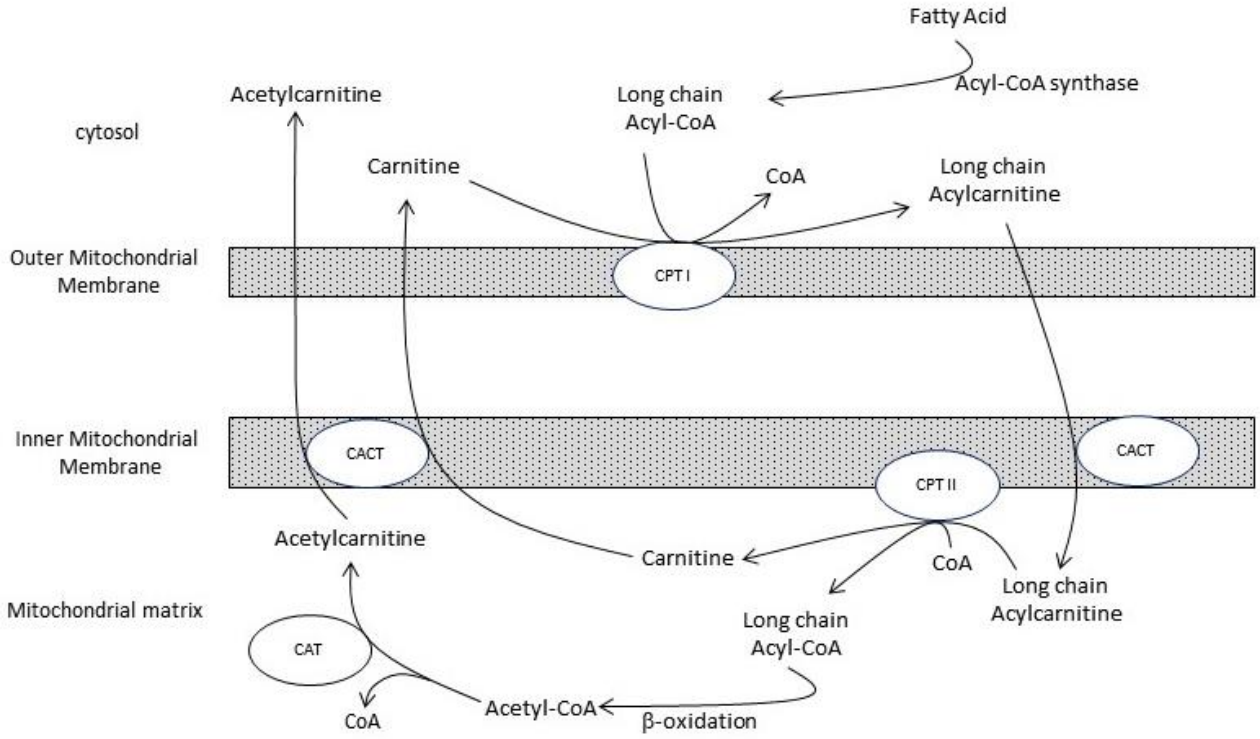


Figure 2.3: Function of carnitine in mitochondrial fatty acid oxidation for energy production.

2.5.2 Carnitine and acylcarnitines as possible markers for chronic fatigue syndrome

The unknown etiology of CFS/ME (Holmes *et al.*, 1988; Afari & Buchwald, 2003; Smits *et al.*; Morch *et al.*, 2013) and the prevalence in absence of diagnostic markers and laboratory tests (Klonoff, 1992; Kumar & Kumar, 2006) to accurately identify CFS/ME opens up an opportunity to develop new methods for diagnosing CFS/ME. A potential starting point for method development would be identifying carnitine and its derivatives as possible diagnostic markers because of the critical role they have in energy production.

Due to the important role carnitine plays in mitochondrial energy metabolism, it can be speculated that carnitine and acylcarnitines metabolite profiles may possibly differentiate between patients diagnosed with CFS/ME and healthy individuals. Although there have been contradicting results with the analysis of carnitines and acylcarnitines in serum and urine samples, a study dating back

to 1994, found an acylcarnitine deficiency in serum samples of positively diagnosed CFS/ME patients (Kuratsune *et al.*, 1994), and soon after, Plioplys and Plioplys (1995) reported significantly lower serum free carnitine, total carnitine and acylcarnitine levels in patients. Vermeulen and Scholte (2004) reported an improvement in fatigue in CFS/ME patients after treatment with acetylcarnitine and propionylcarnitine respectively. In 2010, (Reuter & Evans) reported significantly altered concentrations of certain acylcarnitine species, where long chain species were found on average to be 30-40% lower when compared to healthy individuals.

When one considers previous results found, carnitine and acylcarnitines should definitely be considered as possible diagnostic markers for CFS/ME, together with the aim of producing a reproducible method for the detection of these compounds.

2.5.3 Methods used for analysis of carnitines and acylcarnitines

A wide variety of analytical methods have been developed for the detection, identification and quantification of carnitine and acylcarnitines in biological samples. Popular analytical methods for analysis of carnitines and acylcarnitines are based on chromatography, capillary electrophoresis, mass spectrometry and electrochemistry (Möder *et al.*, 2005; Dabrowska & Starek, 2014). More sophisticated methods apply chromatographic separation techniques such as high performance liquid chromatography (HPLC) with ultra violet (UV) detection, GC-MS and capillary electrophoresis (CE) and electrospray ionisation (ESI) (Möder *et al.*, 2005). Carnitine and acylcarnitne butyl ester formation and tandem mass spectrometry (MS/MS) has become a popular method for detecting carnitines and acylcarnitines because of the method's high sensitivity and rapidity. High-performance liquid chromatography/electrospray ionization tandem mass spectrometry (Maeda *et al.*, 2007; Minkler *et al.*, 2008) is becoming one of the more favourable methods used for detection of carnitine and acylcarnitines.

High-performance liquid chromatography/electrospray ionization tandem mass spectrometry methods are increasingly becoming the more common method of choice for analysis of carnitine and acylcarnitines in urine because of its high selectivity and sensitivity. Acylcarnitines have a wide range of polarities (Vernez *et al.*, 2004) and because of this characteristic HPLC-MS/MS provides the advantage of simultaneous analysis of different compounds, as well as the possibility for analysis of highly polar compounds with or without derivatization.

2.6 Derivatization

Derivatization is used during sample preparation, as it increases sensitivity for analyte detection. Chace *et al.* (2003) compared methods where acylcarnitines were underivatized and derivatized

with butanolic HCl, and found that although the two methods are comparable, detection sensitivity is higher for derivatized acylcarnitines compared to underivatized acylcarnitines. Acylcarnitines are reported as hydrolytic unstable by Johnson (1999), meaning that derivatization with butanolic HCl, hydrolyses acylcarnitines to free carnitine, increasing the amount of free carnitine detected, this can be seen in **Figures 4.8 - 4.19**. This is a concern when it comes to a diagnosis being made based on free carnitine detection.

Butanolic hydrochloric acid (HCl) is used for the derivatization of carboxylic acids and works well with carnitine and acylcarnitines since the structure contains a carboxylic group. Carnitine is ionic in nature and is highly soluble, but solubility of acylcarnitines decreases with an increasing chain length of the ester group. Derivatization of carnitine and its esters with butanolic HCl reduces their polar properties, making them hydrophobic, yielding the product ion at m/z ($[M + H - 56]^+$) as visually represented in **Figure 2.4** after going through the collision cell. It also makes gradient elution a suitable tool for chromatographic separation of carnitine and its esters (Santaella *et al.*, 2004; Möder *et al.*, 2005). Minkler *et al.* (2005) reports that about 30% of acylcarnitines are hydrolysed within 15 minutes of the reaction with butanolic HCl. Chace *et al.* (2003) furthermore reports that derivatization with butanolic HCL for a longer time and higher temperatures, results in more extensive hydrolysis of acylcarnitines and when exposed to colder temperatures and a shorter time, incomplete derivatization is observed.

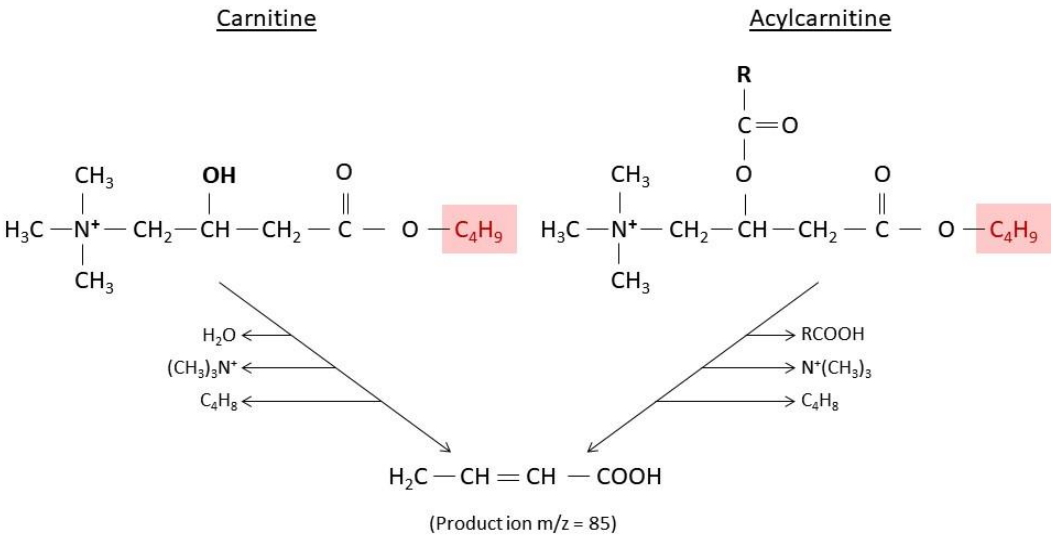


Figure 2.4: Process of derivatization (butylation) of carnitine and acylcarnitines to produce the characteristic product ion of m/z 85.

During derivatization, a C_4H_8 group is added to the carnitine/acylcarnitine on the hydroxyl group and when the butylated carnitine or acylcarnitine passes through the collision cell, the carnitine or acylcarnitine is fragmented into the characteristic product ion of m/z 85. The arrows indicate which parts of the carnitine will be removed to in the end yield this characteristic product ion.

2.7 Mass spectrometry

2.7.1 Multiple Reaction Monitoring

Multiple reaction monitoring (MRM) is a highly sensitive and specific mass spectrometry technique used to selectively quantify compounds in complex matrices. Liquid chromatography tandem mass spectrometry (LC-MS/MS) with MRM mode serves as the basis for precise simultaneous multi-analyte quantitation for large sample sets and is a very powerful technique for identifying and quantifying numerous compounds in complex biological matrices. Using a triple quadrupole LC-MS/MS system with MRM, specific precursor ions and characteristic product ions of each analyte can be detected (Bin *et al.*, 2012). **Figure 3.2** is a schematic representation of how MRM works.

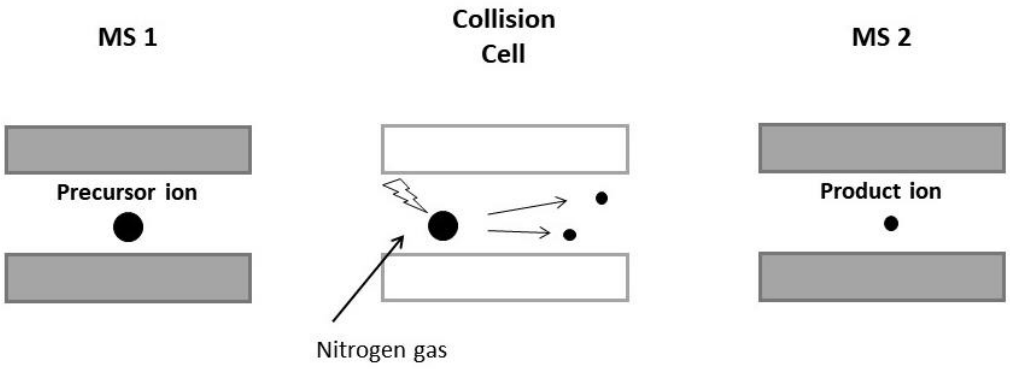


Figure 2.5: Multiple Reaction Monitoring schematic representation.

Quadrupole one (*MS 1) is set in static mode, detecting only the indicated precursor ions' mass-to-charge ratios (m/z), where quadrupole three (MS 2) is also set in static mode, detecting each precursor ions' characteristic product (or daughter) ions' m/z. The collision cell is responsible for fragmenting the precursor ion into the characteristic product ion.*

2.7.2 Chromatographic separation

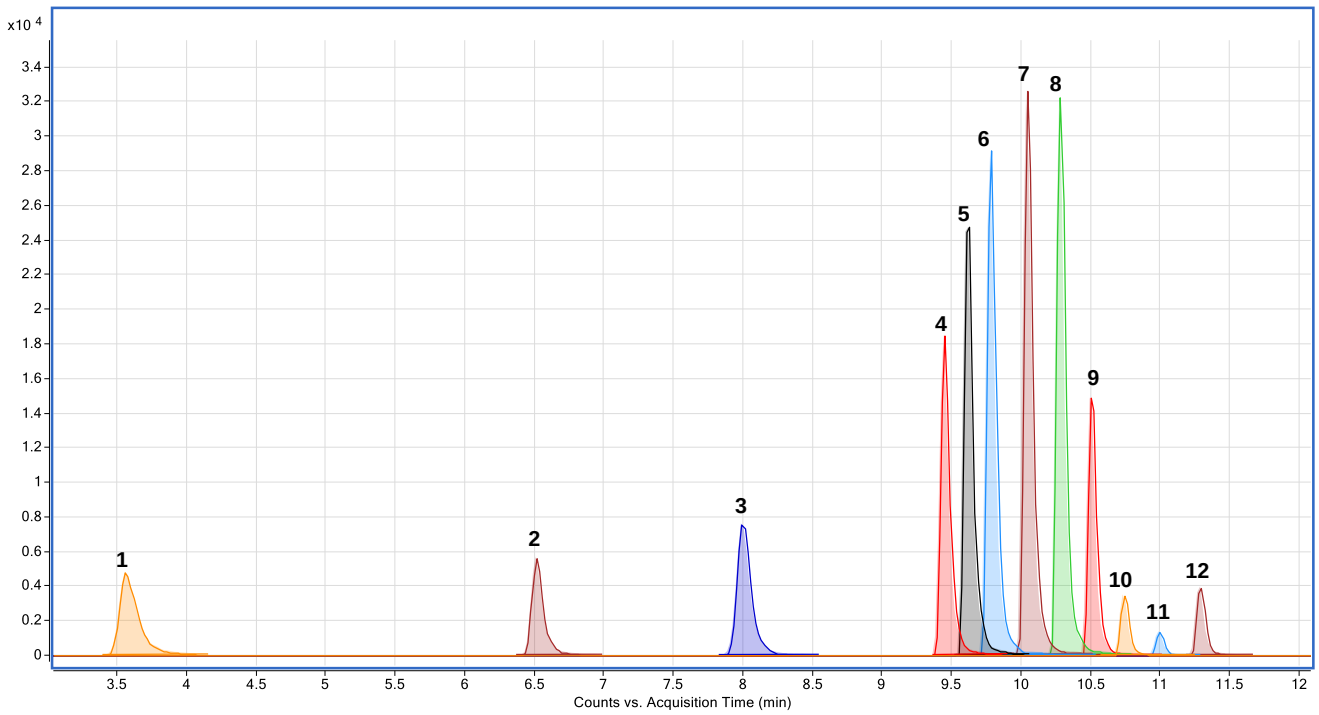


Figure 2.6: The chromatographic separation achieved for carnitine and acylcarnitine esters focused on in this study. Peak information: 1. C0 and C0_IS, 2. C2 and C2_IS, 3. C3 and C3_IS, 4. C4 and C4_IS, 5. C5 and C5_IS, 6. C6 and C6_IS, 7. C8 and C8_IS, 8. C10 and C10_IS, 9. C12 and C12_IS, 10. C14 and C14_IS, 11. C16 and C16_IS, 12. C18 and C18_IS.

CHAPTER 3

3. MATERIALS AND METHODS

3.1 Materials and chemicals

High performance liquid chromatography grade acetonitrile (ACN) was purchased from Honeywell Burdick & Jakson (Morristown, New Jersey) and formic acid (FA) was purchased from Merck Chemical Co. (Darmstadt, Germany). Butanol and acetyl chloride was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). The following carnitine and acylcarnitine standards and deuterated carnitine and acylcarnitine standards were obtained from Dr. H.J. Ten Brink, VU Medical centre (Utrecht, Netherlands): Free carnitine.HCl, [d_3]Free carnitine.HCl, Acetylcarnitine.HCl, [d_3]Acetylcarnitine.HCl, Propionylcarnitine.HCl, [d_3]Propionylcarnitine.HCl, Butyrylcarnitine.HCl, [d_3]Butyrylcarnitine.HCl, Isovalerylcarnitine.HCl, [d_9]Isovalerylcarnitine.HCl, Hexanoylcarnitine.HCl, [d_3]Hexanoylcarnitine.HCl, Octanoylcarnitine.HCl, [d_3]Octanoylcarnitine.HCl, Decanoyl-L-carnitine.HCl, [d_3]Decanoyl-L-carnitine.HCl, Dodecanoyl-L-carnitine.HCl, [d_3]Dodecanoyl-L-carnitine.HCl, Tetradecanoyl-L-carnitine.HCl, [d_3]Tetradecanoyl-L-carnitine.HCl, Palmitoylcarnitine.HCl, [d_3]Palmitoylcarnitine.HCl, Octadecanoyl-L-carnitine.HCl and [d_3]Octadecanoyl-L-carnitine.HCl.

3.1.1 Acylcarnitine standard stock solution preparation

Standard stock solutions of known concentration, which was prepared by the New Born Screening laboratory in the Center for Human Metabolomics, were used to prepare stock concentrations for usage during this practical investigation. Stock solutions of all acylcarnitine standards were prepared separately in methanol, the final stock concentrations and volumes are indicated in **Table 3.1**. The stock solutions were then divided into 500 µL aliquots and stored at -80°C.

Table 3.1: Final stock concentrations for each individual acylcarnitine prepared.

Acylcarnitine	Final Stock Concentration (µmol/L)	Final Volume (µL)
Free carnitine (C0)	1000	400
Acetylcarnitine (C2)	1000	400
Propionylcarnitine (C3)	1000	400
Butyrylcarnitine (C4)	1000	400
Isovalerylcarnitine (C5)	1000	400
Hexanoylcarnitine (C6)	1000	400
Octanoylcarnitine (C8)	1000	400
Decanoylcarnitine (C10)	1000	400
Dodecanoylcarnitine (C12)	1000	400
Tetradecanoylcarnitine (C14)	1000	400
Palmitoylcarnitine (C16)	1000	400
Octadecanoylcarnitine (C18)	1000	400

3.1.2 Acylcarnitine isotope stock solution preparation

Standard isotope stock solutions of known concentration were freshly prepared in water and was then used to prepare a master isotope (IS) stock mixture for usage during this investigation. The stock acylcarnitine isotope mixture was prepared in methanol, the final stock concentrations and volumes are indicated in **Table 3.2**. The stock solution was stored at -20°C. The final concentration of IS added, and also used to calculate concentration values during method validation for linearity needed to be calculated because 250 µL of the final stock concentration was added to each urine sample, dried under nitrogen and then re-suspended in only 100 µL of mobile phase A. For precision, accuracy, stability and sample analysis, 350 µL of the final isotope stock solution were used. This volume was determined by injecting multiple urine samples each with different volumes of isotope mixture and selecting the appropriate volume for best results. **Equation 3.1** was used to calculate the final IS concentration added.

Equation 3.1: Final IS concentration (µmol/L)

$$C_1V_1 = C_2V_2$$

$$C_2 = \frac{C_1V_1}{V_2}$$

Where C_1 is the final stock volume, V_1 is the volume used from the stock and V_2 is the re-suspend volume (100 µL).

Table 3.2: Acylcarnitine isotope stock solution concentrations prepared.

Acylcarnitine	Final Volume (mL)	Final Stock Concentration (µmol/L)	Final Concentration in 250 µl of IS added (µmol/L)	Final Concentration in 350 µl of IS added (µmol/L)
d_3-Acetylcarnitine	100	2.162	5.406	7.569
d_3-Propionylcarnitine	100	1.796	4.491	6.288
d_3-Butyrylcarnitine	100	1.940	4.850	6.790
d_3-Isovalerylcarnitine	100	1.588	3.970	5.558
d_3-Hexanoylcarnitine	100	1.927	4.817	6.744
d_3-Octanoylcarnitine	100	1.771	4.428	6.199
d_3-Decanoylcarnitine	100	1.982	4.956	6.938
d_3-Dodecanoylcarnitine	100	1.986	4.966	6.953
d_3-Tetradecanoylcarnitine	100	1.995	4.987	6.982
d_3-Palmitoylcarnitine	100	2.051	5.128	7.179
d_3-Octadecanoylcarnitine	100	1.988	4.970	6.959

3.1.3 Preparation of calibration curve serial dilutions

To create the serial dilution range, 400 µL of each of the stock concentrations for all acylcarnitines were pooled together in one tube. The pooled sample was then dried under nitrogen for about one hour at 65°C. The residue was then resuspended in 400 µL methanol. The serial dilution range was created by adding a calculated volume of the initial pooled sample and then diluted to 400 µL methanol and then mixed by vortexing the sample. This process was continued until seven different concentration points was obtained, as indicated in **Table 3.3.** Calibration samples were stored at -80°C. For calibration curve sample preparation analysis, each sample was thawed at room temperature and then separately centrifuged at 10000 × g for 10 minutes. 10 µL of the cleaned up calibration sample were transferred to a clean tube and 250µL of the deuterated acylcarnitines-isotope solution was added to each sample and then vortexed. Samples were then dried under nitrogen for ±15 minutes at 65°C, after the samples were dried, 100 µL butanolic HCl was added to each sample and left to incubate for 15 minutes at 65°C. After the 15 minute incubation time, samples were dried again under nitrogen for ±15 minutes at 65°C. Dried samples were re-suspended in 100 µL of mobile phase (as described in Section 3.6). Samples were then placed into inserts in vials and into an auto sampler plate and analysed immediately.

Table 3.3: Concentration ranges for individual acylcarnitines used to prepare a serial dilution range for calibration curves.

Acylcarnitine	Point number	Concentration (µmol/L)	Concentration after preparation (µmol/L)
pooled sample of all carnitine standards	7	1000	100
	6	300	30
	5	100	10
	4	30	3
	3	10	1
	2	3	0.3
	1	1	0.1

Concentration range selection for calibration curves was based on the data obtained through HPLC-MS/MS analysis of 60 patients suffering from chronic fatigue in a preliminary investigation, using multiple reaction monitoring (MR) mode. These results were used to compile a concentration range which falls within the reference values suggested by Mueller *et al.* (2003). Bell curves were constructed for each individual carnitine and used to determine the 10^{th} and 90^{th} percentiles. The 10^{th} and 90^{th} percentiles were also calculated from the processed data, these percentiles were compared to LOD and LOQ determined from the calibration curves as described in Section 3.4.3. Abe *et al.* (2017) described a method to determine acylcarnitines in human urine in which they also state calibration ranges and quality control (QC) sample concentrations and thus this information was used as guidance in selection of calibration range concentrations and QC sample concentrations.

