Quantification of selected energy and redox markers in blood samples of chronic fatigue syndrome patients

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It all starts here ™

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Abstract

Chronic, noncommunicable diseases such as chronic fatigue syndrome (also known as myalgic encephalomyelitis) are rapidly becoming a worldwide epidemic that profoundly affects public health and productivity. Chronic fatigue syndrome (CFS) is characterised by severe and debilitating fatigue and although its etiology is still unknown, recent studies have found considerable evidence that mitochondrial dysfunction and oxidative stress might be responsible for the underlying energy deficit in these patients. Adenine and pyridine nucleotides could be used as potential biomarkers for energy related disorders such as chronic fatigue syndrome because of their various functions in the energy and redox pathways.

The first part of this study focussed on developing a liquid chromatography electrospray-ionisation tandem mass spectrometry (LC-ESI-MS/MS) method for the quantification of these nucleotides in blood samples. Due to the instability of nucleotides in biological matrices it was also necessary to find a suitable extraction method that would be able to stop enzymatic activity via protein precipitation. Out of the four extraction methods investigated during this study, deproteinisation of whole blood samples with perchloric acid produced the highest nucleotide abundances. Although nucleotide standards were found to be stable in perchloric acid, nucleotide levels in blood samples were not stabilised by addition of perchloric acid.

The second part of this study consisted of measuring the nucleotide levels in blood samples of controls and possible CFS patients in order to test the proof of concept of the new LC-ESI-MS/MS method. Despite changes in the nucleotide levels due to perchloric acid and problems with nucleotide instability, it was still possible to distinguish between the two groups based on the results obtained with the new LC-ESI-MS/MS method.

The newly developed LC-ESI-MS/MS method proved to be reliable and adequate for nucleotide quantification in whole blood samples, thus the aim of this study was achieved.

Keywords: nucleotides, LC-ESI-MS/MS, perchloric acid, stability, whole blood, chronic fatigue syndrome

Uittreksel

Kroniese, nie-oordraagbare siektes soos kroniesemoegheidsindroom (ook bekend as mialgiese enkefalomiëlitis) is besig om 'n wêreldwye epidemie te word wat 'n beduidende effek het op die gesondheid en die produktiwiteit van die publiek. Kroniesemoegheidsindroom word gekenmerk deur ernstige verlammende moegheid waarvan die etiologie nog onbekend is. Onlangse studies toon dat mitochondriale disfunksie en oksidatiewe stres die vernaamste oorsake kan wees vir die onderliggende energietekort wat voorkom by kroniesemoegheidsindroom pasiënte. Adenien en piridien nukleotiede kan potensieël gebruik word as biomerkers vir energie-verwante siektes soos kroniesemoegheidsindroom van weë die belangrike funksies wat dit verrig tydens energieproduksie en handhawing van redoksbalans.

Die eerste deel van die studie het gefokus op die ontwikkeling van 'n elektrosproei-ionisasie tandem massaspektrometrie metode wat gebruik kan word vir die kwantifisering van bogenoemde nukleotiede in bloedmonsters. Omdat nukleotiede onstabiel is in biologiese matrikse was dit noodsaaklik om 'n geskikte ekstraksiemetode te vind wat ensiematiese aktiwiteit kan stop d.m.v. proteïenpresipitasie. Uit die vier ekstraksiemetodes wat ondersoek is tydens die studie, het ekstraksie met perchloorsuur die hoogste nukleotiedvlakke tot gevolg gehad. Alhoewel nukleotied-standaarde stabiel was in perchloorsuur, was die nukleotiede teenwoordig in die bloedmonsters nie stabiel na ekstraksie met perchloorsuur nie.

Tydens die tweede deel van die studie is nukleotiedvlakke in bloedmonsters van kontroles en moontlike kroniesemoegheidsindroom pasiënte gemeet met behulp van die nuwe elektrosproei-ionisasie tandem massaspektrometrie metode. Ten spyte van veranderinge in die nukleotiedvlakke a.g.v. perchloorsuur en die onstabiliteit van die nukleotiede, was dit steeds moontlik om te onderskei tussen die twee groepe.

Die nuut ontwikkelde elektrosproei-ionisasie tandem massaspektrometrie metode is dus betroubaar en geskik vir kwantifisering van nukleotiede in bloedmonsters, dus is die doel van die studie bereik.

Sleutelwoorde: nukleotiede, elektrosproei-ionisasie tandem massaspektrometrie, perchloorsuur, stabiliteit, heel bloed, kroniesemoegheidsindroom

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Our greatest weakness lies in giving up. The most certain way to succeed is always to try just one more time.

Thomas A. Edison

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List of Abbreviations

Symbols

α	alpha
β	beta
°C	degrees Celsius
%	percentage
b	slope

Measuring units

g/mol	gram per mole
h	hour(s)
L/min	litre per minute
m/z	mass-to-charge-ratio
µL	microliter
µm	micrometer
µM	micromolar
µmol/L	micromole per litre
mg	milligram
mL	millilitre
mL/min	millilitre per minute
mm	millimeter
mM	millimolar
mmol/L	millimole per liter
min	minutes
M	molar
mol/L	mole per litre
psi	pound per square inch
rpm	revolutions per minute
V	volts

Abbreviations

AA	acetic acid
ACN	acetonitrile
ADP	adenosine diphosphate
Ade	adenine
Adeno	adenosine
AMP	adenosine monophosphate
ATP	adenosine triphosphate
Cat. No.	catalogue number
CBT	cognitive behavioural therapy
CDC	Centres for Disease Control and Prevention
CFS	chronic fatigue syndrome
CFS/ME	chronic fatigue syndrome/ myalgic encephalomyelitis
CV	coefficient of variation
CNS	central nervous system
DBAF	dibutylamonniumformate
DMHA	dimethylhexylamine
ESI-MS	electrospray-ionisation mass spectrometry
FA	formic acid
FDA	Food and Drug Administration
Fig.	figure
GET	graded exercise therapy
H_2O/IS	water/ internal standard mix
HILIC	hydrophilic interaction chromatography
HPA axis	hypothalamic-pituitary-adrenal axis
HPLC	high-performance liquid chromatography
HPLC-UV	high-performance liquid chromatography ultraviolet detection
Hypox	hypoxanthine
IL-1	interleukin-1

IL-6	interleukin-6
Ino	inosine
K_2CO_3	potassium carbonate
KOH	potassium hydroxide
LC	liquid chromatography
LC-ESI-MS/MS	liquid chromatography electrospray-ionisation tandem mass spectrometry
LC-MS	liquid chromatography mass spectrometry
LOD	limit of detection
LOQ	limit of quantification
MRM	multiple reaction monitoring
MS	mass spectrometry
MSQ	medical symptoms questionnaire
NAD	nicotinamide adenine dinucleotide (oxidised)
NADH	nicotinamide adenine dinucleotide (reduced)
NADP	nicotinamide adenine dinucleotide phosphate (oxidised)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced)
NaOH	sodium hydroxide
PCA	perchloric acid principle components analysis
PCA/IS	perchloric acid/ internal standard mix
PFS	Piper fatigue scale
r^2	correlation coefficient value
SD	standard deviation
SIM	selected ion-monitoring
TNF-α	tumor necrosis factor alpha
UV	ultraviolet

Chapter 1: Introduction

1.1. Introduction

According to the World Health Organisation (2010) chronic, noncommunicable diseases are rapidly becoming a worldwide epidemic. Escalating rates of chronic diseases such as neurocognitive, metabolic, autoimmune and cardiovascular disorders have a profound effect on public health and productivity (Sears & Genuis, 2012). Current research is mainly focusing on the etiology, diagnosis and treatment of these chronic diseases. One particular disease known as chronic fatigue syndrome or myalgic encephalomyelitis (CFS/ME) has been discussed in various articles (Afari & Buchwald, 2003; Booth *et al*., 2012; Cho *et al*., 2006; Evengard *et al*., 1999). This disease is characterised by severe and debilitating fatigue. Despite the ongoing research, the etiology of this disease is still unknown. The unknown etiology as well as other factors such as the absence of clinical and diagnostic markers, the vague description of diagnostic criteria and similarities with other ill-defined diseases, complicates diagnosis (Afari & Buchwald, 2003). The current diagnosis of CFS/ME primarily depends on information obtained through clinical interviews resulting in low levels of diagnostic reliability. These challenges provide an opportunity to develop new diagnostic methods in order to accurately diagnose CFS/ME.

1.2. Problem statement and substantiation

A possible starting point for diagnostic method development would be to identify certain key markers that are involved in the energy production pathways. Adenine nucleotides such as ATP, ADP and AMP, and pyridine nucleotides such as NADH, NAD, NADPH and NADP play important roles in the functioning of the metabolic pathways involved in energy production (Kang & Pervaiz, 2012; Nakamura *et al*., 2012.) Carnitine also plays an important role in energy production by transporting long chain fatty acids into the mitochondria for β-oxidation, thus carnitine and acylcarnitine levels are both altered during energy production disorders (Chazotte, 2001). The above mentioned compounds can thus all be used as possible key markers involved in CFS/ME.

In order to measure possible changes in the levels of these markers in biological samples, an accurate and sensitive method is needed. Recent studies specifically focused on finding suitable extraction methods to stabilise nucleotide levels in various biological matrices, as these molecules are rather unstable due to the presence of high energy bonds in their

structures (Klawitter *et al*., 2007; Coolen *et al*., 2008). In a study done by Klawitter *et al.* (2007) it was found that out of the four commonly used nucleotide extraction methods, perchloric acid extraction led to the best absolute recovery and reproducibility for all nucleotides.

Methods that have been used for the quantification of nucleotides in biological matrices include bioluminescence (firefly luciferase enzyme assay) and high performance liquid chromatography (HPLC) methods. Mass spectrometry (MS) coupled with liquid chromatography (LC) has become an important analytical technique for the quantification of nucleotides due to its high sensitivity and selectivity (Khlyntseva *et al*., 2009). The advantages of liquid chromatography mass spectrometry (LC-MS) include simultaneous analysis of different metabolites, its compatibility with commonly used solvents such as methanol and water, and analysis of highly polar molecules (such as nucleotides) without derivatisation (Coulier *et al*., 2006). However, nucleotides present a challenge for standard reversed-phase chromatography due to their high polarity. Various studies have suggested using ion-pairing chromatography as a solution to this problem (Cai *et al*., 2002; Klawitter *et al*., 2007; Qian *et al*., 2004). Using an ion-pairing reagent has its own disadvantages such as the possibility of ion-suppression which would decrease the MS detection sensitivity. It is therefore necessary to look at alternative solutions to the problems encountered during the analysis of nucleotides with LC-MS.

Thus for this study the following research questions will be considered:

1. Can a reliable electrospray-ionisation tandem mass spectrometry (LC-ESI-MS/MS) method be developed to quantify energy and redox status markers in whole blood samples?
2. Can this method then be used to detect possible differences in the energy and redox marker levels of patients with chronic fatigue syndrome and healthy individuals?

1.3. Research aims and objectives

The aim of this study is to develop and validate an electrospray-ionisation tandem mass spectrometry method to quantify selected energy and redox marker levels in whole blood samples.

The objectives of this study include:

- Optimising the LC-ESI-MS/MS conditions for analysis of selected adenine and pyridine nucleotides.

- Optimising the stabilisation and extraction conditions for adenine and pyridine nucleotides.
- Comparing the energy and redox marker levels in whole blood samples of possible chronic fatigue syndrome patients and healthy individuals.

1.4. Experimental design

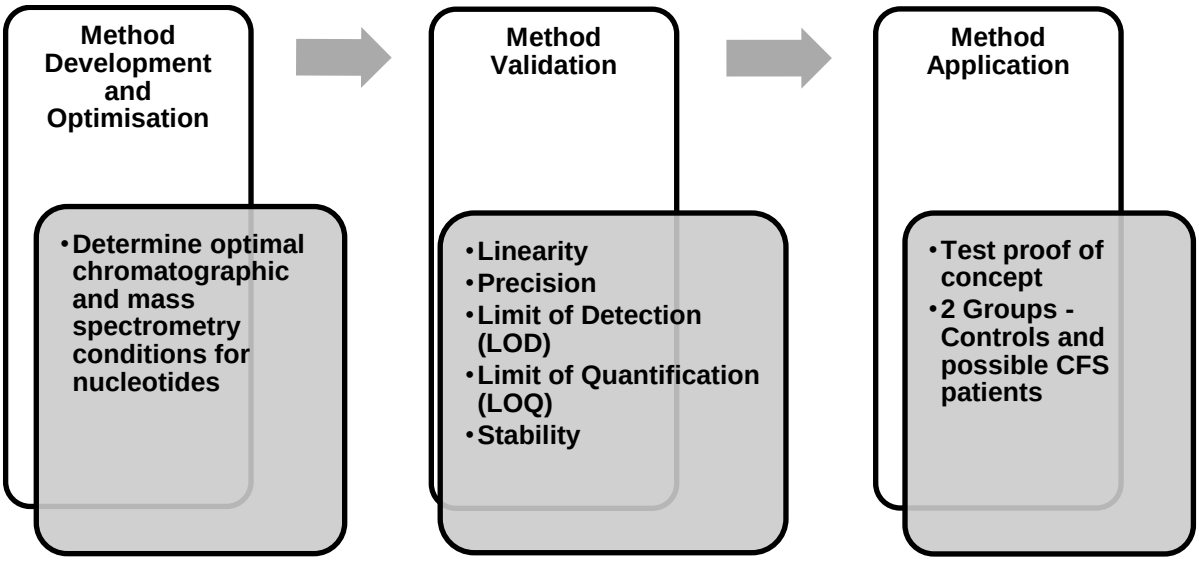


Figure 1.1: Experimental approach followed during this study.

The first step was to develop and optimise an LC-ESI-MS/MS method which can be used to quantify selected adenine and pyridine nucleotides. This entailed finding the optimal chromatographic conditions to separate the nucleotides and the optimal mass spectrometry conditions to detect the nucleotides. The second step was to validate the newly developed LC-ESI-MS/MS method. This was done by determining certain validation parameters such as linearity, precision, limit of detection (LOD) and limit of quantification (LOQ). The stability of nucleotide standards in water and perchloric acid and the stability of nucleotides present in blood were assessed under various conditions. The validated method was then applied to investigate if there is a possible difference in the adenine and pyridine nucleotide levels in the blood samples of relatively healthy volunteers and possible chronic fatigue syndrome patients.

1.5. Outline of dissertation

This dissertation consists of six chapters. The first chapter is an introduction and outlines the problem statement and substantiation, and the research aims and objectives of this

study. The second chapter is an overview of the literature that is currently available on the topic. The literature overview focuses mainly on topics such as chronic fatigue syndrome, the role of adenine and pyridine nucleotides and the possibility that these nucleotides could be used as markers for chronic fatigue syndrome, and the methods used to stabilise, extract and quantify these nucleotides in blood. The third chapter contains all the materials and methods that were used during this study. In the fourth chapter a general discussion of the results obtained during this study, is given. The fifth chapter consists of concluding observations and future recommendations for this study. The last chapter consists of a list of all the references used throughout this dissertation.

Chapter 2: Literature Review

2.1. Chronic fatigue syndrome

Fatigue is a common symptom experienced by many individuals. The fatigue is usually temporary, self-limiting and explained by underlying circumstances such as disease or exertion. However, when an individual suffers from persistent and debilitating fatigue that cannot be explained by any medical condition, it may represent chronic fatigue syndrome also known as myalgic encephalomyelitis (CFS/ME) (Afari & Buchwald, 2003; Norheim *et al*., 2011).

According to the case definition developed by the Centres for Disease Control and Prevention (CDC), chronic fatigue syndrome is a complex and debilitating disorder characterised by severe fatigue lasting for 6 or more consecutive months (Fukuda *et al*., 1994; Holmes *et al*., 1988). The fatigue significantly interferes with daily work and activities and is not caused by ongoing exertion or any other medical conditions associated with fatigue. In addition to fatigue the individual also has 4 or more of the following symptoms during the 6 month period: impaired memory and concentration, sleep disturbances, muscle and joint pain, frequent and recurring sore throat, headaches, tender lymph nodes and post-exertional fatigue lasting for more than 24 hours (Fukuda *et al*., 1994; Holmes *et al*., 1988). Clinical practitioners have found this definition to be too broad and unspecific for use in clinical diagnosis as it focuses only on the presence or absence of symptoms without regarding the severity or frequency. The Canadian expert consensus panel published the first clinical case definition for CFS/ME which can be used for diagnosis (Carruthers *et al*., 2003). In contrast to the CDC's 1994 case definition, this clinical definition not only focuses on fatigue but also on other cardinal CFS/ME symptoms. Thus in order for a patient to be diagnosed with CFS/ME, the patient must exhibit symptoms after exercise and show signs of neurological, neurocognitive, neuroendocrine, dysautonomic circulatory and immune dysfunction.

2.1.1. Etiology and pathophysiology of chronic fatigue syndrome

Despite ongoing research, the etiology of CFS/ME is still unknown and the pathophysiology poorly understood. It is possible that CFS/ME has a complex and multifactorial etiology. Several studies report strong evidence that predisposition to CFS/ME may have a genetic origin (Albright *et al*., 2011; Buchwald *et al*., 2001; Underhill & O'gorman, 2006). Gene

expression studies done on CFS/ME patients and controls, indicated up-regulation of certain genes in CFS/ME patients that are associated with immunological functions (Kaushik *et al*., 2005; Powell *et al*., 2003; Vernon *et al*., 2002). Precipitating factors such as exposure to environmental toxins, physical or emotional trauma and acute or chronic infections may also contribute to the development of CFS/ME. The multi-systemic pathophysiological effects of CFS/ME include immune system abnormalities, central nervous system defects, mitochondrial dysfunction and abnormalities in energy production (IACFS/ME, 2012).

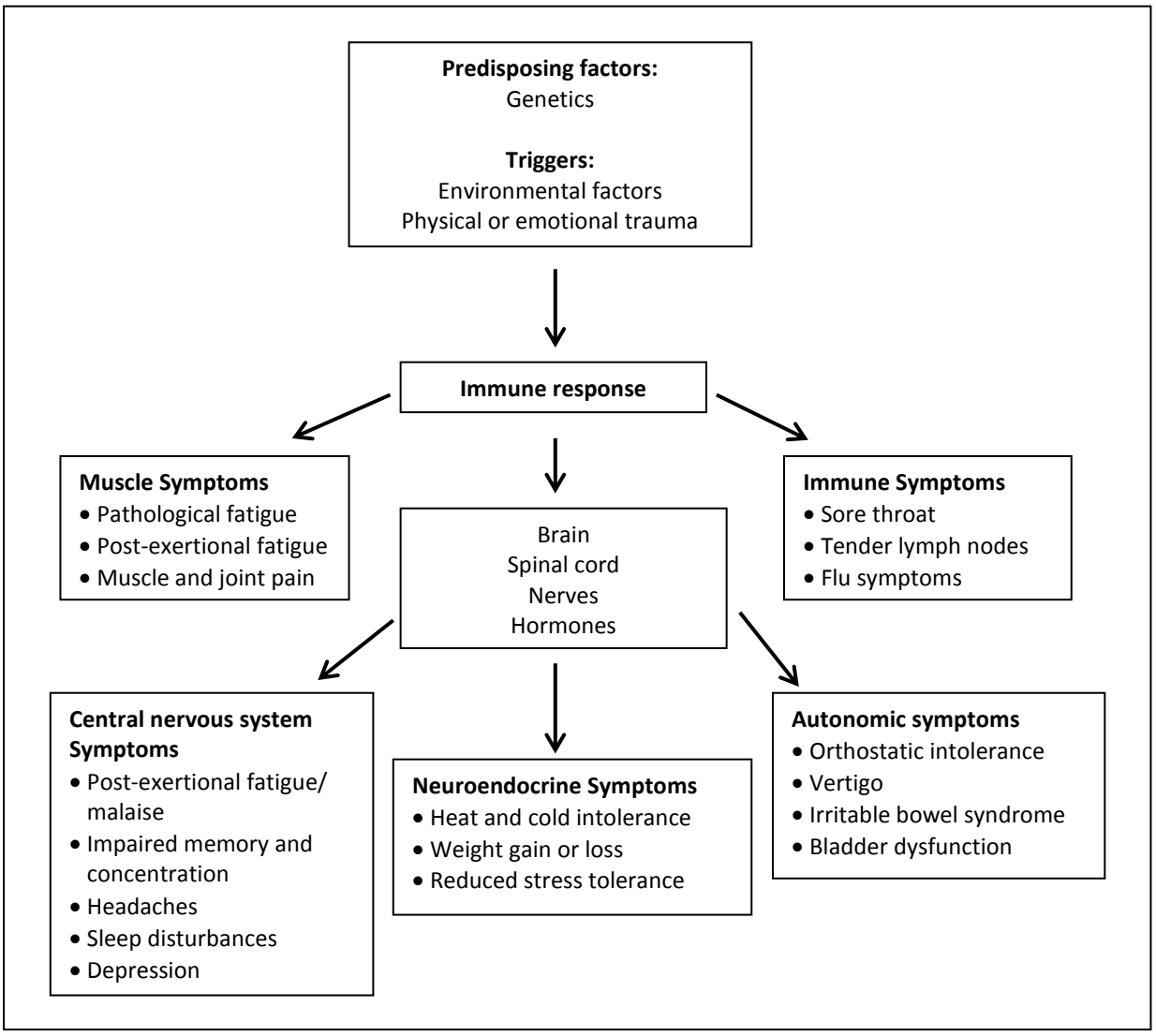


Figure 2.1: The multi-systemic pathophysiological effects of chronic fatigue syndrome. Predisposing factors such as genetics and precipitating factors such as exposure to environmental toxins, physical or emotional trauma, and acute or chronic diseases may contribute to the development of CFS/ME. The pathophysiological effects of CFS/ME are exhibited in various systems of the body including the immune system, central nervous system and neuroendocrine system. This causes the wide variety of symptoms experienced by CFS/ME patients. (Adapted from IACFS/ME, 2012)

2.1.1.1. Immune system

Immunological studies performed on patients with CFS/ME are contradictory and not all of the immune system abnormalities are consistently reported. Immune system abnormalities identified in these patients include immune system activation, an increase in T-lymphocyte numbers and activity, and a decrease in natural killer cell activity (Afari & Buchwald, 2003; Bassi *et al*., 2008; Cho *et al*., 2006; IACFS/ME, 2012). An increase in cytokine production has also been observed. Pro-inflammatory cytokines such as TNF-α, IL-1 and IL-6 are involved in the stress response and induce sickness behaviour such as loss of energy and fatigue, decreased physical activity, changes in sleep patterns, impaired concentration and memory, increased sensitivity for pain, poor appetite and weight loss (Kelley *et al*., 2003). This increase in cytokine production may be linked to the fatigue and flu-like symptoms experienced by CFS/ME patients. Antibodies to certain infectious agents have also been occasionally reported (Ablashi *et al.*, 2000). Infectious agents such as Epstein-Barr virus, human herpesvirus-6, enteroviruses and retroviruses have been proposed to be responsible for the etiology of CFS/ME (Ablashi *et al*., 2000). However, antibody profiles of CFS/ME patients indicate a chronic reactivation of the latent virus infection (Buchwald *et al*., 1992; Jones *et al*., 1985; Straus *et al*., 1985). It is possible that this reactivation is due to immune system dysregulation rather than being the primary cause of CFS/ME.

2.1.1.2. Central nervous system

Symptoms commonly experienced by CFS/ME patients such as fatigue, disrupted sleep, impaired cognitive function, headaches and pain, suggests that the central nervous system (CNS) is involved in the pathophysiology (Afari & Buchwald, 2003; Evengard *et al*., 1999). Neuroimaging studies performed on CFS/ME patients have shown some abnormalities in the CNS. Magnetic resonance imaging studies done by Buchwald *et al.* (1992) and Natelson *et al.* (1993) indicated white matter abnormalities in the subcortical areas of the brain. Single-photon emission computed tomography imaging studies indicate hypoperfusion throughout the brain (Ichise *et al*., 1992) and abnormalities mostly in the frontal and temporal lobes that could be the cause of symptoms such as depression, irritability and decreased memory and mental capacity (Schwartz *et al*., 1994). Autonomic nervous system dysfunction in CFS/ME patients is manifested as orthostatic intolerance. Hypotension, increased heart rate and orthostatic symptoms (light-headedness, dizziness, nausea, fatigue, headache etc.) have been demonstrated during upright tilt-table testing of adult and adolescent CFS/ME patients (Bou-Holaigah *et al*., 1995; Freeman & Komaroff, 1997; Stewart *et al*., 1999).

2.1.1.3. Neuroendocrine system

Central to the abnormalities found in the neuroendocrine system of CFS/ME patients, is the decreased activity of the hypothalamic-pituitary-adrenal (HPA) axis (Cleare, 2004; Demitrack *et al*., 1991) and the up-regulation of the serotonergic pathways (Yamamoto *et al*., 2004). Abnormalities of the HPA axis include mild hypocortisolism, heightened negative feedback and glucocorticoid receptor function, and decreased responses to a variety of stressor challenges (Cleare, 2004; Demitrack *et al*., 1991). Due to the absence of HPA axis abnormalities in the early stages of CFS/ME, it is suggested that the neuro-behavioural changes (inactivity, sleep disturbances, medication and ongoing stress) precipitates HPA axis changes. The HPA axis changes, particularly low cortisol levels, can in turn lead to perpetuation or worsening of fatigue and other symptoms (Cleare, 2004; Papadopoulos & Cleare, 2012).

2.1.2. Diagnosis of chronic fatigue syndrome

Diagnosis of CFS/ME is difficult due to the unknown etiology and pathophysiology. Other factors that also complicate diagnosis include the vague wording of the diagnostic criteria, the absence of clinical or laboratory diagnostic markers, the pattern of remission and relapse exhibited by CFS/ME patients, fatigue being a common symptom of many diseases, and symptoms varying from person to person in type, number and severity (Hawk *et al*., 2006). Diagnosis is also complex due to the similarity of CFS/ME with other ill-defined disorders such as fibromyalgia, multiple chemical sensitivity and posttraumatic stress disorder (Pall, 2001).