3.1.4 Quality control sample preparation

Three concentrations were selected based on data obtained from calibration curves: one low, one middle and one high concentration. The concentration for each individual acylcarnitine for each of the three concentrations selected are indicated in **Table 3.4.** 200 µL of the prepared calibration curve stock solutions for the selected concentrations ranges were added to 400 µL of urine (with a known creatinine value), for the high QC the highest calibration sample were used, for the middle QC calibration point 6 was used and diluted to the desired concentration and for the low QC, calibration point 5 was used and diluted to the desired concentration. The samples were then dried under nitrogen for one hour at 65°C. The residue was re-suspended in 600 µL methanol and then stored at -80°C. The QC samples were injected with each run, and treated the same as the patient and control samples. For QC sample preparation for analysis, each

sample was thawed at room temperature and then separately centrifuged (to remove crystals that might have formed) at 10000 × g for 10 minutes. 30 µL of the cleaned up QC sample were transferred to a clean tube and 250 µL of the deuterated acylcarnitines-isotope solution was added to each QC sample and then vortexed. Samples were then dried under nitrogen for ±15 minutes at 65°C, after the samples were dried, 100 µL butanolic HCl was added to each sample and left to incubate for 15 minutes at 65°C. After the 15 minute incubation time, samples were dried again under nitrogen for ±20 minutes at 65°C. Dried samples were re-suspended in 100 µL of mobile phase (as described in Section 3.6). Samples were then placed into inserts in vials and into an auto sampler plate and analysed immediately.

Table 3.4: Low, middle and high quality control samples selected

Acylcarnitine concentrations present:	Low Concentration (µmol/L)	Middle Concentration (µmol/L)	High concentration (µmol/L)
L – carnitine (C0)	5	20	80
Acetylcarnitine (C2)	5	20	80
Propionylcarnitine (C3)	5	20	80
Butyrylcarnitine (C4)	5	20	80
Isovalerylcarnitine (C5)	5	20	80
Hexanoylcarnitine (C6)	5	20	80
Octanoylcarnitine (C8)	5	20	80
Decanoylcarnitine (C10)	5	20	80
Dodecanoylcarnitine (C12)	5	20	80
Tetradecanoylcarnitine (C14)	5	20	80
Palmitoylcarnitine (C16)	5	20	80
Octadecanoylcarnitine (C18)	5	20	80

3.1.5 Mobile phase preparation

Mobile phase A consisted of HPLC grade water with 0.1% formic acid. Mobile phase B consisted of HPLC grade acetonitrile with 0.1% formic acid. Mobile phase for sample re-suspension consisted of 5% acetonitrile in water with 0.1% formic acid, as this is the composition of mobile phase at the start of the gradient elution for analysis.

3.2 Biological Samples

A total of 39 urine samples were collected from patients' preliminary diagnosed with chronic fatigue. All patients were adult females with ages ranging between 30 and 55 years. These patients were diagnosed on the following criteria: fatigue scores ranging between 8.0 and 10.00 out of a possible score of 10.00 based on the PFS, and on the MSQ patient scores were above 100. This was generated on the basis of a combined questionnaire – Biotransformation and Oxidative Stress Status profile (BOSS) - compiled by the Centre for Human Metabolomics, North West University, Potchefstroom Campus. This questionnaire is a combination of the Piper Fatigue Scale, the Medical Symptoms Questionnaire, as well as the inclusion of the life style questionnaire. Control samples were collected from 34 healthy individuals; all were adult females with ages ranging from 30 and 57 years. The healthy individuals completed the same BOSS questionnaire, and obtained scores between 1 and 2 out of a possible score of 10.00 for the PFS, and scores below 100 for the MSQ, thus these individuals was selected as controls according to their fatigue scores.

3.3 Method development and optimization

The method used for analysis was adapted from the pre-existing method for carnitine and acylcarnitine analysis compiled at the Centre for Human Metabolomics, North West University, Potchefstroom Campus by the BOSS laboratory. Even though this method was adapted from a pre-existing method, optimisation for the acylcarnitine standards and isotopes was done to ensure that the highest possible sensitivity is achieved. An application note of Agilent Technologies (George *et al.*, 2010) was used as a starting point for this optimization, where after optimization was done on the Agilent technologies optimizer program by injecting individually prepared (as described in Section 2.5) acylcarnitine standards and deuterated acylcarnitine isotopes. After optimization, a sample containing all acylcarnitines with their deuterated acylcarnitine isotopes were injected, where separation of all butylated acylcarnitines with their respective acylcarnitine isotopes were obtained. All acylcarnitines were analysed in positive ion mode. Specifications for carnitine and all acylcarnitines can be seen in **Table 3.5**.

3.3.1 Carnitine and acylcarnitines analysed

Table 3.5: Carnitine and acylcarnitines with their isotope information used for analysis

Acylcarnitine	Butylated Formula	Molecular weight (g/mole)	Butylated weight (g/mole)	Weight, positive ion mode (g/mole)
C0	$C_{11}H_{23}NO_3$	161	217.2	218.2
C0_IS	$C_{11}D_3H_{20}NO_3$	164.2	220.19	221.2
C2	$C_{13}H_{25}NO_4$	203.2	259.18	260.2
C2_IS	$C_{13}D_3H_{22}NO_4$	206.2	262.2	263.2
C3	$C_{14}H_{27}NO_4$	217	273.19	274.2
C3_IS	$C_{14}D_3H_{24}NO_4$	220.2	276.21	277.2
C4	$C_{15}H_{29}NO_4$	231	287.21	288.2
C4_IS	$C_{15}D_3H_{26}NO_4$	234.2	290.23	291.2
C5	$C_{16}H_{31}NO_4$	245	301.23	302.2
C5_IS	$C_{16}D_9H_{22}NO_4$	254.2	310.28	311.3
C6	$C_{17}H_{33}NO_4$	259	315.24	316.2
C6_IS	$C_{17}D_3H_{30}NO_4$	262	318.26	319.3
C8	$C_{19}H_{37}NO_4$	287	343.27	344.3
C8_IS	$C_{19}D_3H_{34}NO_4$	290.2	346.29	347.3
C10	$C_{21}H_{41}NO_4$	315	371.3	372.3
C10_IS	$C_{21}D_3H_{38}NO_4$	318.2	374.32	375.3
C12	$C_{23}H_{45}NO_4$	343	399.33	400.3
C12_IS	$C_{23}D_3H_{42}NO_4$	346.3	402.3	403.4
C14	$C_{25}H_{49}NO_4$	371	427.37	428.4
C14_IS	$C_{25}D_3H_{46}NO_4$	374	430.38	431.4
C16	$C_{27}H_{53}NO_4$	399	455.4	456.4
C16_IS	$C_{27}D_3H_{50}NO_4$	402.4	458.42	459.4
C18	$C_{29}H_{57}NO_4$	427	483.43	484.4
C18_IS	$C_{29}D_3H_{54}NO_3$	430.5	486.45	487.5

3.3.2 HPLC-MS/MS Specifications

The HPLC system used during this study consisted of an Agilent 1200 Infinity series coupled to an Agilent 6460 triple quadrupole mass spectrometer (Santa Clara, CA, USA), with a Jet Stream electrospray ionisation (ESI) source. Data acquisition of the analytes were done using Agilent MassHunter Data Acquisition (B.04.00) software and analyte quantification were done using Quantitative Analysis (B.04.00) and Qualitative analysis (B.04.00) software.

The source parameters for the MS were as follows: a gas temperature of 300°C, a gas flow of 7.5 L/min, the nebulizer was set at 30 psi and the capillary voltage was 3500 V. The column used for chromatographic separation was a C18 (Zorbax SB-Aqua, 1.8 µm, 2.1 x 100 mm) from Agilent Technologies (Santa Clara, CA, USA). A gradient elution was used with the column

chromatography to ensure separation of all compounds of interest. The summary of the mobile phase composition as a gradient elution is given in **Table 3.6**. A summary of all analysis parameters for each compound is given in **Table 3.7**.

3.3.3 Chromatographic separation

Reversed phase chromatography are generally used with a gradient elution, in which the mobile phase being used starts out more polar and gradually becomes more a-polar as the analytical run continues. This means that over time, the percentage organic solvent increases, increasing the elution strength of the eluent over time, allowing for all compounds of interest to elute within an acceptable time range. Gradient elution allows for compounds with a large range of polarity to be separated and eluted efficiently, without losing resolution of peaks eluting earlier and preventing broadening of peaks eluting at a later time. During this study, the gradient started with the same composition as the solvent in which the samples were suspended (5% acetonitrile in water with 0.1% formic acid), over time increased in organic solvent composition to achieve separation of closely eluting compound. The gradient is then increased to 95% of mobile phase B (organic solvent) to elute highly retained compounds and was held at an isocratic gradient to ensure elution of all compounds. After the isocratic hold, the gradient is returned to the initial gradient composition for conditioning (Snyder *et al.*, 1983; Jandera & Hájek, 2018). MRM chromatography achieved during this study can be seen in **Figure 3.1**.

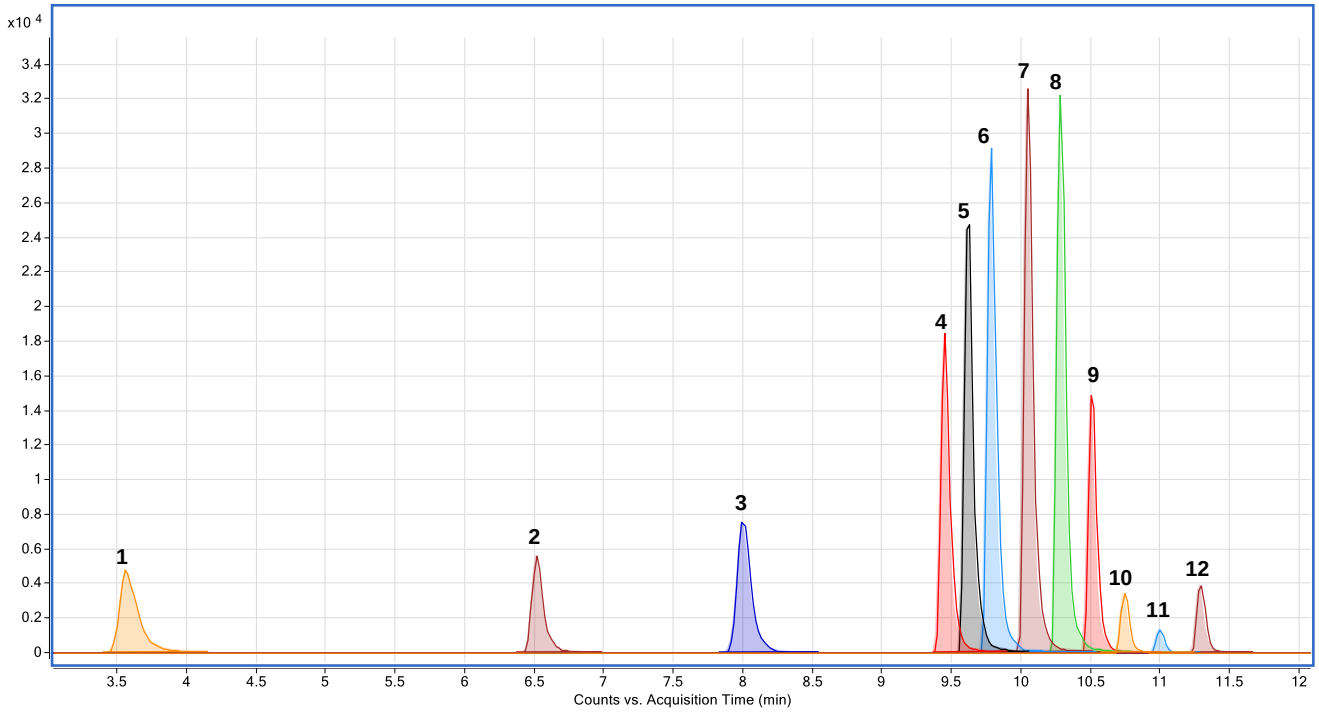


Figure 3.1: The chromatographic separation achieved for carnitine and acylcarnitine esters focused on in this study. Peak information: 1. C0 and C0_IS, 2. C2 and C2_IS, 3. C3 and C3_IS, 4. C4 and C4_IS, 5. C5 and C5_IS, 6. C6 and C6_IS, 7. C8 and C8_IS, 8. C10 and C10_IS, 9. C12 and C12_IS, 10. C14 and C14_IS, 11. C16 and C16_IS, 12. C18 and C18_IS.

Table 3.6: Mobile phase composition of gradient elution.

Time (minutes)	% Mobile phase A	% Mobile phase B	Flow (mL/min)
0.0	95.0	5.0	0.400
1.0	95.0	5.0	0.400
2.0	80.0	20.0	0.400
6.00	80.0	20.0	0.400
6.50	15.0	85.0	0.400
7.50	15.0	85.0	0.400
8.50	10.0	90.0	0.400
9.00	5.0	95.0	0.400
10.00	5.0	95.0	0.400
10.10	5.0	95.0	0.500
12.00	5.0	95.0	0.500
12.50	95.0	5.0	0.500

Table 3.7: Summary of all acylcarnitines analysed with their specifications.

Acylcarnitine	Precursor ion (m/z)	Product ion (m/z)	Dwell	Fragmentor voltage	Collision Energy (CE)	Retention Time (minutes)
C0	218.2	103	45	103	16	3.525
C0_IS	221.2	103	45	113	16	3.524
C2	260.2	85.1	45	103	20	5.950
C2_IS	263.2	85.1	45	98	24	5.930
C3	274.2	85.1	45	113	20	7.207
C3_IS	277.2	85	45	103	20	7.166
C4	288.2	85.1	45	103	24	8.922
C4_IS	291.2	85	45	128	24	8.921
C5	302.2	85.1	45	113	24	9.120
C5_IS	311.3	85.1	45	123	24	9.100
C6	316.2	85.	45	128	20	9.260
C6_IS	319.3	85	45	118	24	9.259
C8	344.3	85.1	45	142	24	9.517
C8_IS	347.3	85.1	45	118	28	9.516
C10	372.3	85.1	45	118	28	9.775
C10_IS	375.3	85.1	45	122	28	9.774
C12	400.3	85.1	45	127	32	10.033
C12_IS	403.4	85.1	45	132	28	10.032
C14	428.4	85.1	45	141	28	10.311
C14_IS	431.4	85.1	45	147	32	10.310
C16	456.4	85.1	45	146	32	10.589
C16_IS	459.4	85.1	45	137	32	10.588
C18	484.4	85.1	45	171	36	10.888
C18_IS	487.5	85.1	45	132	32	10.887

For free carnitine (C0), the product ion of m/z 103 was used instead of the usual m/z 85 fragment. Derivatised free carnitine yield both m/z 85 and 103 product ions, the m/z 103 product ion includes the aliphatic hydroxyl group compared to the acylcarnitines where this aliphatic hydroxyl group forms part of fatty acid that is lost, the formation of both fragments are indicated in ***Figure 3.2****. Chace et al. (2003) reported that there is no other advantage than a higher sensitivity for the precursor ion of 103 Da. The fragment of m/z 85.1 is the result of the optimizer program.*

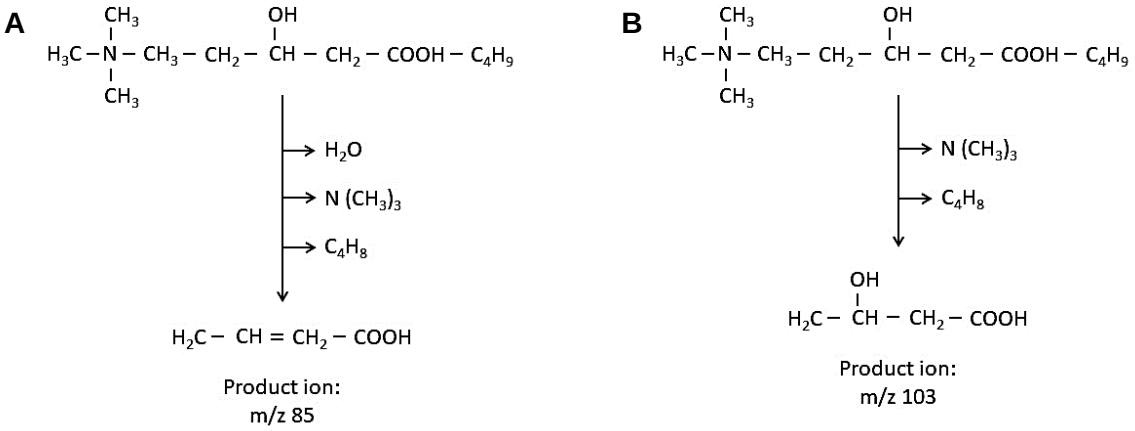


Figure 3.2: (A) shows the formation of the m/z 85 ion with the aliphatic hydroxyl group removed and (B) shows the formation of the m/z 103 ion where the aliphatic hydroxyl group is included in the fragment.

3.4 Method Validation

Validation involves the documenting of the performance features of an analytical method, to determine if the characteristics are suitable and reliable for the intended analytical method application and to assure that the method offers accurate and reproducible results (FDA, 2013; Gonzalez *et al.*, 2014).

Three types of validation is considered namely, (1) full validation, which include all fundamental parameters namely selectivity and sensitivity, precision, accuracy, stability, calibration curves and reproducibility. Normally a pre-validation will be carried out to identify some parameters, especially for the lower limit of quantification (LLOQ) and the upper limit of quantification (ULOQ), before the full validation process is started, (2) partial validation, will be done when minor changes are made to a pre-existing method which was already validated, during this validation process parameters can range from only doing accuracy (inter- and intraday) validation to almost a full validation, and (3) cross validation which will be carried out in the case where a comparison needs to be made between validation parameters of two or more different analytical methods used in the same study, or when different instruments are being used within the same laboratory (Gonzalez *et al.*, 2014).

For this study, because the method used was adapted from a pre-existing method, partial validation was done almost to the full validation extent. Parameters validated include, selectivity, precision, accuracy, stability, and linearity (calibration curves).

3.4.1 Selectivity

Selectivity of an analytical method relies on its ability to detect analytes of interest without the interference from other compounds found in the sample. For this study, four new compounds were added to the pre-existing method, which had to be assessed for sensitivity, these compounds included butyrylcarnitine (C4), d_3-butyrylcarnitine.HCl (C4_IS), hexanoylcarnitine (C6) and d_3-hexanoylcarnitine.HCl (C6_IS). For this study, three QC samples were prepared in triplicate as described in Section 3.1.4 and analysed, together with a non-spiked human urine sample (only containing isotope mixture) used for preparing the QC samples.

3.4.2 Linearity (calibration curves)

Calibration curves correlate the relationship between the known concentration of a compound and the normalised response of the instrument, which should be continuous and reproducible. A calibration curve should be generated for each compound of interest and should consist of at least six different concentration points covering the whole calibration range (FDA, 2013; Gonzalez *et al.*, 2014)

During this study, a concentration range of six different concentrations were used as described in Section 3.1.3. All compounds analysed were within linear range.

3.4.3 Limit of detection and quantification

Limit of detection (LOD) is defined by the FDA (2013) as the lowest concentration of an analyte that can reliably be differentiated from background noise by the analytical method. Different approaches to determine LOD are described by the FDA (1996) and Shrivastava and Gupta (2011) namely: by visual evaluation which can be used for both instrumental and non-instrumental methods; signal-to-noise ratio calculations (comparing signals measured from samples with known concentrations to the signals of blank sample, where for LOD a ratio of 3:1 is acceptable); standard deviation of response and the slope, where LOD is calculated as 3.3 times the standard deviation of the response divided by the slope of the calibration curve. Standard deviation of the blank and the calibration curves can be used to determine the standard deviation. LOD was determined using the standard deviation of response and the slope, where the calibration curves were used to determine standard deviation.

Limit of quantification (LOQ) on the other hand is defined as the lowest concentration of an analyte that can be determined with acceptable accuracy and precision of the data set (Shrivastava & Gupta, 2011). LOQ can be determined by visual evaluation which can be used for both instrumental and non-instrumental methods; signal-to-noise ratio calculations (comparing signals measured from samples with known concentrations to the signals of blank sample, where for LOQ a ratio of 10:1 is acceptable); standard deviation of response and the slope, where LOD is calculated as ten times the standard deviation of the response divided by the slope of the calibration curve. Standard deviation of the blank and the calibration curves can be used to determine the standard deviation. LOQ was determined using the standard deviation of response and the slope, where the calibration curves were used to determine standard deviation as described by the FDA guidance for industry (1996).

3.4.4 Precision and Accuracy

Precision is the closeness of individual measures of a specific analyte when analysed in multiple aliquots by the same method repeatedly. A minimum of three concentrations which fall within the suspected range of the analyte is recommended by the FDA (2013). The %CV (or %RSD) for precision determinations should not exceed 15%, except for LLOQ where it should not exceed 20% of the CV. Precision are further divided into within-run (intraday) and between-run (interday) precision.

Interday precision measures the precision of the method when applied to one sample, prepared in triplicate, during one single analytical run. This will demonstrate the consistency in sample preparation by the analyst and together with this, one sample was injected in triplicate, which will demonstrate the reproducibility of the instrument used. Intraday precision is the measurement of precision over a period of time, which means the same sample is prepared fresh, in triplicate, over a set time period of a minimum of five days (Gonzalez *et al.*, 2014). For this study, three different concentrations covering the calibration range were selected (QC samples), prepared in triplicate and analysed accordingly.

Accuracy of a method can be described as the closeness of agreement between the measured concentration and the true concentration detected. Standard deviation serves as the measurement for accuracy and the mean value of the analytes should be within 15% of the nominal, except for LLOQ where it should not deviate by more than 20%. Accuracy also expresses recovery of the analytes because when determining accuracy the detector response obtained for the amount of analyte added to a sample is compared to the true concentration of the analyte in the solvent and indicates the percentage of the analyte recovered. The recovery does not need to be 100%, but should be consistent, precise and reproducible (FDA, 2013). For

determination of percentage recovery, three different concentrations covering the calibration curve range (low, middle and high QC samples) were selected, prepared in triplicate and analysed.

3.4.5 Stability

Defined as the chemical stability of the analytes to be analysed in a biological sample under the specific conditions for given time intervals by Gonzalez *et al.* (2014). Conditions should be selected according to the type of biological sample and all the possible scenarios the sample could encounter during sample transportation, preparation and analysis as stated by the FDA (2013). Guidelines state that the calculated value of the stability samples should not deviate more than ± 15% from the nominal value (Gonzalez *et al.*, 2014)

During this study, five different storage possibilities for stability validation were selected. Freeze and thaw stability was evaluated by storing low, middle and high QC samples at temperatures of -80°C and -20°C respectively for 24 hours and one week respectively. Short-term stability was evaluated by storing low and high QC samples at 4°C, for 24 hours and one week respectively. Bench-top stability was assessed by storing low, middle and high value QC samples at ambient temperature for 24 hours and 1 week respectively. Auto-sampler stability was evaluated by storing the low, middle and high QC samples in the compartment of the HPLC-MS/MS instrument for 24 hours and one week respectively. All stability QC samples were prepared as described in Section 3.1.4.