There is currently no available diagnostic test which is sufficiently sensitive or specific enough for diagnosing CFS/ME. Diagnosis primarily consists of a detailed clinical evaluation in order to rule out other possible causes for the severe fatigue. The first step of the clinical evaluation is to compile a detailed medical and social history of the patient. Previous medical records, lab tests, medication and family history are assessed. Information is gathered on the daily activities and functioning of the patient prior to the illness in order to determine the severity and impact of disease symptoms. This information can be gathered with the use of medical questionnaires such as the Piper fatigue scale (PFS) and the medical symptom questionnaire (MSQ). Secondly, a thorough physical and mental examination is performed. Through the mental examination other psychiatric disorders that present similar symptoms, such as major depression, can be ruled out. The findings for the physical examination are usually subtle and not clearly evident. If the patient has symptoms of widespread pain and musculoskeletal tenderness, fibromyalgia can be confirmed or ruled

out with a tender point examination. The clinical evaluation is concluded with basic laboratory screening tests. In addition to the routine laboratory tests, more specific tests should be considered depending on particular symptoms that the patient is experiencing. The results obtained through the clinical evaluation can then be used for differential diagnosis in order to exclude other diseases and disorders with similar symptoms.
(Afari & Buchwald, 2003; Carruthers *et al*., 2003; IACFS/ME, 2012)

The various challenges involved in the diagnosis of CFS/ME are likely to produce low levels of diagnostic reliability and accuracy. This provides new opportunities for researchers to develop accurate and sensitive methods that can be used for CFS/ME diagnosis.

2.1.3. Reliability and validity of fatigue rating scales

In order to determine the effects and improve management of fatigue, reliable and valid methods of assessment are needed. The increased recognition of fatigue as a major problem in many clinical conditions has led to the development of a large number of unidimensional (e.g. the fatigue severity scale) and multidimensional (e.g. Piper fatigue scale) scales that attempt to measure the nature, severity and impact of fatigue (Dittner *et al*., 2004). The information obtained from these self-report scales is subjective to the developer's understanding of fatigue and the respondent's interpretation of the questions asked. Thus different scales may be measuring different aspects of fatigue which means that a scale which is developed for one specific clinical condition cannot necessarily be used for patients with another clinical condition. It is thus important for any clinician or researcher to ensure that the scale used measures the correct aspects of fatigue that is required for their specific study (Dittner *et al*., 2004; Hjollund *et al*., 2007).

Dittner *et al.* (2004) described and evaluated a number of different fatigue rating scales. It was found that basic data on the reliability and validity were not reported and few suggest cut-off scores which can be used to distinguish between fatigue within the general population and fatigue as a result of clinical conditions. Furthermore, not all scales take the effects of age, gender, ethnic and cultural factors into consideration. It was concluded that due to the wide range of mechanisms responsible for fatigue and the different manifestations of symptoms, it is unlikely that one fatigue scale will be sufficient enough to measure fatigue in all clinical conditions. Thus new scales need to be developed and existing scales need further validation.

2.1.3.1. The Piper fatigue scale

The Piper fatigue scale (PFS) is a multidimensional visual analogue scale which is used to measure several dimensions of fatigue including: duration, severity/ intensity, sensory and cognitive/ mood. The PFS was initially validated with a group of 50 cancer patients during their first week of radiation therapy (Piper *et al*., 1987; Piper *et al*., 1989). The revised version of this scale consists of 27 items of which 22 items are scored numerically and the other 5 items provide qualitative information (Piper *et al*., 1998). Due to the research interests of the developers, the PFS has predominately been used to assess fatigue in cancer patients. However, the PFS has been used to subjectively measure fatigue in a variety of populations including HIV patients (Breitbart *et al*., 2001), patients with postpolio syndrome (Strohschein *et al*., 2003), iron deficient woman (Patterson *et al*., 2001), chronic fatigue syndrome patients (Nicolson & Ellithorpe, 2006) and healthy individuals (Libbus *et al*., 1995). High internal consistency as well as high concurrent validity and good test-retest reliability have been reported for the PFS (Dittner *et al*., 2004; Piper *et al*., 1998).

2.1.3.2. The medical symptoms questionnaire

The medical symptoms questionnaire (MSQ) is used as a clinical tool for evaluating a patient's general physical signs and symptoms. It is used for both initial assessment and to monitor the patient's response to therapy. The MSQ was derived from the Cornell Medical Index Health Questionnaire and consists of a 71-item symptoms checklist that is divided into separate domains which measure various physical, mental and emotional symptoms. The MSQ is completed using a 5-point Likert scale for each symptom. A total score of above 75 is generally associated with extensive symptomology while a score below 30 indicates few, low intensity symptoms. Although the validity of the MSQ is still undetermined, it is frequently used by many medical practitioners and clinical detoxification programs. (Bland & Bralley, 1992; Bennett *et al*., 2010; Mallar, 2008)

2.1.4. Treatment of chronic fatigue syndrome

Due to the unknown etiology and the heterogeneity of the CFS/ME population there is no established treatment or therapy with a guaranteed positive outcome. Various pharmacological and non-pharmacological interventions have been used for treatment of CFS/ME (Afari & Buchwald, 2003; IACFS/ME, 2012). Non-pharmacological interventions include cognitive behavioural therapy (CBT), graded exercise therapy (GET) and self-help techniques. CBT is a form of psychotherapy which is used by therapists to change the patient's thought patterns and behaviour to enable the patient to better cope with the

emotional impact of their symptoms (Prins *et al*., 2006). GET focuses on developing a structured aerobic exercise program that aims to gradually increase the patient's physical activity levels. These exercise programs are adapted to the patient's physical capabilities, needs and limits (Prins *et al*., 2006). Patient self-management is also an important aspect of treatment. The patient needs to be educated on CFS/ME in order to make certain lifestyle changes and avoid specific known aggravators to minimise impairment caused by the disease. Medications are prescribed to CFS/ME patients to reduce the severity of their most troubling symptoms. As many CFS/ME patients are hypersensitive to medications it is important to start at low dosages and gradually increase, in order to determine the patient's tolerability (Carruthers *et al*., 2003). The goal of all the above mentioned treatments is to reduce symptoms and to improve functioning and quality of life of the CFS/ME patient.

2.2. Nucleotides

2.2.1. Nucleotides as possible markers for chronic fatigue syndrome

Recent studies have found considerable evidence that mitochondrial dysfunction and oxidative stress might be important causes for the underlying energy deficit in CFS/ME patients (Booth *et al*., 2012; Fulle *et al*., 2000; Kennedy *et al*., 2005; Myhill *et al*., 2009). According to Booth *et al.* (2012) partial blocking of the adenine nucleotide transporter proteins in the mitochondria and a shortage of essential substrates and co-factors, causes the mitochondrial dysfunction. Mitochondrial dysfunction impairs aerobic energy production and causes a disturbance in ATP/ADP metabolic recycling. When aerobic capacity is exceeded during physical and mental activity, the anaerobic pathways are activated which are far less efficient at energy production than the aerobic pathways. During the process of anaerobic energy production, lactic acid is produced which can cause the muscle pain experienced by CFS/ME patients (IACFS/ME, 2012). The impaired ATP/ADP recycling will result in a lower rate of ATP synthesis after moderate physical activity which may be the cause of the prolonged post-exertional fatigue experienced by CFS/ME patients (Myhill *et al*., 2009).

Mitochondrial dysfunction also causes an increase in oxidative stress which will result in further oxidative damage to the mitochondria. In a study done by Fulle *et al.* (2000) it was found that this increase in oxidative stress was not due to a decline in the efficiency of the antioxidant defence systems but due to an increase in the production of reactive oxygen species. Fulle *et al.* (2000) and Myhill *et al.* (2009) found a significant decrease in the serum acylcarnitine concentrations of CFS/ME patients. A decrease in serum carnitine and acylcarnitine levels may contribute to mitochondrial dysfunction since carnitine and

acylcarnitine are involved in energy production (Fulle *et al*., 2000). Due to the important roles of adenine nucleotides, pyridine nucleotides, carnitine and acylcarnitines in the metabolic pathways involved in energy production, these compounds can be considered as possible markers for chronic fatigue syndrome.

2.2.2. Pyridine nucleotides

Pyridine nucleotides such as NADH, NAD^+, NADPH and $NADP^+$, are a group of coenzymes that are involved in various critical metabolic pathways in living organisms (Markuszewski *et al*., 2003). They are synthesised from nicotinamide (vitamin B3) and consist of two mononucleotides, adenosine monophosphate and nicotinamide mononucleotide. These nucleotides play an important role in energy production and maintenance of the cellular redox status as illustrated in Figure 2.2. Pyridine nucleotides are also involved in a wide variety of other cellular functions such as cell survival and cell death, ion channel regulation, and cell signalling under normal and pathological conditions (Nakamura *et al*., 2012). The reduced forms (NADH and NADPH) act as electron donors while the oxidised forms (NAD^+ and $NADP^+$) act as electron acceptors, in several biological electron-transfer reactions. NAD^+ is used as a coenzyme during glycolysis, the Krebs cycle and β-oxidation for the oxidation of fuel molecules. During these processes NADH is produced which is used for ATP production by the mitochondrial electron transport chain and oxidative phosphorylation. $NADP^+$ is used as a coenzyme by the pentose phosphate pathway to produce NADPH and ribose-5-phosphate for the synthesis of nucleotides and nucleic acids. NADPH is used for the reductive biosynthesis of fatty acids, cholesterol and reduced glutathione (Markuszewski *et al*., 2003; Nakamura *et al*., 2012).

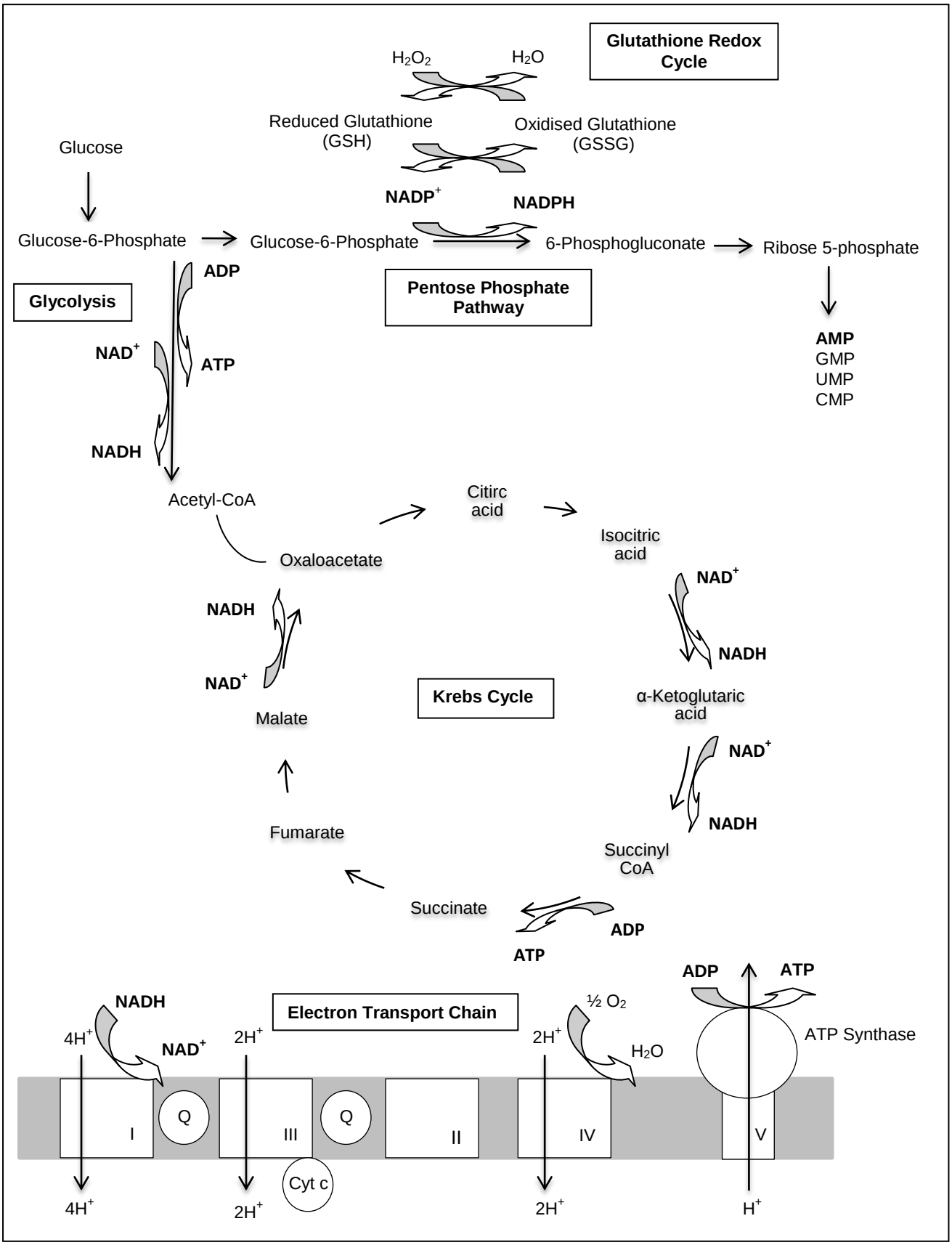


Figure 2.2: The role of pyridine and adenine nucleotides in energy metabolism. Pyridine nucleotides are coenzymes that play an important role in energy production and maintenance of cellular redox balance. NADH and NADPH act as electron donors while NAD^+ and $NADP^+$ act as electron acceptors in several biological electron-transfer reactions. Adenine nucleotides such as ATP, ADP and AMP are the primary intracellular energy carriers which supply energy for biological reactions. (Adapted from Salway, 2004)

2.2.3. Adenine nucleotide

Adenine nucleotides such as adenosine triphosphate (ATP), adenosine diphosphate (ADP) and adenosine monophosphate (AMP) are the primary intracellular energy carriers for energy requiring biological reactions within the cell (Markuszewski *et al*., 2003). The structure of these nucleotides consists of an adenine base, a 2-deoxyribose sugar and one or more phosphate group. The phosphoric anhydride bonds between the phosphate groups are a prime source of chemical energy (Garrett & Grisham, 2005). During energy requiring reactions, ATP is hydrolysed to ADP and inorganic phosphate (P). The energy that is released during this reaction is used for mechanical work such as cellular movements, active transport of molecules and ions and biosynthesis reactions (Markuszewski *et al*., 2003).

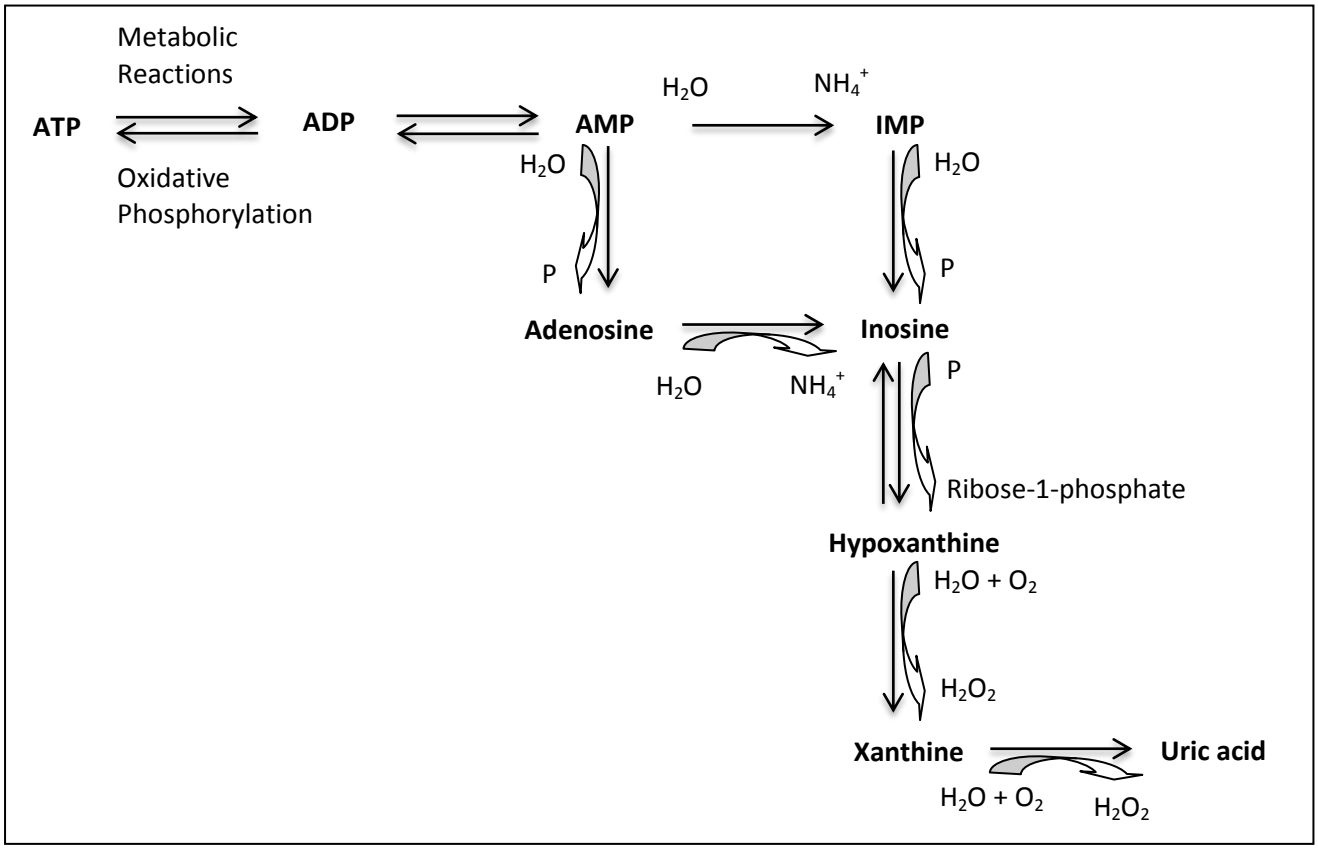


Figure 2.3: Recycling of adenine nucleotides. During biological reactions ATP is hydrolysed to ADP in order to release energy. ADP can either be recycled back to ATP or be broken down further to AMP. Recycling of AMP back to ADP is a slow process thus most AMP is finally broken down to uric acid which is excreted in the urine. (Adapted from Garrett & Grisham, 2005)

As illustrated in Figure 2.3, ADP can be recycled back to ATP through a phosphorylation reaction. If the energy demand is high and ATP levels are low, ADP can be further hydrolysed to AMP in order to release more energy. The recycling of AMP back to ADP is a slow process and therefore most AMP is converted to adenosine and finally to uric acid which is excreted in the urine (Garrett & Grisham, 2005; Markuszewski *et al*., 2003).

2.3. Extraction and stabilisation of nucleotides

The pre-analytical phase is a critical factor that can greatly affect analytical data. In order to accurately measure the *in vivo* concentration of nucleotides, correct sample preparation is required to achieve nucleotide stability before analytical analysis (Caruso *et al*., 2004). A suitable extraction method should be able to recover the nucleotides quantitatively through protein precipitation and/or nucleotidase inactivation (Klawitter *et al*., 2007). Adenine nucleotides have a very short half-life due to rapid degradation by the enzymes present in biological matrices. Thus slow sample processing cannot be used for the extraction of adenine nucleotides as this causes a decrease in their actual concentrations and an increase in the concentrations of their respective degradation products (Lazzarino *et al*., 2003). Pyridine nucleotides are present in both their oxidised and reduced forms in biological samples. Extraction methods involving fast deproteinisation with strong acids (perchloric acid, trichloro- or trifluoroacetic acids) will cause oxidation or degradation of the pyridine nucleotides, leading to changes in the ratios of the oxidised and reduced forms (Lazzarino *et al*., 2003; Wu *et al*., 1986).

In the literature many different extraction methods for intracellular metabolites have been studied including dilute solutions of acids, detergents and organic solvents. Many studies (Coolen *et* al., 2008; Klawitter *et al*., 2007; Lazzarino *et al*., 2003) have focused specifically on the extraction and measurement of nucleotides in different biological matrices. Different concentrations of organic solvents such as acetonitrile, methanol, ethanol and chloroform have been used to extract nucleotides from cell cultures and animal tissues (Au *et al.*, 1989; Grob *et al.*, 2003; Ritter *et al.*, 2008; Vogt *et al.*, 1998). Results obtained by Grob *et al.* (2003) showed that extraction with 20-80% acetonitrile or 40-60% ethanol in water led to acceptable nucleotide recovery and reproducibility for animal cells. Vogt *et al.* (1998) efficiently extracted organic acids, phosphorylated sugars and nucleotides from rat heart tissue with 60% acetonitrile. Klawitter *et al.* (2007) compared organic solvent extraction with acid extraction of nucleotides from animal tissue samples. Their results indicated that perchloric acid extraction led to the best absolute recovery and reproducibility of all adenine nucleotides as well as NAD^+, compared to acetonitrile, methanol or methanol/chloroform extractions. Throughout the literature perchloric acid is frequently used for the extraction of adenine nucleotides from various matrices including human blood samples (Caruso *et al*., 2004; Coolen *et al*., 2008) and cell cultures (Cai *et al*., 2002; Qian *et al*., 2004). The above mentioned studies all concluded that perchloric acid extraction produced high levels of nucleotide recovery from the various matrices. In contrast to these results, Au *et al.* (1989) and Ritter *et al.* (2008) reported that perchloric acid extraction yielded the lowest ATP concentration when compared to organic solvent extraction. Ushimura and Fukushima

(2003) also suggested the use of other extraction methods instead of perchloric acid extraction as the perchloric ions may form complexes with the nucleotides that interfere with chromatography. Other acid extraction methods described in the literature include formic acid extraction (Buckstein *et al.*, 2008; Ritter *et al.*, 2008) and acetic acid extraction (Nazar *et al.*, 1970) of nucleotides from cell cultures. Several studies have also aimed to develop extraction methods that are suitable for both redox and energy state metabolites. Caruso *et al.* (2004) applied different extraction conditions for oxidised and reduced pyridine nucleotides. Acid extraction (with perchloric acid) was used for the adenine and oxidised pyridine nucleotides while alkaline extraction (with potassium hydroxide) was used for the reduced pyridine nucleotides. Lazzarino *et al.* (2003) developed a method for simultaneous extraction of adenine and pyridine nucleotides from tissue samples. This method involves fast deproteinisation under non-oxidising conditions by homogenising tissue in ice-cold, nitrogen saturated acetonitrile and monopotassium phosphate.

2.4. Methods used for the quantification of nucleotides

Bioluminescence assays using the firefly luciferase enzyme are one of the most popular and commonly used methods for quantitative determination of ATP in biological matrices due to its high sensitivity, selectivity and relative easy application (Chida *et al*., 2012; Khlyntseva *et al*., 2009). The firefly luciferase enzyme catalyses the oxidation of luciferin in the presence of ATP. This reaction produces a bioluminescence signal which is directly proportional to the ATP concentration in the biological sample (Khan, 2003). The initial step of this assay requires the extraction of ATP from the biological matrix. Reagents that are commonly used for ATP extraction are chaotropic reagents such as trichloroacetic acid, perchloric acid (PCA) and ethylene glycol (Chida *et al*., 2012). The disadvantage of this assay is that most of the reagents that are used for ATP extraction interfere with the luciferase reaction and thus causes a decrease in the sensitivity of the assay (Khlyntseva *et al*., 2009).

Chromatographic methods such as anion-exchange HPLC, reversed-phase HPLC and ion-pair reversed-phase HPLC are frequently used for separation and simultaneous analysis of nucleosides, nucleotides and nitrogen bases. Ultraviolet (UV) detection is commonly used in combination with HPLC (Khlyntseva *et al*., 2009). UV detectors have relatively high sensitivity and can be used for both quantitative and qualitative analysis of trace compounds in a mixture. However, endogenous substances in complex matrices that absorb at the same wavelength as the analyte of interest can cause interference (Georgita *et al*., 2010). Various studies have applied HPLC-UV detection for nucleotide analysis in different biological matrices (Caruso *et al*., 2004; Coolen *et al*., 2008; Matyska *et al*., 2010). Liquid chromatography coupled with electrospray-ionisation mass spectrometry is another

technique that has become widely used for the qualitative and quantitative analysis of nucleotides (Cai *et al*., 2002; Claire, 2000; Klawitter *et al*., 2007; Qian *et al*., 2004).

2.4.1. LC-ESI-MS analysis of nucleotides

Liquid chromatography (LC) combined with tandem mass spectrometry (MS) is an analytical tool that is frequently used due to its high specificity and sensitivity (Nordström *et al*., 2004). The advantages of LC-MS include the simultaneous analysis of different metabolites, its compatibility with commonly used solvents such as water and methanol, and analysis of highly polar molecules, such as nucleotides, without derivatisation (Coulier at al., 2006). These advantages make LC-MS an attractive alternative technique for trace analysis of nucleotides.

2.4.1.1. Liquid chromatography

Nucleotides present a challenge to conventional reversed-phase HPLC. Due to their strong polarity, nucleotides cannot be analysed using standard reverse-phase conditions (Coulier *et al*., 2006; Fung *et al*., 2001; Matyska *et al*., 2010). Ion-exchange HPLC and ion-pair reversed-phase chromatography have been the methods of choice for nucleotide analysis. However, these chromatographic methods are often not compatible with mass spectrometric detection. Ion-exchange chromatography requires the use of mobile phases with high salt concentrations to elute the nucleotides from the column. This causes salt precipitation in the ion source that affects the detection sensitivity (Wang *et al*., 2009).