3.4.6 Recovery

The detector response obtained for the amount of analyte added to a sample compared to the true concentration of the analyte in the solvent indicates the percentage of the analyte recovered. The recovery does not need to be 100%, but should be consistent, precise and reproducible (FDA, 2013). For determination of percentage recovery, three different concentrations covering the calibration curve range were selected, prepared in triplicate and analysed.

3.5 Sample Preparation

Urine samples were thawed at room temperature. The urine samples were then separately centrifuged (to remove crystals that might have formed) at 10000 × g for 10 minutes. 20 µL of the cleaned up urine samples were transferred to a clean tube and 350 µL of the deuterated acylcarnitine-isotope solution was added to each urine sample and then vortexed. Samples were then dried under nitrogen for ±20 minutes at 65°C, after the samples were dried, 100 µL butanolic HCl was added to each sample and left to incubate for 15 minutes at 65°C. After the 15 minute incubation time, samples were dried again under nitrogen for ±15 minutes at 65°C. Dried samples were re-suspended in 100 µL of mobile phase A (as described in Section 3.6). Samples were then placed into inserts in vials and into an auto sampler plate and analysed immediately.

3.6 Sample Analysis by HPLC-MS/MS

Chromatographic separation was performed on an Agilent 1290 Infinity HPLC coupled to an Agilent 6460 Triple Quadrupole Mass Spectrometer.

The Agilent 1290 Infinity HPLC was used for sample handling as well as mobile phase delivery. A 2 µL sample aliquot was injected onto the column. A gradient elution with two mobile phases was used: mobile phase A consisted of water with 1% formic acid and mobile phase B consisted of acetonitrile with 1% formic acid.

MS/MS analysis was performed on an Agilent 6460 Triple Quad in positive ion electrospray mode. A precursor ion scan after controlled collision induced dissociation, with a fragmentor voltage specific for each compound (range of 98-171 V) and a collision energy specific for each compound (range of 16-36 V) was used for acylcarnitine analysis.

MRM mode was used to quantify carnitine and acylcarnitines. Both the first and third quadrupole mass analysers are held static at the m/z of the precursor ions and the most intense product ion,

respectively for all individual compounds with all other specifications are summarised in **Table 3.7**. A schematic representation of MRM mode is given in **Figure 2.5**.

3.7 Statistical methods

Multivariate (PCA) and univariate (unpaired *t*-test) statistical analysis were applied using MetaboAanalyst, a metabolomics web-based server (version 3.0).

Data files were composed with the concentrations of the patient and control samples, as specified by MetaboAnalyst, and was uploaded in comma separated values (.csv) format. A data integrity check was performed and no missing values were detected. No sample normalization or data scaling was performed, but a log transformation was carried out for data transformation. This is done to apply the data to the unpaired *t*-test.

CHAPTER 4

4. RESULTS AND DISCUSSION

4.1 Validation parameters results:

4.1.1 Selectivity

The four new analytes that needed to be tested included butyrylcarnitine (C4), d_3-butyrylcarnitine (C4_IS), hexanoylcarnitine (C6) and d_3-hexanoylcarnitine (C6_IS). Both C4 and C6 with their isotope solution were detected with no interference from other analytes. **Figures 4.1 – 4.5** shows that these analytes are detected without interference in all three QC samples as well as in a patient urine sample. **Figure 4.6** shows the MRM chromatogram of the isotope mixture.

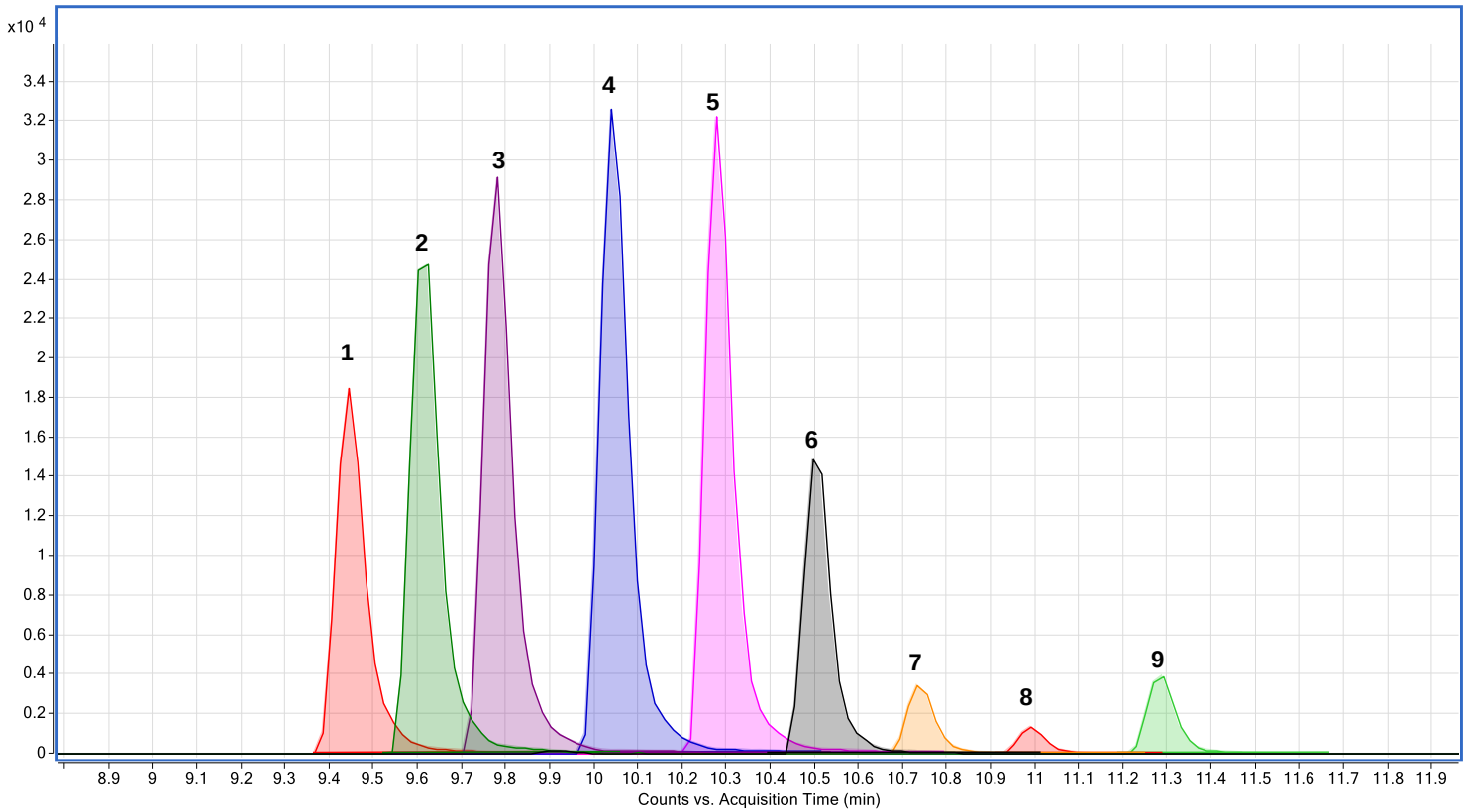


Figure 4.1: MRM of compounds C4 to C18 with their isotopes in a urine sample with only isotope mixture added. Peak information: **1.** C4 and C4_IS, **2.** C5 and C5_IS, **3.** C6 and C6_IS, **4.** C8 and C8_IS, **5.** C10 and C10_IS, **6.** C12 and C12_IS, **7.** C14 and C14_IS, **8.** C16 and C16_IS, **9.** C18 and C18_IS.

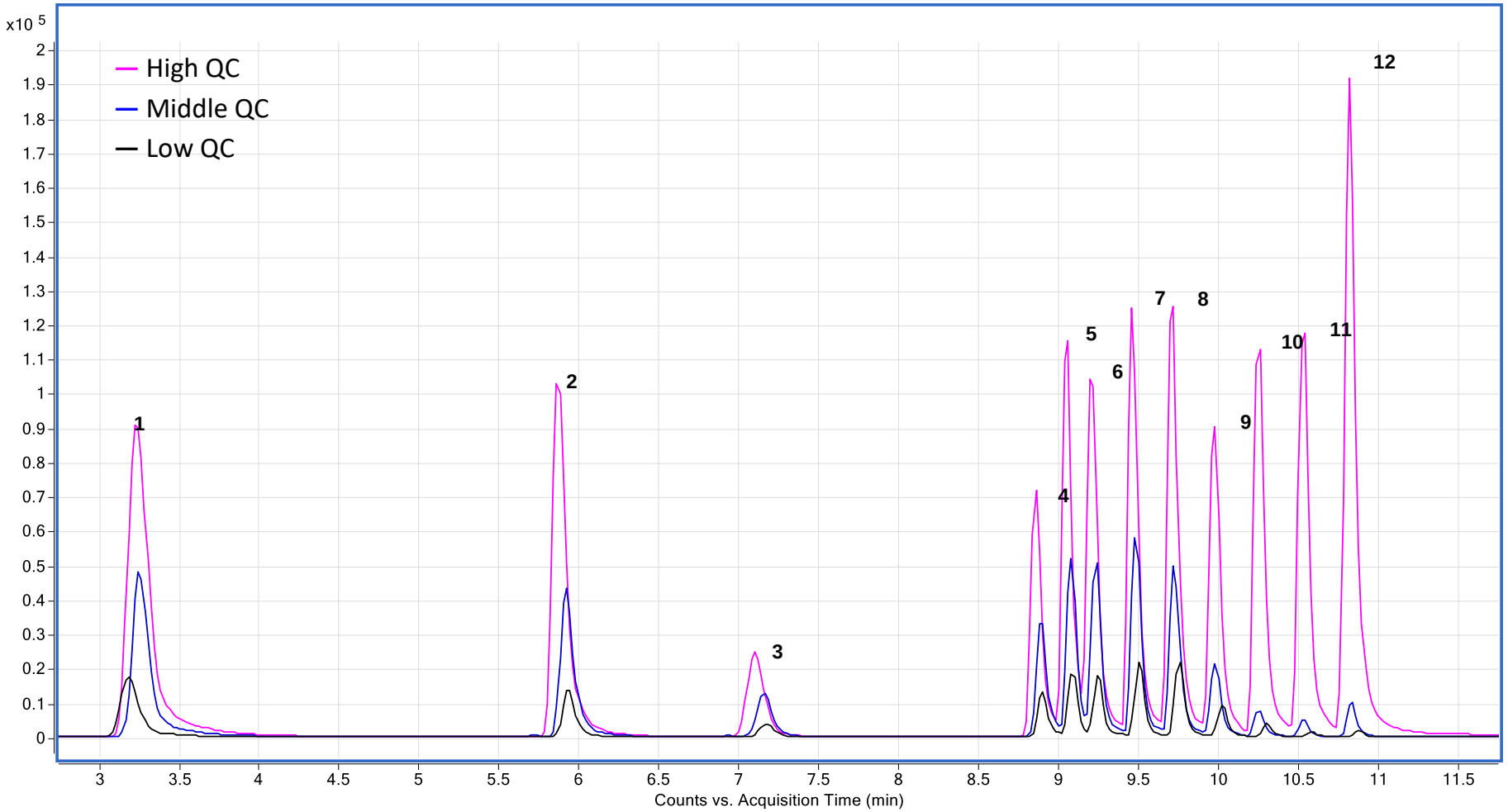


Figure 4.2: TIC of the urine sample spiked with acylcarnitine standard and isotope mixture (QC samples overlay). Peak information: **1.** C0 and C0_IS, **2.** C2 and C2_IS, **3.** C3 and C3_IS, **4.** C4 and C4_IS, **5.** C5 and C5_IS, **6.** C6 and C6_IS, **7.** C8 and C8_IS, **8.** C10 and C10_IS, **9.** C12 and C12_IS, **10.** C14 and C14_IS, **11.** C16 and C16_IS, **12.** C18 and C18_IS.

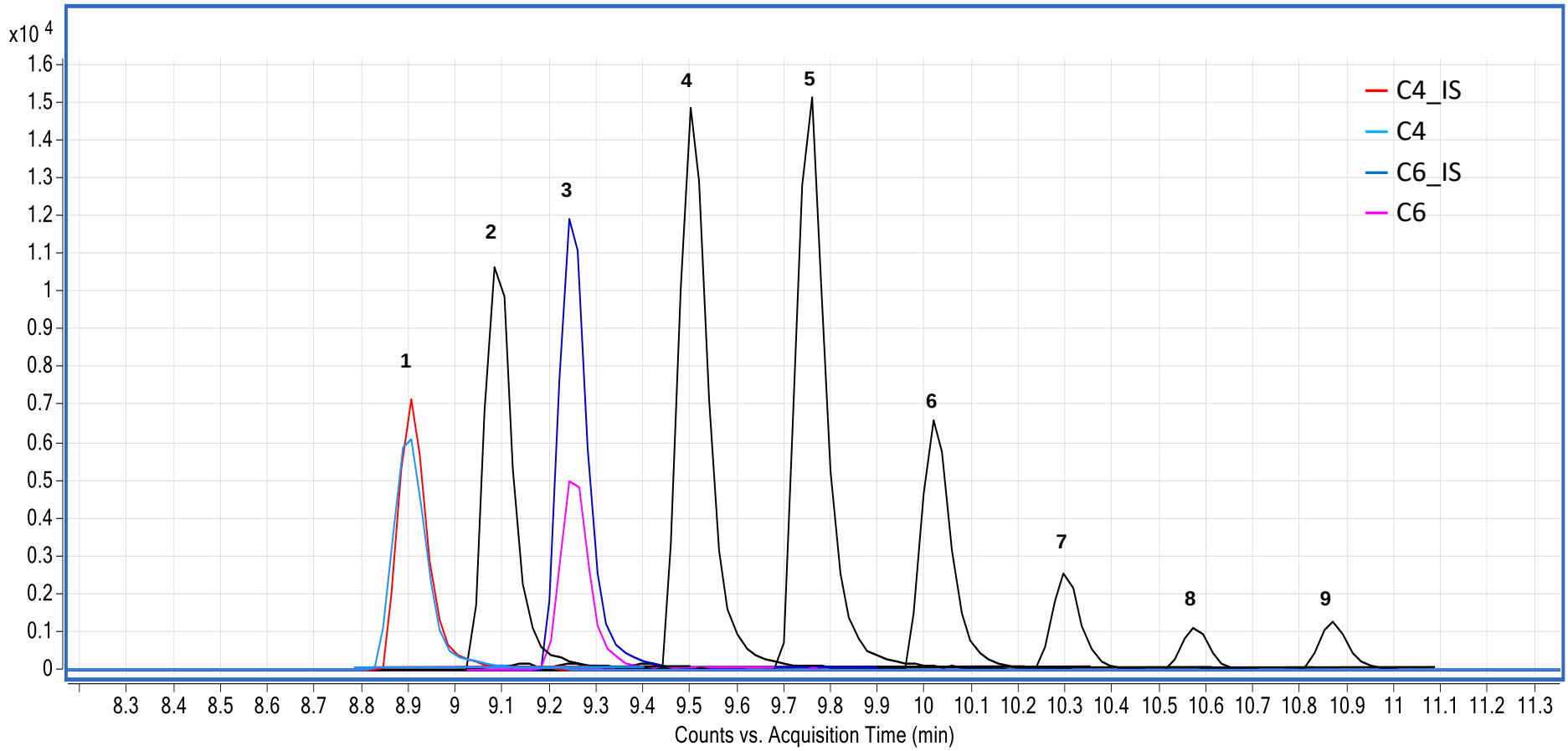


Figure 4.3: Individual compounds, C4, C4_IS, C6 and C6_IS in the low QC sample. Peak information: **1.** C4 and C4_IS, **2.** C5 and C5_IS, **3.** C6 and C6_IS, **4.** C8 and C8_IS, **5.** C10 and C10_IS, **6.** C12 and C12_IS, **7.** C14 and C14_IS, **8.** C16 and C16_IS, **9.** C18 and C18_IS.

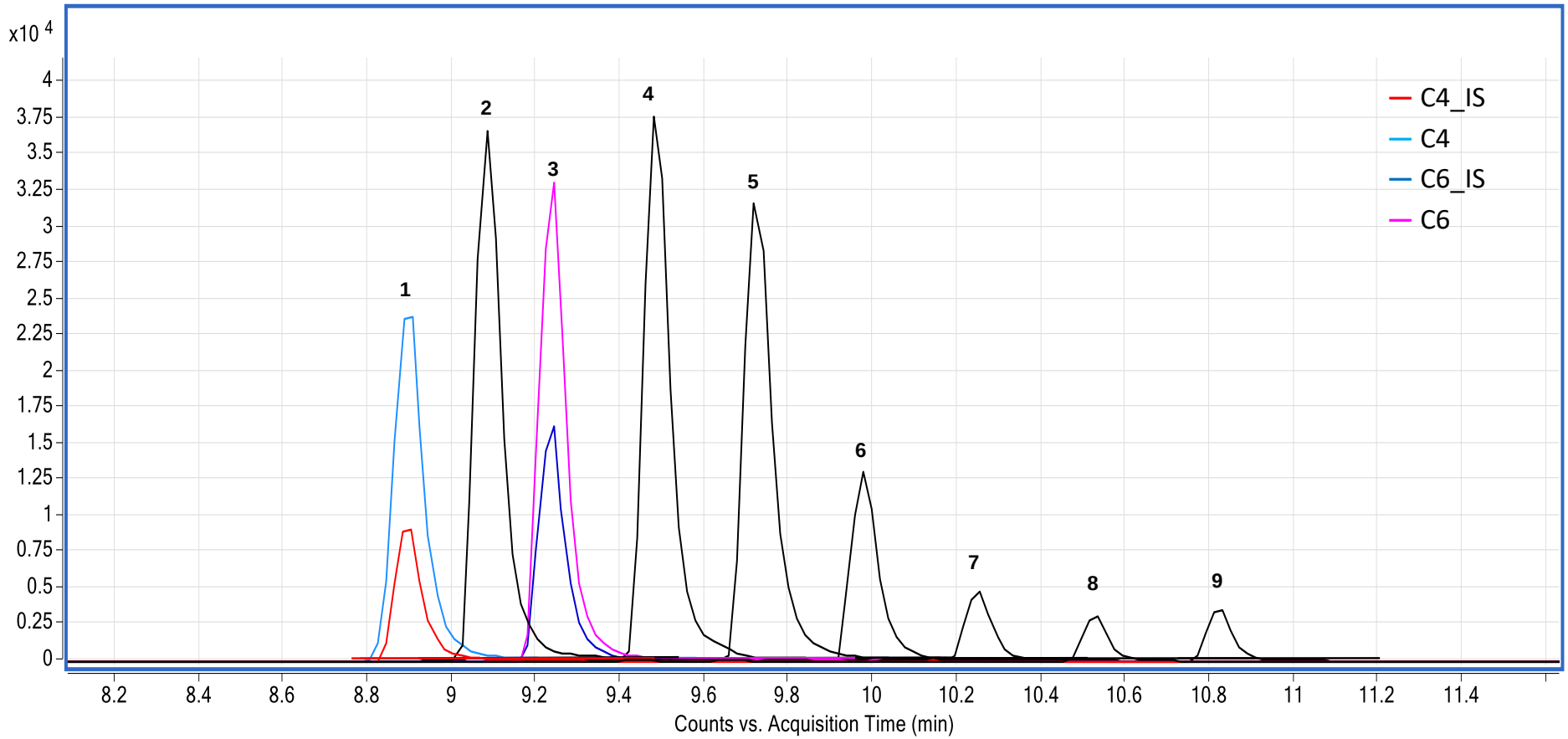


Figure 4.4: Individual compounds, C4, C4_IS, C6 and C6_IS in the middle QC sample. Peak information: **1.** C4 and C4_IS, **2.** C5 and C5_IS, **3.** C6 and C6_IS, **4.** C8 and C8_IS, **5.** C10 and C10_IS, **6.** C12 and C12_IS, **7.** C14 and C14_IS, **8.** C16 and C16_IS, **9.** C18 and C18_IS.

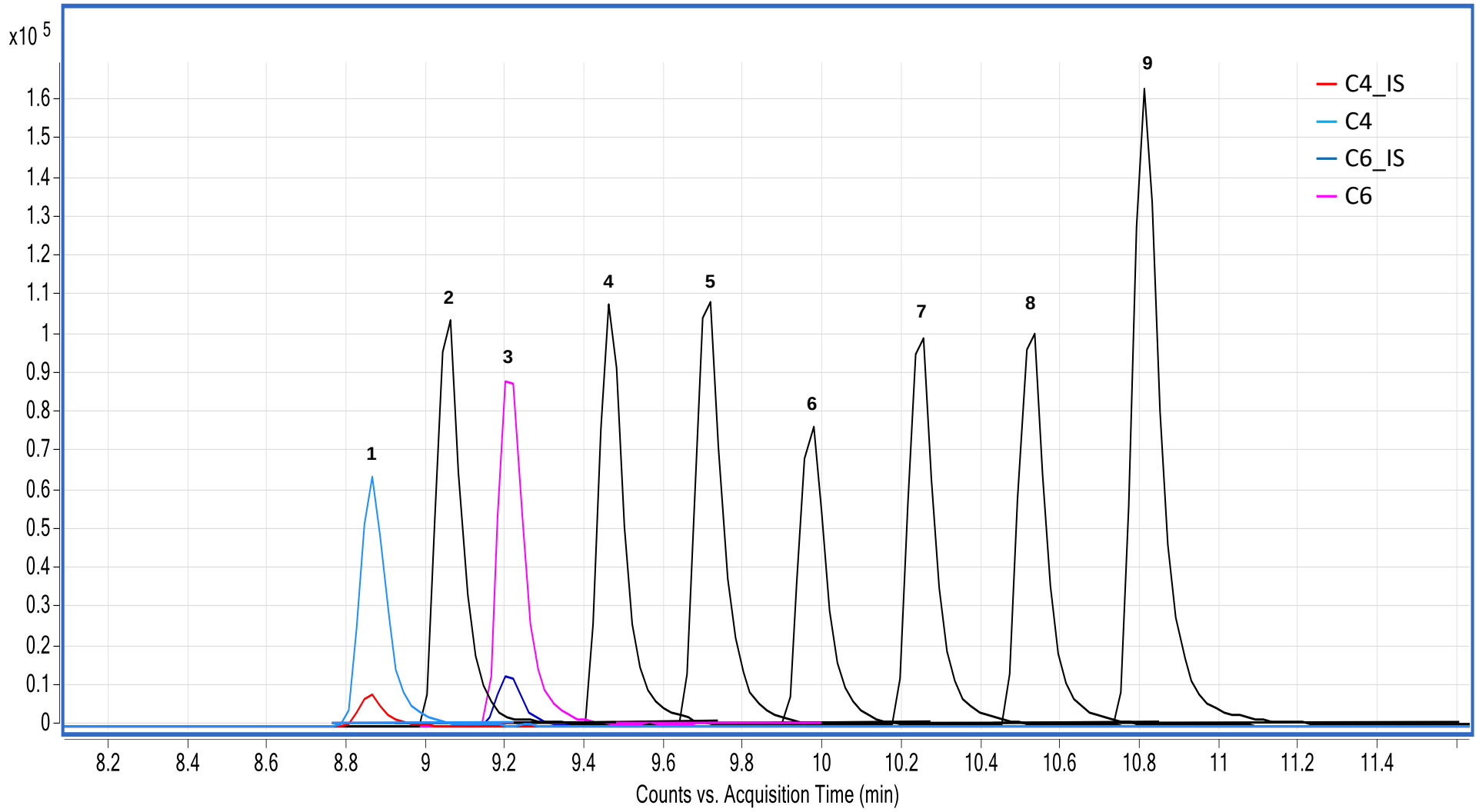


Figure 4.5: Individual compounds, C4, C4_IS, C6 and C6_IS in the high QC sample. Peak information: **1.** C4 and C4_IS, **2.** C5 and C5_IS, **3.** C6 and C6_IS, **4.** C8 and C8_IS, **5.** C10 and C10_IS, **6.** C12 and C12_IS, **7.** C14 and C14_IS, **8.** C16 and C16_IS, **9.** C18 and C18_IS.

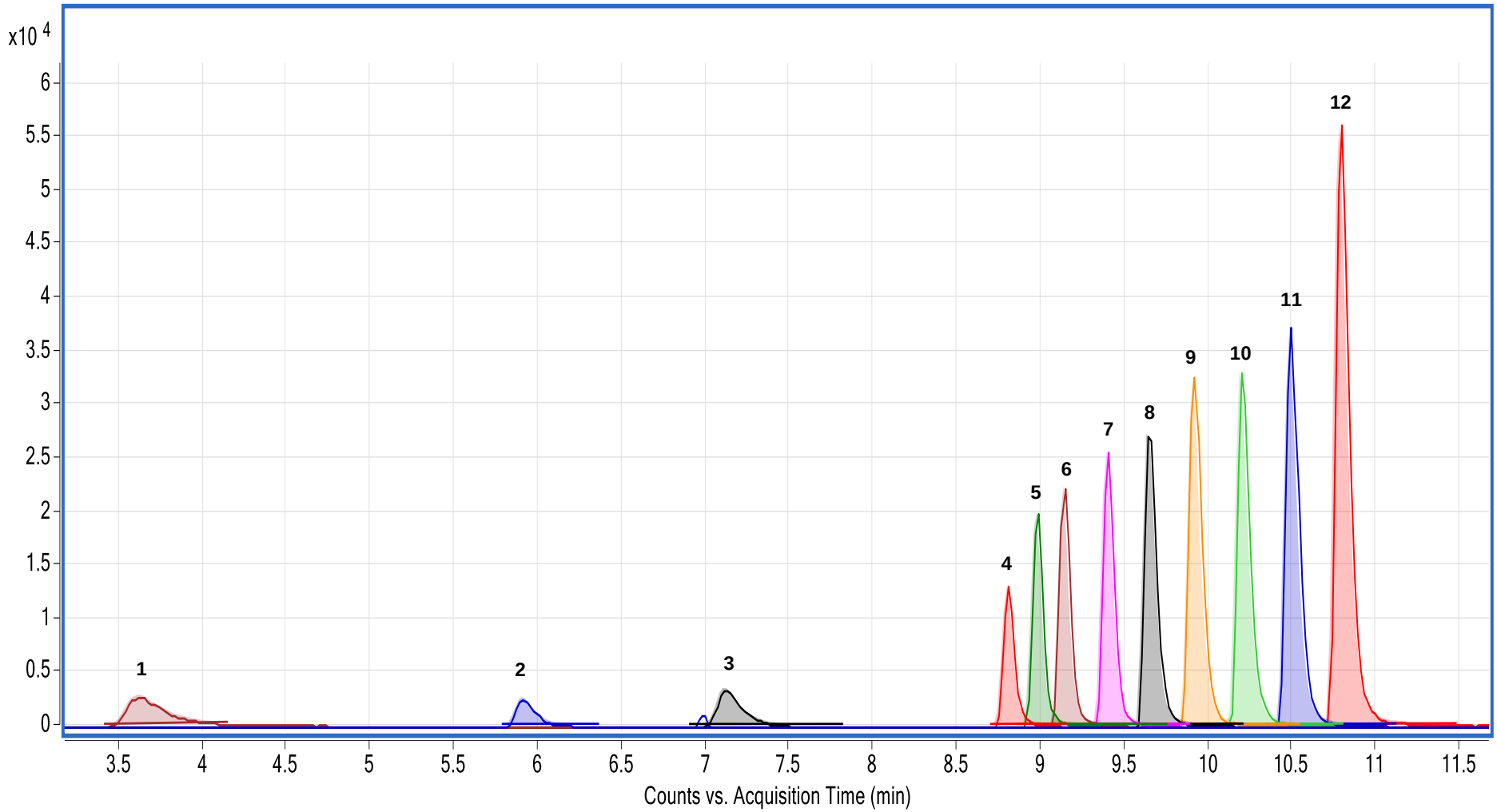


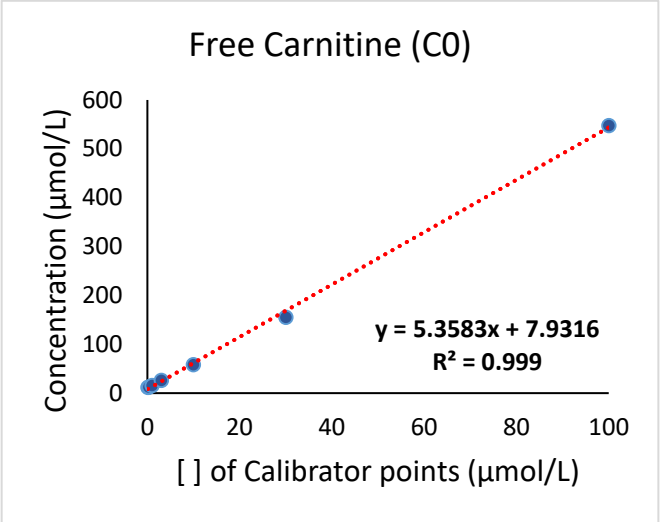
Figure 4.6: MRM of isotope mixture only. Peak information: **1.** C0 and C0_IS, **2.** C2 and C2_IS, **3.** C3 and C3_IS, **4.** C4 and C4_IS, **5.** C5 and C5_IS, **6.** C6 and C6_IS, **7.** C8 and C8_IS, **8.** C10 and C10_IS, **9.** C12 and C12_IS, **10.** C14 and C14_IS, **11.** C16 and C16_IS, **12.** C18 and C18_IS.

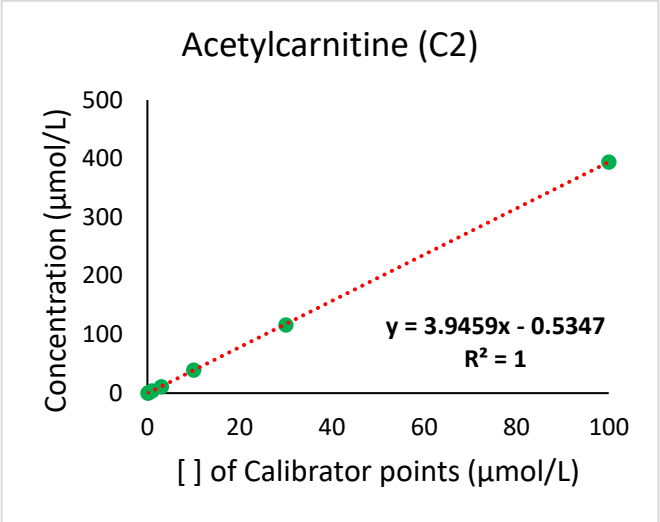
4.1.2 Linearity

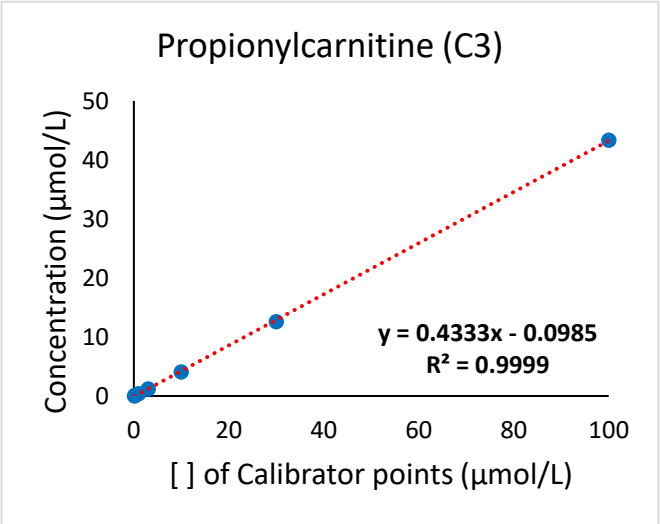
The linearity of the method was determined by a standard concentration range specific to each individual carnitine. The correlation coefficient (R^2) and linear ranges for individual carnitines are summarised in **Table 4.1**, with all calibration graphs included in **Figure 4.7.** Correlation coefficient (R^2) values ranged from 0.999 to 1 with a wide range of linearity over the concentration ranges.

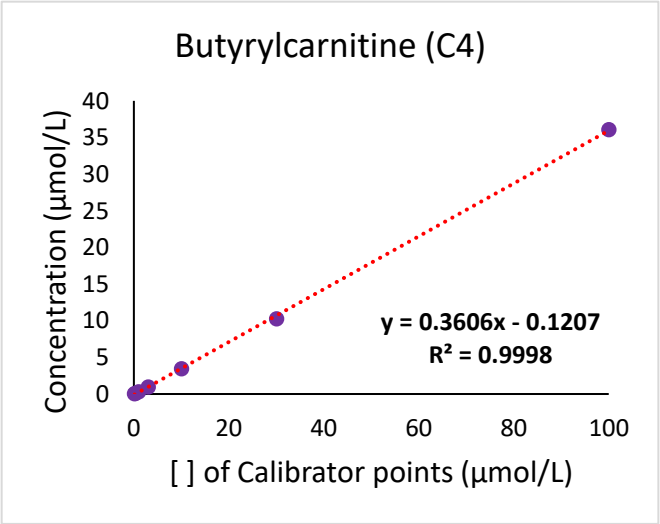
Table 4.1: Concentration ranges for individual acylcarnitines analysed with their linear regions and corresponding correlation coefficients.

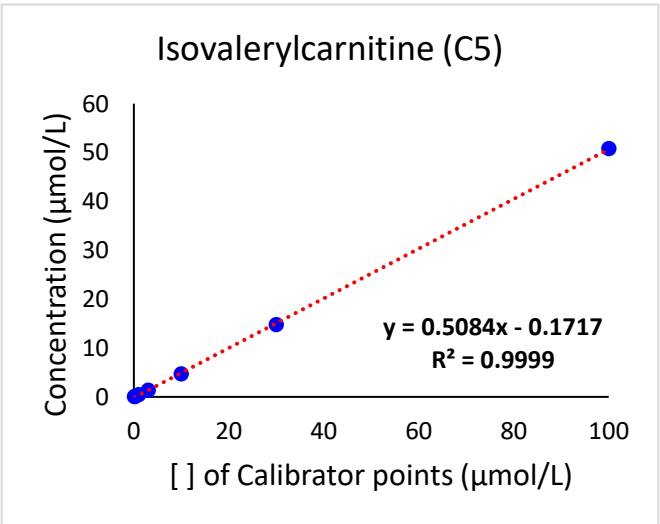
Acylcarnitine	Range analysed (µmol/L)	Correlation Coefficient (R^2)
C0	0.1 – 80.0	0.999
C2	0.1 – 80.0	1
C3	0.1 – 80.0	0.9999
C4	0.1 – 80.0	0.9998
C5	0.1 – 80.0	0.9999
C6	0.1 – 80.0	1
C8	0.1 – 80.0	0.9997
C10	0.1 – 80.0	0.9999
C12	0.1 – 80.0	0.9999
C14	0.1 – 80.0	0.9999
C16	0.1 – 80.0	0.9998
C18	0.1 – 80.0	1

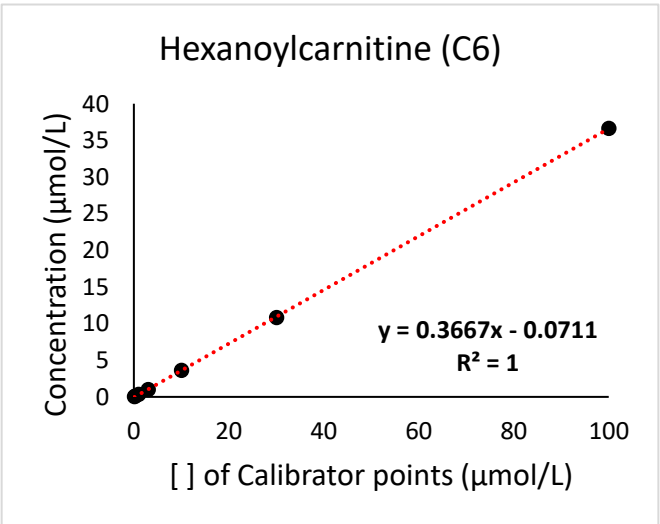

Free Carnitine (C0)
Concentration (µmol/L)
600
500
400
300
200
100
0
0
20
40
60
80
100
[ ] of Calibrator points (µmol/L)
y = 5.3583x + 7.9316
R² = 0.999


Acetylcarnitine (C2)
Concentration (µmol/L)
500
400
300
200
100
0
0
20
40
60
80
100
[ ] of Calibrator points (µmol/L)
y = 3.9459x - 0.5347
R² = 1


Propionylcarnitine (C3)
Concentration (µmol/L)
50
40
30
20
10
0
0
20
40
60
80
100
[ ] of Calibrator points (µmol/L)
y = 0.4333x - 0.0985
R² = 0.9999


Butyrylcarnitine (C4)
Concentration (µmol/L)
40
35
30
25
20
15
10
5
0
0
20
40
60
80
100
[ ] of Calibrator points (µmol/L)
y = 0.3606x - 0.1207
R² = 0.9998


Isovalerylcarnitine (C5)
Concentration (µmol/L)
60
50
40
30
20
10
0
0
20
40
60
80
100
[ ] of Calibrator points (µmol/L)
y = 0.5084x - 0.1717
R² = 0.9999


Hexanoylcarnitine (C6)
Concentration (µmol/L)
40
35
30
25
20
15
10
5
0
0
20
40
60
80
100
[ ] of Calibrator points (µmol/L)
y = 0.3667x - 0.0711
R² = 1

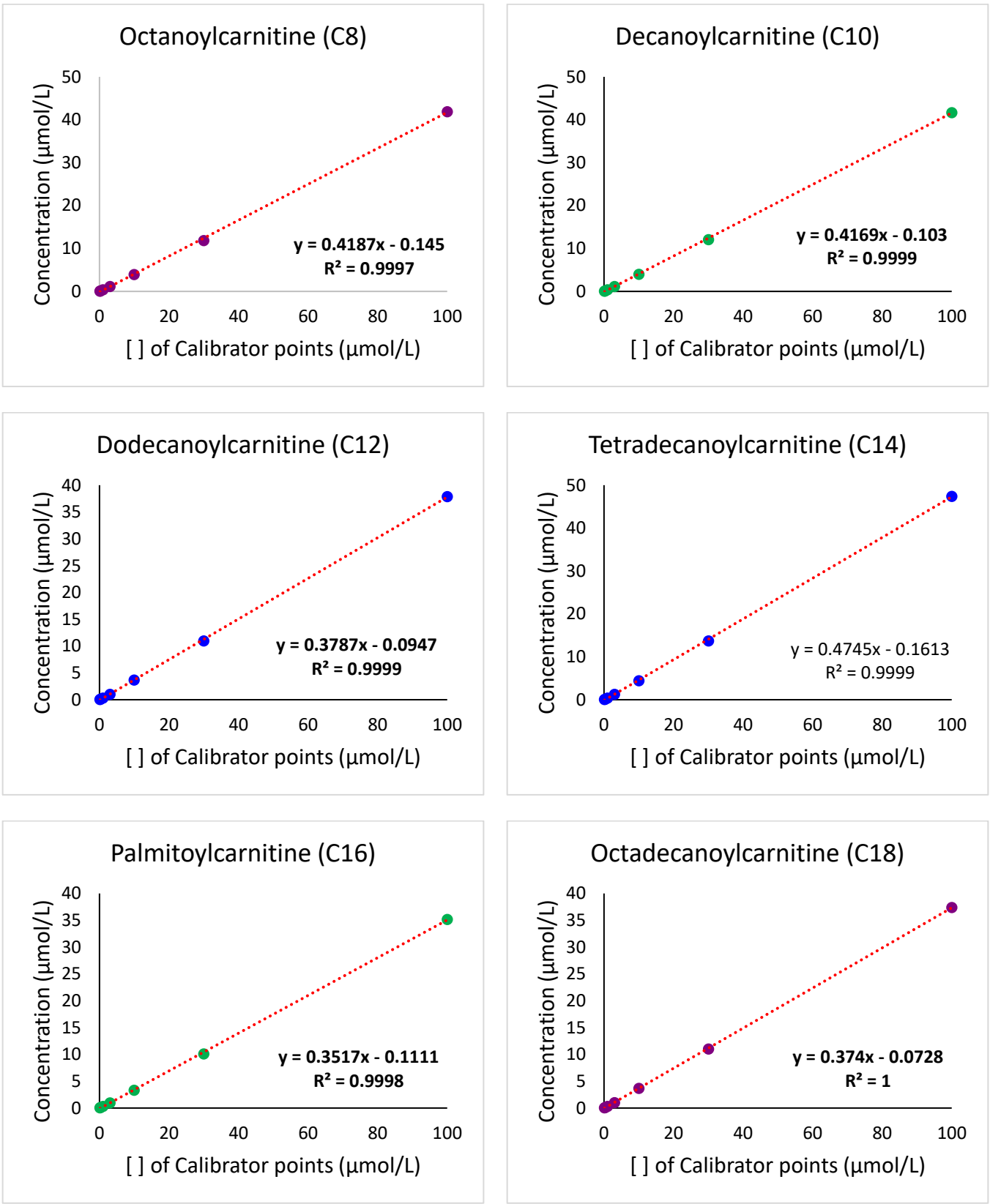


Figure 4.7: Calibration curves for carnitine and individual acylcarnitines.

4.1.3 LOD and LOQ

Residual standard deviation were calculated using the STEYX function in excel, which calculates the residual standard deviation of regression. The formula for determining the LOD and LOQ is given in **Equation 4.1** and **Equation 4.2** respectively, results are shown in **Table 4.2**. Overall the LOD and LOQ corresponded well with the linear ranges of the calibration curves and no problems with analyte detection was encountered.

Equation 4.1: Limit of detection

$$\text{LOD} = \frac{\sigma}{S} \times 3.3$$

Where σ is the residual standard deviation of the regression line and S the slope of the calibration curve.

Equation 4.2: Limit of quantification

$$\text{LOQ} = \frac{\sigma}{S} \times 10$$

Where σ is the residual standard deviation of the regression line and S the slope of the calibration curve.

Table 4.2: Limit of detection and limit of quantification.

compound name	slope	STEYX	LOD (µmol/L)	LOQ (µmol/L)
C0	5.358	6.937	4.27	12.95
C2	3.946	0.797	0.67	2.02
C3	0.433	0.167	1.27	3.85
C4	0.361	0.209	1.92	5.81
C5	0.508	0.209	1.36	4.12
C6	0.367	0.079	0.71	2.14
C8	0.419	0.306	2.41	7.30
C10	0.417	0.176	1.39	4.22
C12	0.379	0.146	1.28	3.87
C14	0.475	0.215	1.50	4.54
C16	0.352	0.191	1.79	5.44
C18	0.374	0.085	0.75	2.28

4.1.4 Precision and Accuracy

Precision was determined as %RDS, also known as coefficient of variance (%CV) and accuracy was determined as a percentage of the nominal concentration (FDA, 1996; Bae *et al.*, 2008), **Equation 4.3** and **Equation 4.5** were used for the calculation of precision and accuracy, respectively. **Equation 4.4** was used to calculate concentration of analytes. Precision and accuracy results obtained are summarized in **Table 4.3**.

Equation 4.3: Precision (%RSD)

$$\text{RSD} = \frac{Standard\ deviation}{mean} \times 100$$

Equation 4.4: Concentration calculation (µmol/L)

$$\text{Concentration} = \frac{Acylcarnitne\ response}{Acylcarnitine\ Isotope\ response} \div \text{Slope of calibration curve} \times \text{Isotope stock concentration added}$$

Equation 4.5: Accuracy (%)

$$\text{Accuracy} = \frac{mean\ observed\ concentration}{nominal\ concentration} \times 100$$

Interday results obtained showed good overall precision for all analytes with all RSD values within 15% of the nominal. This shows consistency in sample preparation by the analyst with minimal variance between individual samples prepared. The low QC sample display good precision with RDS values between 0.6% and 9.42%. Values between 0.97% and 8.12% were obtained for the middle QC sample, which indicates good precision and the high QC sample also indicates good precision with all %RSD values within prescribed limits of the nominal.

Interday accuracy was within 15% of the nominal for all acylcarnitines in all QC samples, as described in Section 3.4.4, except for free carnitine (C0), which has a very high accuracy percentage for the low QC sample, as well as high accuracy percentages for the middle and high QC samples. All three QC samples has accuracy percentage that is higher than the nominal and is not reproducible considering the big difference in the percentage accuracy going from the low QC to the middle QC to the high QC sample.

A possible explanation for this could be due to hydrolysis of acylcarnitines to free carnitine as stated in Section 3.1.6. The chromatograms in **Figures 4.8** to **4.19** illustrated hydrolysis that took place of each acylcarnitine to free carnitine. The reason why the low QC sample has a higher percentage accuracy remains unclear, but a possible reason could be that with low concentrations, the effect of hydrolysis is more visible due to the initial low concentration of free carnitine in the sample in comparison to when free carnitine is present in high concentrations in the sample. In other words, the higher the initial concentration of free carnitine in a sample, the less effect hydrolysis will have on the free carnitine concentration.

Intraday results obtained over five days display good precision for all three QC samples. All %RSD values are below 15% of the nominal, showing good analytical preparation within the five days. Accuracy obtained shows good reproducibility between days from the low QC sample to the middle QC sample and the high QC sample, with all acylcarnitines yielding acceptable accuracy percentages, except for free carnitine. This correlates with the interday accuracy for free carnitine, which further supports the theory of acylcarnitine hydrolysis to free carnitine.