Ion-pairing chromatography has been successfully used in various studies for the separation of nucleotides (Cai *et al*., 2002; Klawitter *et al*., 2007; Qian *et al*., 2004). Ion-pairing reagents such as tetra-alkyl ammonium salts and alkylamines are added to the mobile phase. The positive charge of the ion-pairing reagent reacts with the negatively charged phosphate groups of the nucleotides, forming ion-pairs. The hydrophobic region of the ion-pairing reagent allows interaction with the stationary phase and thus increases the retention of the nucleotides on the reversed-phase column (Fung *et al*., 2001; Miller & Fischer, 2000). The use of ion-pairing reagents in combination with LC-MS has several possible disadvantages including the incompatibility of non-volatile reagents with electrospray ionisation, contamination of the MS source and the formation of adducts. The main disadvantage of using ion-pairing reagents is the possibility of ion-suppression. This combined with the ion-suppressive properties that nucleotides exhibit themselves, will decrease the MS detection sensitivity and significantly affect the results. It is thus critical to

optimise the type and concentration of the ion-pairing reagent prior to analysis (Coulier *et al*., 2006; Fung *et al*., 2001).

An alternative to using ion-pairing chromatography for reversed-phase separation of nucleotides is derivatisation of the nucleotides prior to LC-MS analysis. The derivatisation method used by Nordström *et al.* (2004) involves the esterification of the nucleotides' free hydroxyl groups with propionyl or benzoyl acid anhydride. The more hydrophobic derivatives that are formed during derivatisation, increases the retention of the nucleotides on the reversed-phase column without the use of ion-pairing reagents or acetate/ formate buffers. This will eliminate the ion-suppression caused by ion-pairing reagents and enhance ionisation and the MS response. Increasing the retention time of the nucleotides will also avoid suppression problems due to salts and other interfering compounds in the matrix, that elute early in the chromatographic analysis. Nordström *et al.* (2004) demonstrated that the retention, separation and sensitivity of the benzoylated ATP, ADP and AMP were much better than those of the underivatised nucleotides.

Callahan *et al.* (2009) and Matyska *et al.* (2010) demonstrated aqueous normal phase retention of a variety of polar compounds including nucleotides, on a diamond hydride column. The surface composition of hydride columns differ from pure HILIC (hydrophilic interaction chromatography) columns. The surface of the HILIC columns consist of highly polar silanol (-Si-OH) groups while the surface of the hydride columns consist of silica-hydride (-Si-H) groups which is weakly hydrophobic. This unique property of the hydride columns allows for retention of both polar and non-polar compounds (Pesek & Matyska, 2005).

2.4.1.2. Electrospray-ionisation mass spectrometry

Negative-ion mode electrospray-ionisation mass spectrometry (ESI-MS) is commonly used for the detection of nucleotides. Klawitter *et al.* (2007) found that detection of nucleotides in negative-ion mode was more effective compared to positive-ion mode due to the negatively charged phosphate groups present in the nucleotide's structure. Yamaoka *et al.* (2010) found that negative-ion mode ESI-MS was more effective for the detection of nucleotides when compared to the detection of nucleosides that does not have any phosphate groups in their structures. Wang *et al.* (2009) observed enhanced MS sensitivity when analysing adenine nucleotides in negative-ion mode under basic mobile phase conditions (ammonium acetate and acetonitrile) without using an ion-pairing reagent. Coulier *et al.* (2006) used negative-ion mode in combination with hexylamine as ion-pairing reagent. It was observed that the ion-pairing reagent caused no interference during analysis as hexylamine is only

visible in positive-ion mode. Furthermore, no significant adduct formation was observed when using negative-ion mode which aided the quantification and identification process.

Recent studies have also applied positive-ion mode ESI-MS coupled with ion-pairing chromatography for the quantitative analysis of nucleotides. Fung *et al.* (2001) compared positive-ion mode to negative-ion mode for the simultaneous analysis of nucleoside drug analogs and their corresponding nucleotides. It was observed that positive-ion mode was more sensitive for nucleotide analysis when using dimethylhexylamine (DMHA) as ion-pairing reagent. DMHA enhanced the protonation of the nucleotides which made detection in positive-ion mode possible. Cai *et al.* (2002) and Dodbida *et al.* (2010) also used DMHA as ion-pairing reagent for the analysis of nucleotides. The negatively charged phosphate groups of the nucleotides associate with the cationic ion-pairing reagent, creating an overall positively charged complex which can be detected in positive-ion mode ESI-MS. Claire (2000) used positive-ion mode ESI-MS for the detection of a nucleotide analog as a unique and abundant product ion could not be found when using negative-ion mode ESI-MS.

2.5. Method development and validation

New analytical methods require careful and thorough method development in order to obtain reliable analytical data. The process of LC-MS method development, as illustrated in Figure 2.4, involves the evaluation and optimisation of the various stages of sample preparation, chromatographic separation, detection and quantitation (Causon, 1997).

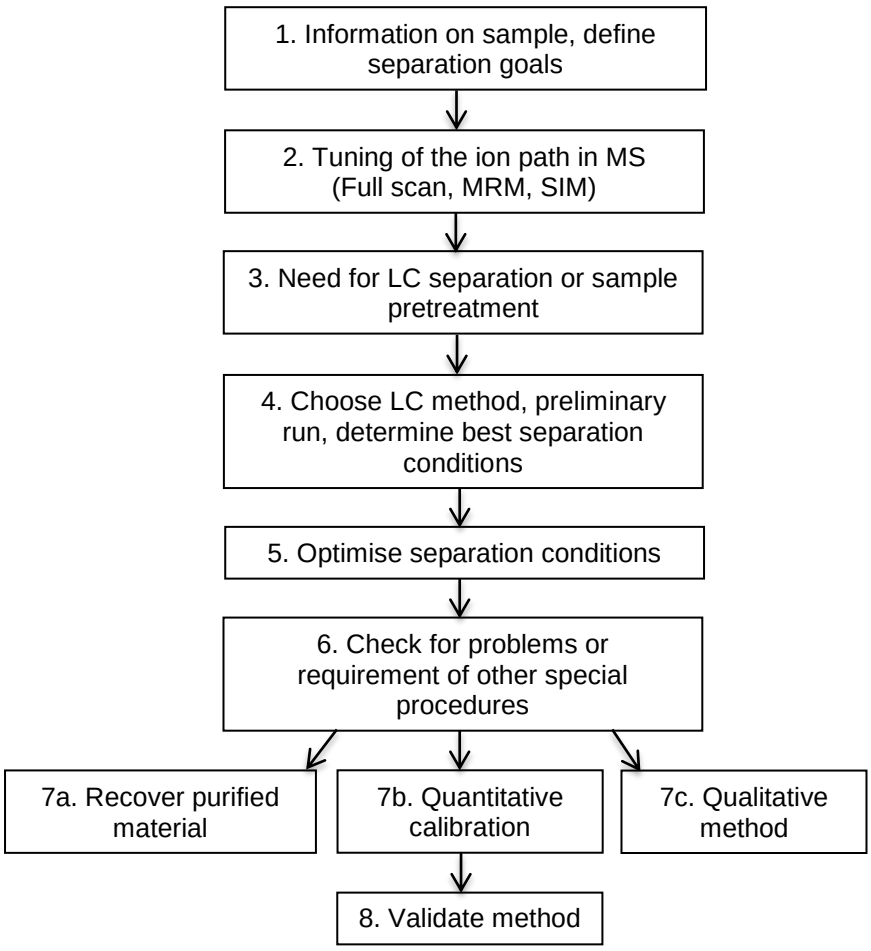


Figure 2.4: Steps involved in LC-MS method development. The process of method development includes research on the analyte/s of interest, optimisation of mass spectrometry conditions, and optimisation of chromatographic separation. MRM = Multiple reaction monitoring mode; SIM = Selected-ion monitoring mode. (Adapted from Wal *et al*., 2010)

Method development requires extensive knowledge about the analyte/s of interest namely, type of biological matrix in which the analyte/s are found, molecular weight, polarity, solubility etc., in order to determine preliminary separation and detection conditions. The next step of method development is to optimise the mass spectrometry conditions for analyte detection. This includes determining precursor and product ions for each analyte, selecting the best ionisation mode and optimising source parameters (voltages, gas flow, temperatures etc.). Some methods may require additional separation of the analytes by using a HPLC procedure and/ or sample pretreatment such as solid phase or liquid-liquid extraction. Chromatographic conditions such as type of column, flow rate and mobile phases need to be optimized.

Method development is followed by method validation in order to assess the suitability of the new analytical method for its intended use. In general agreement, the following validation

parameters should be determined for quantitative bioanalytical methods: specificity, linearity, accuracy, precision, stability and lower limit of quantification. Other validation parameters which may also be relevant include lower limit of detection, recovery, reproducibility and robustness of the method (EURACHEM, 1998; Shah *et al*., 2000; Thompson *et al*., 2002). The definitions, according to the International Conference on Harmonization reports (1995, 1997), of each of these validation parameters are given in Table 2.1.

Table 2.1: Validation parameters as defined by the International Conference on Harmonization (ICH) reports (1995, 1997).

Validation Parameter	Definition
Specificity	The ability to differentiate and quantify the analyte of interest in the presence of other components in the sample such as impurities, degradants, matrix etc.
Accuracy	The closeness of agreement between the test result value and the accepted reference value.
Precision	The closeness of agreement (degree of scatter) between a series of measurements from multiple sampling of the same homogenous sample under the prescribed conditions. Precision can be considered at three levels: repeatability, intermediate precision and reproducibility.
Repeatability (Intra-day precision)	The precision under the same operating conditions over a short interval of time
Intermediate precision	Expresses within-laboratory variations such as analysis on different days, different analysts, different equipment etc.
Reproducibility	Expresses the precision between different laboratories
Detection limit (LOD)	The lowest concentration of analyte in a sample which can be detected and reliably differentiated from background noise, but not necessarily quantified as an exact value.
Quantitation limit (LOQ)	The lowest concentration of analyte in a sample which can be quantitatively determined with acceptable accuracy and precision.
Linearity	The ability of an analytical method (within a given range) to obtain results that are directly proportional to the concentration of analyte in the sample.
Robustness	The capacity of an analytical procedure to remain unaffected by small, but deliberate variations in method parameters. This provides an indication of the method's reliability during normal usage.
Stability	The chemical stability of an analyte in a given matrix under specific conditions for given time intervals.

Chapter 3: Materials and Methods

3.1. Introduction

Adenine and pyridine nucleotides play important roles in energy production, energy transport and maintenance of cellular redox status. (Kang & Pervaiz, 2012; Nakamura, 2012) Thus these nucleotides can possibly be used as diagnostic biomarkers for energy related disorders such as chronic fatigue syndrome (CFS).

Due to their instability in biological matrices these nucleotides are yet to be included in a routine laboratory test for diagnosing energy related disorders. It is thus necessary to find a suitable extraction method to stabilise the nucleotides and to develop a sensitive and accurate method to quantify them. Some commonly used extraction methods to stabilise nucleotides include dilute solutions of acids, detergents and organic solvents. This study mainly focused on the use of perchloric acid for nucleotide extraction.

It was decided to develop an LC-ESI-MS/MS method for nucleotide quantification as this technology was available in our laboratory and it is described in the literature as one of the more sensitive and accurate methods for the detection of nucleotides (Coulier et al., 2006; Nordström *et al*., 2004). Method development and optimisation consisted of determining the optimal chromatographic conditions for nucleotide separation and the optimal mass spectrometric conditions for nucleotide detection. Various parameters such as linearity, precision, limits of detection and quantification, and stability under different conditions were studied during method validation. The newly developed and validated method was then applied to investigate the possible differences in adenine and pyridine levels in blood samples of relatively healthy individuals and possible chronic fatigue syndrome patients.

3.2. Reagents, standards and solutions

All nucleotide standards were purchased from Sigma-Aldrich (St. Louis, MO, USA): Adenosine 5'-triphosphate disodium salt (ATP) (Cat. No. A2383), adenosine 5'-diphosphate sodium salt (ADP) (Cat. No. A2754), adenosine 5'-monophosphate disodium salt (AMP) (Cat. No. 01930), adenosine (Cat. No. A9251), adenine (Cat. No. A8626), inosine (Cat. No. I4125), uric acid (Cat. No. U2625), hypoxanthine (Cat No. H9377), β-nicotinamide adenine dinucleotide 2'-phosphate reduced tetrasodium salt hydrate (NADPH) (Cat. No. N1630), β-

nicotinamide adenine dinucleotide phosphate hydrate (NADP) (Cat. No. N5755), β-nicotinamide adenine dinucleotide reduced disodium salt hydrate (NADH) (Cat. No. N8129) and β-nicotinamide adenine dinucleotide hydrate (NAD) (Cat. No. N7004).

Honeywell Burdick & Jackson, spectrometry -grade acetonitrile (Cat. No. 015-4), methanol (Cat. No. 230-4) and water (Cat. No. 365-4) were purchased from Anatech Instruments Ltd (Gauteng, SA). Ammonia was purchased from BDH Chemicals (Radnor, PA, USA) (Cat. No. 100115Q). Acetic acid (Cat. No. 27225), potassium hydroxide (KOH) (Cat. No. 06005), potassium carbonate (K_2CO_3) (Cat. No. 209619), 2-Acetamidophenol (Cat. No. A7000), formic acid (Cat. No. 94318), 70% perchloric acid (Cat. No. 77227), dibutylamine (Cat. No. 34490) and ammonium acetate (Cat. No. 32301) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium hydroxide solution (NaOH) (Cat. No. 5062-8575) was purchased from Agilent technologies (Santa Clara, CA, USA).

3.2.1. Preparation of standard stock solutions

Stock solutions of all nucleotide standards were prepared separately in water with a final concentration of 10 mmol/L. The uric acid stock solution was prepared in 0.1 M of NaOH instead of water. The standard stock solutions were then divided into 500 µL aliquots and stored at - 80°C.

3.2.2. Preparation of KOH, K_2CO_3 and perchloric acid stock solutions

Klawitter *et al.* (2007) used 12% perchloric acid for extraction of nucleotides. The perchloric acid was then neutralised to pH 7 with 6 M potassium hydroxide (KOH) and 2 M potassium carbonate (K_2CO_3).

The 6 M KOH stock solution was prepared in a 100 mL volumetric flask by dissolving KOH in water. The 2 M K_2CO_3 stock solution was prepared in a 100 mL volumetric flask by dissolving K_2CO_3 in water. All perchloric acid solutions (12%, 18%, 30%, and 52%) were prepared by diluting the 70% stock solution with water.

3.2.3. Preparation of the internal standard

2-Acetamidophenol was used as internal standard during sample analysis to compensate for volume differences between samples and for losses due to pipetting errors. An internal standard stock solution with a concentration of 14.99 mol/L was prepared by dissolving

226.6 mg of 2-acetamidophenol in 100 mL of methanol: water (60:40). 200 µL of the internal standard stock solution was then added to 5 mL of perchloric acid and 5 mL of water. The perchloric acid/ internal standard mix (PCA/IS) and the water/internal standard mix (H_2O/IS) were stored at 4°C.

3.2.4. Preparation of mobile phases

3.2.4.1. Ammonium acetate mobile phases (pH 7.2 and pH 6.5)

Matyska *et al.* (2010) used 5 mM ammonium acetate (mobile phase A) and 90% acetonitrile/ 10% water containing 10 mM ammonium acetate (mobile phase B) for LC-ESI-MS/MS analysis of nucleotides. The mobile phases were prepared as follows:

A 100 mM ammonium acetate stock solution was prepared by dissolving ammonium acetate in water. Mobile phase A was prepared by dilution of the 100 mM ammonium acetate stock solution and adjusting the pH to 7.2 with ammonia. Mobile phase B was prepared by adjusting the pH of the 100 mM ammonium acetate stock solution to 6.5 with acetic acid and then diluting the stock solution with 90% acetonitrile.

3.2.4.2. Dibutylammonium formate buffer (pH 6.0)

Klawitter *et al.* (2007) used dibutylamine as ion-pairing reagent during LC-ESI-MS/MS analysis of nucleotides. The dibutylammonium formate (DBAF) buffer (mobile phase A) was prepared as follows: A 20 mM dibutylamine stock solution was prepared in a 100 mL volumetric flask. The pH of the stock solution was then adjusted to 6.0 with formic acid. Mobile phase B consisted of 100% methanol.

3.3. Method development and optimisation

3.3.1. Nucleotides analysed

The pyridine nucleotides, adenine nucleotides and their degradation products that were analysed during this study are given in Table 3.1.

Table 3.1: Chemical properties of the adenine nucleotides, ATP degradation products and pyridine nucleotides analysed.

Name	Formula	Molecular weight (g/mol)
Adenosine 5'-triphosphate (ATP)	$C_{10}H_{16}N_5O_{13}P_3$	507.18
Adenosine 5'-diphosphate (ADP)	$C_{10}H_{15}N_5O_{10}P_2$	427.20
Adenosine 5'-monophosphate (AMP)	$C_{10}H_{14}N_5O_7P$	347.22
Adenosine	$C_{10}H_{13}N_5O_4$	267.24
Adenine	$C_5H_5N_5$	135.13
Inosine	$C_{10}H_{12}N_4O_5$	268.23
Hypoxanthine	$C_5H_4N_4O$	136.11
Uric acid	$C_5H_4N_4O_3$	168.11
Reduced β-nicotinamide adenine dinucleotide (NADH)	$C_{21}H_{29}N_7O_{14}P_2$	665.44
β-nicotinamide adenine dinucleotide (NAD)	$C_{21}H_{27}N_7O_{14}P_2$	663.43
Reduced β-nicotinamide adenine dinucleotide phosphate (NADPH)	$C_{21}H_{30}N_7O_{17}P_3$	745.43
β-nicotinamide adenine dinucleotide phosphate (NADP)	$C_{21}H_{28}N_7O_{17}P_3$	743.41
2-Acetamidophenol (Internal Standard)	$CH_3CONHC_6H_4OH$	151.16

3.3.2. LC-MS/MS Specifications

An Agilent 1200 series LC system (Santa Clara, CA, USA) coupled to an Agilent 6410 Triple quadrupole mass spectrometer (Santa Clara, CA, USA) was used during this study. The source parameters for the MS were as follows: a gas temperature of 300°C, a gas flow of 10 L/min, the nebulizer was set at 45 psi and the capillary voltage was 4000 V.

3.3.3. Mass spectrometry optimisation (Direct infusions)

The first step of method development was to optimise the MS conditions necessary for the detection of adenine and pyridine nucleotides. Masshunter optimiser software from Agilent (Santa Clara, CA, USA) was used to optimise the data acquisition parameters for multiple reaction monitoring (MRM) mode for each nucleotide. These parameters include selection of precursor and product ions, fragmentor voltages for each precursor ion, and collision energy needed to form characteristic product ions.

One 500 µL stock solution for each standard was defrosted at room temperature. The ATP, ADP, AMP, adenosine, adenine, inosine, hypoxanthine, uric acid, NAD and NADP stock solutions were diluted to a concentration of 500 µM and the NADH and NADPH stock solutions were diluted to a concentration of 250 µM. 100 µL of each standard was pipetted into separate auto-sampler vials. A 1 mL standard mix was also prepared using the defrosted stock solutions. The concentration for each standard in the mix was the same as mentioned above.

For optimisation of the MS conditions 10 µL of each standard was separately infused into the MS. Ammonium acetate (pH 7.2) and Ammonium acetate/ Acetonitrile (pH 6.5) were used as mobile phases during optimisation. Method optimisation was carried out in both positive and negative ionisation modes. It was determined that negative ionisation mode was more favourable for nucleotide analysis than positive ionisation. The results obtained from optimisation in negative ionisation mode are given in Table 3.2.

Table 3.2: Optimal MRM conditions for the quantification of adenine and pyridine nucleotides in negative ionisation mode.

Compound	Precursor Ion (m/z)	Fragmentor voltage	Most abundant product ion (m/z)	Collision Energy (V)
ATP	506	149	159	36
ADP	426	143	159	24
AMP	346	148	79	52
NADH	664	205	79	60
NAD	662	117	79	60
NADPH	744	210	159	60
NADP	742	143	620	12
Adenosine	266	96	134	12
Adenine	134	117	107	16
Inosine	267	148	135	16
Hypoxanthine	135	120	92	12
Uric acid	167	105	124	8
2-Acetamidophenol	150	65	108	8

3.3.4. Optimisation of chromatographic separation

After optimisation of the MS conditions the nucleotides could be chromatographically separated. Chromatographic separation of nucleotides was necessary in order to prevent ion-suppression that is caused by the nucleotides themselves. Due to their strong polarity, the nucleotides could not successfully be retained on a C18 column during standard reversed-phase chromatography. It was thus necessary to consider other alternatives for chromatographic separation such as columns that are more suitable to retain polar compounds or the use of ion-pairing reagents.

3.3.4.1. Nucleotide separation using a diamond hydride column

The method proposed by Matyska *et al.* (2010), was used for nucleotide separation. A 1 mL standard mix was prepared from the frozen standard stock solutions. The standard mix had a final concentration of 500 µM for ATP, ADP, AMP, adenosine, adenine, inosine, hypoxanthine, uric acid, NAD and NADP; and a final concentration of 250 µM for NADH and NADPH. 5 µL of the standard mix was injected onto a diamond hydride column (Cogent Diamond Hydride, 4 µm, 2.1 x 150 mm, MicroSolv Technology Corporation, USA). 5 mM Ammonium acetate pH 7.2 (mobile phase A) and 10 mM ammonium acetate/ 90% acetonitrile pH 6.5 (mobile phase B) were used as mobile phases with a flow rate of 0.3 mL/min. The column temperature was 25°C. The total run time was 20 minutes and the column was re-equilibrated for 5 minutes between runs. The mobile phase gradient that was used is given in Table 3.3 and the retention time for each nucleotide is given in Table 3.4.

Table 3.3: The mobile phase gradient used for chromatographic separation of nucleotides on a diamond hydride column.

Time (min)	% 10 mM Ammonium acetate/ 90% Acetonitrile (Mobile phase B)
0.0	95
1.0	95
10.0	70
15.0	30
20.0	95

Figure 3.1 illustrates the separated nucleotides on the diamond hydride column. The nucleotides were not retained very well by the diamond hydride column and most of the

nucleotides did not exhibit good peak shapes (peak tailing). Complete separation of all twelve nucleotides was not possible.

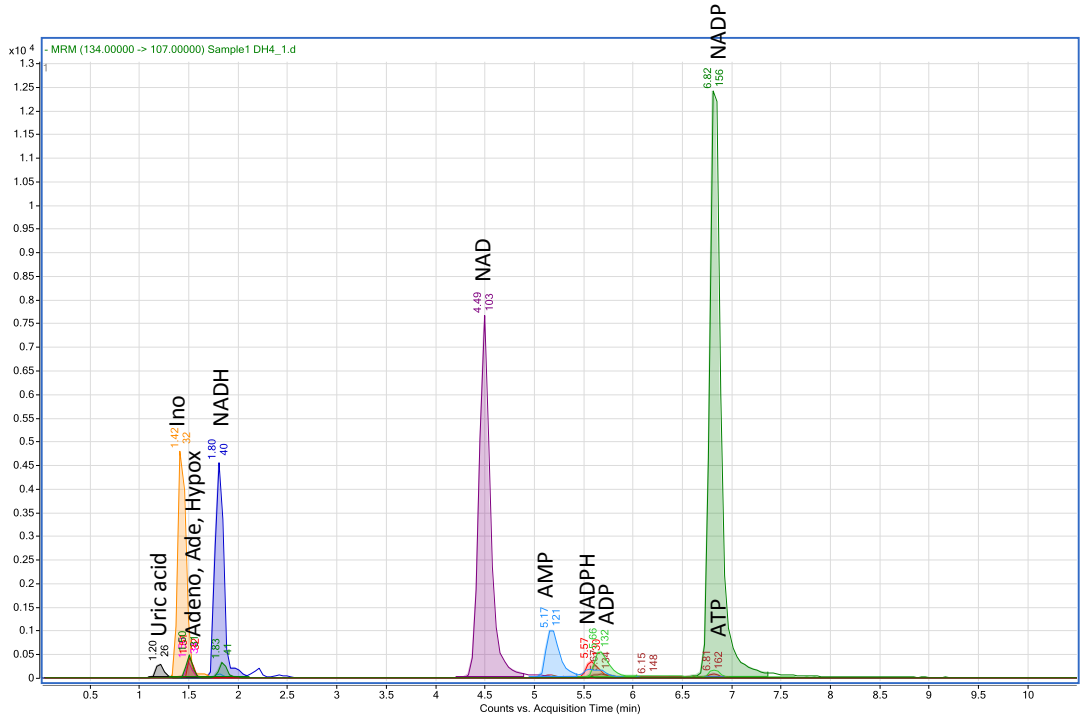


Figure 3.1: Chromatogram illustrating nucleotide separation on a diamond hydride column.
Ino = Inosine; Adeno = Adenosine; Ade = Adenine; Hypox = Hypoxanthine.

Table 3.4: Nucleotide retention times on a Diamond hydride column.

Compound	Retention time (min)
ATP	6.81
ADP	5.67
AMP	5.19
NADH	1.80
NAD	4.49
NADPH	5.57
NADP	6.80
Adenosine	1.49
Adenine	1.50
Inosine	1.41
Hypoxanthine	1.50
Uric acid	1.21

3.3.4.2. Nucleotide separation using an Aqua-C18 column

A 1 mL standard mix was prepared from the frozen standard stock solutions. The standard mix had a final concentration of 500 µM for ATP, ADP, AMP, adenosine, adenine, inosine, hypoxanthine, uric acid, NAD and NADP and a final concentration of 250 µM for NADH and NADPH. 5 µL of the standard mix was injected onto an Aqua-C18 column (Zorbax SB-Aqua, 3.5 µm, 2.1 x 150 mm, Agilent Technologies, Santa Clara, CA, USA). 5 mM Ammonium acetate pH 7.2 (mobile phase A) and 10 mM ammonium acetate/ 90% acetonitrile pH 6.5 (mobile phase B) were used as mobile phases with a flow rate of 0.2 mL/min. The column temperature was 35°C. The total run time was 23 minutes and the column was re-equilibrated for 6 minutes between runs. The mobile phase gradient that was used is given in Table 3.5 and the retention time for each nucleotide is given in Table 3.6.