Table 4.3: Precision and accuracy result summary for interday precision.

Interday						
Compound	Low QC		Middle QC		High QC	
	RSD (%)	Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)	Accuracy (%)
C0	3.58	162.65	0.97	126.37	1.23	118.67
C2	5.63	90.75	2.61	92.08	2.31	111.68
C3	5.38	91.76	1.92	101.35	3.26	106.33
C4	5.10	118.69	1.33	93.91	2.73	112.87
C5	8.36	115.11	2.05	94.44	1.45	108.12
C6	9.42	106.42	4.89	116.41	2.10	106.64
C8	8.97	105.54	3.53	111.99	1.75	105.05
C10	0.6	101.8	5.86	112.53	1.42	104.74
C12	0.9	101.6	2.55	112.06	4.62	107.04
C14	2.1	97.5	4.15	108.63	0.68	105.44
C16	6.88	95.39	8.12	111.13	1.63	105.02
C18	9.31	104.72	1.66	113.80	3.76	103.84
Intraday						
Compound	Low QC		Middle QC		High QC	
	RSD (%)	Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)	Accuracy (%)
C0	5.51	160.50	5.32	119.18	2.14	120.23
C2	7.68	95.39	8.71	98.93	7.25	98.75
C3	7.29	101.48	5.20	98.32	6.53	102.19
C4	13.94	108.20	5.50	101.35	6.17	103.75
C5	8.16	101.74	6.57	98.80	5.35	101.19
C6	11.58	101.78	11.14	98.56	4.26	101.02
C8	12.08	101.24	8.75	99.51	5.22	98.68
C10	11.51	99.58	8.38	103.44	6.68	99.68
C12	10.99	96.68	7.51	99.56	6.08	99.97
C14	8.47	97.90	6.24	101.12	6.12	99.16
C16	12.70	104.06	6.95	102.58	7.22	97.70
C18	8.69	105.62	9.46	100.87	9.08	102.41

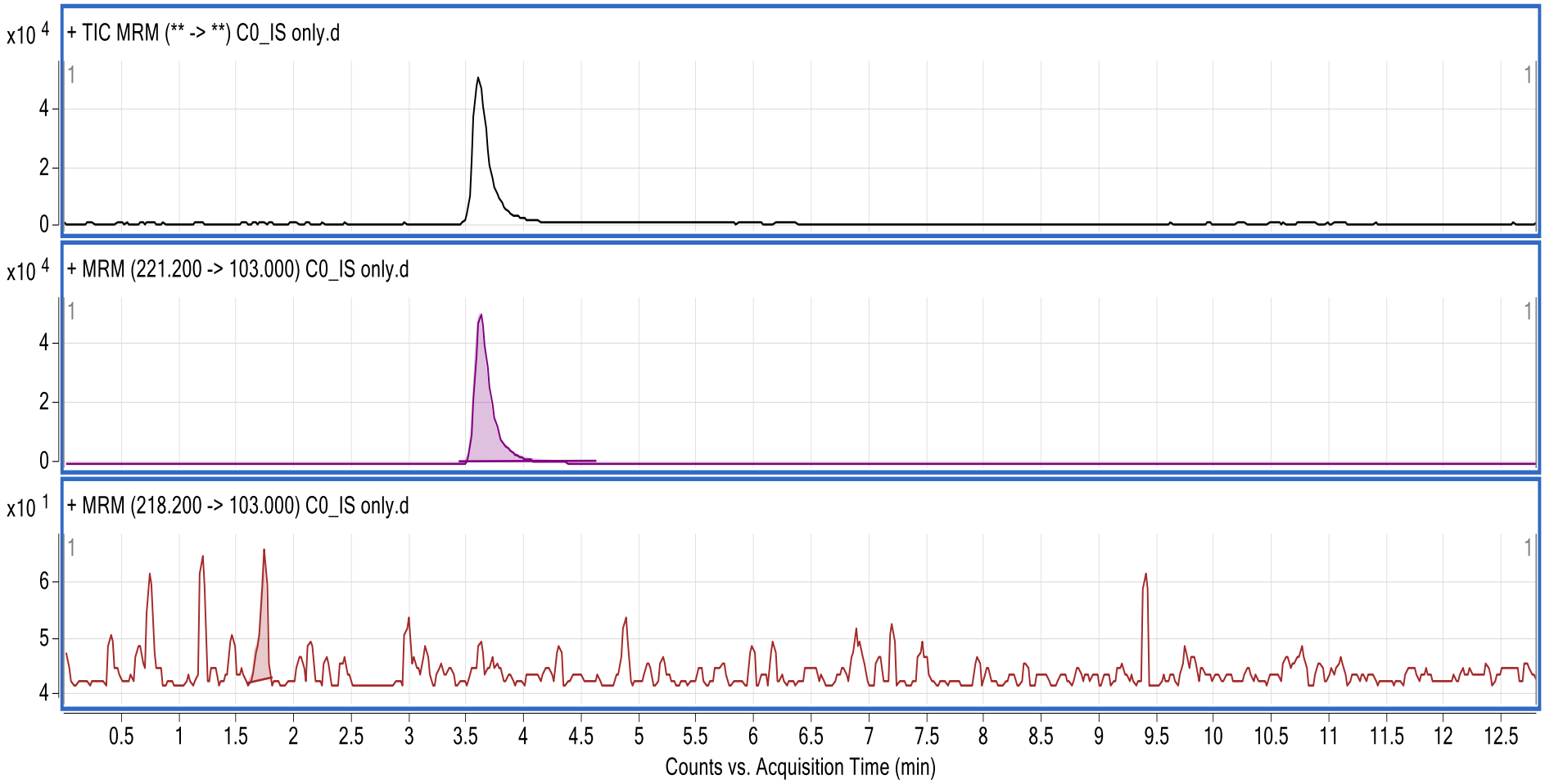


Figure 4.8: TIC and MRM of individual compound C0 isotope with MRM transitions for C0 and C0 isotope. As can be seen above, there is no evidence of C0 isotope breaking down to C0.

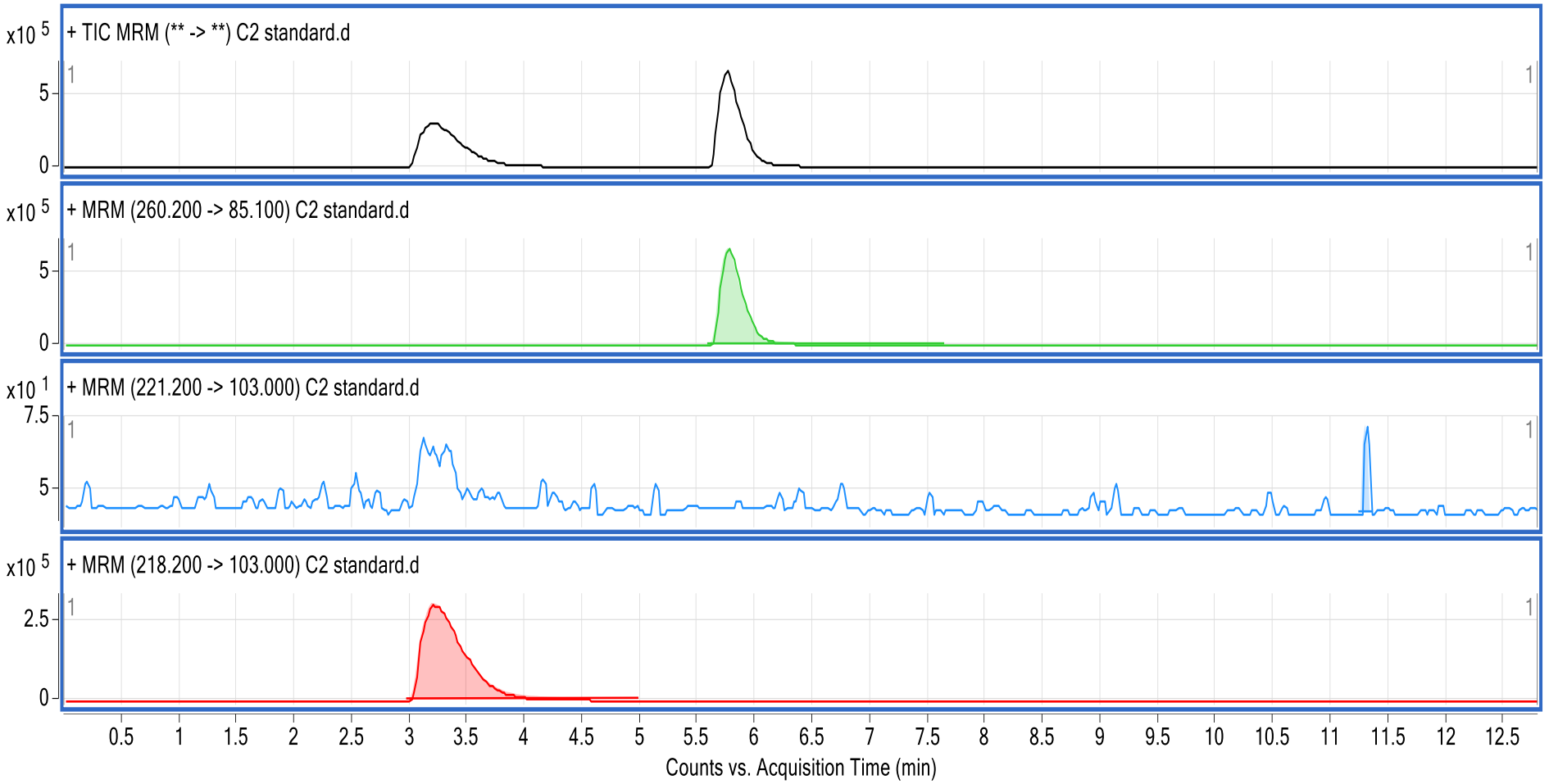


Figure 4.9: TIC and MRM of C2 standard solution showing MRM transitions for C2, C2 isotope and C0. As can be seen above, the C2 standard also produces a peak at MRM transition 218.2 – 103.0, which represents compound C0.

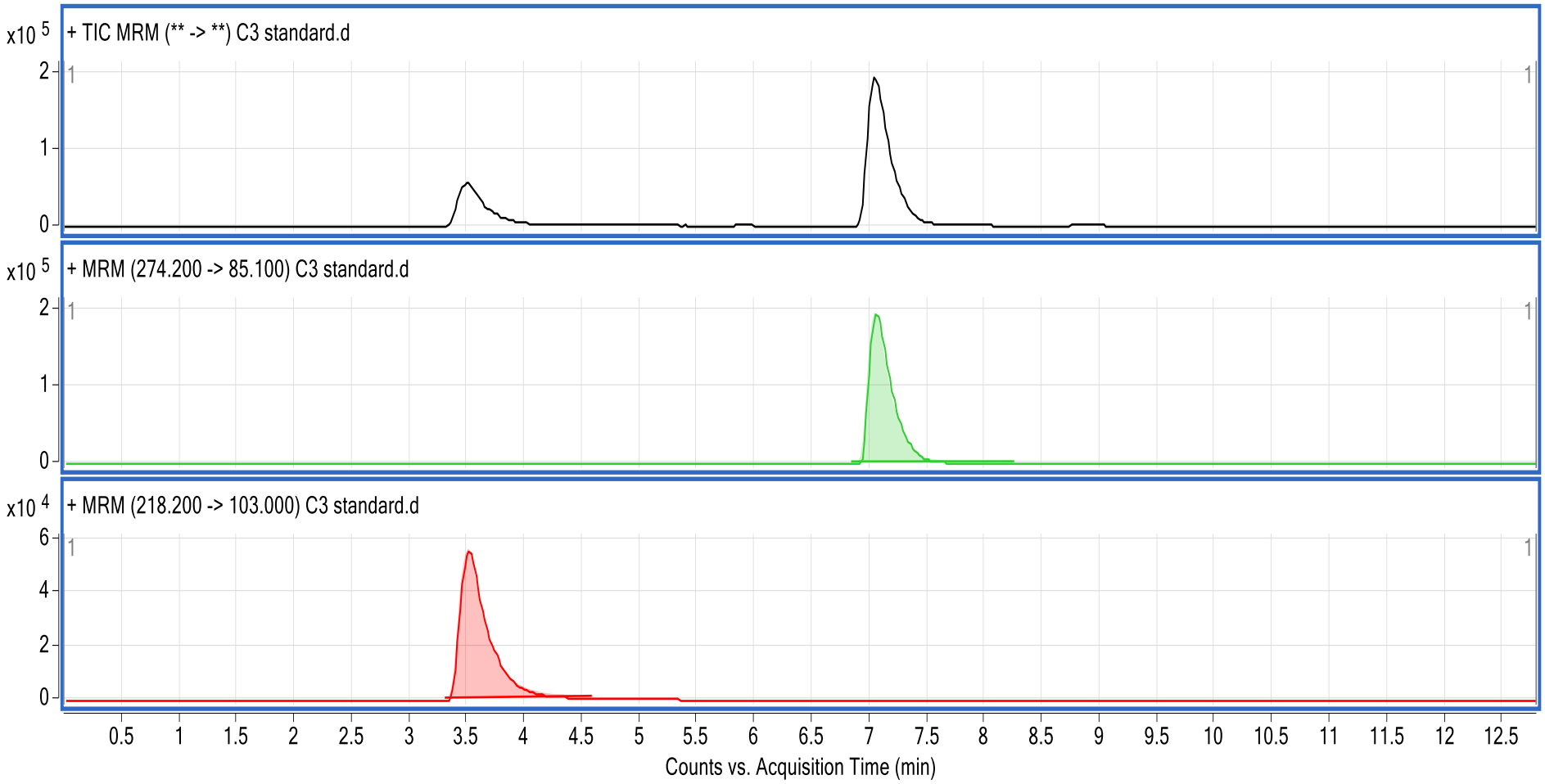


Figure 4.10: TIC and MRM of C3 standard solution showing MRM transitions for C3, C3 isotope and C0. As can be seen above, the C3 standard also produces a peak at MRM transition 218.2 – 103.0, which represents compound C0.

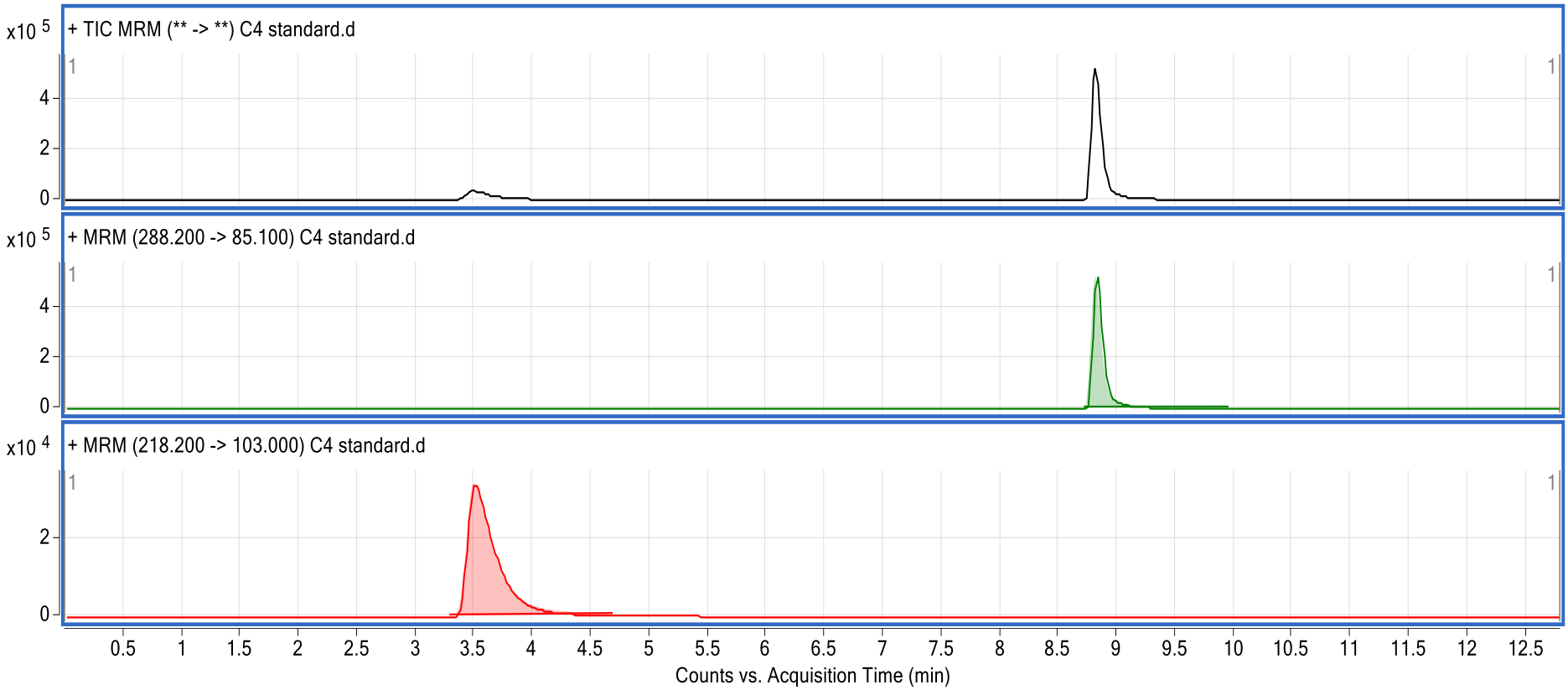


Figure 4.11: TIC and MRM of C4 standard solution showing MRM transitions for C4, C4 isotope and C0. As can be seen above, the C4 standard also produces a peak at MRM transition 218.2 – 103.0, which represents compound C0.

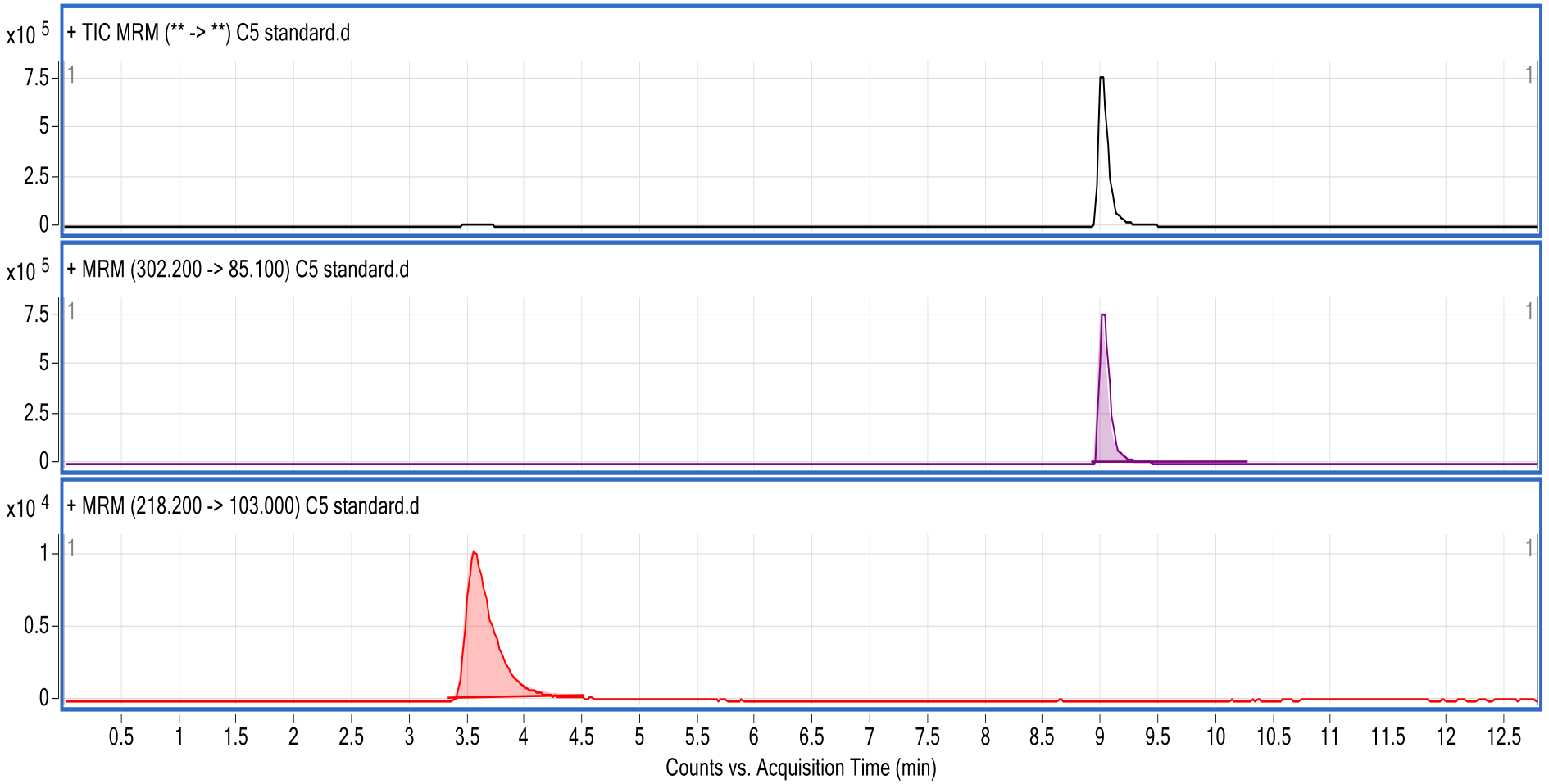


Figure 4.12: TIC and MRM of C5 standard solution showing MRM transitions for C5, C5 isotope and C0. As can be seen above, the C5 standard also produces a peak at MRM transition 218.2 – 103.0, which represents compound C0.

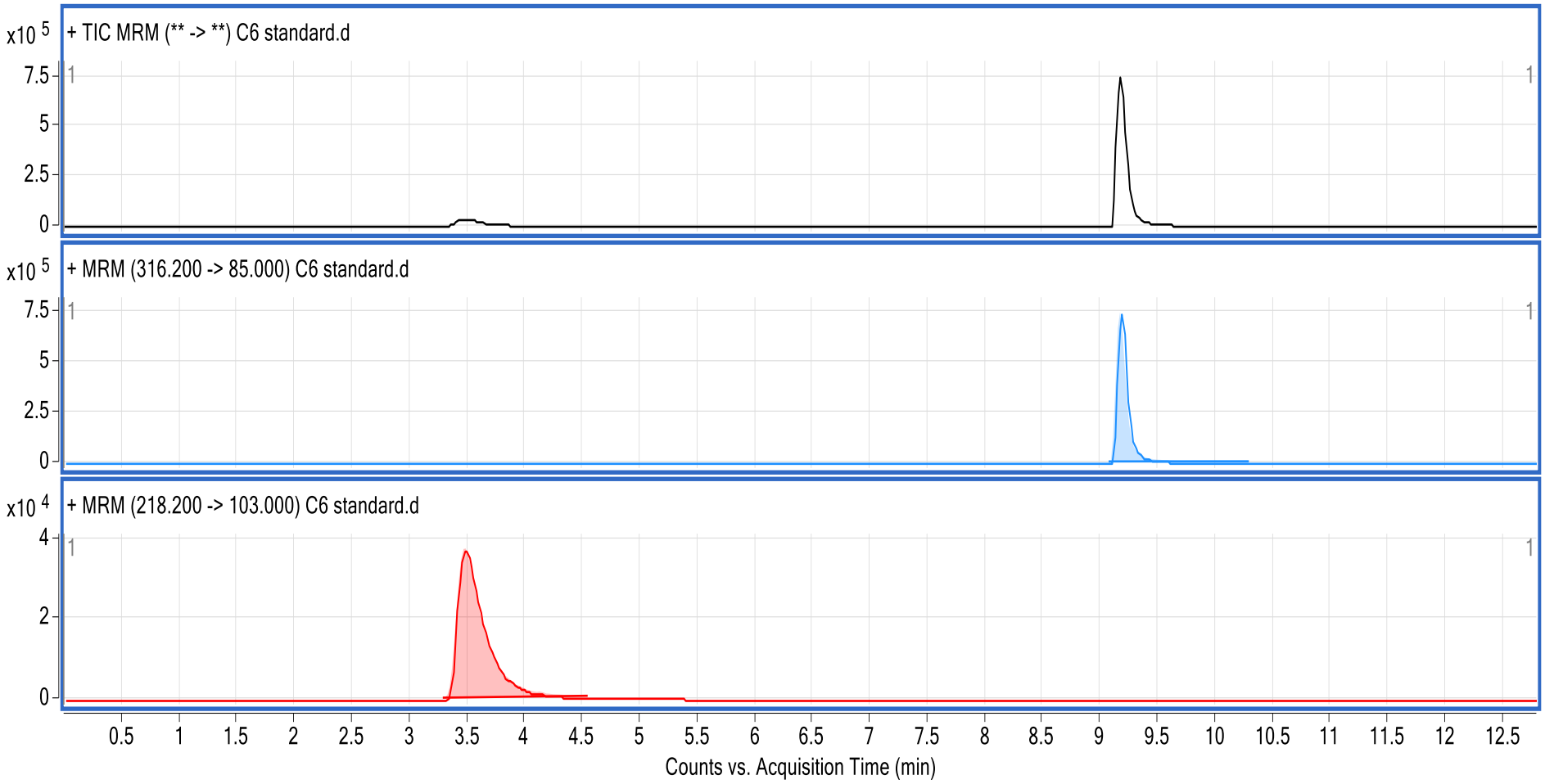


Figure 4.13: TIC and MRM of C6 standard solution showing MRM transitions for C6, C6 isotope and C0. As can be seen above, the C6 standard also produces a peak at MRM transition 218.2 – 103.0, which represents compound C0.

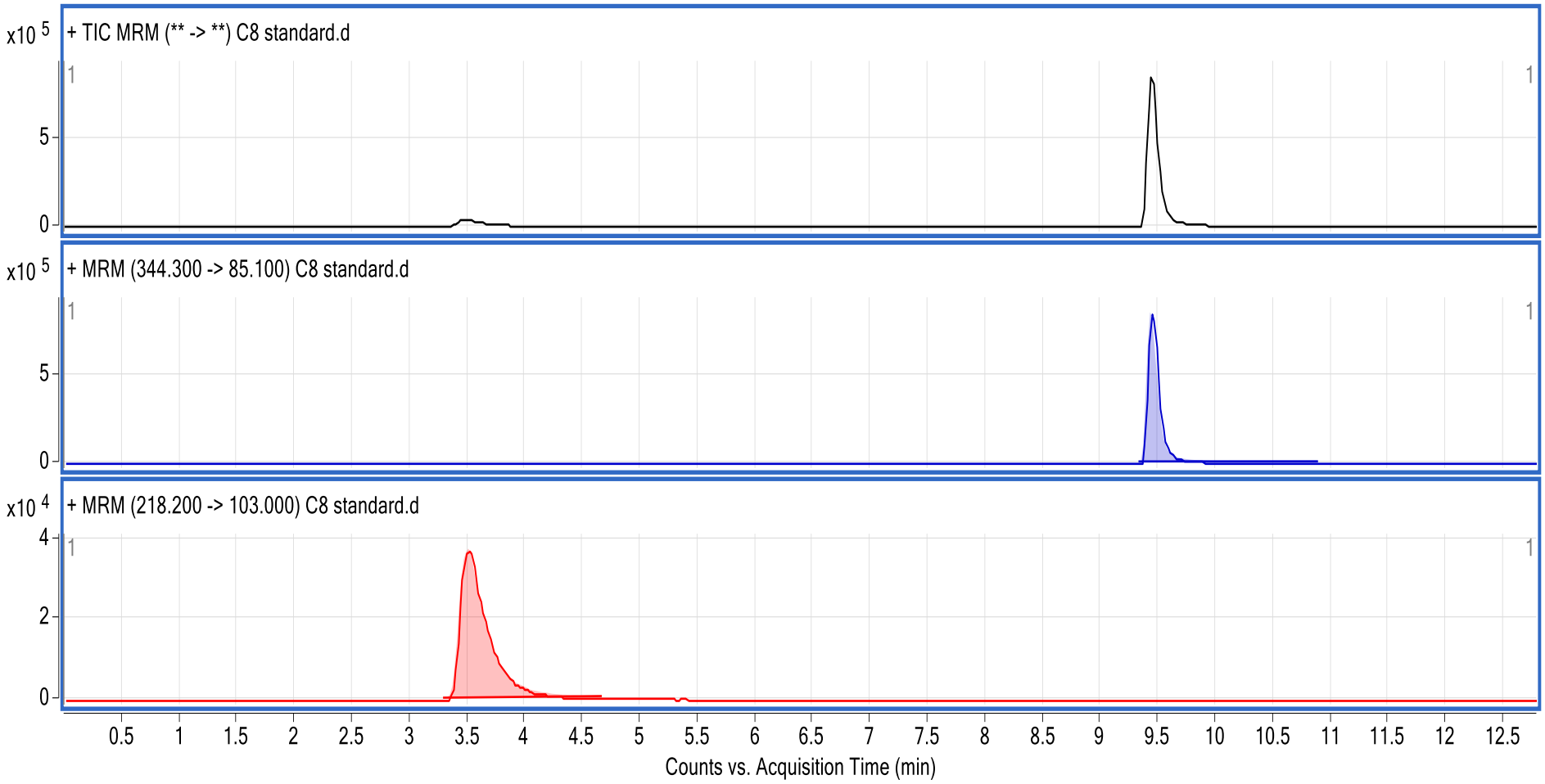


Figure 4.14: TIC and MRM of C8 standard solution showing MRM transitions for C8, C8 isotope and C0. As can be seen above, the C8 standard also produces a peak at MRM transition 218.2 – 103.0, which represents compound C0.

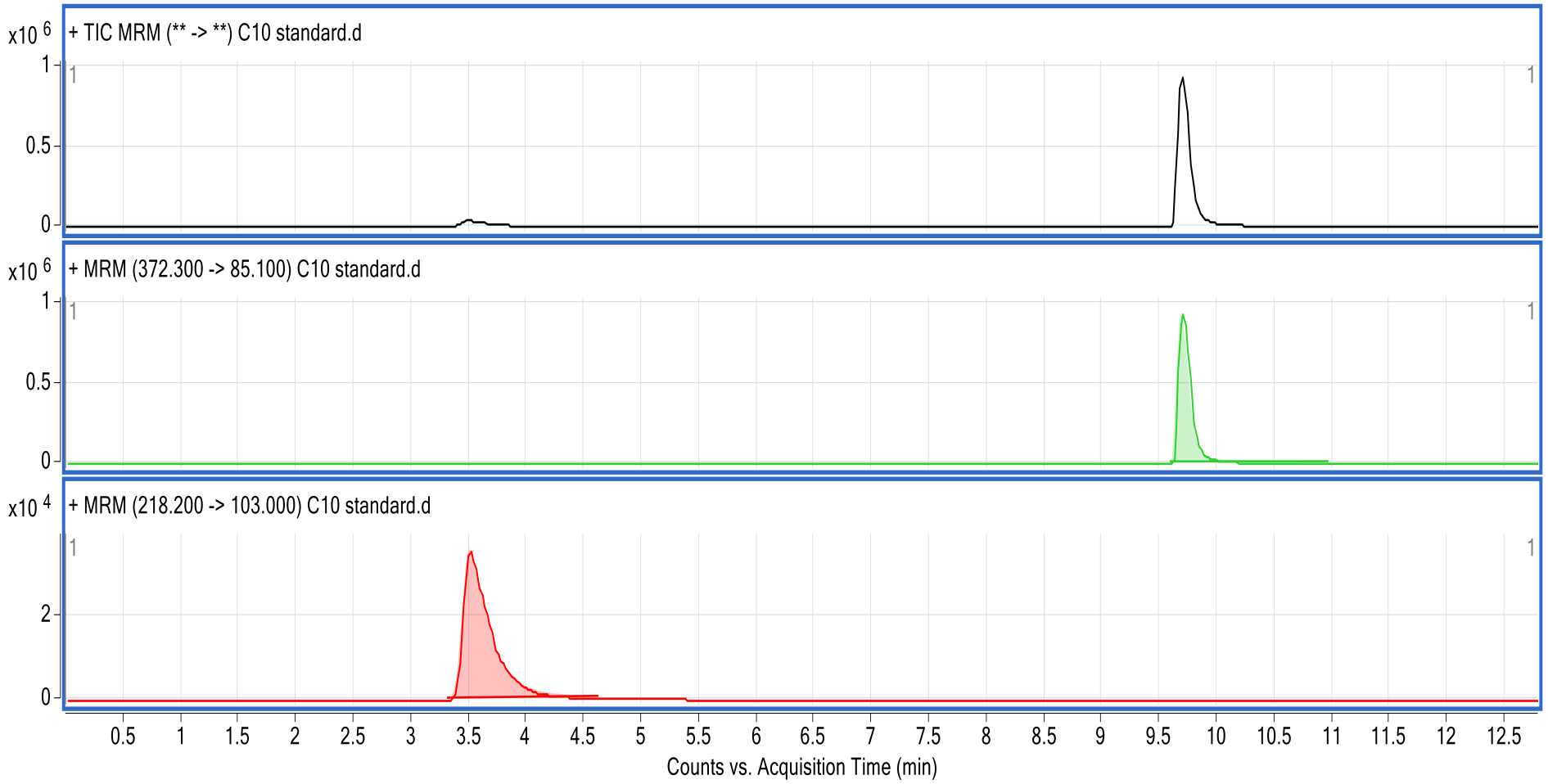


Figure 4.15: TIC and MRM of C10 standard solution showing MRM transitions for C10, C10 isotope and C0. As can be seen above, the C10 standard also produces a peak at MRM transition 218.2 – 103.0, which represents compound C0.

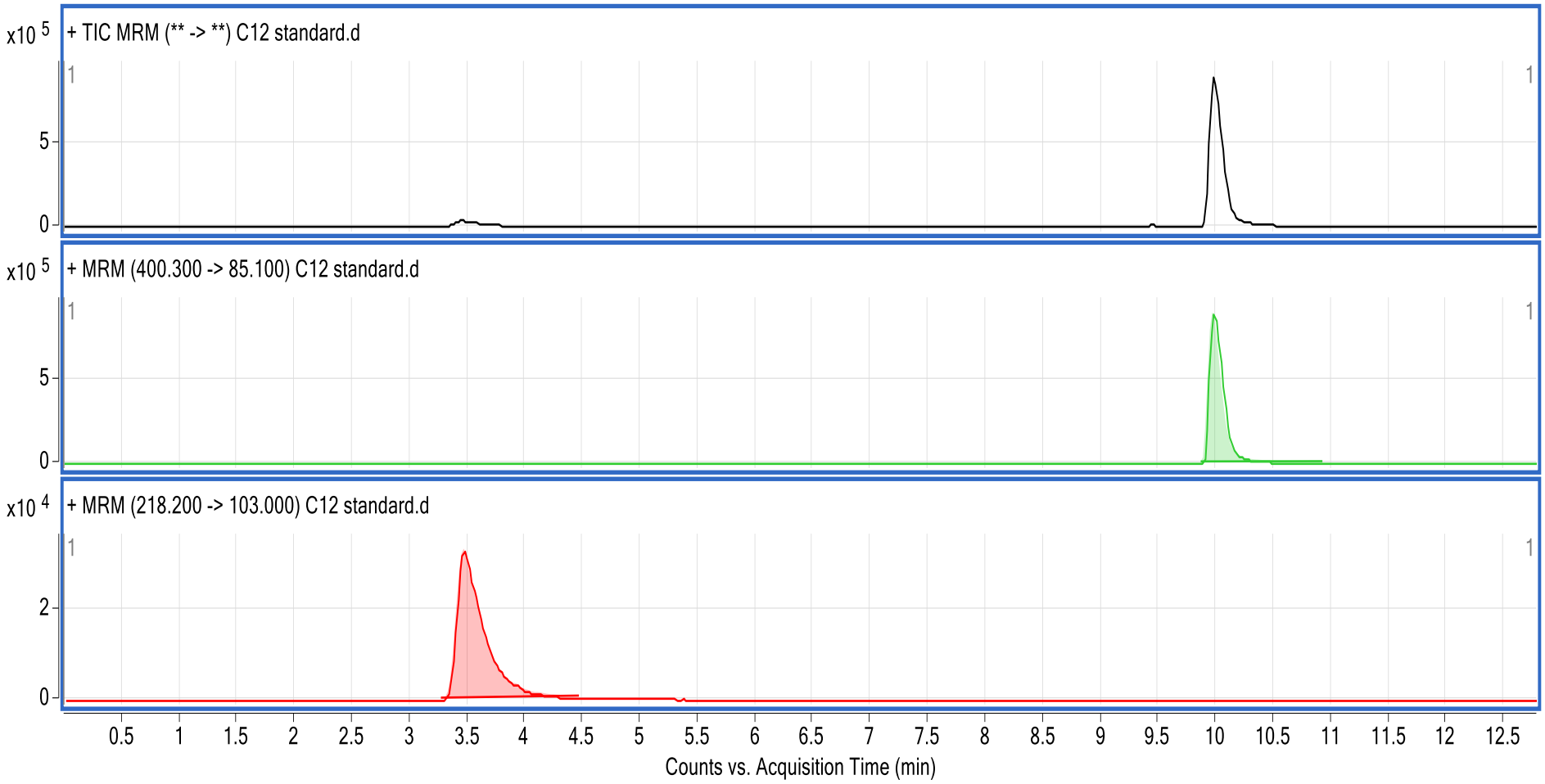


Figure 4.16: TIC and MRM of C12 standard solution showing MRM transitions for C12, C12 isotope and C0. As can be seen above, the C12 standard also produces a peak at MRM transition 218.2 – 103.0, which represents compound C0.

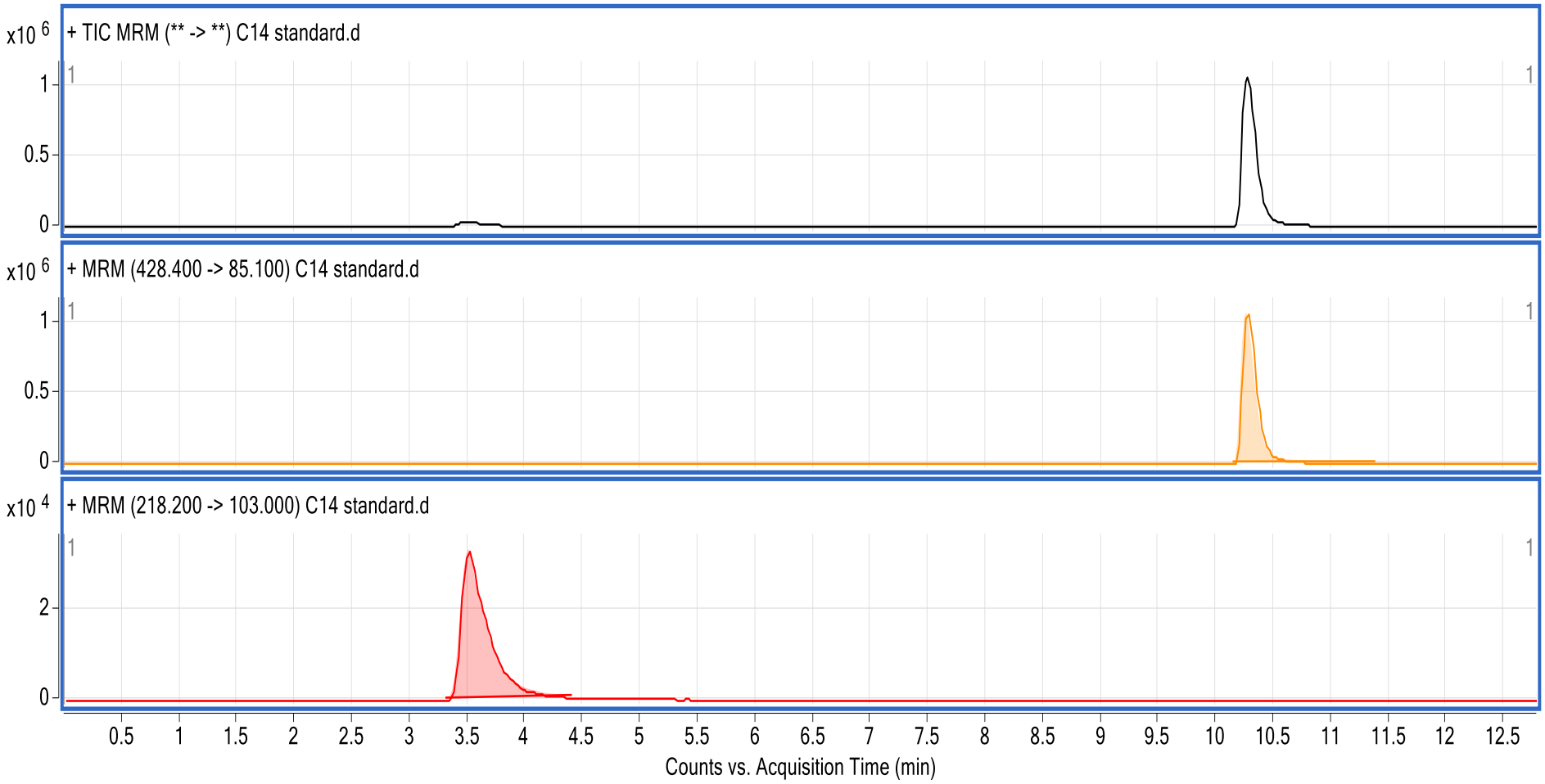


Figure 4.17: TIC and MRM of C14 standard solution showing MRM transitions for C14, C14 isotope and C0. As can be seen above, the C14 standard also produces a peak at MRM transition 218.2 – 103.0, which represents compound C0.

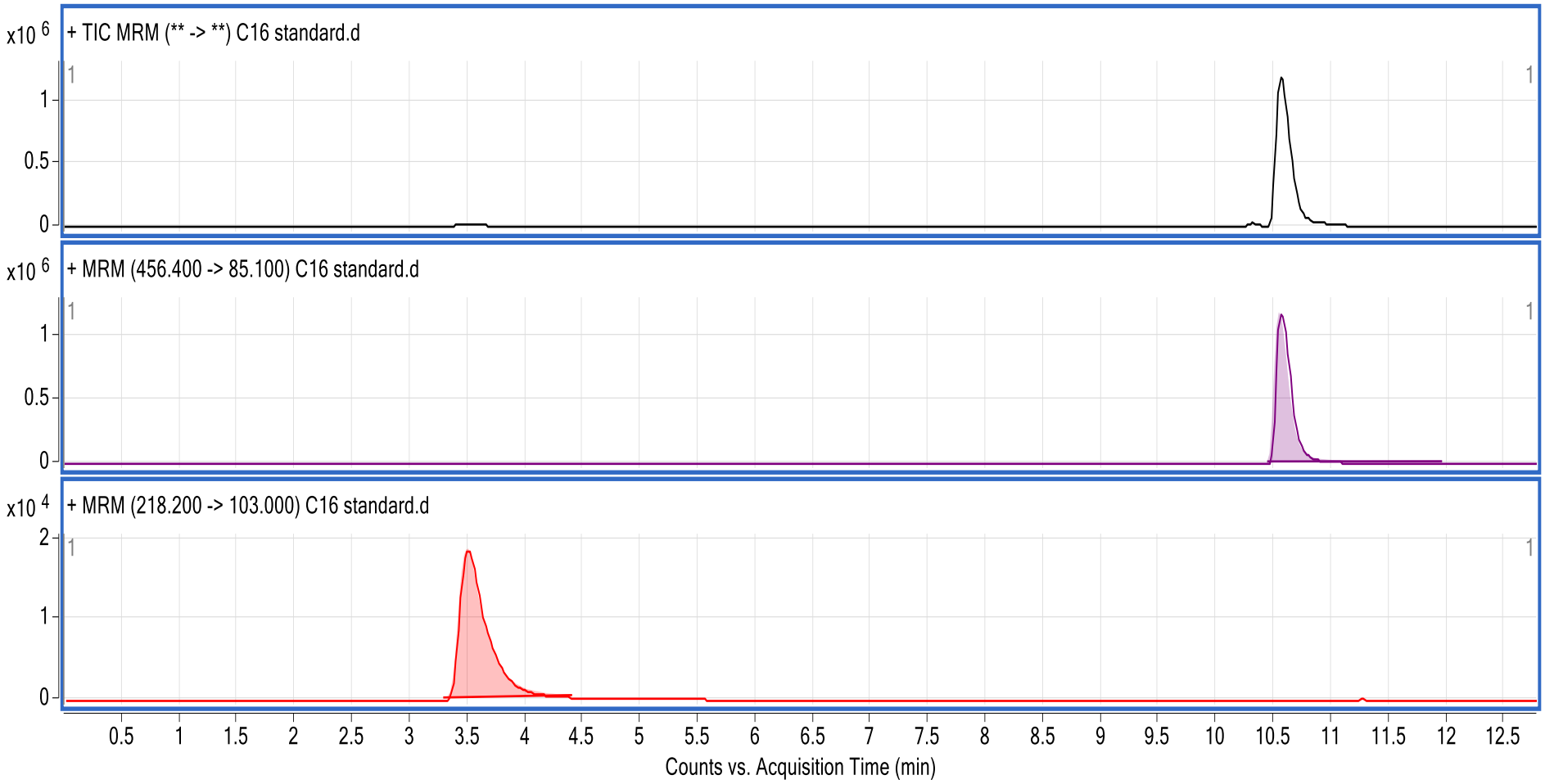


Figure 4.18: TIC and MRM of C16 standard solution showing MRM transitions for C16, C16 isotope and C0. As can be seen above, the C16 standard also produces a peak at MRM transition 218.2 – 103.0, which represents compound C0.