Table 3.5: The mobile phase gradient used for chromatographic separation of nucleotides on an Aqua-C18 column.

Time (min)	% 10 mM Ammonium acetate/ 90% Acetonitrile (Mobile phase B)
0.0	3.0
2.0	3.0
5.4	12.0
6.4	12.0
10.0	17.5
15.0	17.5
17.0	25.0
19.0	80.0
21.0	80.0
23.0	3.0

Figure 3.2 illustrates the separated nucleotides on the Aqua-C18 column. When compared to the diamond hydride column separation (Figure 3.1) most of the nucleotides showed improved peak shapes (less peak tailing). Although the Aqua-C18 column provided better retention than the diamond hydride column, complete separation of all twelve nucleotides was still not possible.

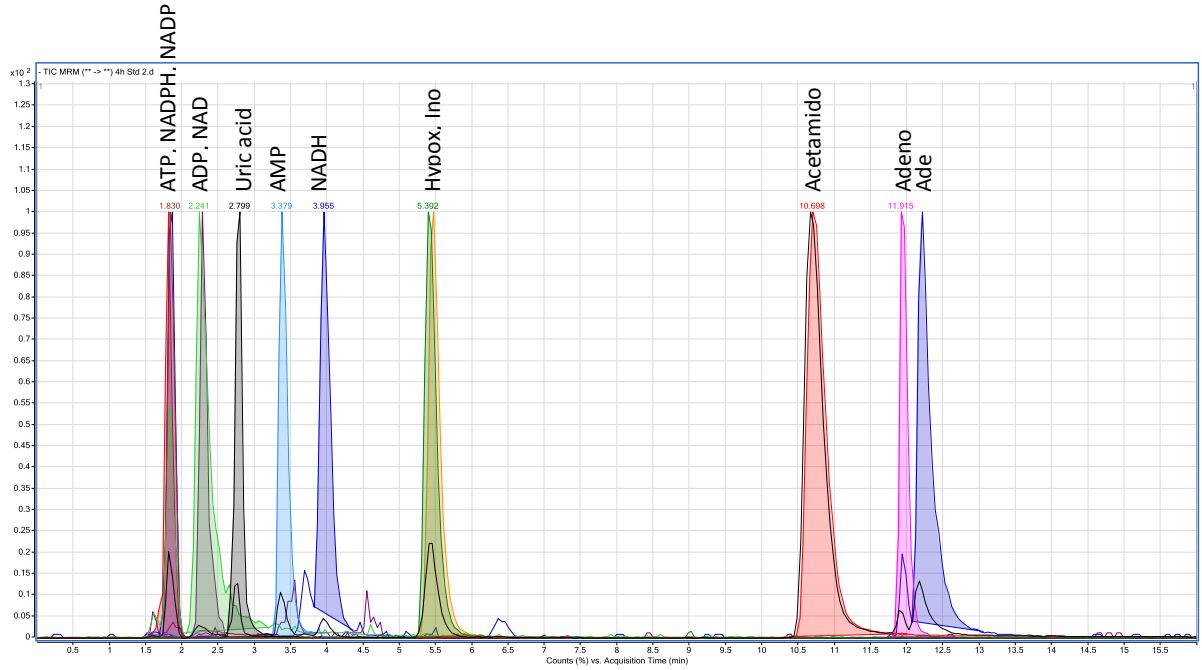


Figure 3.2: Chromatogram illustrating nucleotide separation on an Aqua-C18 column.

Hypox = Hypoxanthine; Ino = Inosine; Acetamido = 2-Acetamidophenol; Adeno = Adenosine; Ade = Adenine.

Table 3.6: Nucleotide retention times on an Aqua-C18 column.

Compound	Retention time (min)
ATP	1.8
ADP	2.2
AMP	3.3
NADH	3.9
NAD	2.3
NADPH	1.8
NADP	1.8
Adenosine	11.9
Adenine	12.2
Inosine	5.4
Hypoxanthine	5.3
Uric acid	2.7
2-Acetamidophenol	10.6

3.3.4.3. Nucleotide separation using ion-pairing reversed-phase chromatography

Ion-pairing reversed-phase chromatography is frequently used for the separation of nucleotides (Cai *et al*., 2002; Klawitter *et al*., 2007; Qian *et al*., 2003). For this study,

dibutylammonium formate buffer (DBAF) was used as ion-pairing reagent. A C18 column (Zorbax Bonus-RP, 3.5 µm, 2.1 x 150 mm, Agilent Technologies, Santa Clara, CA, USA) as well as an Aqua-C18 column (Zorbax SB-Aqua, 3.5 µm, 2.1 x 150 mm, Agilent Technologies, Santa Clara, CA, USA) was used to determine which column would provide better retention and separation during ion-pairing chromatography.

A 1 mL standard mix was prepared from the frozen standard stock solutions. The standard mix had a final concentration of 500 µM for ATP, ADP, AMP, adenosine, adenine, inosine, hypoxanthine, uric acid, NAD and NADP; and a final concentration of 250 µM for NADH and NADPH. 5 µL of the standard mix was injected onto the column. Different concentrations (0.5 mM, 1 mM and 2 mM) of DBAF were used in order to determine the optimal concentration for the ion-pairing reagent. Mobile phase B consisted of 100% methanol. The column temperature was 35°C. The total run time was 23 minutes and the column was re-equilibrated for 6 minutes between runs. The mobile phase gradient and the flow rate that was used are given in Table 3.7 and the retention time for each nucleotide is given in Table 3.8.

Table 3.7: The mobile phase gradient used for ion-pairing reversed-phase chromatographic separation of nucleotides.

Time (min)	% Methanol (Mobile phase B)	Flow (mL/min)
0.0	8.0	0.20
2.0	8.0	0.20
5.4	12.0	0.20
6.4	12.0	0.20
10.0	17.5	0.25
15.0	17.5	0.25
17.0	25.0	0.25
19.0	80.0	0.25
21.0	80.0	0.25
23.0	8.0	0.20

The Aqua-C18 column provided significantly better retention than the C18 column when used in combination with the ion-pairing reagent. Complete nucleotide separation was not possible on either column. Nucleotide separation on an Aqua-C18 column using 1 mM DBAF (optimal concentration) is illustrated in Figure 3.3. The use of the ion-pairing reagent resulted in more efficient nucleotide retention and separation than with the other two chromatographic methods. DBAF also significantly increased the abundances of ATP, ADP, AMP and NADH when compared to the ammonium acetate mobile phases. The

abundances of the other nucleotides decreased or remained the same when DBAF was used. The main disadvantage of using DBAF was that it remained in the source of the MS long after analysis and caused interference with the routine laboratory work done in positive-ion mode.

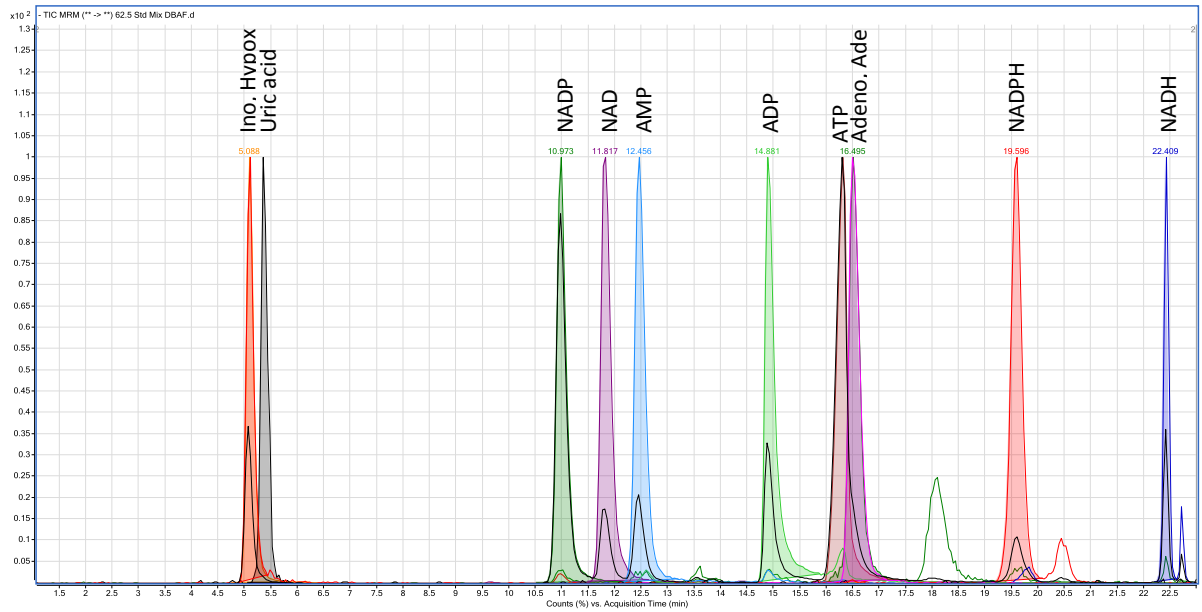


Figure 3.3: Chromatogram illustrating nucleotide separation with ion-pairing reversed-phase chromatography. Ino = Inosine; Hypox = Hypoxanthine; Adeno = Adenosine; Ade = Adenine.

Table 3.8: Nucleotide retention times during ion-pairing reversed-phase chromatography.

Compound	Retention time (min)
ATP	16.30
ADP	14.88
AMP	12.45
NADH	22.40
NAD	11.81
NADPH	19.59
NADP	10.97
Adenosine	16.48
Adenine	16.49
Inosine	5.08
Hypoxanthine	5.09
Uric acid	5.34

3.3.5. Optimal chromatographic conditions for nucleotide separation

Comparing the chromatograms and the nucleotide abundances of the different chromatographic separations, it was decided to use the Aqua-C18 column in combination with the ammonium acetate mobile phases for further nucleotide separation. Complete nucleotide separation was not necessary because MRM mode was used for nucleotide detection. The main goal of the chromatographic separation was to prevent the nucleotides from going into the source all at once and causing ion-suppression. It was also done to separate the nucleotides from the salts present in the blood samples.

The chromatographic conditions described in 3.3.4.b. were used for further method validation and method application. The only adjustment made to these conditions was the pH of the 90% acetonitrile/ 10% water containing 10 mM ammonium acetate (mobile phase B). The pH was adjusted from 6.5 to 7.9 with ammonia as the higher pH resulted in higher nucleotide abundances.

3.4. Method validation

3.4.1. Linearity

The linearity was investigated using a concentration range of 0.12 – 62.5 µM for ATP, ADP, AMP, adenosine, adenine, inosine, hypoxanthine, NAD and NADP; a concentration range of 0.06 – 31.25 µM for NADH and NADPH; and a concentration range of 31.25 µM – 1 mM for uric acid. A 5 mL standard mix was prepared by weighing the standards, except uric acid, and dissolving it in water. All nucleotide standards in the mix had a concentration of 250 µM except for NADH and NADPH which had concentrations of 125 µM. A 2 mM Uric acid solution was prepared separately from the standard mix by dissolving it in 0.1 M of NaOH. The 250 µM standard mix was first diluted with water to a concentration of 125 µM and then further diluted with the 2 mM uric acid solution to a concentration of 62.5 µM (uric acid had a final concentration of 1 mM in the mix). The concentration range was then prepared by serial dilution of the 62.5 µM standard mix with water. Each concentration level was then analysed in triplicate.

3.4.2. Limits of detection and quantification

The limit of detection (LOD) and the limit of quantification (LOQ) were determined with the linear regression method (Sanagi *et al.*, 2009). According to this method the LOD and LOQ

can be calculated based on the standard deviation of the response and the slope of the calibration curve as shown in Equation 3.1 and 3.2.

Equation 3.1: Calculating Limit of Detection (LOD).

$$LOD = \frac{3SD}{b}$$

Equation 3.2: Calculating Limit of Quantification (LOQ).

$$LOQ = \frac{10SD}{b}$$

Were SD is the standard deviation of the response and b is the slope of the calibration curve. The values of SD and b were calculated as described in section 3.6.1.

3.4.3. Intraday and interday variation

Three concentration levels for each nucleotide standard, given in Table 3.9, were chosen for analysis to determine intra- and interday variation.

Table 3.9: The three concentration levels used to determine intra- and interday variation.

Nucleotide Standard	Concentration level 1	Concentration level 2	Concentration level 3
ATP, ADP, AMP, Adeno, Ade, Ino, Hypox NAD, NADP	62.50 µM	31.25 µM	15.60 µM
NADH, NADPH	31.25 µM	15.60 µM	7.80 µM
Uric acid	250.00 µM	125.00 µM	62.50 µM

A 5 mL standard mix was prepared by weighing the standards and dissolving it in water. Uric acid was prepared separately from the standard mix by dissolving it in 0.1 M of NaOH. The three concentration levels were then prepared by serial dilution of the standard mix and uric acid solution with water.

For the intraday variation, each concentration level was analysed in triplicate immediately after preparation. For the interday variation, each concentration level was analysed in triplicate over a period of five consecutive days.

3.4.4. Nucleotide standard stability in water

The stability of all nucleotide standards in water was evaluated over a 24 hour period. A 5 mL standard mix was prepared by weighing all the nucleotide standards, except for uric acid, and dissolving it in water. All nucleotide standards in the mix had a concentration of 250 µM except for NADH and NADPH which had concentrations of 125 µM. A 3 mL of 2 mM uric acid solution was prepared separately from the standard mix by dissolving it in 0.1 M NaOH. 2.5 mL of the standard mix was then diluted with 1.25 mL of uric acid solution and 1.25 mL of water (Dilution 1 in Figure 3.4). 500 µL of water/IS was then added to 500 µL of the diluted standard mix (Dilution 2 in Figure 3.4). The standard mix was divided into two aliquots. One aliquot was analysed in triplicate at the following time intervals: immediately after preparation (T0 samples), two hours, four hours and 24 hours. The samples were kept at room temperature (25°C) in the auto-sampler for the entire 24 hour period. The second aliquot was frozen at - 80°C for 24 hours, before being defrosted at room temperature and analysed in triplicate.

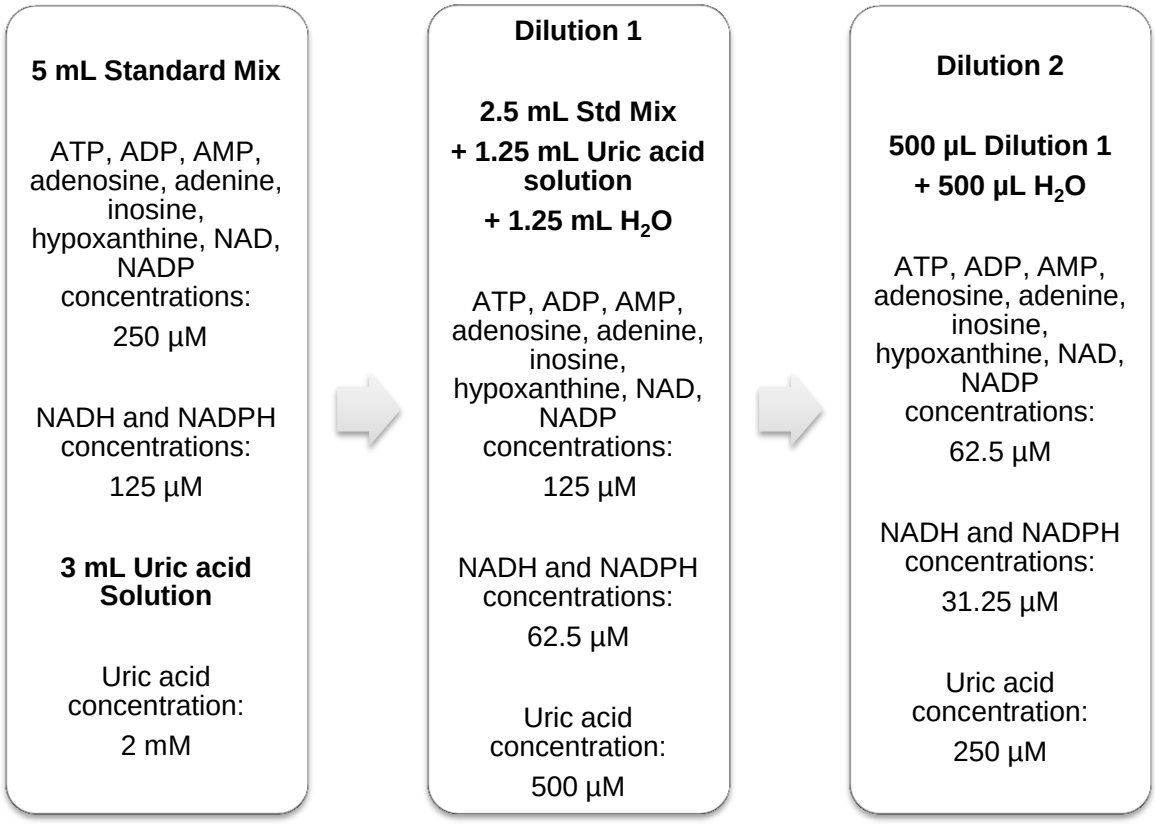


Figure 3.4: Serial dilution of the nucleotide standard mix with water.

3.4.5. Nucleotide standard stability in perchloric acid

The stability of all nucleotide standards in 12% perchloric acid was evaluated over a 24 hour period. A 5 mL standard mix was prepared by weighing all the nucleotide standards, except for uric acid, and dissolving it in water. All nucleotide standards in the mix had a concentration of 250 µM except for NADH and NADPH which had concentrations of 125 µM. A 3 mL of 2 mM uric acid solution was prepared separately from the standard mix by dissolving it in 0.1 M NaOH. 2.5 mL of the standard mix was then diluted with 1.25 mL of uric acid solution and 1.25 mL of water (Dilution 1 in Figure 3.5). 500 µL of ice-cold 12% PCA/IS was then added to 500 µL of the diluted standard mix (Dilution 2 in Figure 3.5). The perchloric acid was then neutralised with 6 M KOH and 2 M K_2CO_3 to pH 6. The neutralised supernatant was divided into two aliquots. One aliquot was analysed in triplicate at the following time intervals: immediately after preparation (T0 samples), two hours, four hours and 24 hours. The samples were kept at room temperature (25°C) in the auto-sampler for the entire 24 hour period. The second aliquot was frozen at - 80°C for 24 hours, before being defrosted at room temperature and analysed in triplicate.

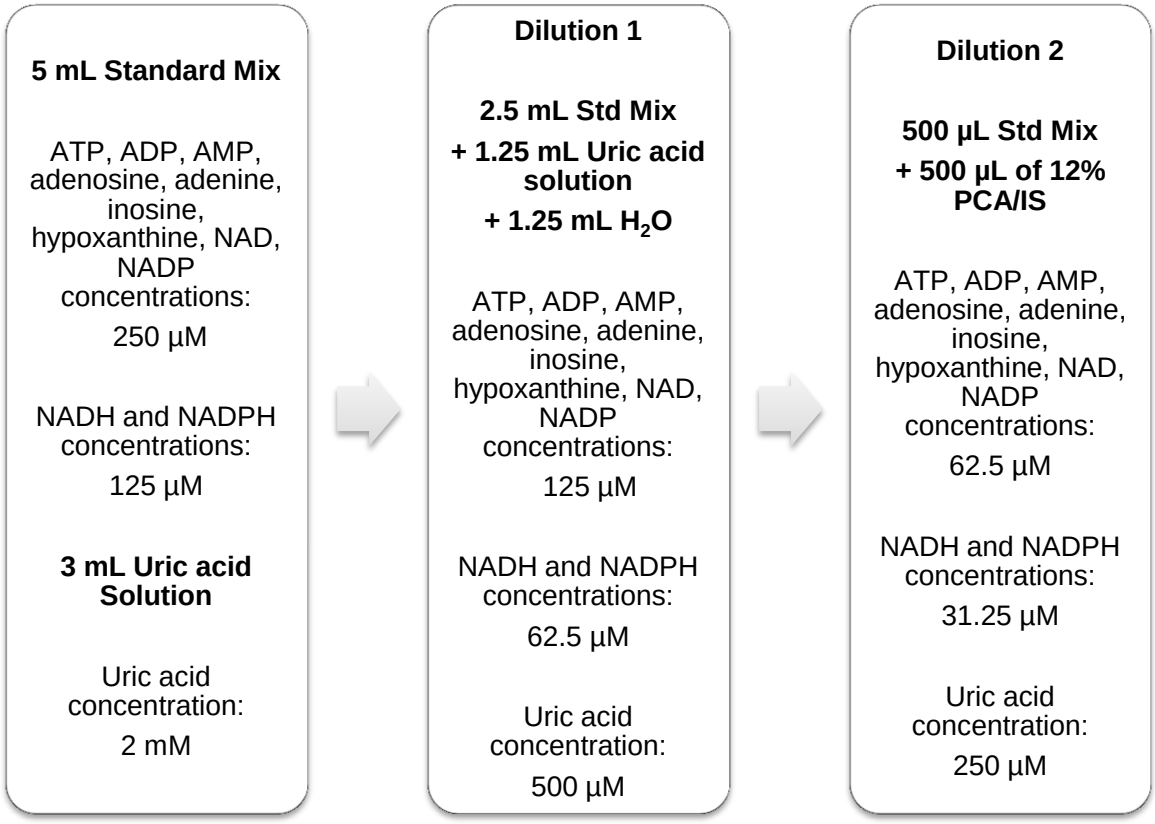


Figure 3.5: Serial dilution of the nucleotide standard mix with 12% perchloric acid.

3.4.6. Different deproteinisation methods for whole blood samples

3.4.6.1. Blood sample collection

Blood was collected from a healthy volunteer by venepuncture in EDTA-containing Vacutainer® tubes (Becton Dickinson Ltd., Johannesburg, SA). Fresh blood was collected for each experiment. The blood was used immediately after collection.

3.4.6.2. Deproteinisation with perchloric acid

The method described by Coolen *et al.* (2008) was used as a starting point for blood sample preparation. Blood was gravimetrically divided into three 500 µL aliquots in 1.5 mL Eppendorf tubes. To each of the three samples 500 µL of ice-cold 12% PCA/IS was added. The samples were vortex-mixed and centrifuged (14 000 rpm, 10 min, 4°C) to precipitate the proteins present in the blood samples. After centrifugation, 650 µL of the supernatant of each sample was transferred to separate Eppendorf tubes. The perchloric acid was then neutralised to pH 6 by adding 65 µL of 6 M KOH and 60 µL of 2 M K_2CO_3 to the supernatant of each sample. The insoluble perchlorate was removed by centrifugation (14 000 rpm, 10 min, 4°C) before transferring 200 µL of each sample to separate auto-sampler vials. Each sample was then analysed in triplicate.

3.4.6.2.a. Neutralisation of perchloric acid

In the literature different solutions have been applied for the neutralisation of perchloric acid in the supernatant. Coolen *et al.* (2008) used both KOH and K_2CO_3 for neutralisation while others used only KOH for neutralisation (Nazar *et al.,* 1970; Qian *et al.,* 2004; Ritter *et al.,* 2008; Goulard *et al.,* 1999). The effect of neutralisation on the ionisation process was determined using only KOH, only K_2CO_3 and both KOH and K_2CO_3 for sample neutralisation.

A 3 mL standard mix was prepared from the frozen nucleotide standard stock solutions. The concentrations of ATP, ADP, AMP, adenosine, adenine, inosine, hypoxanthine, NAD and NADP in the mix were 125 µM; the concentrations of NADH and NADPH in the mix were 62.5 µM; and the concentration of uric acid in the mix was 250 µM. The standard mix was divided into three 500 µL aliquots in 1.5 mL Eppendorf tubes. To each of the three samples 500 µL of ice-cold 12% PCA/IS was added and the samples were vortex-mixed. 650 µL of supernatant of each sample was transferred to separate Eppendorf tubes. The first sample was then neutralised with 6 M KOH and 2 M K_2CO_3 to pH 6. The second sample was

neutralised with 2 M K_2CO_3 to pH 6. The third sample was neutralised with 6 M KOH to pH 6. After centrifugation (14 000 rpm, 10 min, 4°C), 200 µL of each sample was transferred to separate auto-sampler vials and the samples were analysed in triplicate.

3.4.6.2.b. Sample pH effect

Different articles in the literature (Caruso *et* al., 2004; Coolen *et al*., 2008; Klawitter *et al*., 2007) suggest neutralising the perchloric acid in the supernatant to different pH values. To determine how the pH of the sample affected the ionisation process, samples with a pH of 3 and pH of 6 - 7 were analysed.

A 3 mL standard mix was prepared from the frozen nucleotides standard stock solutions. The concentrations of ATP, ADP, AMP, adenosine, adenine, inosine, hypoxanthine, NAD and NADP in the mix were 125 µM; the concentrations of NADH and NADPH in the mix were 62.5 µM; and the concentration of uric acid in the mix was 250 µM. The standard mix was divided into three 500 µL aliquots in 1.5 mL Eppendorf tubes. To each of the three samples 500 µL of ice-cold 12% PCA/IS was added and the samples were vortex-mixed. 650 µL of supernatant of each sample was transferred to separate Eppendorf tubes. The first sample was neutralised with 6 M KOH to pH 7. The second sample was neutralised with 6 M KOH to pH 7 before bringing the pH back down to three with formic acid. The third sample was neutralised with 6 M KOH to pH 7 before bringing the pH back down to three with acetic acid. After centrifugation (14 000 rpm, 10 min, 4°C), 200 µL of each sample was transferred to separate auto-sampler vials and the samples were analysed in triplicate.

3.4.6.2.c. Different concentrations of perchloric acid

Blood was gravimetrically divided into aliquots in 1.5 mL Eppendorf tubes (± 500 µL samples). The concentration and volume of PCA/IS added to each sample is given in Table 3.10.

Table 3.10: Concentration and volume of PCA/IS added to each blood sample.

Sample	Initial PCA concentration (%)	Volume of PCA added (µL)	Final PCA concentration (%)
1	12	500	6
2	18	250	6
3	30	125	6
4	52	65	6
5	70	47	6

After adding the ice-cold PCA/IS, the samples were vortex-mixed and centrifuged (14 000 rpm, 10 min, 4°C). The volume of supernatant removed from each sample is given in Table 3.11.

Table 3.11: Volume of supernatant removed from each sample.

Sample	PCA concentration (%)	Volume of supernatant (µL)
1	12	700
2	18	500
3	30	400
4	52	350
5	70	300

The supernatant from each sample was then neutralised with 6 M KOH to pH 6. 200 µL of each sample was transferred to separate auto-sampler vials and analysed in triplicate.

3.4.6.3. Deproteinisation with acetonitrile

Blood was gravimetrically divided into aliquots in 1.5 mL Eppendorf tubes (± 500 µL samples). 1 mL of ice-cold 100% acetonitrile was added to two of the aliquots. 500 µL of ice-cold 12% PCA/IS was added to one of the aliquots. The samples were then vortex-mixed. The two acetonitrile samples were centrifuged (14 000 rpm, 10 min, 4°C) and 1.3 mL of supernatant from each sample was transferred to new Eppendorf tubes. The samples were then dried under a stream of nitrogen at 20°C, frozen at -80°C for 2 hours and then freeze dried overnight. The perchloric acid sample was stored at 4°C overnight. The freeze dried acetonitrile samples were then resuspended in 500 µL of water, vortex-mixed and centrifuged (14 000 rpm, 2 min, 4°C). The PCA sample was centrifuged (14 000 rpm, 10

min, 4°C) and 700 µL of the supernatant was neutralised with 6 M KOH to pH 6. 200 µL of each sample was transferred to separate auto-sampler vials and analysed in triplicate.

3.4.6.4. Deproteinisation with formic acid and acetic acid

In the literature formic acid and acetic acid have been used for protein precipitation during nucleotide extraction but only in cell cultures (Buckstein *et al.*, 2008; Nazar *et al.*, 1970; Ritter *et al.*, 2008). It was decided to attempt protein precipitation in blood samples by use of formic acid and acetic acid.

Blood was divided into two 500 µL aliquots in 1.5 mL Eppendorf tubes. 500 µL of ice-cold 98% formic acid was added to one tube and 500 µL of ice-cold 100 % acetic acid was added to the other tube. The samples were vortex-mixed and centrifuged (14 000 rpm, 10 min, 4°C). During centrifugation no pellet was formed in either of the tubes. Thus it is not possible to precipitate the proteins from blood samples using either formic acid or acetic acid.

3.4.7. Nucleotide stability in blood samples

The stability of the nucleotides present in blood was evaluated over a 24 hour period.
Blood was gravimetrically divided into aliquots in 1.5 mL Eppendorf tubes (± 500 µL samples). 500 µL of ice-cold 12% PCA/IS was added to the blood samples at the time intervals described in Table 3.12.

Table 3.12: Time intervals at which 12% perchloric acid was added to the blood samples.

Samples	Time Intervals
3x T0 Samples	12% PCA/IS was added immediately, samples were neutralised and analysed in triplicate.
6x 2h Samples	12% PCA/IS was immediately added to three samples and the samples were stored at 4°C for two hours. The samples were then neutralised and analysed in triplicate.
	Three samples were stored at 4°C for two hours. 12% PCA/IS was then added, the samples were neutralised and analysed in triplicate.
6x 4h Samples	12% PCA/IS was immediately added to three samples and the samples were stored at 4°C for four hours. The samples were then neutralised and analysed in triplicate.
	Three samples were stored at 4°C for four hours. 12% PCA/IS was then added, the samples were neutralised and analysed in triplicate.
6x 24h Samples	12% PCA/IS was immediately added to three samples and the samples were stored at 4°C for 24 hours. The samples were then neutralised and analysed in triplicate.
	Three samples were stored at 4°C for 24 hours. 12% PCA/IS was then added, the samples were neutralised and analysed in triplicate.

3.5. Method application

To test the proof of concept of the newly developed and validated LC-ESI-MS/MS method, the adenine and pyridine nucleotide levels of a small group of healthy individuals and possible chronic fatigue syndrome patients, were compared. Ethical approval for this pilot study was obtained from the Ethics Committee of the North-West University, Department of Research Support (NWU-00102-12-S1).

3.5.1. Selecting patient and control groups

All participants for this study completed both the Piper fatigue scale (PFS) and the medical symptoms questionnaire (MSQ). The control group and possible CFS patients were then selected based only on the results of the PFS. A score between zero and three on the Piper fatigue scale was used for the control group and a score between seven and ten was used for the possible CFS patient group. The control group consisted of 14 healthy volunteers

(seven males and seven females) within an age range of 23 – 50. The CFS group consisted of three possible CFS patients (one male and two females) within an age range of 25 – 40. Informed consent was obtained from all participants prior to blood collection.

3.5.2. Blood sample collection and analysis

3 mL of blood was collected by venepuncture in an EDTA-containing Vacutainer® tube (Becton Dickinson Ltd., Johannesburg, SA) from each volunteer. Immediately after collection, each volunteer's blood was gravimetrically divided into three 500 µL aliquots. 12% of ice-cold PCA/IS was added to each of the samples. The samples were vortex-mixed and centrifuged (14 000 rpm, 10 min, 4°C). 700 µL of supernatant from each sample was transferred to separate 1.5 mL Eppendorf tubes and neutralised to pH 6 with 6 M KOH. 200 µL of each sample was transferred to separate auto-sampler vials and analysed in triplicate.

3.6. Statistical data analysis

3.6.1. Limit of detection and quantification

To determine the linear concentration range for each nucleotide (goodness of fit of the linear model), the Fisher test was used. The calculated F-value and the degrees of freedom (df2) was determined by using the LINEST function in Microsoft Excel. The critical F-value was obtained from the F-table (α=0.05, df1=1) (Sanagi *et al*., 2009; Westgard, 2008).

The limit of detection (LOD) and limit of quantification (LOQ) for each nucleotide were determined using the linear regression method (Sanagi *et al.,* 2009). The linear regression parameters (standard deviation (SD) and slope (b) of calibration curve) required for Equation 3.1 and 3.2 were also calculated using the LINEST function.

3.6.2. Stability and method application data

The raw data were first adjusted with a dilution factor (to correct for differences in sample volumes) and the internal standard (to correct for losses during sample preparation) prior to statistical analysis. Statistical analysis of the data was performed using Statistica 11.0 (Statsoft Inc., Tulsa, Oklahoma, USA) and MetaboAnalyst 2.0 (www.metaboanalyst.ca/). The Shapiro-Wilk test indicated that normality of the data was rejected ($p<0.05$) thus non-parametric tests were used for further data analysis. Log transformation was used in order to normalise the data distribution. The data was further subjected to one-way analysis of

variance (ANOVA) followed by post-hoc analysis with Tuckey's honestly significant difference test (Tuckey's HSD). *p* values smaller than 0.05 were considered to be statistically significant.

Chapter 4: Results and Discussion

4.1. Introduction

The aim of this study was to develop and validate an electrospray-ionisation tandem mass spectrometry method to quantify selected energy and redox marker levels in blood samples. The energy and redox markers included pyridine nucleotides, adenine nucleotides and their degradation products all of which can be used as possible diagnostic markers for energy related disorders such as chronic fatigue syndrome.

The newly developed method consisted of chromatographic separation on an Aqua-C18 column using 5 mM ammonium acetate (pH 7.2) and 10 mM ammonium acetate/ acetonitrile (pH 7.9) as mobile phases, and nucleotide detection in MRM mode. Various validation parameters such as linearity, limits of detection and quantification, and stability under different conditions, were studied to assess the suitability of the new method for its intended use. The proof of concept of the method was then tested using the blood samples of a small group of controls and possible CFS patients.

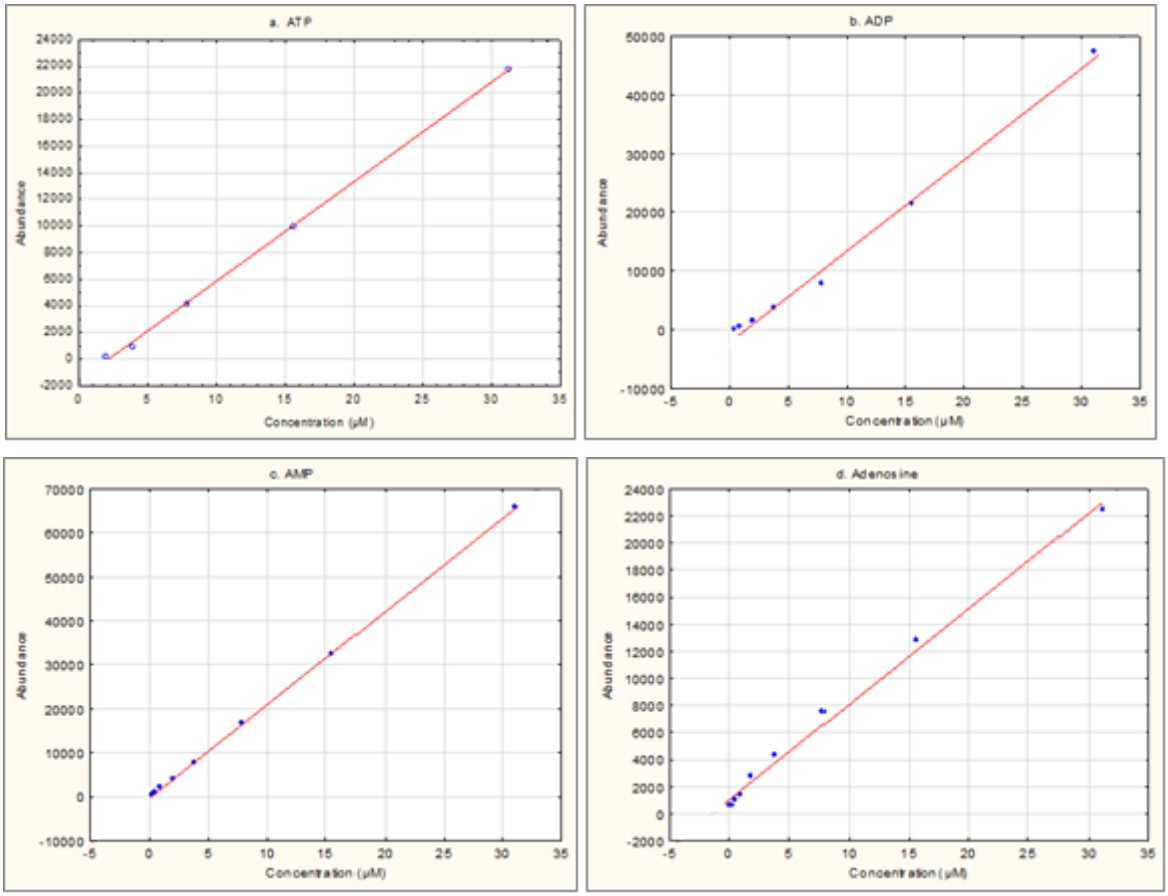
4.2. Method Validation

4.2.1. Linearity

The linearity of the method was investigated using a concentration range of 0.12 – 62.5 µM for ATP, ADP, AMP, adenosine, adenine, inosine, hypoxanthine, NAD and NADP; a concentration range of 0.06 – 31.25 µM for NADH and NADPH; and a concentration range of 31.25 µM – 1 mM for uric acid. A lower concentration range was used for NADH and NADPH because the reduced pyridine nucleotide levels are much lower in biological samples than the oxidised pyridine nucleotide levels. The normal uric acid levels in blood range between 142 µmol/L to 416 µmol/L (Chernecky & Berger, 2001; Fischbach, 2003) thus a higher concentration range was used for determining linearity. Due to the wide variety of analytical methods used to quantify adenine and pyridine nucleotides, accurate reference range values for these nucleotides can not be reported here. The calibration curve for each nucleotide standard is illustrated in Figure 4.1.a-k. The linear range and the correlation coefficient value (r^2) for each nucleotide standard is summarised in Table 4.1.

Table 4.1: The correlation coefficient and linear range for each nucleotide.

Nucleotide standard	Correlation coefficient value (r^2)	Linear range (µM)
ATP	0.999	1.95 – 31.25
ADP	0.995	0.98 – 31.25
AMP	0.999	0.12 – 31.25
Adenosine	0.995	0.12 – 31.25
Adenine	0.995	0.12 – 31.25
Inosine	0.997	0.48 – 31.25
Hypoxanthine	0.995	0.48 – 31.25
Uric acid	0.997	31.25 – 250.00
NADH	0.996	0.12 – 15.60
NAD	0.999	0.12 – 31.25
NADPH	0.997	0.24 – 15.60
NADP	0.998	0.12 – 31.25



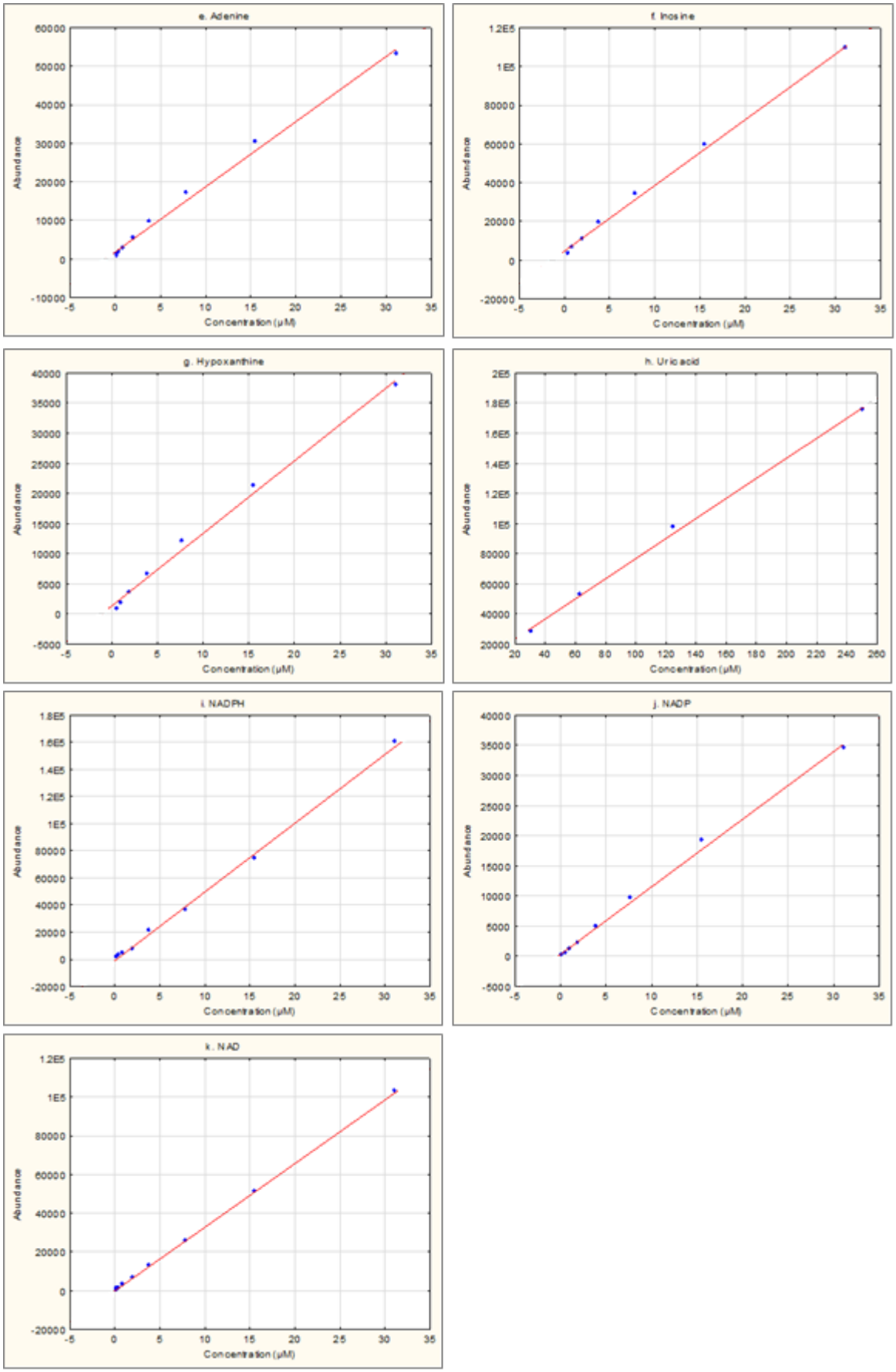


Figure 4.1: Nucleotide calibration curves. The calibration curves illustrate the linear range for each nucleotide. Linearity was assessed using a concentration range of 0.12 – 62.5 µM for ATP, ADP, AMP, adenosine, adenine, inosine, hypoxanthine, NAD and NADP, a concentration range of 0.06 – 31.25 µM for NADH and NADPH and a concentration range of 31.25 µM – 1 mM for uric acid.

4.2.4. Limits of detection and quantification

The limit of detection (LOD) and limit of quantification (LOQ) for each nucleotide were determined using the linear regression method (Sanagi *et al.,* 2009). To determine the goodness of fit of each linear calibration curve, the calculated and critical F-values were determined as described in section 3.6.1. The calculated F-value for each nucleotide was significantly larger than the critical F-value ($\alpha = 0.05$) thus the instrumental response significantly correlates to the concentrations of the nucleotide standards (Sanagi *et al*., 2009). The results of the statistical evaluation of the linear regression curve and the LOD and LOQ for each nucleotide are given in Table 4.2.

Table 4.2: Limit of detection and limit of quantification determined using the linear regression method.

Compound	Calculated F-value (α=0.05)	Critical F-value (α=0.05)	Limit of Detection (µmol/L)	Limit of Quantification (µmol/L)
ATP	4227.72	10.13	1.2	3.7
ADP	841.73	7.71	2.9	8.9
AMP	120267.32	5.59	0.3	0.9
Adenosine	985.74	5.59	3.1	9.4
Adenine	994.68	5.59	3.1	9.4
Inosine	1787.14	6.61	2.1	6.5
Hypoxanthine	1013.89	6.61	2.8	8.6
Uric acid	906.47	18.51	18.4	55.7
NADH	1807.11	5.59	2.3	6.9
NAD	126646.62	5.59	0.3	0.8
NADPH	1994.71	7.71	1.9	5.8
NADP	4379.57	5.59	1.5	4.5

4.2.3. Intraday and interday variation

The precision of the analytical method (Intraday variation) was accessed by analysing three concentrations for each nucleotide standard in triplicate on the same day. The precision of the analyst (Interday variation) was accessed by analysing three concentrations for each nucleotide standard in triplicate over a period of five days. The intraday and interday precision at each of the three concentration levels are summarised in Table 4.3.

Table 4.3: Interday and Intraday precision.

Compound	Concentration (µM)	Mean of Abundances N=3	Intraday precision (% CV)	Mean of Abundances N=5	Interday precision (% CV)
ATP	15.60	23829	5.0	27645	10
	31.25	73202	1.1	69043	12
	62.50	189562	0.6	192390	7
ADP	15.60	8542	4.0	8109	12
	31.25	28943	1.6	27706	10
	62.50	74299	1.8	76463	2
AMP	15.60	27804	1.8	35549	9
	31.25	65346	0.8	69296	5
	62.50	136737	0.7	133601	5
Adenosine	15.60	17831	2.9	14139	10
	31.25	27012	1.7	18837	13
	62.50	43043	1.0	39467	8
Adenine	15.60	14967	0.2	19726	13
	31.25	29079	0.3	31002	5
	62.50	53918	2.4	58731	10
Inosine	15.60	52562	1.9	53161	6
	31.25	103568	1.0	105380	3
	62.50	210244	0.6	214130	2
Hypoxanthine	15.60	2798	2.1	16445	3
	31.25	5066	1.0	32068	3
	62.50	8712	1.3	62440	4
NAD	15.60	22800	0.7	20304	4
	31.25	46084	1.4	42269	3
	62.50	89373	0.8	86842	2
NADP	15.60	46001	4.9	52592	10
	31.25	103839	1.0	114434	3
	62.50	225135	0.7	230032	3
NADH	7.80	6631	0.4	5222	8
	15.60	16410	1.2	15638	11
	31.25	44596	0.6	42391	4
NADPH	7.80	2863	0.1	3112	15
	15.60	6938	3.5	5120	14
	31.25	10866	3.9	15057	12
Uric acid	62.50	97381	1.7	56186	2
	125.00	174960	0.9	105901	2
	250.00	157775	2.3	140482	10

The intraday and interday precision, expressed as coefficient of variation (% CV), were calculated using Equation 4.1. The mean and standard deviation (SD) values for the intraday precision were calculated using the abundances of the triplicates of each concentration level. For the interday precision the mean value of the triplicates was also calculated for each nucleotide on each of the five days. The mean and the SD values used in Equation 4.1 were then calculated using the five mean values for each nucleotide.

Equation 4.1: Calculating coefficient of variation (% CV).

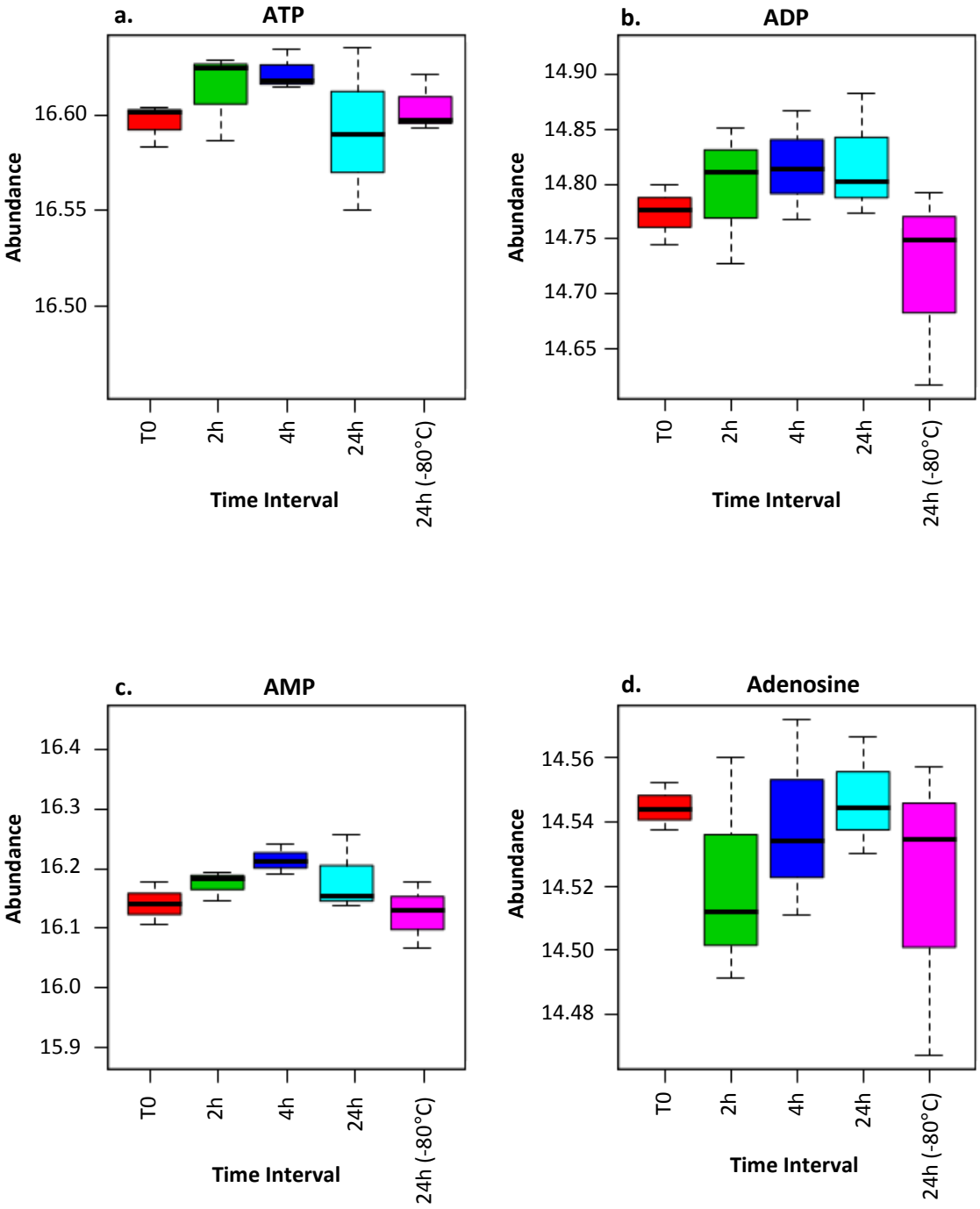
$$\% \text{ CV} = \frac{\text{Standard deviation}}{\text{Mean}} \times 100$$

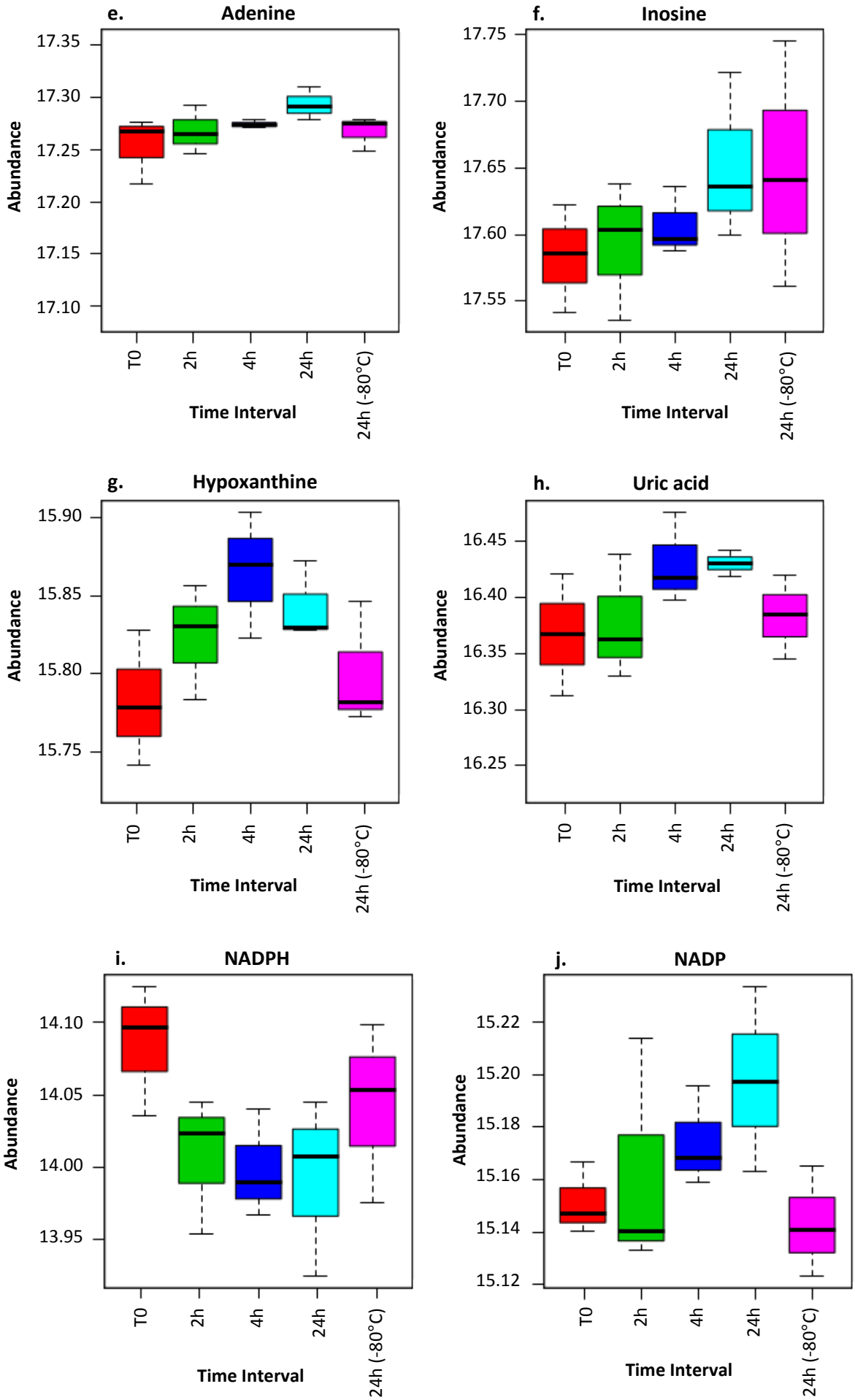
The intraday precision was ≤ 5% for all nucleotides at all three concentration levels. The interday precision was ≤ 15% for all nucleotides at all three concentration levels. According to the U.S. Food and Drug Administration guidelines for bioanalytical method validation (2001): "the precision determined at each concentration level should not exceed 15% of the coefficient of variation (CV) except for the LOQ, where it should not exceed 20% of the CV". The precision of the analytical method is thus compliant with the FDA's acceptance criteria. These results also correlate with the findings of other articles that used perchloric acid to extract nucleotides from whole blood samples. Coolen *et al.* (2008) reported a within-day CV range of 0.7 – 5.9% and a between-day CV range of 2.6 – 15.3%. Caruso *et al.* (2004) reported a within-day CV range of 1.0 – 3.9% for ATP, ADP, AMP, NAD and NADP. The between-day CV range was 0.6 – 17.6% except for AMP which had a range of 26.8 – 43.3%.