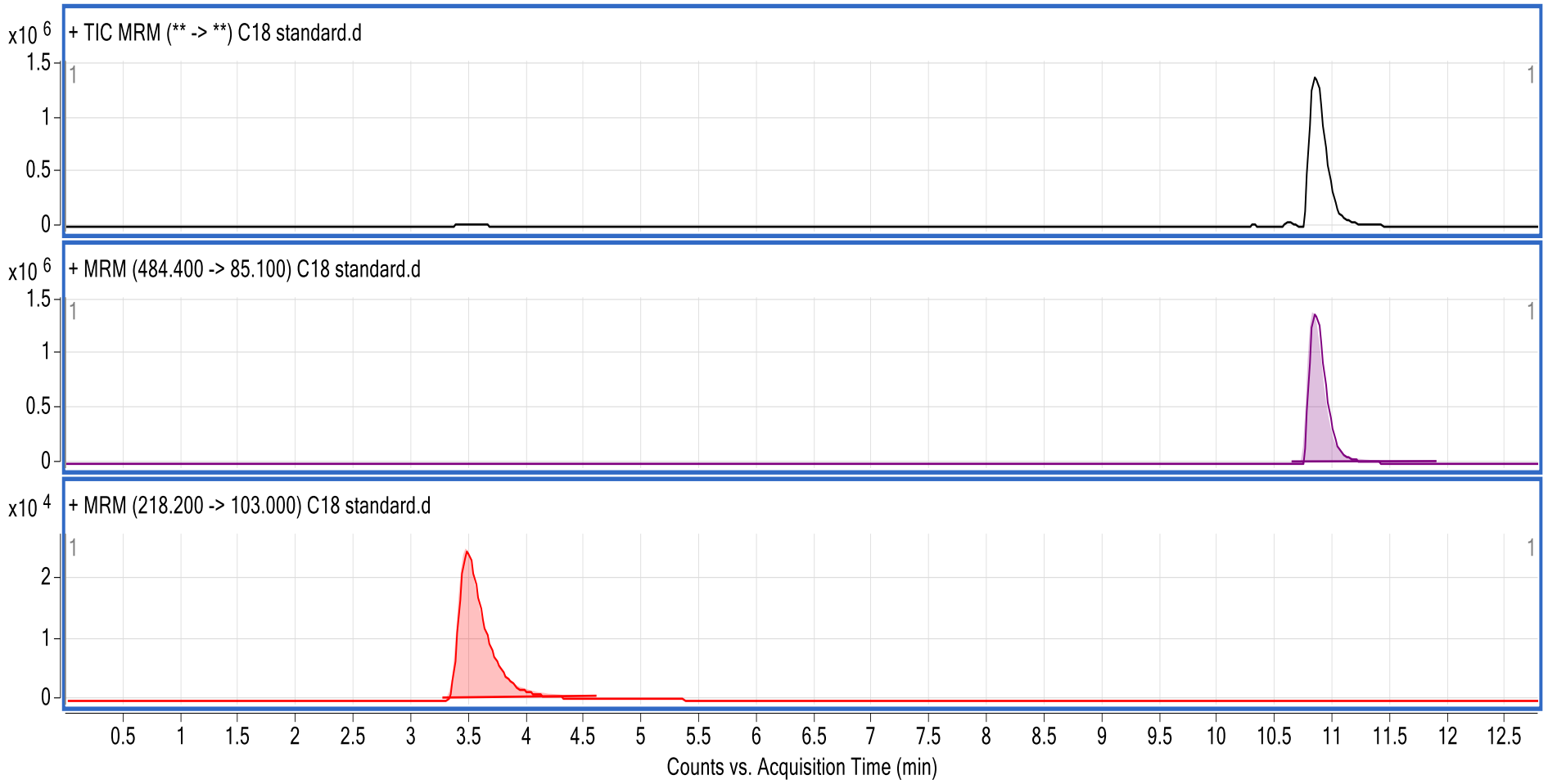


Figure 4.19: TIC and MRM of C18 standard solution showing MRM transitions for C18, C18 isotope and C0. As can be seen above, the C18 standard also produces a peak at MRM transition 218.2 – 103.0, which represents compound C0.

4.1.5 Stability

Stability can be calculated by dividing the response of the stability sample by the response of the freshly prepared sample and multiplying by 100 to get percentage. **Equation 4.6** was used to calculate stability as a percentage of the freshly prepared samples. The results for 24 hour and 1 week stability are recorded in **Table 4.4** and **Table 4.5**, respectively.

Equation 4.6: Stability (%)

$$\text{Stability} = \frac{response\ of\ stability\ sample}{response\ of\ freshly\ prepared\ sample} \times 100$$

For 24 hour stability, the low QC sample shows poor stability for all acylcarnitines for all five storage conditions, except for free carnitine which has a stability between 64 and 79 percent. Hydrolysis of acylcarnitines can play a role in storage stability of samples, considering that all acylcarnitines can hydrolyse to free carnitine, the increased free carnitine compared to other acylcarnitines can be a reason for the lower acylcarnitine percentages. The middle QC sample shows good stability for acylcarnitines C0 to C14 for storage at bench top, the auto sampler and the refrigerator (4°C). Acylcarnitines C16 and C18 have a low percentage stability and can possibly be due to hydrolysis during storage conditions. For storage conditions at -20°C, C16 and C18 show very good stability with percentages within 15% of the nominal. As for -80°C storage conditions, C16 and C18 shows good stability, but with lower percentages than the suggested nominal. High QC samples have an overall good stability for carnitine C0 and acylcarnitines C2 to C12 at all storage conditions. Bench top stability of C14, C16 and C18 are lower than the suggested nominal, whereas the auto sampler and refrigerator (4°C) stability of C14, C16 and C18 are well below the suggested nominal. Freezer storage at -20°C and -80°C shows acceptable stability for acylcarnitines C14, C16 and C18.

In general, acylcarnitines does not show good stability when low concentrations are stored (low QC sample) at any of the five storage conditions for 24 hours. The middle QC samples showed the best stability when stored in the freezer at -20°C or -80°C for all acylcarnitines, including the long chain acylcarnitines. As for the high QC samples, the best storage conditions for a period of 24 hours would be in the freezer at -20°C and -80°C as this is where all acylcarnitines show good stability.

In conclusion, the best overall storage location for 24 hours would be in the freezer at -20°C or -80°C, keeping in mind that if the sample has low concentrations of the specific acylcarnitine present, stability will not be very good. For samples with low concentrations of acylcarnitines, the best storage location would be in the refrigerator at 4°C.

For one week stability, low QC samples showed acceptable stability for carnitine C0 and acylcarnitines C2 to C12 and poor stability for acylcarnitines C14 to C18, except for storage in the refrigerator at 4°C which yields a higher than the nominal percentage stability. The middle QC sample shows good stability for acylcarnitines C0 to C12 when stored at bench top, in the auto sampler and in the refrigerator at 4°C. For long chain acylcarnitines C14, C16 and C18, poor stability is shown for the aforementioned conditions. For -20°C and -80°C, carnitine C0 and acylcarnitines C2 to C14 shows good stability, but for acylcarnitines C16 and C18, stability percentages are higher than the nominal for storage at -20°C and stability percentages at -80°C are lower than the nominal. High QC samples have a higher than the nominal stability percentage for free carnitine at all storage conditions, which might be due to hydrolysis of all other acylcarnitines to free carnitine. Bench top and freezer (-20°C) storage conditions shows remarkable stability for all acylcarnitines, except for free carnitine. Auto sampler and refrigerator (4°C) storage shows good stability for acylcarnitines C2 to C12, whereas C14 to C18 show poor stability at these storage conditions. Stability of acylcarnitines C2 to C12 at storage conditions of -80°C are within the nominal percentage and are higher than the nominal percentage for acylcarnitines C14 to C18.

In conclusion, the best option for one week storage of samples for best results, would be in the freezer at -20°C, or on the bench top for samples with high concentrations of acylcarnitines. For samples with low concentrations of acylcarnitines, the best storage location would be in the auto sampler, but keeping in mind that long chain acylcarnitines might be hydrolysed to free carnitine.

Table 4.4: Results of sample stability experiments as a percentage of freshly prepared samples for 24 hour stability.

Sample	Bench top			Auto sampler			Refrigerator (4°C)			Freezer (-20°C)			Freezer (-80°C)		
	Low QC	Middle QC	High QC	Low QC	Middle QC	High QC	Low QC	Middle QC	High QC	Low QC	Middle QC	High QC	Low QC	Middle QC	High QC
C0	78.72	119.12	106.07	63.58	113.67	135.13	70.96	117.02	124.14	70.93	109.91	119.10	68.54	117.73	110.76
C2	47.46	113.80	104.40	41.65	111.97	128.58	47.63	113.72	111.37	43.69	104.06	110.60	46.29	111.18	108.46
C3	37.50	117.33	104.62	31.56	107.43	127.75	36.71	108.49	112.27	35.50	102.93	110.85	34.51	106.25	107.59
C4	48.47	117.36	104.12	42.86	108.62	121.63	50.28	112.08	113.05	46.99	106.94	104.92	45.52	104.37	103.61
C5	35.36	115.25	104.16	30.82	106.16	120.79	36.93	104.46	106.61	35.04	104.79	103.40	33.89	111.39	100.22
C6	27.34	102.65	105.27	25.39	106.27	119.76	27.22	100.88	111.25	29.44	108.46	101.26	28.05	111.14	104.39
C8	29.74	106.93	101.45	22.95	92.03	113.89	28.89	101.30	107.58	26.28	100.68	102.63	29.07	111.93	101.66
C10	31.99	101.69	103.45	23.78	95.91	105.34	38.14	98.89	98.31	27.84	100.42	104.60	28.30	105.09	101.86
C12	23.42	96.27	92.69	25.23	92.00	90.27	32.09	87.54	94.25	24.86	98.05	97.05	23.58	101.17	97.50
C14	15.17	83.46	78.02	20.25	77.74	53.77	37.74	82.74	65.89	17.35	96.19	83.25	15.17	87.67	92.38
C16	10.06	54.68	73.99	5.79	37.42	43.64	50.18	58.55	55.11	11.48	95.32	78.62	6.00	71.74	98.23
C18	8.78	50.10	76.88	3.55	24.66	45.78	33.72	42.35	58.52	12.56	102.04	88.63	3.71	65.23	100.96

Table 4.5: Results of sample stability experiments as a percentage of freshly prepared samples for 1 week stability.

Sample	Bench top			Auto sampler			Refrigerator (4°C)			Freezer (-20°C)			Freezer (-80°C)		
	Low QC	Middle QC	High QC	Low QC	Middle QC	High QC	Low QC	Middle QC	High QC	Low QC	Middle QC	High QC	Low QC	Middle QC	High QC
C0	173.50	137.46	126.89	136.86	145.21	135.67	151.94	129.16	134.38	178.81	131.52	134.08	141.59	139.68	131.24
C2	129.74	108.81	108.40	115.08	129.19	117.87	137.64	122.16	113.72	145.72	115.57	118.30	152.46	128.15	117.65
C3	118.16	107.59	106.62	104.92	127.80	117.87	129.95	120.43	115.14	149.80	113.60	119.77	144.67	118.59	117.38
C4	119.30	110.75	108.20	113.13	121.76	110.82	127.11	118.12	107.19	141.61	112.51	111.35	137.85	113.26	108.45
C5	124.12	111.40	103.69	113.56	118.15	109.63	122.97	110.40	104.46	135.97	109.11	112.09	110.68	112.93	105.51
C6	115.30	96.01	105.27	106.87	112.20	105.15	122.23	102.99	100.53	517.73	105.73	103.64	133.06	108.14	103.11
C8	111.94	103.33	103.59	104.96	98.11	108.85	115.29	108.46	103.46	127.38	106.39	111.37	107.79	116.71	107.72
C10	113.44	100.06	111.75	100.09	100.57	110.68	121.39	109.84	104.53	126.17	104.51	115.07	128.99	115.15	107.77
C12	104.60	97.58	112.31	77.37	98.28	83.53	116.99	93.03	105.83	101.75	100.32	114.11	111.18	104.35	116.95
C14	74.01	59.40	112.65	44.13	66.41	25.22	127.82	59.94	75.88	64.17	94.85	94.31	70.12	91.33	127.34
C16	39.93	26.19	114.96	47.40	22.15	9.88	174.11	18.47	67.39	57.59	135.58	96.74	23.47	54.13	132.82
C18	27.33	13.03	117.31	42.81	8.18	8.92	141.54	8.66	66.44	54.64	146.89	102.52	18.75	61.44	130.53

4.2 Sample application

The collected patient and urine samples were analysed as described in Section 3.2. Concentration was determined from the obtained data and expressed as a concentration of the creatinine value of each individual sample from patients or control, respectively. **Equation 4.7** was used to calculate the concentrations and the results for patient and control subjects are given in **Table 4.6** and **Table 4.7**, respectively (*Patient samples are indicated by P1, P2, etc. and control samples are indicated by C1, C2, etc.)*. Calculated values was also compared to reference values as reported by Mueller *et al.* (2003).

Equation 4.7: Concentration / mmol creatinine

$$\text{concentration} = \frac{concentration\ (\mu mol/L)}{patient\ creatinine\ value}$$

The concentration (µmol/L) was calculated with **Equation 4.4** in Section 4.1.4.

In general, there are a lot of patient samples which had higher concentrations than the proposed reference values. The most common acylcarnitine which falls outside of the recommended reference value is free carnitine (this includes both values higher than the reference range, as well as lower than the reference range). Unfortunately this does not distinguish the patient and control group from each another, as the control group shows similar results.

In the patient group, there were nine individual patients that had six or more acylcarnitines with values higher than the reference ranges, but again, not all patients had the same acylcarnitines with higher than the expected values. As for the control group, a total of three samples had six or more acylcarnitines with values higher than the reference ranges.

Not one acylcarnitine could be identified in the patient group that is different than that of the control group. This is also confirmed by the PCA and Mann-Whitney test performed during statistical analysis, which could not distinguish between the patient and control group, nor were any significant p-values obtained. It is therefore not possible to make accurate conclusions based on information obtained to differentiate between the patient and control group.

Table 4.6: Patient sample concentration values calculated compared to reference values.

Compound	Reference value (µmol/mol creatinine)	Concentration (µmol/mol creatinine)																	
		P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12	P13	P14	P15	P16	P17	P18
C0	1.32 - 17.02	1.98	1.14	0.62	2.95	2.41	0.27	0.60	0.36	0.50	1.60	1.22	0.20	0.06	1.54	2.02	0.31	0.89	0.05
C2	<4.92	2.78	0.45	2.68	4.66	3.38	0.09	0.85	0.30	0.53	4.44	2.05	0.03	0.03	4.93	2.67	0.09	1.04	0.21
C3	<0.49	1.04	0.21	0.25	2.27	1.38	0.06	0.57	0.26	0.19	0.51	0.68	0.03	0.02	0.75	1.11	0.03	0.47	0.02
C4	<1.50	1.02	1.17	1.70	5.01	1.64	0.69	3.25	0.65	0.47	4.55	3.89	0.35	0.19	4.01	2.91	0.09	1.93	1.98
C5	<0.42	0.05	0.03	0.03	0.04	0.04	0.03	0.10	0.01	0.01	0.15	0.09	0.04	0.00	0.11	0.13	0.00	0.04	0.10
C6	<0.14	0.06	0.03	0.03	0.05	0.05	0.03	0.13	0.01	0.02	0.20	0.11	0.04	0.00	0.14	0.17	0.00	0.05	0.12
C8	<0.58	0.25	0.34	0.14	0.28	0.24	0.13	0.54	0.05	0.13	0.68	0.25	0.07	0.03	0.25	0.47	0.13	0.34	0.36
C10	<0.14	0.11	0.12	0.00	0.11	0.11	0.05	0.45	0.02	0.04	0.47	0.17	0.03	0.01	0.11	0.27	0.04	0.11	0.25
C12	<0.16	0.02	0.06	0.01	0.01	0.02	0.01	0.04	0.00	0.01	0.05	0.05	0.01	0.00	0.01	0.43	0.01	0.02	0.10
C14	<0.20	0.00	0.04	0.01	0.59	0.03	0.96	0.82	0.00	0.01	2.20	2.45	0.38	0.14	2.43	4.61	0.01	0.03	1.05
C16	<0.13	0.01	0.03	0.01	0.06	0.02	3.60	0.48	0.00	0.00	0.20	0.81	0.03	0.01	0.20	0.40	0.03	0.07	7.66
C18	<0.05	0.02	0.05	0.01	0.04	0.01	0.14	0.02	0.00	0.00	0.09	1.38	0.01	0.00	0.46	1.92	0.00	0.08	23.44

Compound	Reference value (µmol/mol creatinine)	Concentration (µmol/mol creatinine)																	
		P19	P20	P21	P22	P23	P24	P25	P26	P27	P28	P29	P30	P31	P32	P33	P34	P35	P36
C0	1.32 - 17.02	0.61	0.72	0.81	0.44	0.28	0.30	0.79	1.87	1.92	0.54	0.48	2.72	2.04	1.88	0.65	1.31	0.62	0.80
C2	<4.92	1.19	0.45	0.86	0.50	0.12	0.29	1.08	1.81	1.93	1.14	0.52	6.01	3.57	1.96	1.24	1.44	0.88	0.78
C3	<0.49	0.09	0.18	0.39	0.57	0.06	0.18	0.67	0.92	0.82	0.13	0.19	1.63	1.40	1.18	0.54	0.77	0.45	0.31
C4	<1.50	1.55	2.14	1.97	2.42	0.18	0.61	2.24	5.49	1.71	0.32	0.66	1.84	0.08	1.48	1.95	10.85	1.17	3.41
C5	<0.42	0.06	0.03	0.07	0.01	0.01	0.03	0.04	0.10	0.04	0.01	0.03	0.12	0.07	0.03	0.04	0.08	0.04	0.07
C6	<0.14	0.08	0.04	0.09	0.02	0.01	0.04	0.05	0.13	0.05	0.01	0.03	0.15	0.09	0.04	0.06	0.10	0.05	0.09
C8	<0.58	0.23	0.22	0.23	0.12	0.08	0.08	0.19	0.43	0.44	0.11	0.15	0.31	0.28	0.27	0.21	0.39	0.12	0.26
C10	<0.14	0.14	0.12	0.10	0.05	0.03	0.04	0.11	0.21	0.16	0.02	0.07	0.18	0.10	0.09	0.10	0.15	0.04	0.11
C12	<0.16	0.05	0.02	0.88	0.01	0.01	0.00	0.02	0.05	0.05	0.01	0.01	0.04	0.01	0.05	0.02	0.01	0.00	0.04
C14	<0.20	0.59	0.37	3.98	0.02	0.02	0.15	0.02	0.45	0.07	0.01	0.01	0.63	1.50	0.01	0.05	2.10	0.18	0.05
C16	<0.13	0.02	0.02	0.39	0.01	0.06	0.02	0.11	4.17	0.08	0.01	0.01	0.56	0.27	0.01	0.21	2.61	0.04	0.18
C18	<0.05	0.00	0.01	2.44	0.01	0.12	0.00	0.03	7.16	0.20	0.01	0.00	0.01	0.04	0.01	0.23	0.87	0.02	0.22

Table 4.7: Control sample concentration values calculated compared to reference values.

Compound	Reference value (µmol/mol creatinine)	Concentration (µmol/mol creatinine)																
		C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12	C13	C14	C15	C16	C17
C0	1.32 - 17.02	1.11	0.38	0.50	5.73	0.27	0.62	0.92	0.37	0.16	0.18	0.52	0.40	0.45	0.28	0.70	0.67	0.50
C2	<4.92	1.97	0.79	3.79	13.51	0.48	1.08	1.23	0.77	0.07	1.72	0.31	0.47	0.66	0.31	1.47	0.82	1.35
C3	<0.49	0.95	0.96	0.26	7.57	0.20	0.67	0.85	0.40	0.03	0.07	0.21	0.17	0.39	0.24	0.88	0.39	0.46
C4	<1.50	3.75	1.82	1.60	10.29	0.67	2.08	2.91	1.25	0.79	1.23	0.99	0.55	1.49	0.92	2.68	1.87	4.02
C5	<0.42	0.09	0.02	0.03	0.17	0.01	0.03	0.04	0.02	0.02	0.03	0.03	0.03	0.03	0.01	0.03	0.05	0.07
C6	<0.14	0.11	0.03	0.04	0.22	0.01	0.04	0.05	0.03	0.02	0.03	0.04	0.04	0.03	0.02	0.04	0.07	0.09
C8	<0.58	0.52	0.09	0.17	0.91	0.06	0.10	0.30	0.16	0.09	0.13	0.15	0.21	0.12	0.07	0.18	0.26	0.17
C10	<0.14	0.44	0.03	0.07	0.38	0.04	0.05	0.10	0.11	0.05	0.05	0.08	0.08	0.06	0.03	0.06	0.11	0.09
C12	<0.16	0.04	0.01	0.01	0.07	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.02	0.01	0.01	0.01	0.02	0.03
C14	<0.20	0.39	0.14	0.93	3.17	0.00	0.33	0.03	0.60	1.53	1.34	0.02	0.01	0.01	0.01	0.02	1.72	1.94
C16	<0.13	0.07	0.01	0.25	0.29	0.00	0.03	0.02	0.09	0.18	0.06	0.03	0.01	0.03	0.03	0.04	0.57	7.57
C18	<0.05	0.03	0.01	0.05	0.11	0.00	0.02	0.01	0.03	0.25	0.03	0.00	0.01	0.01	0.06	0.01	1.31	0.14

Compound	Reference value (µmol/mol creatinine)	Concentration (µmol/mol creatinine)																
		C18	C19	C20	C21	C22	C23	C24	C25	C26	C27	C28	C29	C30	C31	C32	C33	C34
C0	1.32 - 17.02	0.23	0.77	0.45	0.91	1.49	1.15	0.59	1.18	0.91	4.19	0.22	0.10	1.64	1.41	0.51	1.20	1.16
C2	<4.92	0.12	1.46	0.04	1.89	3.36	3.29	1.84	1.25	1.92	3.28	0.19	0.02	4.02	2.20	0.60	2.70	4.83
C3	<0.49	0.11	0.59	0.02	0.81	1.88	1.91	0.79	0.59	1.02	1.05	0.14	0.02	1.54	1.18	0.26	1.46	0.71
C4	<1.50	0.53	1.99	0.36	5.87	9.71	2.87	1.92	1.08	1.80	1.30	1.00	0.38	6.10	3.19	1.64	1.78	1.92
C5	<0.42	0.00	0.13	0.02	0.12	0.02	0.04	0.04	0.03	0.04	0.07	0.03	0.00	0.08	0.09	0.03	0.03	0.05
C6	<0.14	0.00	0.16	0.02	0.16	0.02	0.06	0.06	0.04	0.05	0.08	0.03	0.01	0.10	0.12	0.04	0.04	0.07
C8	<0.58	0.04	0.30	0.17	0.35	0.24	0.17	0.09	0.17	0.20	0.30	0.30	0.04	0.45	0.40	0.20	0.14	0.18
C10	<0.14	0.01	0.19	0.07	0.20	0.26	0.07	0.05	0.05	0.10	0.15	0.06	0.02	0.13	0.22	0.09	0.08	0.10
C12	<0.16	0.00	0.01	0.02	0.02	0.01	0.01	0.00	0.01	0.01	0.01	0.02	0.00	0.01	0.61	0.02	0.01	0.01
C14	<0.20	0.00	0.13	0.03	0.55	0.10	0.07	0.64	0.01	0.49	0.04	0.88	0.01	0.08	0.87	9.07	0.14	1.12
C16	<0.13	0.01	0.03	0.09	0.21	0.55	0.13	0.07	0.02	0.23	0.08	0.13	0.02	0.68	0.79	4.66	0.07	0.10
C18	<0.05	0.01	0.04	0.07	0.03	1.08	0.11	0.39	0.01	0.02	0.06	0.12	0.01	0.13	0.04	2.12	0.02	0.12

4.3 Statistical analysis

4.3.1 Principal Components Analysis

Principal component analysis (PCA) is a multivariate statistical analytical procedure that can be used to visually illustrate if natural grouping exist between different groups based on differences or similarities. Multivariate data is referred to as datasets including two or more variables (Saccenti *et al.*, 2013).