4.2.4. Nucleotide standard stability in water

The stability of all nucleotides in water was assessed over a 24 hour period. The effect of temperature, either at room temperature (25 °C in the auto-sampler) or at -80°C for 24 hours, was also evaluated. The data obtained were subjected to statistical analysis as described in section 3.6.2.

As seen in Figure 4.2.a – l. all the nucleotides remained stable in water for the entire 24 hour period. Temperature had no effect as there were no statistical difference in the nucleotide abundances of the samples left at room temperature and the samples stored at -80°C for 24 hours.


a.
ATP
Abundance
16.60
16.55
16.50
T0
2h
4h
24h
24h (-80°C)
Time Interval
b.
ADP
Abundance
14.90
14.85
14.80
14.75
14.70
14.65
T0
2h
4h
24h
24h (-80°C)
Time Interval
c.
AMP
Abundance
16.4
16.3
16.2
16.1
16.0
15.9
T0
2h
4h
24h
24h (-80°C)
Time Interval
d.
Adenosine
Abundance
14.56
14.54
14.52
14.50
14.48
T0
2h
4h
24h
24h (-80°C)
Time Interval


e.
Adenine
Abundance
17.35
17.30
17.25
17.20
17.15
17.10
T0
2h
4h
24h
24h (-80°C)
Time Interval
f.
Inosine
Abundance
17.75
17.70
17.65
17.60
17.55
T0
2h
4h
24h
24h (-80°C)
Time Interval
g.
Hypoxanthine
Abundance
15.90
15.85
15.80
15.75
T0
2h
4h
24h
24h (-80°C)
Time Interval
h.
Uric acid
Abundance
16.45
16.40
16.35
16.30
16.25
T0
2h
4h
24h
24h (-80°C)
Time Interval
i.
NADPH
Abundance
14.10
14.05
14.00
13.95
T0
2h
4h
24h
24h (-80°C)
Time Interval
j.
NADP
Abundance
15.22
15.20
15.18
15.16
15.14
15.12
T0
2h
4h
24h
24h (-80°C)
Time Interval

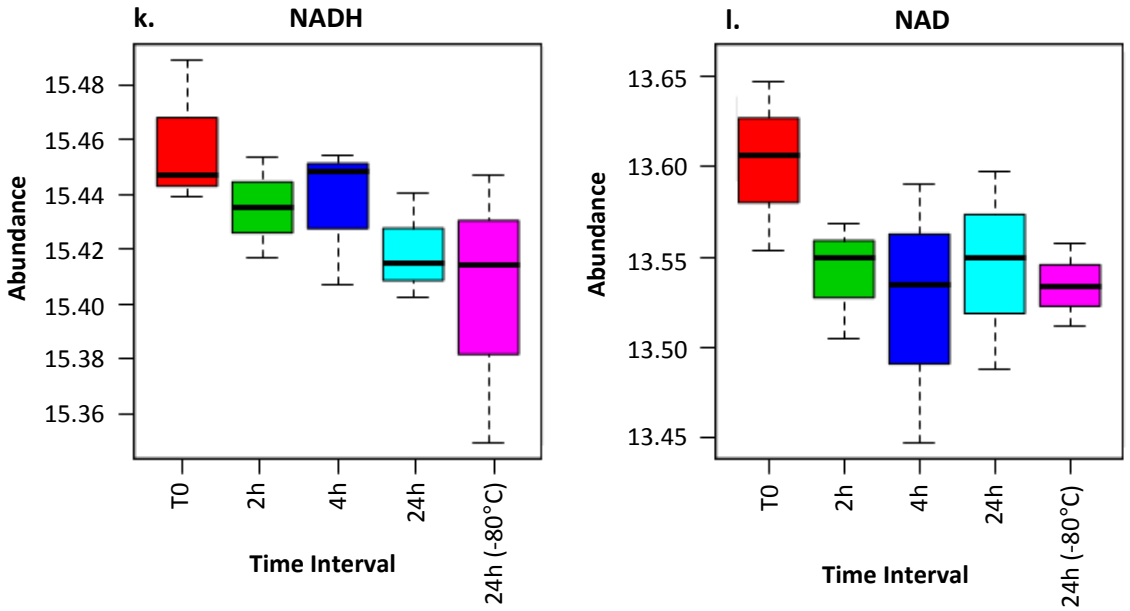


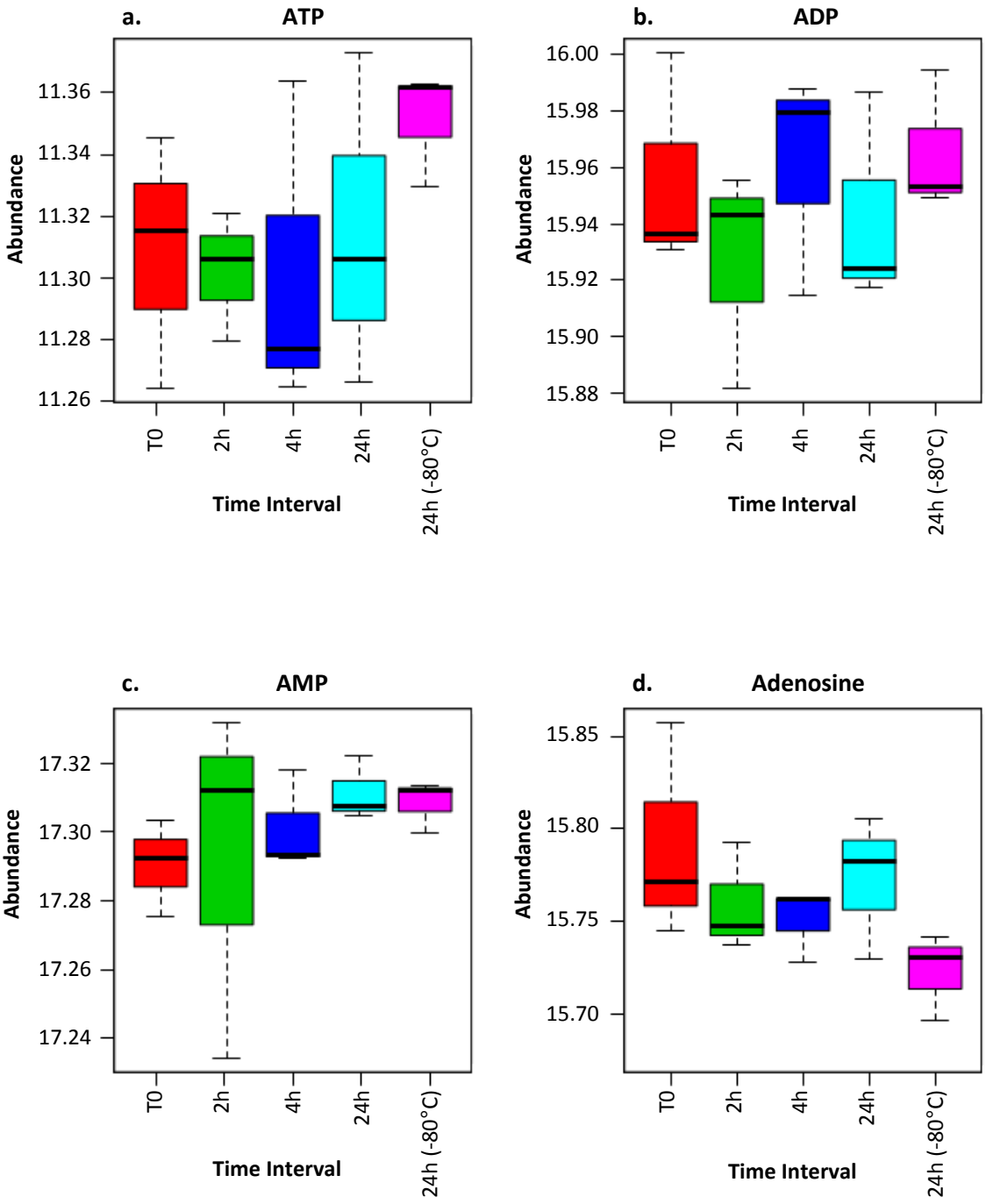
Figure 4.2: Nucleotide stability in water over a 24 hour period. All nucleotides were stable in water over the 24 hour period as there were no statistical differences ($p>0.05$) between the different time intervals. There was also no statistical difference ($p>0.05$) between the standard mix left at room temperature for 24 hours and the standard mix stored at -80°C for 24 hours.

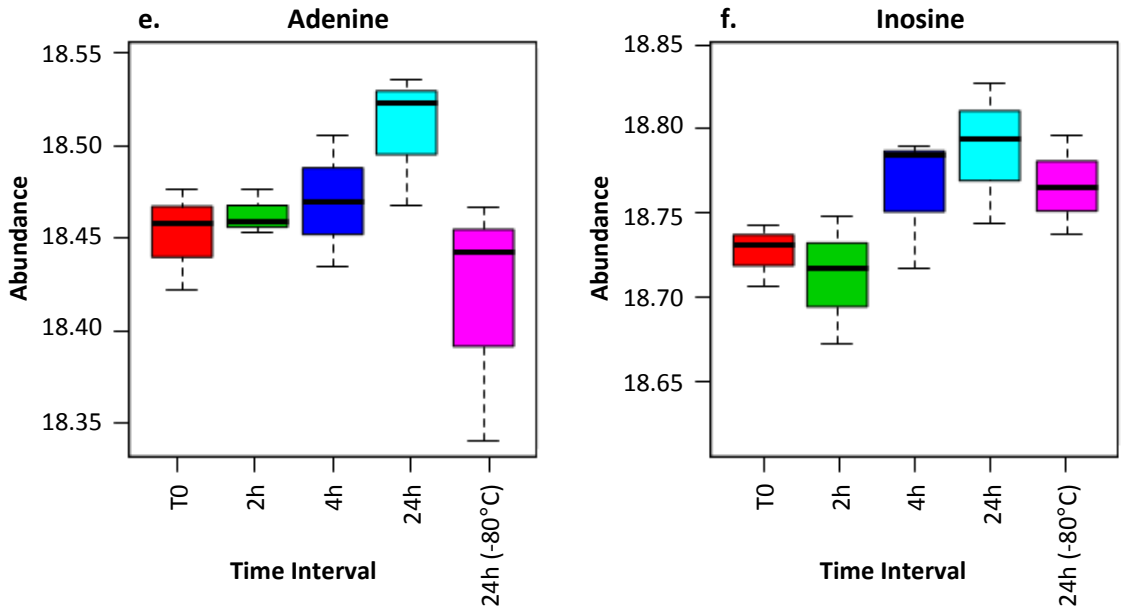
4.2.5. Nucleotide standard stability in perchloric acid

The stability of all nucleotides in 6% perchloric acid was evaluated over a 24 hour period. The effect of temperature, either at room temperature (25 °C in the auto-sampler) or at -80°C for 24 hours, was also evaluated. The data obtained were subjected to statistical analysis as described in section 3.6.2.

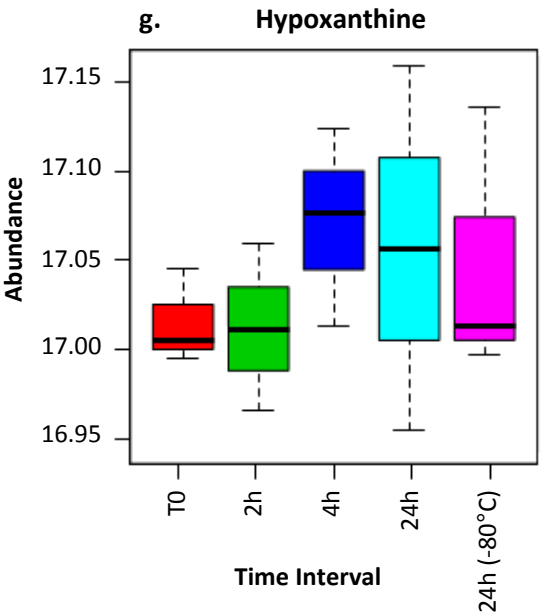
Adding perchloric acid to the standard mix caused a decrease in the abundances of ATP, NADPH (Fig. 4.3.a. and i.) and NADH The abundance of NADH in the presence of perchloric acid was very close to the baseline and could not be positively distinguished from the background. Due to the complete hydrolysis of NADH its results are not reported in further analyses. The abundances of all the other nucleotides (Fig. 4.3.a – k.) increased significantly due to the degradation of ATP, NADH and NADPH caused by the perchloric acid.

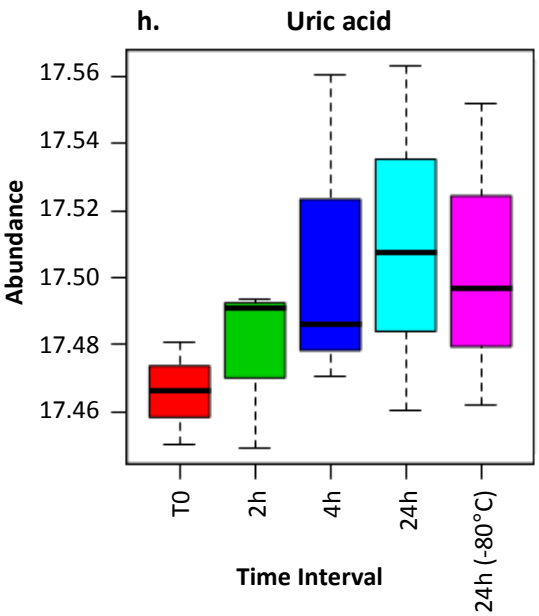
Although the perchloric acid caused changes in the abundances of the nucleotides, the nucleotides remained stable for the entire 24 hour period. Temperature (25°C or -80°C) had no effect on the stability of the nucleotides in perchloric acid.

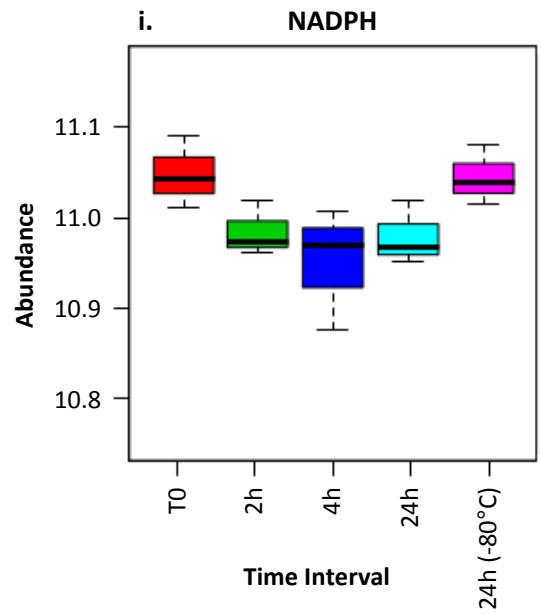


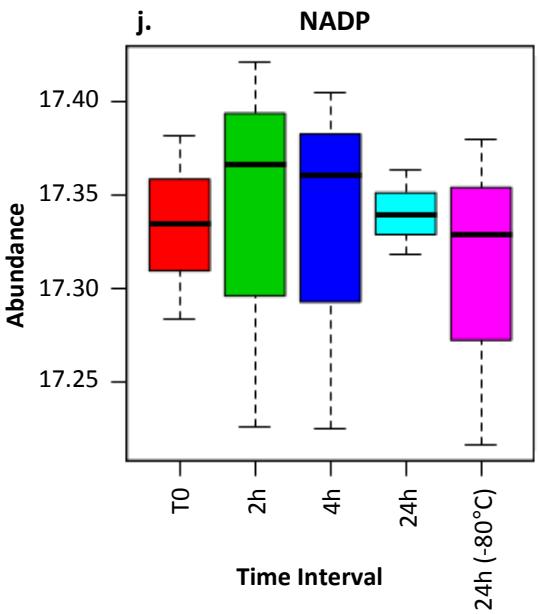

e.
Adenine
Abundance
18.55
18.50
18.45
18.40
18.35
T0
2h
4h
24h
24h (-80°C)
Time Interval

f.
Inosine
Abundance
18.85
18.80
18.75
18.70
18.65
T0
2h
4h
24h
24h (-80°C)
Time Interval


g.
Hypoxanthine
Abundance
17.15
17.10
17.05
17.00
16.95
T0
2h
4h
24h
24h (-80°C)
Time Interval


h.
Uric acid
Abundance
17.56
17.54
17.52
17.50
17.48
17.46
T0
2h
4h
24h
24h (-80°C)
Time Interval


i.
NADPH
Abundance
11.1
11.0
10.9
10.8
T0
2h
4h
24h
24h (-80°C)
Time Interval


j.
NADP
Abundance
17.40
17.35
17.30
17.25
T0
2h
4h
24h
24h (-80°C)
Time Interval

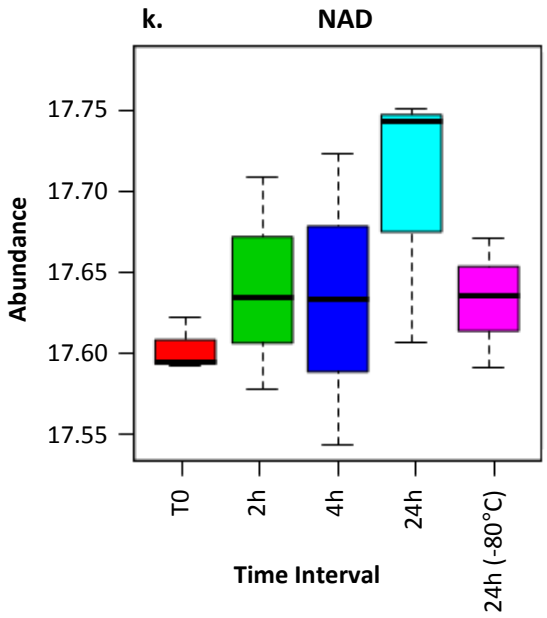


Figure 4.3: Nucleotide stability in perchloric acid over a 24 hour period. All nucleotides were stable in 6% perchloric acid over the 24 hour period as there were no statistical differences ($p>0.05$) between the different time intervals. There was also no statistical difference ($p>0.05$) between the standard mix left at room temperature for 24 hours and the standard mix stored at -80°C for 24 hours.

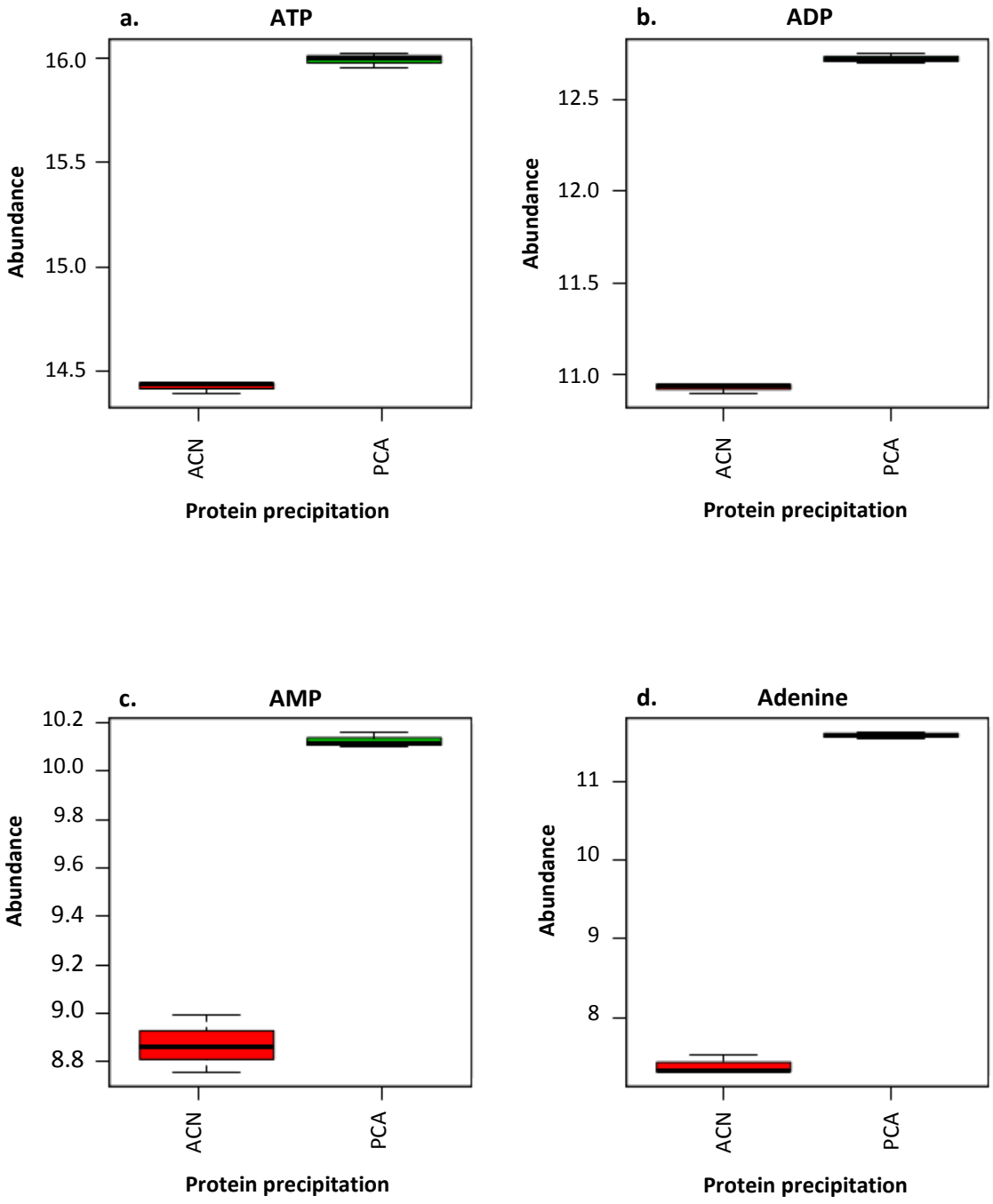
4.2.6. Deproteinisation of whole blood samples

4.2.6.1. Different deproteinisation methods

Proteins were precipitated from blood samples with either 66% acetonitrile, 6% perchloric acid, 49% formic acid or 50% acetic acid to determine the effect on nucleotide abundances. As described in section 3.4.6.4. neither the formic acid nor acetic acid samples formed a pellet during centrifugation, thus protein precipitation was not possible. The data obtained from the acetonitrile and perchloric acid samples were subjected to statistical analysis as described in section 3.6.2.