Large datasets are common amongst many disciplines, thus methods are required to statistically interpret large datasets effectively without losing information. PCA is described as a key tool for analysing large datasets. PCA reduces dimensionality and makes large datasets more easily interpretable while preserving the data (Jolliffe & Cadima, 2016). Multivariate statistical methods make use of all variables simultaneously and includes the relationship between variables. The intercorrelated variables are reduced into a few dimensions which gather a big amount of the variability of the original variables, and are called principal components (PCs) (Zhang & Castelló, 2017). PC 1 accounts for the highest variance in the date, while each following PC accounts for the next largest variance in the remaining data (Saccenti *et al.*, 2013).

PCA plots for this study was constructed using MetaboAnalyst, a metabolomics web-based server. The PCA scores plot was used to determine if natural grouping exists between the control group and the patient group based on their acylcarnitine profiles (**Figure 4.20**).

The PCA scores plot shows no natural separation between the patient and the control group, however there are one patient sample and one control sample which does not form part of the two main groups. Since the analysis were done in two batches another PCA was done to see if there might be a batch effect, but the PCA indicated no batch effect between the two analytical runs.

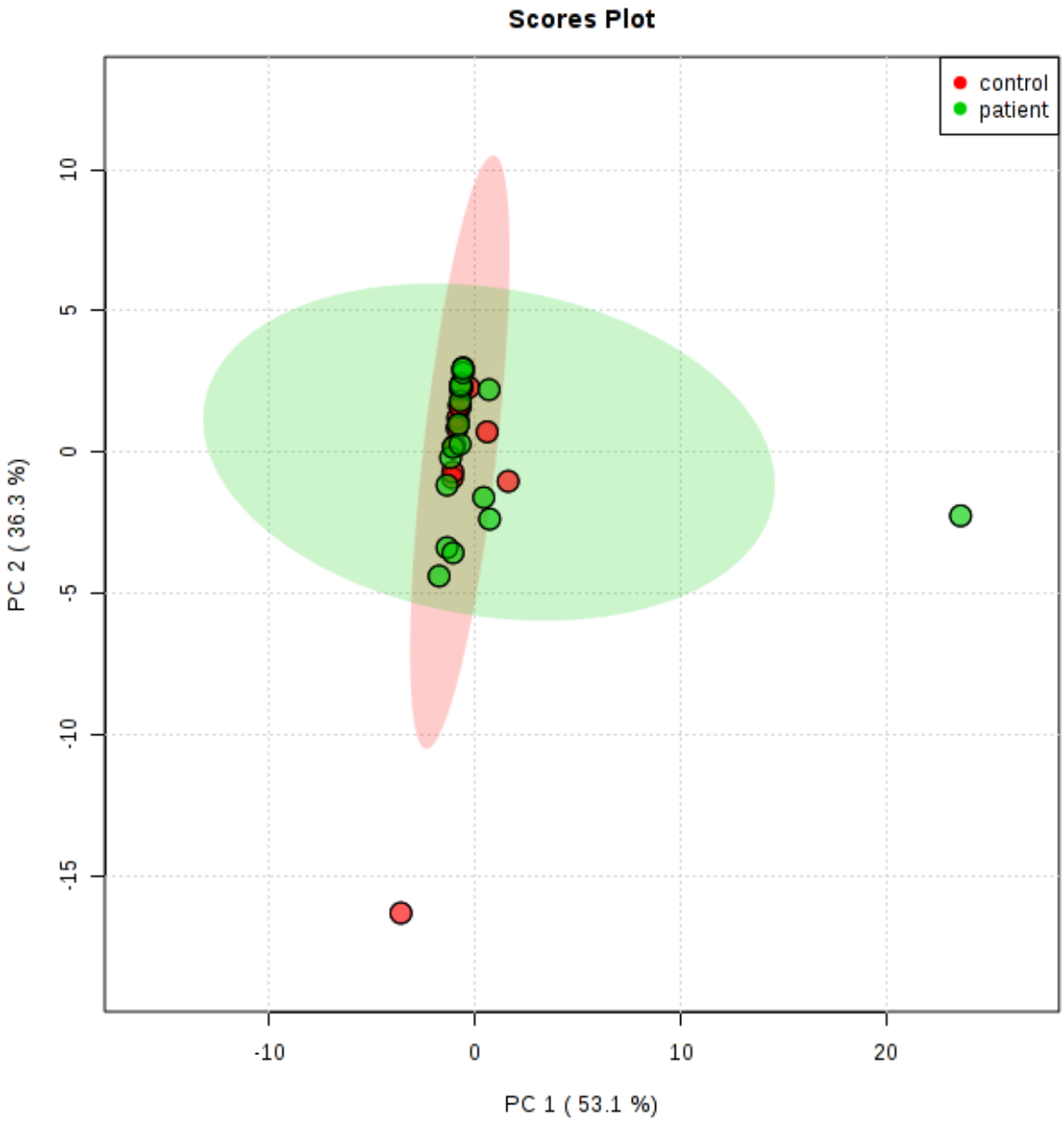


Figure 4.20: Principle Component Analysis scores plot of the acylcarnitine profiles of patient and control groups.

4.3.2 Parametric *t*-test

The Mann-Whitney test is considered the non-parametric alternative to the t-test, when independent samples are being assessed. The unpaired *t*-test for this study was constructed using MetaboAnalyst, a metabolomics web-based server. The unpaired *t*-test establishes statistical significance (p-value) by determining whether or not the averages of two groups differ. Conventionally, a p-value <0.05 is considered statistically significant. The *t*-test resulted in no significant p-values.

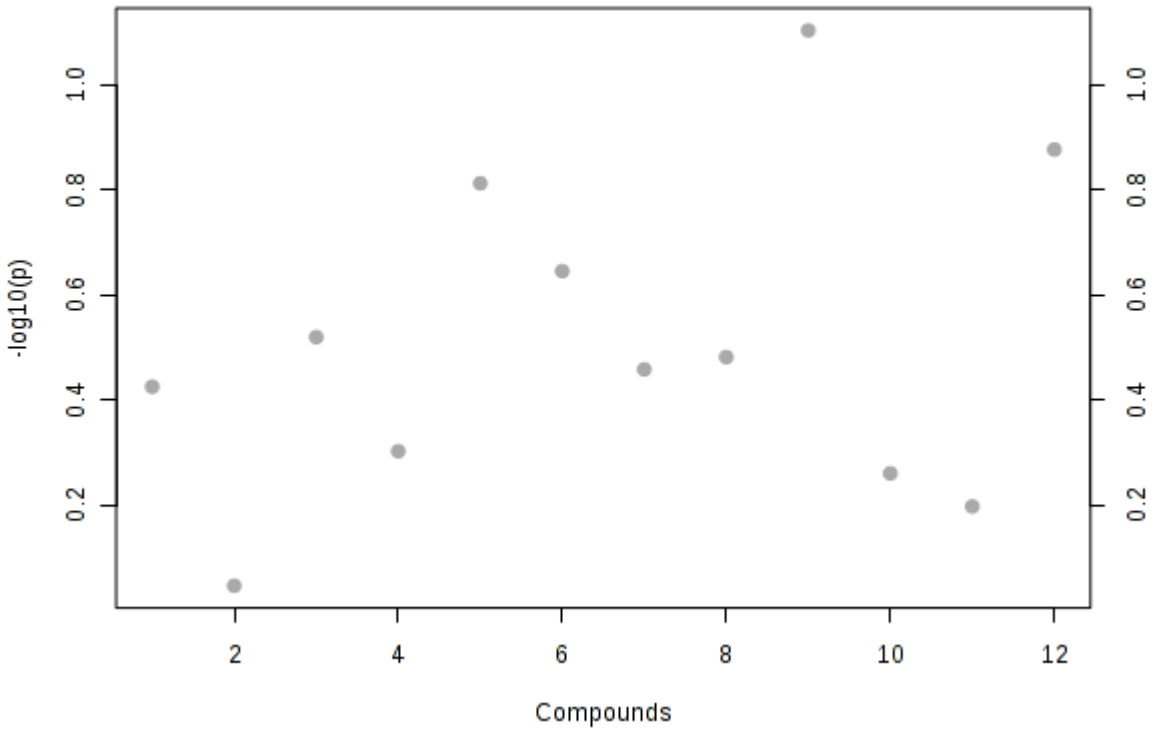


Figure 4.21: t-test applied to the data group resulted in no significant p-values.

4.3.3 Box and whiskers diagrams

The box and whiskers diagram is one way to visually represent the distribution of the data. The whiskers represent the minimum and maximum values, while the box part are divided into the first quartile (Q1 or 25%) which is represented by the line closest to the minimum value, the median (Q2 or 50%) which is the line in the middle of the box, and quartile 3 (Q3 or 75%) which is the line closest to the maximum value. Outliers are represented by dots above or below the maximum or minimum value, respectively. Outliers are calculated in terms of Q1 and Q3 values. First the interquartile range is calculated by subtracting Q1 from Q3. To calculate outliers less than Q1, the interquartile range is multiplied by 1.5 and then subtracted from Q1, if the value is lower than that of Q1, it is considered a 'low' outlier. To calculate outliers higher than Q3, the interquartile range is multiplied by 1.5 and then subtracted from Q3 and if the value is higher than that of Q3, it is considered a 'high' outlier.

There were no significant p-values in this data set, however there were one difference when ratios of certain acylcarnitines were calculated. According to reference values published by Mueller *et al.* (2003), they included acylcarnitine ratios with diagnostic value, this ratio with its reference

range, as well as the calculated patient and control ratios, can be found in **Table 4.8**. The graph representing the ratio showing different values than suggested are given in **Figure 4.22**.

Table 4.8: Diagnostically relevant acylcarnitine reference ratios with calculated patient and control ratios.

Acylcarnitine ratios	Reference values (mmol/mol creatinine)	Patient value (mmol/mol creatinine)	Control value (mmol/mol creatinine)
C3/C16	1.27 - 9.03	0.91	1.68

According to the data in **Table 4.8**, the patient group has a lower value than the indicated reference value ratio C3/C16 compared to the control group.

In **Figure 4.22** it can be seen that the combined C3/C16 ratio of the patient group is lower than the suggested reference value when compared to the C3/C16 ratio of the control group. However, when the ratios are determined for individual patient and control samples, there is no clear difference between the two groups.

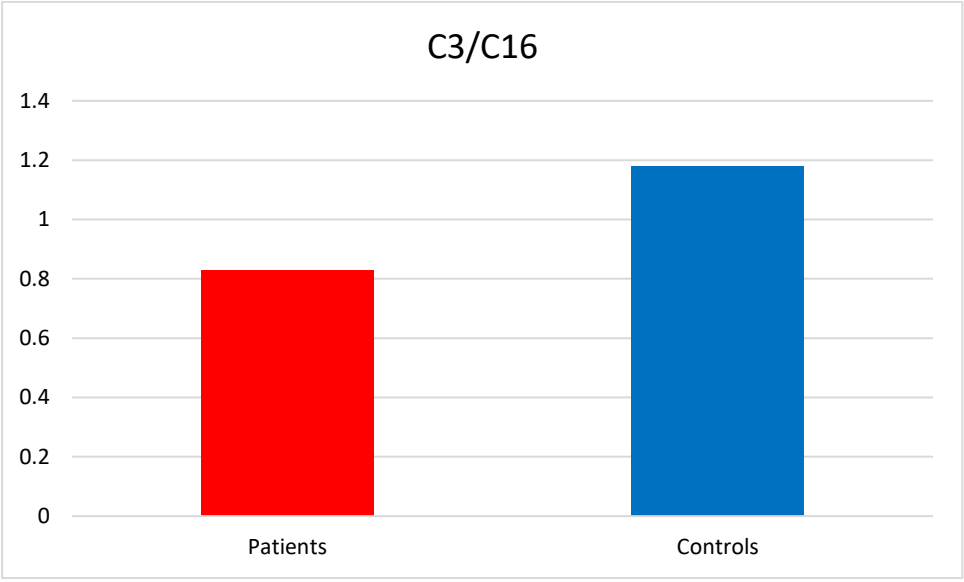


Figure 4.22: C3/C16 ratio column graph for the combined patient and control groups

Box and whiskers diagrams were created for the two acylcarnitines which is included in the diagnostic reference ratios.

Figure 4.23 shows the box and whiskers diagram for C3 patient and control samples; an uneven distribution of data points for the patients can be observed, with the median closer to the first quartile and in general showing lower valued data points (concentrations) for the patient group compared to the sample group (the box plot is positioned lower on the Y-axis). The control sample group shows more evenly distributed data with the median being more central. The minimum value of the control group however, is much lower than that of the patient group. Both groups have outliers above the maximum value, the patient group has higher values outliers compared to the control group. Overall, the control group has higher valued data points (concentrations) compared to the patient group (the box plot is positioned higher on the Y-axis).

Figure 4.24 shows the box and whiskers diagrams for C16 patient and control samples; an uneven distribution of data points (concentrations) for the patient group can be observed, with the median being closer to the first quartile. The control group shows more even distribution of data points (concentrations), with the median being more central. In general, the concentrations of both groups are within the same range when compared to position in the Y-axis, but the patient group shows a lot of outliers higher than the maximum compared to the control group.

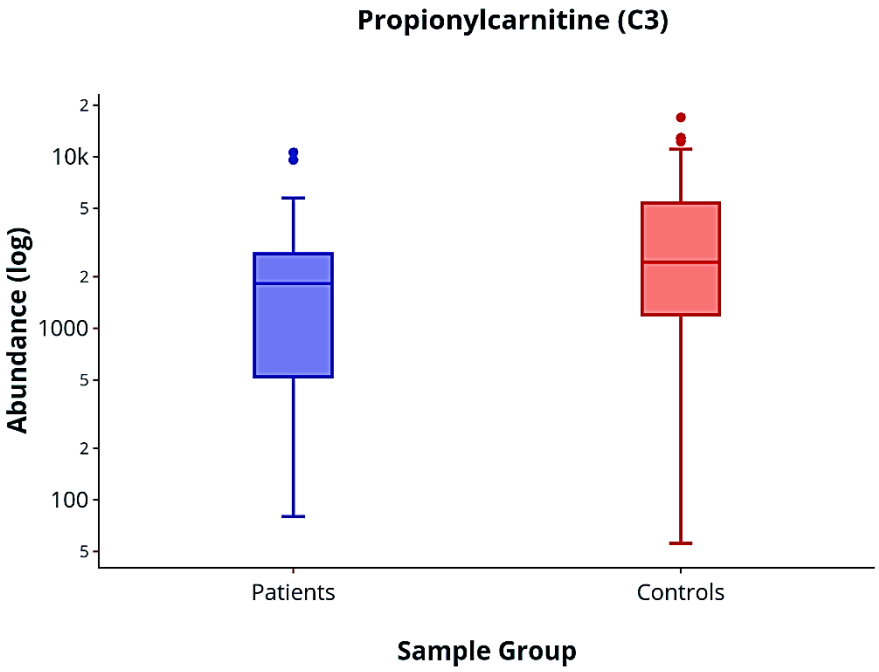


Figure 4.23: C3 box and whiskers diagram for patient and control group used in the C3/C16 diagnostically relevant ratio.

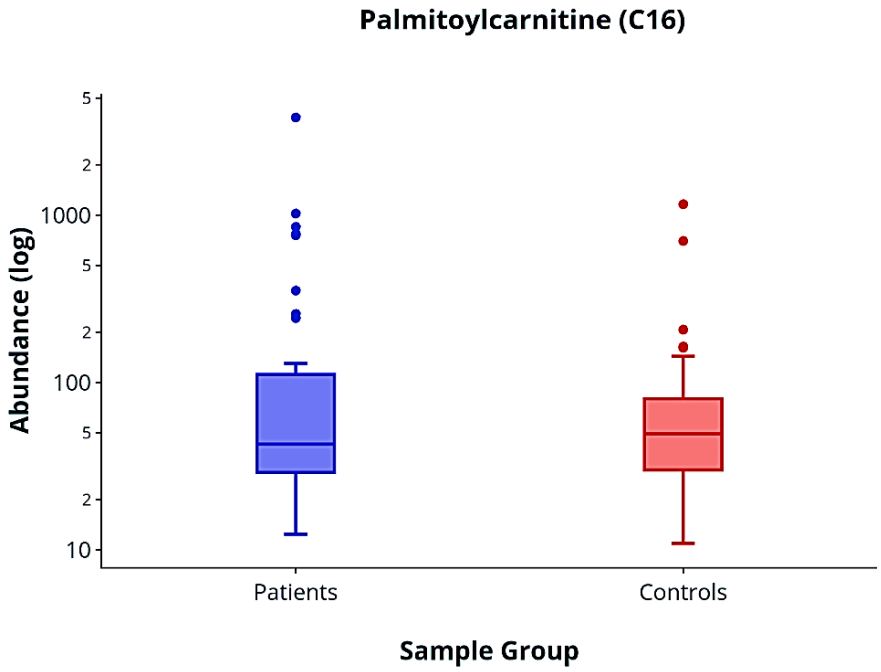


Figure 4.24: C16 box and whiskers diagram for patient and control group used in the C3/C16 diagnostically relevant ratio.

CHAPTER 5

5. CONCLUSION AND FUTURE PROSPECTS

The broad aim of this study was to identify altered urinary acylcarnitine profiles that are associated with individuals diagnosed with chronic fatigue. Although no clear differences could be established between patient and control groups during this study, some results indicate that acylcarnitines may yet be used as possible diagnostic markers to differentiate between identified chronic fatigue patients and control groups.

In this study, acylcarnitine analysis was standardised and optimised to detect all metabolites of interest, however, there were some problems with the detection of long-chain acylcarnitines (C14 to C18) in the urine samples, since they are generally not found in high concentrations in urine samples. During method validation, recovery of the long-chain acylcarnitines was within recommended ranges. It could be possible that in human urine, these long-chain acylcarnitines could be in low concentrations and could lead to detection problems, the other possibility being that there is interfering compounds present in the urine samples which have an influence on the detection of these acylcarnintines.

Method validation was achieved with acceptable accuracy and precision of all compounds and it can thus be said the method can be used to detect and identify all short and medium chain acylcarnitines examined during this study and can be applied to urine samples for routine analysis. It is unclear as to whether the method can be accurately applied for diagnostic purposes, due the detection problems with the long chain acylcarnitines.

Problems with urinary long chain acyl carnitines can be explained with *log-p* values. The ratio of the compound concentration in a homogenous mixture is referred to as the partition coefficient (P) and indicates the solubility of compounds in different immiscible phases. This indicates the hydrophobic (non-water soluble) or hydrophilic (water soluble) nature of compounds. Positive *log-p* values indicates the hydrophobicity (compounds have a higher solubility in lipid phase) of compounds, whereas a negative *log-p* value indicates the hydrophilicity (compounds have a higher solubility in aqueous phases) of compounds (Kujawski *et al.*, 2012). **Table 5.1** indicates the *log-p* values of each acylcarnitine, these are predicted values obtained from the Human Metabolome Data Base (Wishart *et al.*, 2017). Short and medium chain acylcarnitines have negative (lower) *log-p* values, whereas long chain acylcarnitines have positive (higher) *log-p* values. This explains why the long chain acylcarnitines are difficult to detect in urine samples, as they are very apolar compounds which mean they will be more soluble in apolar solvents and not

in polar solvents such as water. Urine is polar of nature and therefore not a good solvent for the apolar long chain acylcarnitines. Urine was chosen as sample matrix for this analysis because it is a non-invasive procedure to collect samples from volunteers, as well as for the reason that substances released after beta oxidation ends up in urine, which might lead to better insights about the energy metabolism taking place during beta oxidation.

Because of the apolar nature of long chain acylcarnitines, blood samples would be a better sample matrix for analysing the long chain acylcarnitines more accurately.

Table 5.1: *Log-p* values of acylcarnitines analysed

	C0	C2	C3	C4	C5	C6	C8	C10	C12	C14	C16	C18
Log p value	-5.48	-4.62	-3.7	-3.3	-3.0	-2.4	-1.5	-0.63	0.26	1.14	2.03	2.92

According to the diagnostic ratio reference ranges, only one ratio value calculated were not within the suggested range. An abnormal C3/C16 ratio is normally associated with methylmalonic aciduria, but is usually characterised by high concentrations of C3 and methylmalonylcarnitine, but the C3/C16 ratio gives the best reflection of methylmalonic aciduria (Mueller *et al.*, 2003). This however needs to be ruled out as a diagnosis before any assumptions can be made regarding information associated with chronic fatigue.

Future prospects: Due to the chemical nature of the long chain acylcarnitines special sample extraction methods for example solid phase extraction or liquid-liquid extraction can be implemented specifically for the extraction of long-chain acylcarnitines. Another possibility is to also look at hydroxyl-carnitines, as isotope hydroxyl-carnitines has become available the past year which can be used for improved optimisation and selectivity during method development.

Other studies conducted to identify acylcarnitines in urine samples with HPLS-MS/MS also reported low recovery percentages for the long chain acylcarnitines, C14, C16 and C18 (Heinig & Henion, 1999; Mueller *et al.*, 2003). Because of this problem, it would be suggested to use blood samples as matrix instead of urine samples to ensure more accurate detection of long chain acylcarnitines. A promising study conducted by Peng *et al.* (2013), included analysis of underivatized plasma acylcarnitines using ultra-fast liquid chromatography and reported an increased recovery percentage for long chain acylcarnitines, but a higher recovery percentage for C18 (113% – 140%), which they attribute to incomplete dissolution of long chain acylcarnitines in a methanol-water solution. Other studies involving identification of acylcarnitines was done using UPLC-MS/MS, which showed promising results for the long chain acyl carnitines (Reuter & Evans, 2011; Peng *et al.*, 2013).

CHAPTER 6

6. REFERENCES

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