As seen in Figure 4.4.a – h. there were statistically significant differences ($p<0.05$) between the acetonitrile and the perchloric acid deproteinisation methods for all nucleotides. Protein precipitation with perchloric acid resulted in higher nucleotide abundances than protein precipitation with acetonitrile. This could be due to possible losses during the extra sample preparation steps (nitrogen drying and freeze drying). For both deproteinisation methods the abundances of adenosine and inosine were very close to the baseline and could thus not be positively distinguished from the background. Harkness *et al*. (1984) and Gorman *et al.* (2007) reported that the EDTA in the blood collection tubes prevented adenine nucleotide

conversion to adenosine and inosine. Therefore the results for adenosine and inosine are not reported in further analysis of blood samples. It was decided to use perchloric acid for deproteinisation of all whole blood samples.



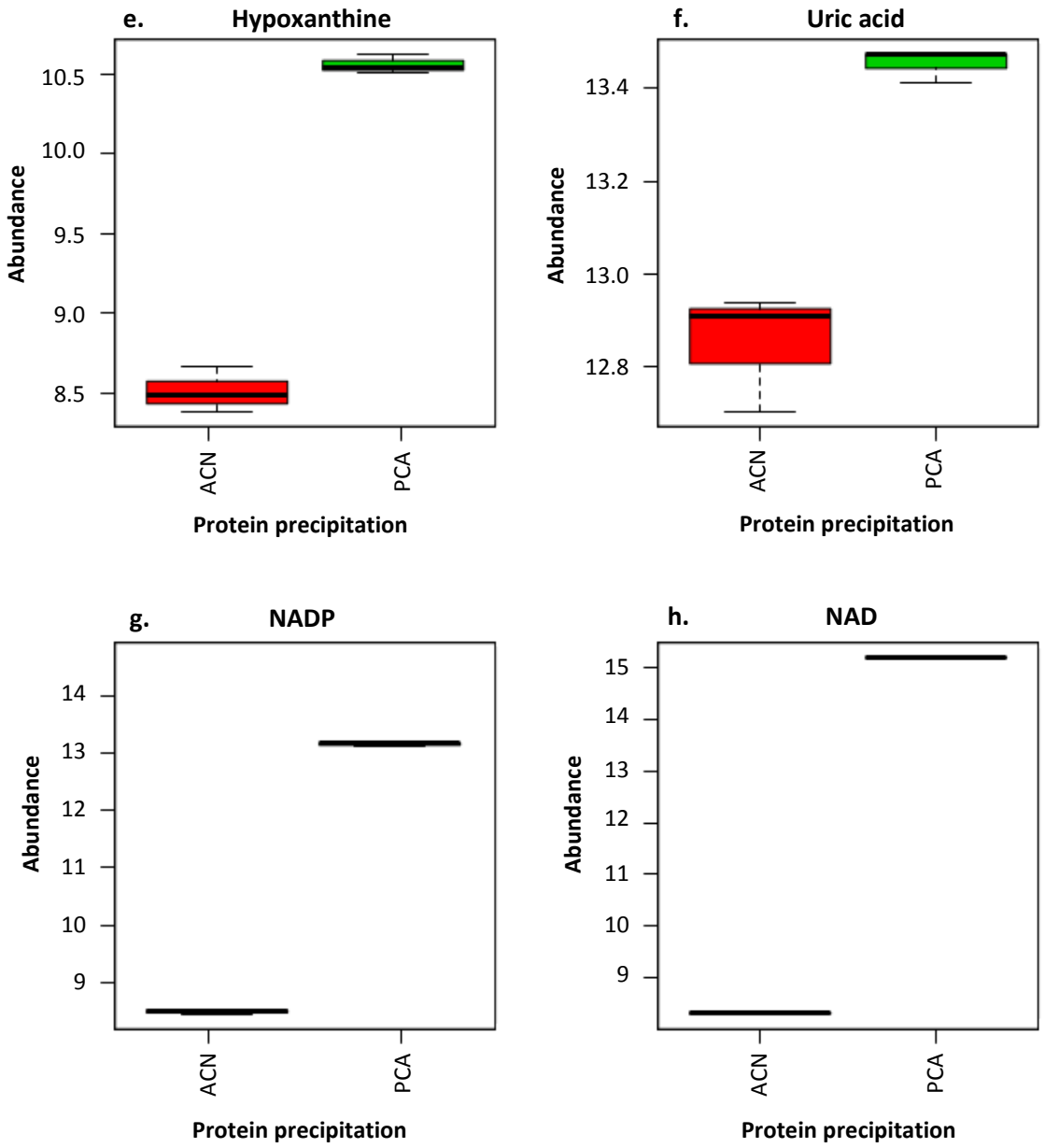


Figure 4.4: Deproteinisation of whole blood samples with acetonitrile and perchloric acid. There were statistically significant differences ($p<0.05$) between the acetonitrile and the perchloric acid deproteinisation methods for all nucleotides. Protein precipitation with perchloric acid resulted in higher nucleotide abundances.

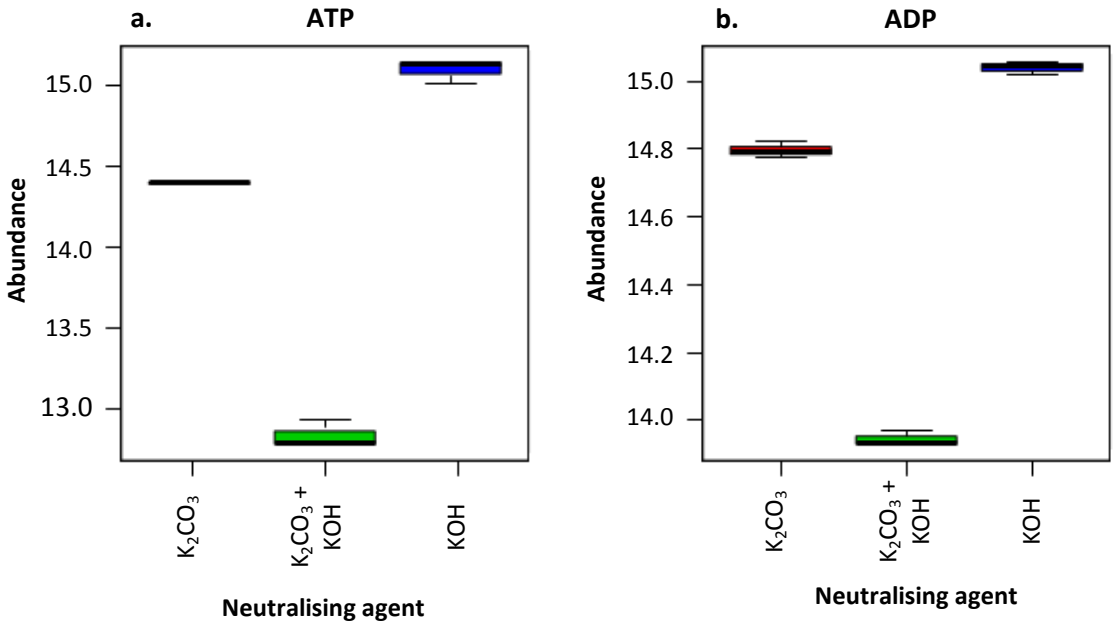
4.2.6.2. Neutralisation of perchloric acid

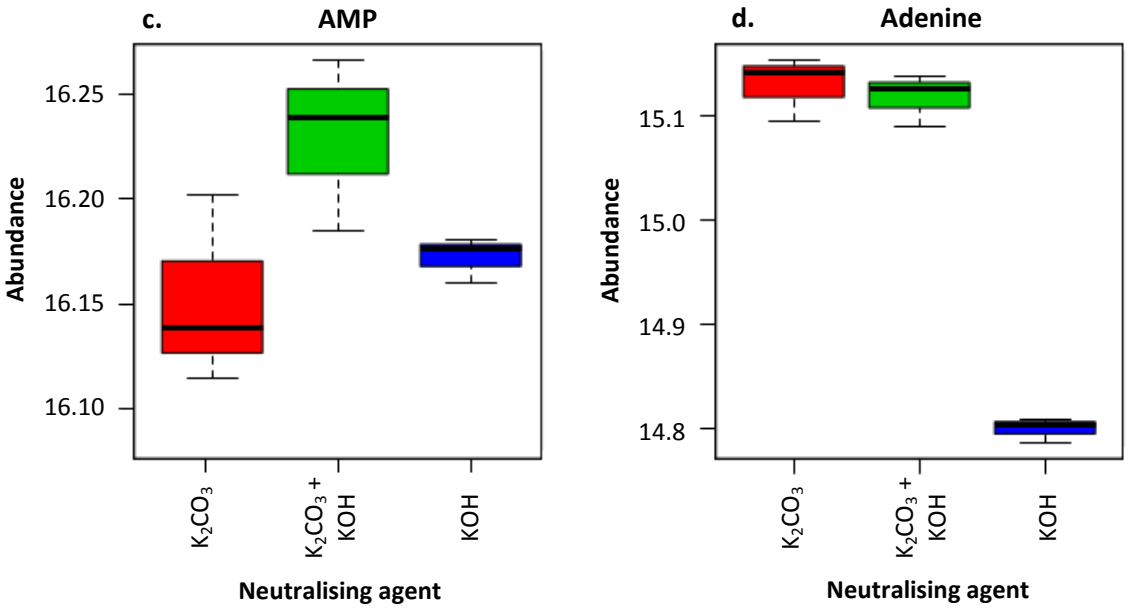
In the literature different neutralising agents have been applied to neutralise the perchloric acid after protein precipitation (Coolen *et al.* 2008; Nazar *et al.,* 1970; Qian *et al.,* 2004; Ritter *et al.,* 2008; Goulard *et al.,* 1999). In order to determine the effect of neutralisation on the nucleotide abundances, samples were neutralised to pH 6 -7 using either KOH, K_2CO_3 or

both KOH and K_2CO_3. The data obtained with the three different neutralising agents were subjected to statistical analysis as described section 3.6.2.

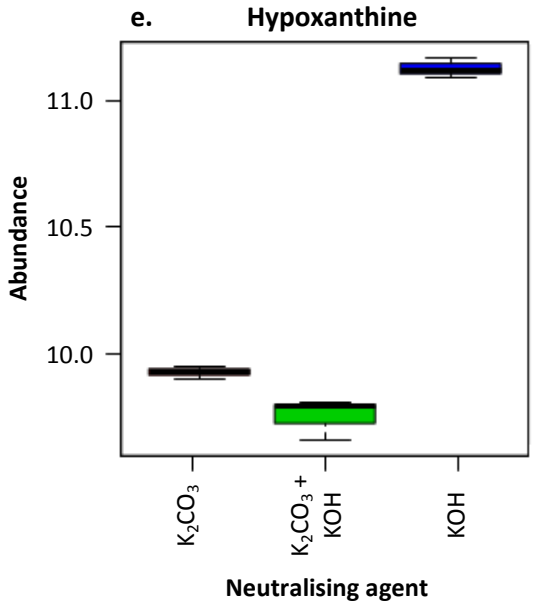
For ATP, ADP, hypoxanthine, uric acid, NADP and NAD (Fig. 4.5.a., b., e., f., h. and i.) there were distinct differences between the three neutralising agents. Neutralisation of the samples with KOH resulted in higher abundances of these nucleotides than the other two neutralising agents. The type of neutralising agent did not affect AMP (Fig. 4.5.c.) as there were no differences in the AMP abundance between the three neutralising agents. There were no differences in the NADPH (Fig. 4.5.g.) and adenine (Fig. 4.5.d.) levels of samples neutralised with K_2CO_3 and neutralised with K_2CO_3 + KOH. Neutralisation with KOH resulted in a higher abundance of NADPH while neutralisation with K_2CO_3 and K_2CO_3 + KOH resulted in a higher abundance of adenine.

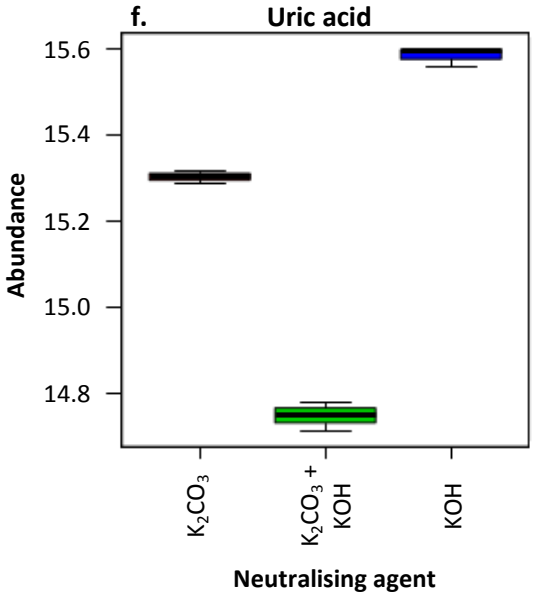
As the majority of nucleotides had higher abundances when the perchloric acid was neutralised with KOH, it was decided to use KOH for neutralisation in all further analyses.

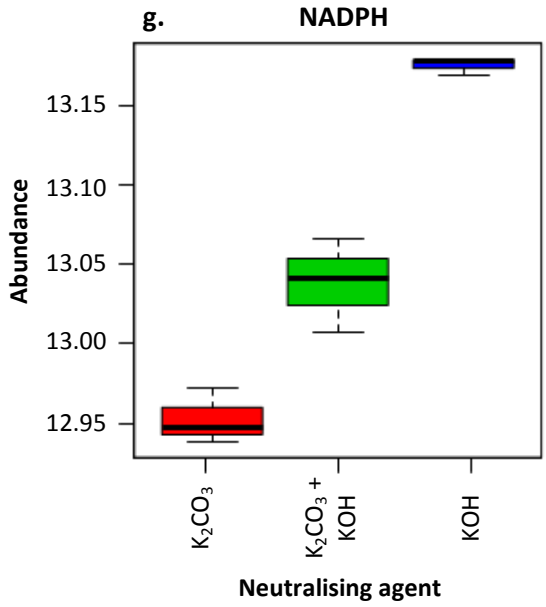


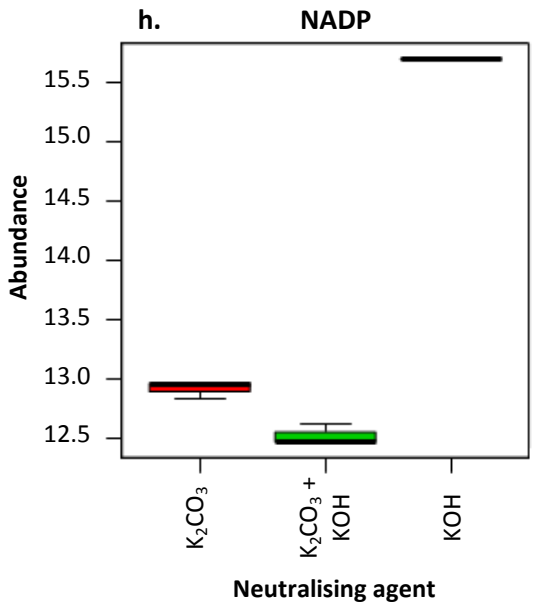

c.
AMP
Abundance
16.25
16.20
16.15
16.10
K2CO3
K2CO3 + KOH
KOH
Neutralising agent

d.
Adenine
Abundance
15.1
15.0
14.9
14.8
K2CO3
K2CO3 + KOH
KOH
Neutralising agent


e.
Hypoxanthine
Abundance
11.0
10.5
10.0
K2CO3
K2CO3 + KOH
KOH
Neutralising agent


f.
Uric acid
Abundance
15.6
15.4
15.2
15.0
14.8
K2CO3
K2CO3 + KOH
KOH
Neutralising agent


g.
NADPH
Abundance
13.15
13.10
13.05
13.00
12.95
K2CO3
K2CO3 + KOH
KOH
Neutralising agent


h.
NADP
Abundance
15.5
15.0
14.5
14.0
13.5
13.0
12.5
K2CO3
K2CO3 + KOH
KOH
Neutralising agent

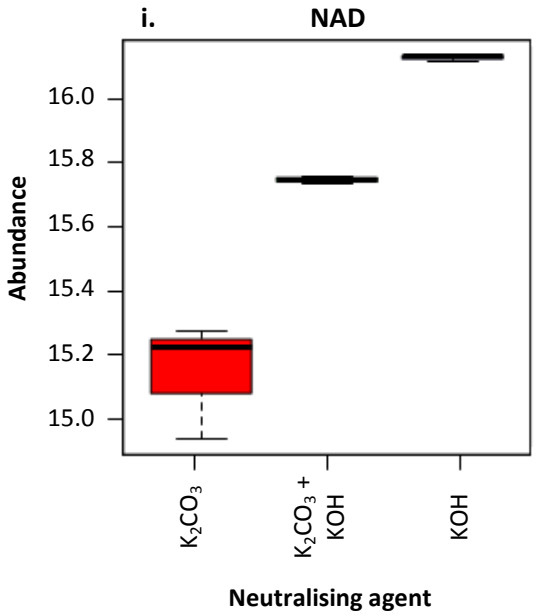
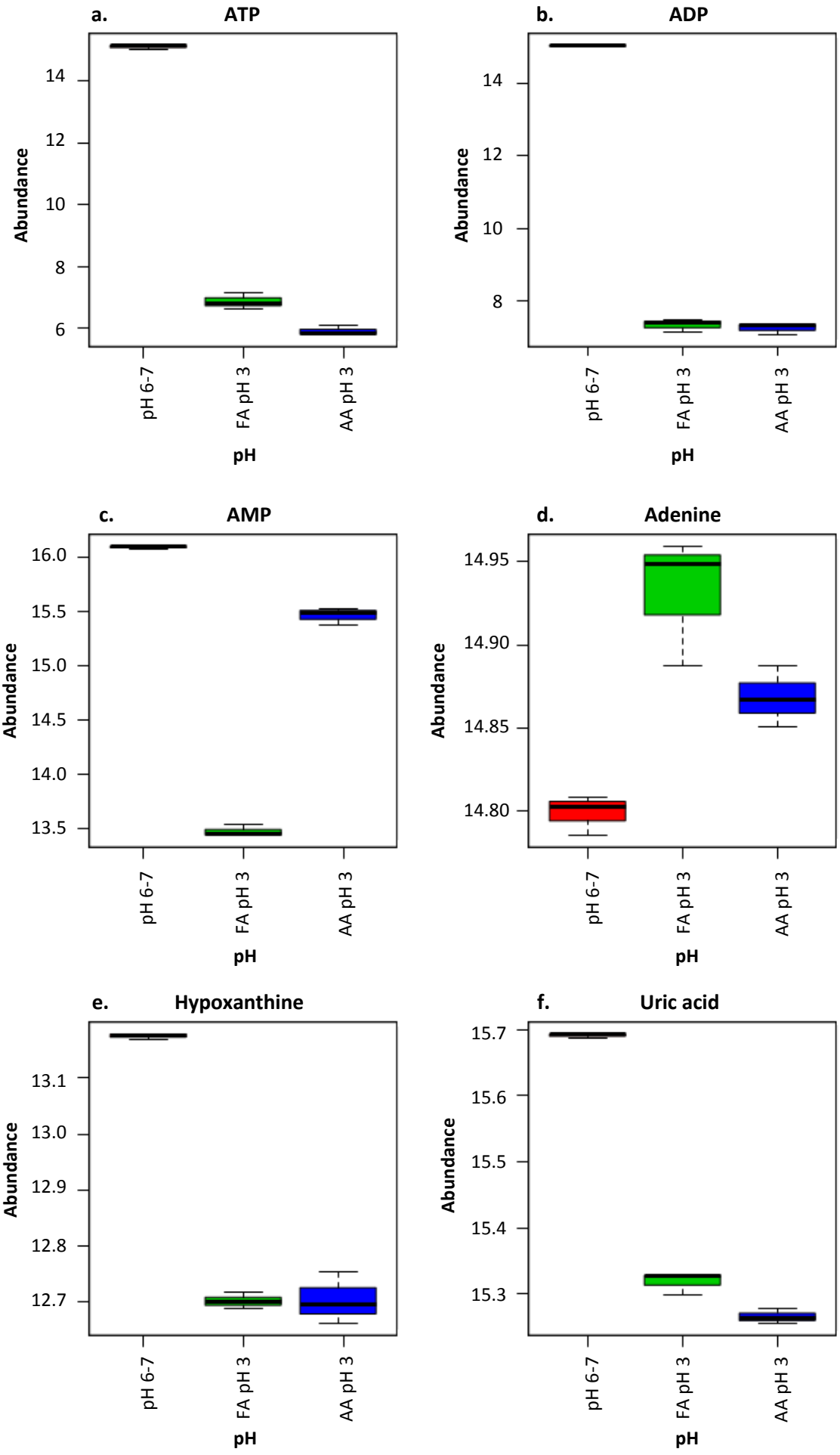


Figure 4.5: Different neutralising agents used to neutralise perchloric acid. The perchloric acid had to be neutralised after protein precipitation to prevent interference during ionisation. The samples were either neutralised with K_2CO_3, KOH or both K_2CO_3 and KOH to pH 6-7.

4.2.6.3. Sample pH effect

As the sample pH can greatly affect the ionisation process it was necessary to determine if nucleotides are better detected at a low or high pH. All the samples were first neutralised to pH 6-7 with KOH. The samples were then either analysed at pH 6-7 or the pH was lowered to 3 with formic acid or acetic acid before analysis. The data obtained was subjected to statistical analysis as described in section 3.6.2.

For the majority of nucleotides (Fig. 4.6. a., b., c., e., f., g. and h.), a higher pH (neutralisation with KOH to pH 6-7) resulted in higher abundances. Adenine (Fig. 4.6.d.) showed higher abundance when the pH was lowered with either formic acid or acetic acid. NADPH could not be detected when the pH was lowered with formic acid or acetic acid. It was decided to neutralise all samples to pH 6-7 for further analysis.


a.
ATP
Abundance
14
12
10
8
6
pH 6-7
FA pH 3
AA pH 3
pH
b.
ADP
Abundance
14
12
10
8
pH 6-7
FA pH 3
AA pH 3
pH
c.
AMP
Abundance
16.0
15.5
15.0
14.5
14.0
13.5
pH 6-7
FA pH 3
AA pH 3
pH
d.
Adenine
Abundance
14.95
14.90
14.85
14.80
pH 6-7
FA pH 3
AA pH 3
pH
e.
Hypoxanthine
Abundance
13.1
13.0
12.9
12.8
12.7
pH 6-7
FA pH 3
AA pH 3
pH
f.
Uric acid
Abundance
15.7
15.6
15.5
15.4
15.3
pH 6-7
FA pH 3
AA pH 3
pH

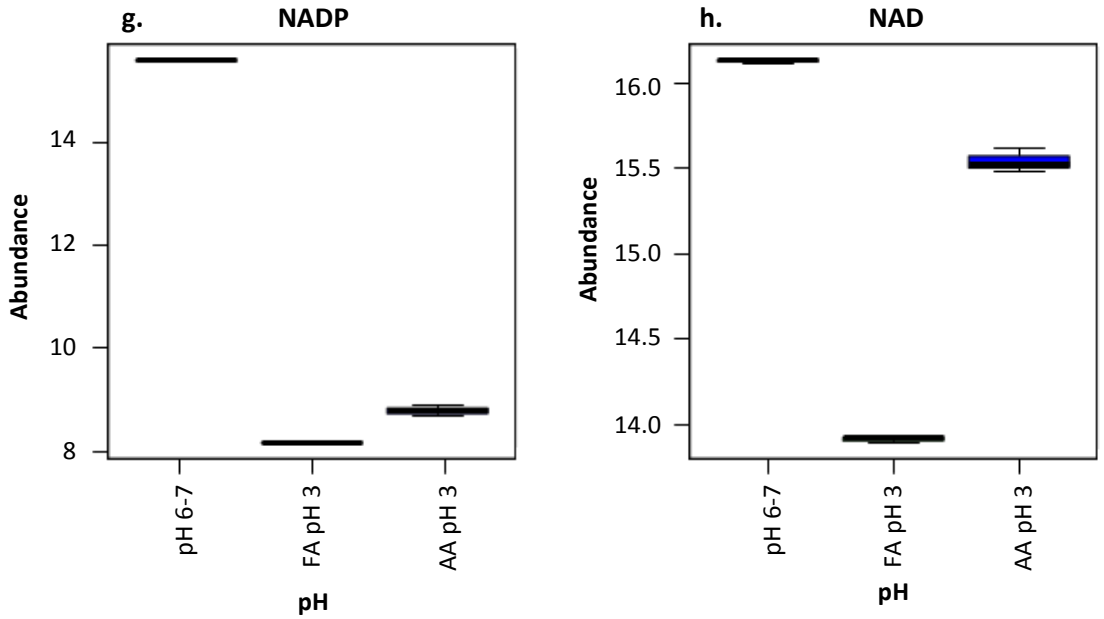


Figure 4.6: The effect of sample pH on nucleotide abundance. All samples were first neutralised with 6 M KOH to pH 6-7. The pH of some of the samples was then adjusted to pH 3 with either formic acid (FA) or acetic acid (AA).

4.2.6.4. Different concentrations of perchloric acid

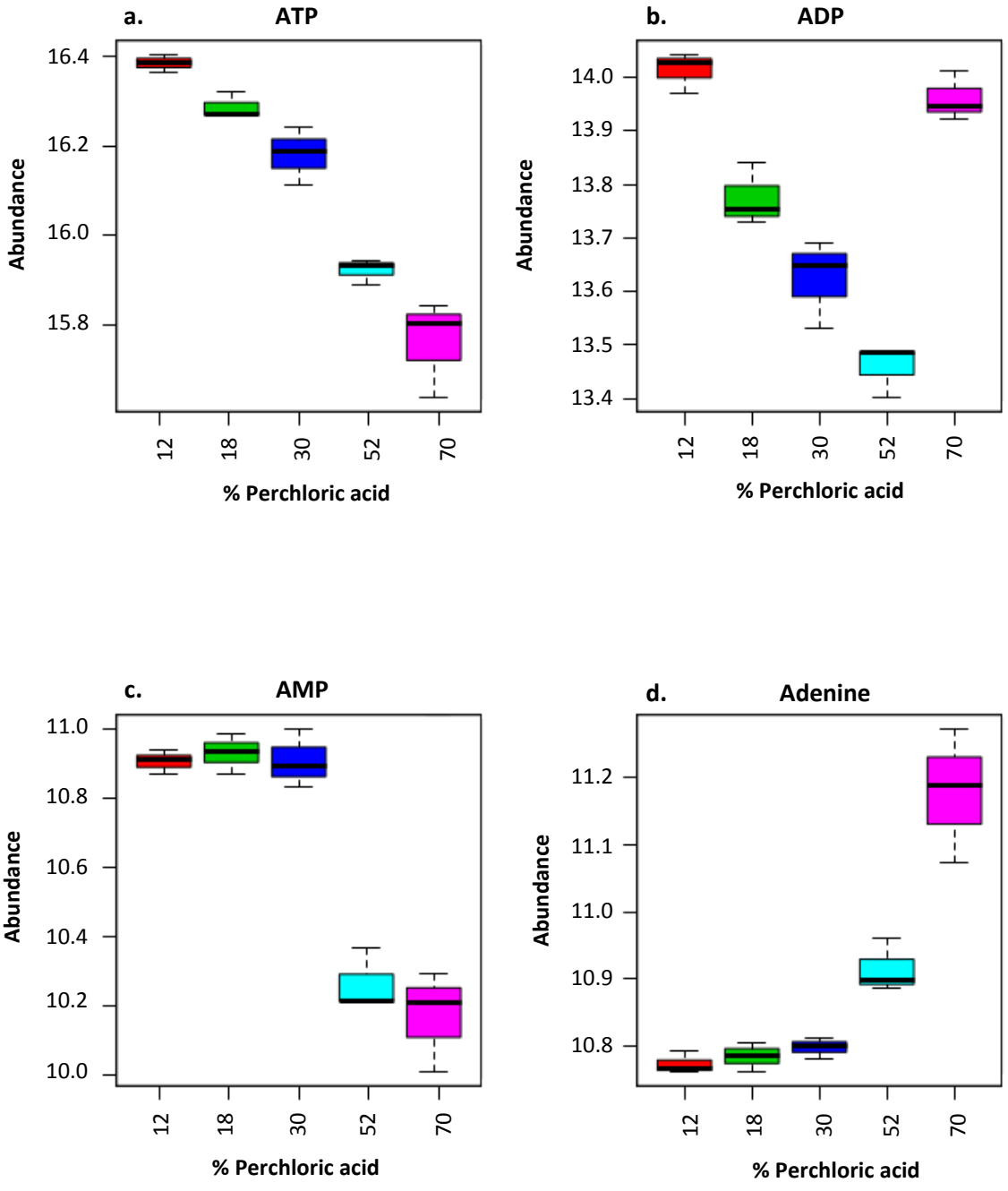
In an attempt to concentrate the nucleotides present in blood, decreasing volumes of different concentrations of perchloric acid were added to blood samples. The final perchloric acid concentration (6%) was the same for all the samples. The data obtained was subjected to statistical analysis as described in section 3.6.2.

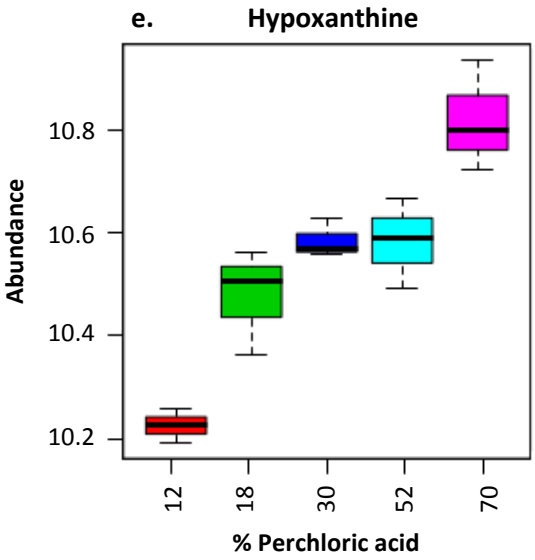
It was expected that the less diluted samples would have higher nucleotide abundances. However, the general trend seen in the data indicated that there was no difference between the 12%, 18%, 30% and 52% samples and that the nucleotide abundances either increased or decreased when 70% perchloric acid (lowest volume) was used.

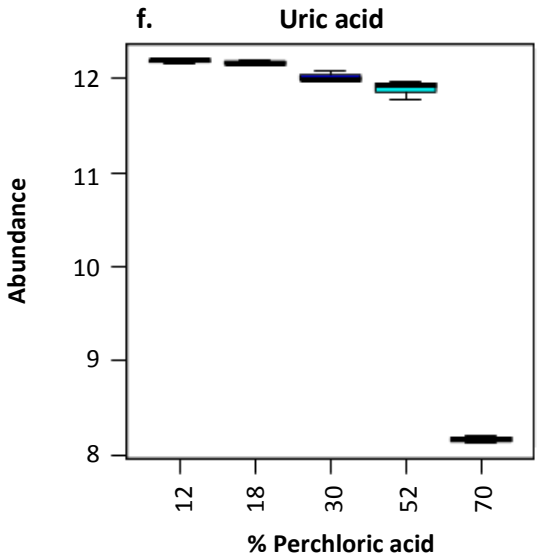
ATP, uric acid and NADPH (Fig. 4.7.a., f. and g.) showed no significant difference between the 12% and 18% perchloric acid samples and showed decreased abundances when higher concentrations of perchloric acid were used. Hypoxanthine (Fig. 4.7.e.) also showed no significant difference between the 12% and 18% perchloric acid samples but showed increased abundance when higher concentrations of perchloric acid were used. Adenine and NADP (Fig. 4.7.d. and h.) both showed no difference between the 12%, 18%, 30% and 52% perchloric acid samples however NADPH abundance decreased and adenine abundance increased when 70% perchloric acid was used. No difference was seen in the AMP (Fig. 4.7.c.) abundance when 12%, 18% and 30% perchloric acid were used. The

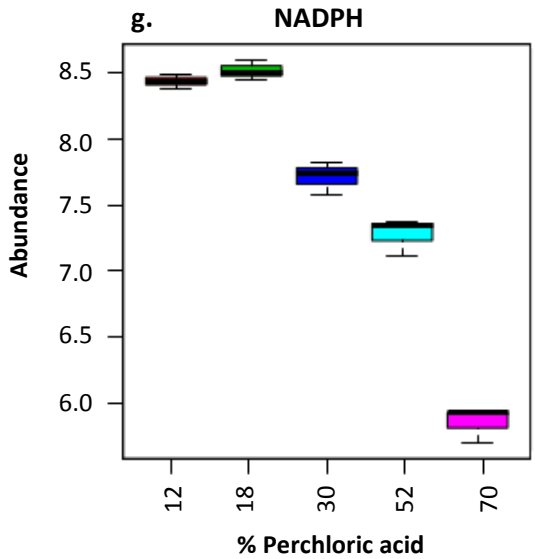
abundance of AMP decreased when higher concentrations of perchloric acid were used. NAD (Fig. 4.7.i.) had the highest abundance when 12% perchloric acid was used. Its abundance decreased when higher concentrations of perchloric acid were used.

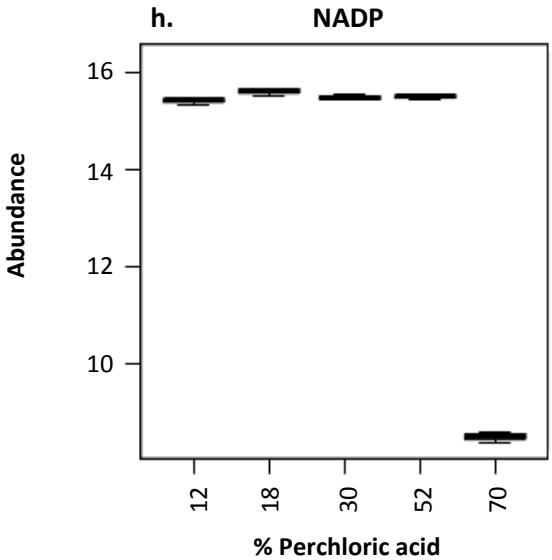
It can be concluded that the 47 µL of 70% perchloric acid added to the samples was a too low volume to thoroughly mix with all the blood thus resulting in some losses. As there was little increase in the abundances of the nucleotides when 18%, 30% or 52% of perchloric acid was used, it was decided to use 12% perchloric acid for further analyses.




e.
Hypoxanthine
Abundance
10.2
10.4
10.6
10.8
12
18
30
52
70
% Perchloric acid


f.
Uric acid
Abundance
8
9
10
11
12
12
18
30
52
70
% Perchloric acid


g.
NADPH
Abundance
6.0
6.5
7.0
7.5
8.0
8.5
12
18
30
52
70
% Perchloric acid


h.
NADP
Abundance
10
12
14
16
12
18
30
52
70
% Perchloric acid

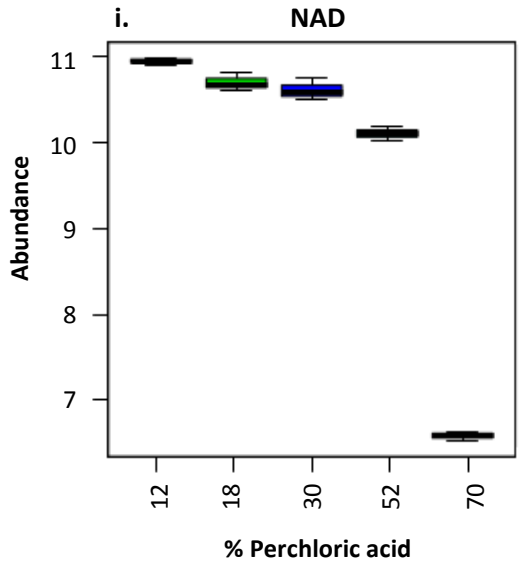


Figure 4.7: Deproteinisation of whole blood with different initial concentrations of perchloric acid. Decreasing volumes (500 µL, 250 µL, 125 µL, 65 µL and 47 µL) of increasing initial perchloric acid concentrations (12%, 18%, 30%, 52% and 70%) were added to blood samples in an attempt to concentrate the nucleotides. The final perchloric acid concentration (6%) was the same for all the samples.

4.2.6.5. Nucleotide stability in blood samples

Nucleotide stability in blood was analysed in two batches over a 24 hour period. The first batch was used to determine if perchloric acid could stabilise the nucleotide levels. This batch (referred to in text as samples with perchloric acid) was prepared by immediately adding perchloric acid to the blood sample aliquots after blood collection. The samples were then stored at 4°C and analysed at zero, two, four and 24 hours. The second batch was used to determine how long the nucleotide levels would remain stable in whole blood samples without perchloric acid. This batch (referred to in text as samples without perchloric acid) was prepared by dividing a whole blood sample into aliquots after collection and then storing these aliquots at 4°C. Perchloric acid was then added to different aliquots at zero, two, four and 24 hours after collection, before LC-ESI-MS/MS analysis. The data obtained from these two batches was subjected to statistical analysis as described in section 3.6.2.

ATP (Fig. 4.8.a.) levels remained stable for two hours after preparation in samples stored without perchloric acid while the ATP levels decreased over the two hour period in samples stored with perchloric acid. After four hours the ATP levels in the perchloric acid samples had decreased about 15% more than in the samples without perchloric acid. Although ATP decreased faster in the perchloric acid samples, there was no difference between the

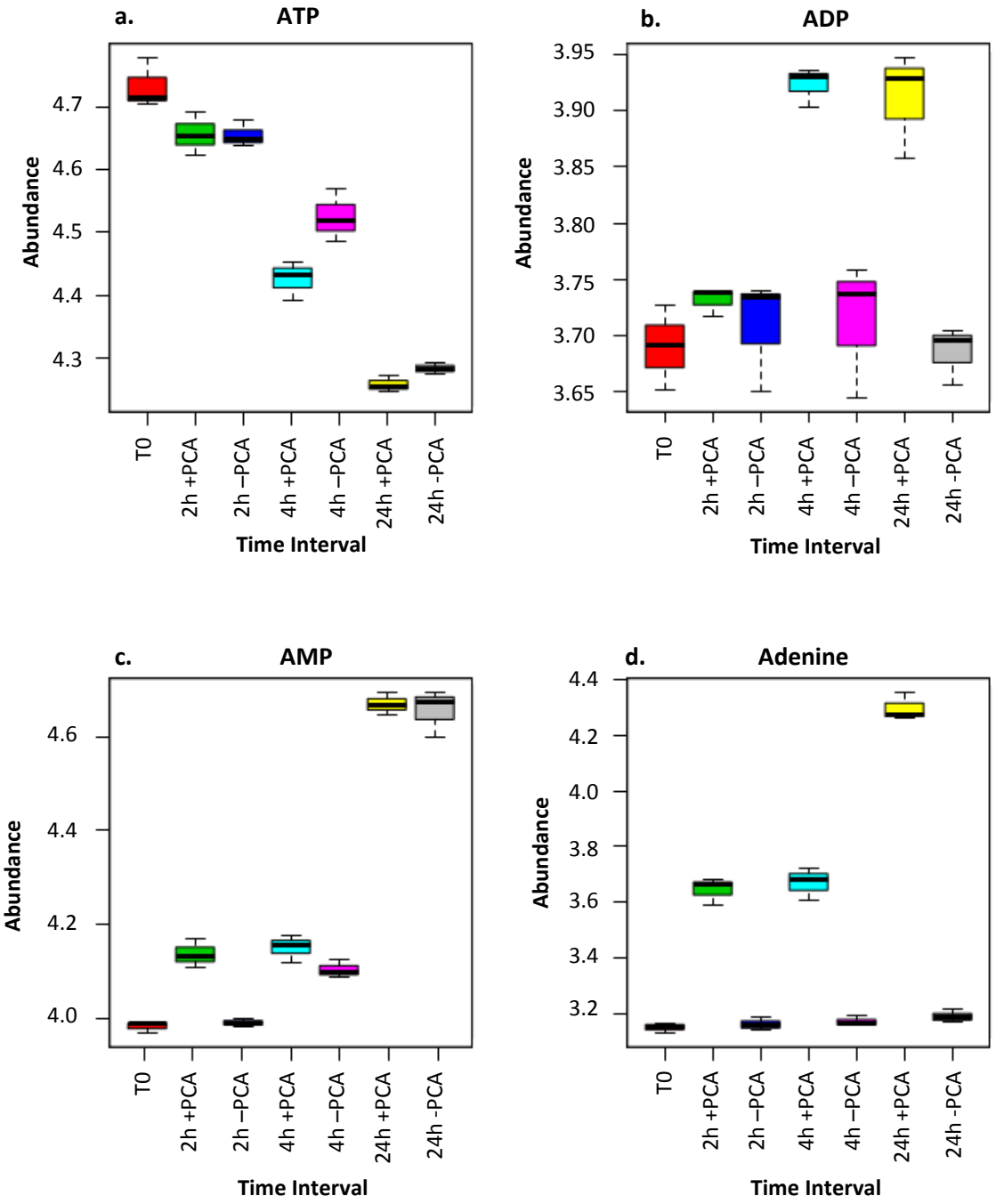
samples with and without perchloric acid after 24 hours. ADP (Fig. 4.8.b.) levels remained stable for the entire 24 hour period in samples without perchloric acid. However, the variation between the triplicate samples increased as ATP was hydrolysed to ADP. The ADP levels in samples with perchloric acid remained stable for two hours after preparation and then increased about 34% over the next two hours. The ADP levels stabilised after four hours and remained stable for the 24 hour period. AMP (Fig. 4.8.c.) levels were stable for two hours after preparation in samples without perchloric acid while it increased over the two hour period in samples with perchloric acid. After two hours there was no difference in the rate of increase between the samples with and without perchloric acid over the 24 hour period. Adenine, hypoxanthine and uric acid levels (Fig. 4.8.d., e. and f.) remained stable for the entire 24 hour period in samples without perchloric acid. In the samples with perchloric acid, adenine, hypoxanthine and uric acid levels started to increase over the two hour period after preparation.

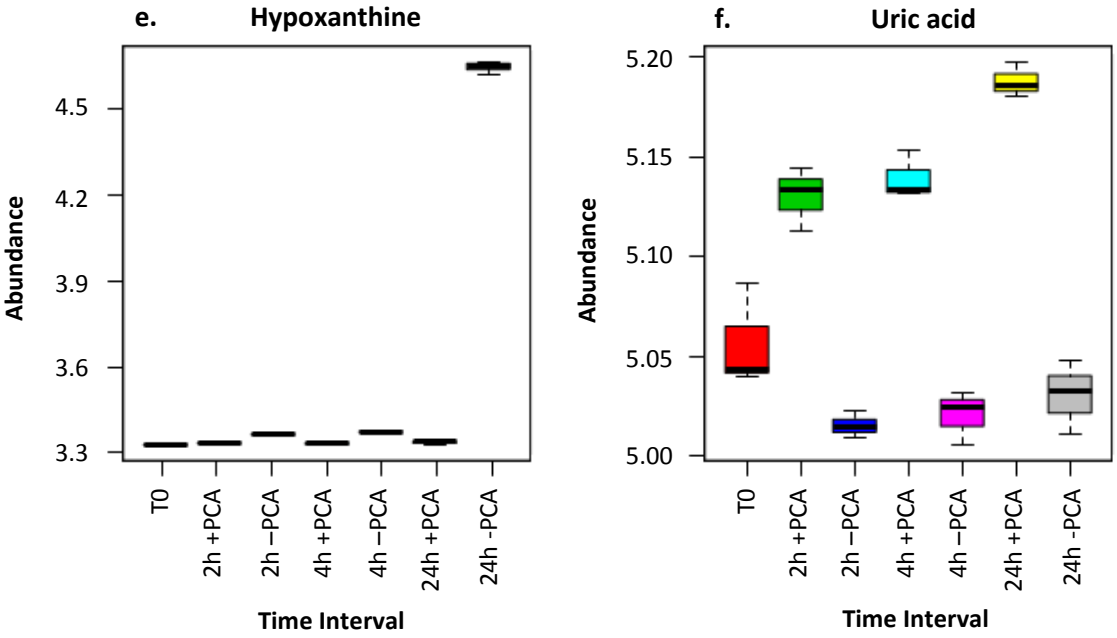
NADPH (Fig. 4.8.g.) remained stable for two hours and then decreased in samples without perchloric acid. In the samples with perchloric acid the NADPH levels decreased during the two hour period after preparation due to hydrolysis in the acidic conditions. Although NADPH levels decreased in both samples with and without perchloric acid, the NADPH levels decreased more over the 24 hour period in samples with perchloric acid. NADP (Fig. 4.8.h.) increased during the two hour period after preparation in both samples with and without perchloric acid. Although the NADP levels in the perchloric acid samples stabilised after two hours and remained stable for 24 hours, the variation between triplicate samples were very large due to hydrolysis of NADPH to NADP. After 24 hours there was no difference in the NADP levels of the samples with and without perchloric acid. NAD (Fig. 4.8.i.) remained stable in samples without perchloric acid for the 24 hour period. In the perchloric acid samples the NAD levels increased during the two hour period after preparation due to the hydrolysis of NADH to NAD in the acidic conditions. After two hours the NAD levels in the perchloric acid samples stabilised and then remained stable for 24 hours.

In the literature (Coolen *et al*., 2008; Klawitter *et al*., 2007) perchloric acid is frequently used for nucleotide extraction and the majority of articles report high levels of nucleotide recovery and precision. Others report significant degradation of ATP and the reduced pyridine nucleotides due to the perchloric acid (Au *et al.*, 1989; Grob *et al.*, 2003; Lazzarino *et al.*, 2003). However, very few of these studies tested the stability of nucleotides in the biological matrix after perchloric acid extraction. Klawitter *et al.* (2007) reported that nucleotides (ATP, ADP, AMP and NAD) extracted from rat kidney tissue with perchloric acid were stable for at least 24 hours at 4°C. Storage of the extracted samples at 4°C for five days caused a significant decrease in the ATP levels. Caruso *et al.* (2004) determined the stability of

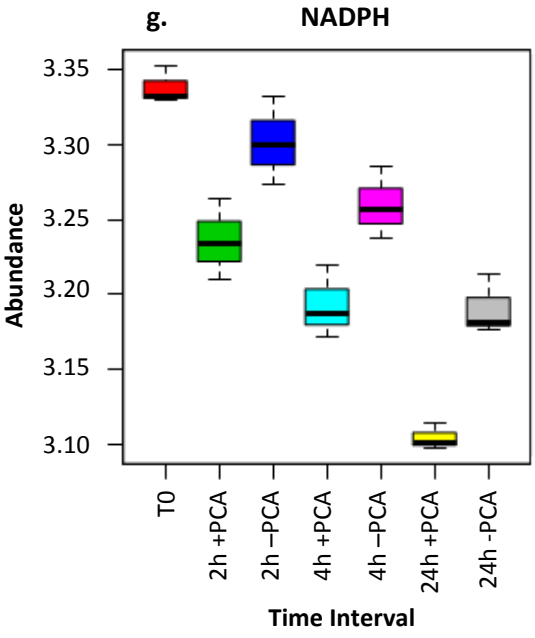
nucleotides (ATP, ADP, AMP, NADP and NAD) extracted from whole blood samples with perchloric acid. ATP levels were stable for 30 days when stored at -80°C while ADP and AMP levels increased progressively during the first day after blood sampling. NADP and NAD were stable for 4 days when samples were stored at -80°C or -20°C. Coolen *et al.* (2008) also extracted nucleotides from whole blood samples and reported efficient protein precipitation with perchloric acid and no significant loss of ATP.

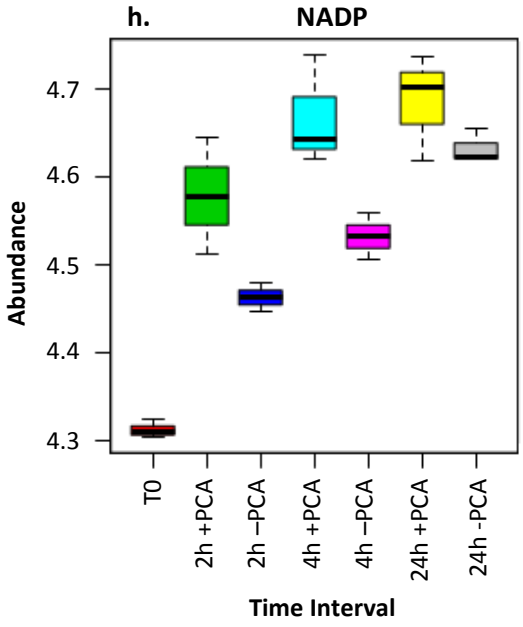
The results obtained from this study showed that perchloric acid did not stabilise ATP levels but resulted in faster degradation. The perchloric acid also caused increased oxidation of NADH and NADPH which correlates with the results reported by Caruso *et al.* (2004) and Lazzarino *et al.* (2003).




e.
Hypoxanthine
Abundance
4.5
4.2
3.9
3.6
3.3
T0
2h +PCA
2h −PCA
4h +PCA
4h −PCA
24h +PCA
24h -PCA
Time Interval

f.
Uric acid
Abundance
5.20
5.15
5.10
5.05
5.00
T0
2h +PCA
2h −PCA
4h +PCA
4h −PCA
24h +PCA
24h -PCA
Time Interval


g.
NADPH
Abundance
3.35
3.30
3.25
3.20
3.15
3.10
T0
2h +PCA
2h −PCA
4h +PCA
4h −PCA
24h +PCA
24h -PCA
Time Interval


h.
NADP
Abundance
4.7
4.6
4.5
4.4
4.3
T0
2h +PCA
2h −PCA
4h +PCA
4h −PCA
24h +PCA
24h -PCA
Time Interval

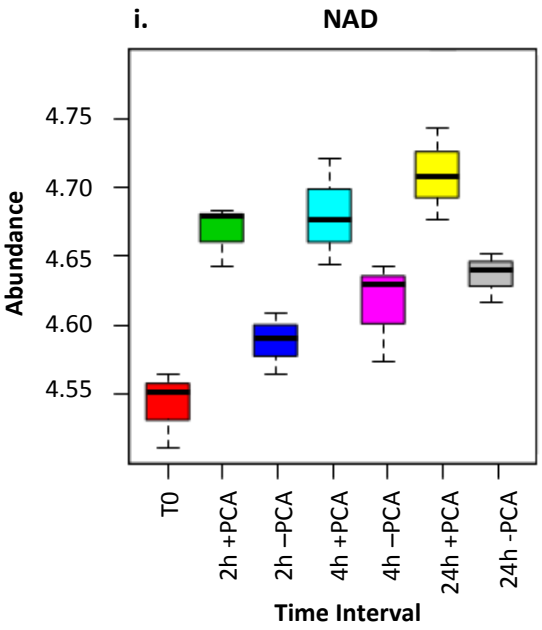


Figure 4.8: Nucleotide stability in blood samples at different time intervals. The stability of the nucleotides present in blood was evaluated at different time intervals over a 24 hour period. 12% Perchloric acid was added to the + PCA samples immediately after blood collection and stored at 4°C until analysis at 0h, 2h, 4h and 24h. The – PCA samples were stored at 4°C until 12% perchloric acid was added at 2h, 4h and 24h after blood collection.

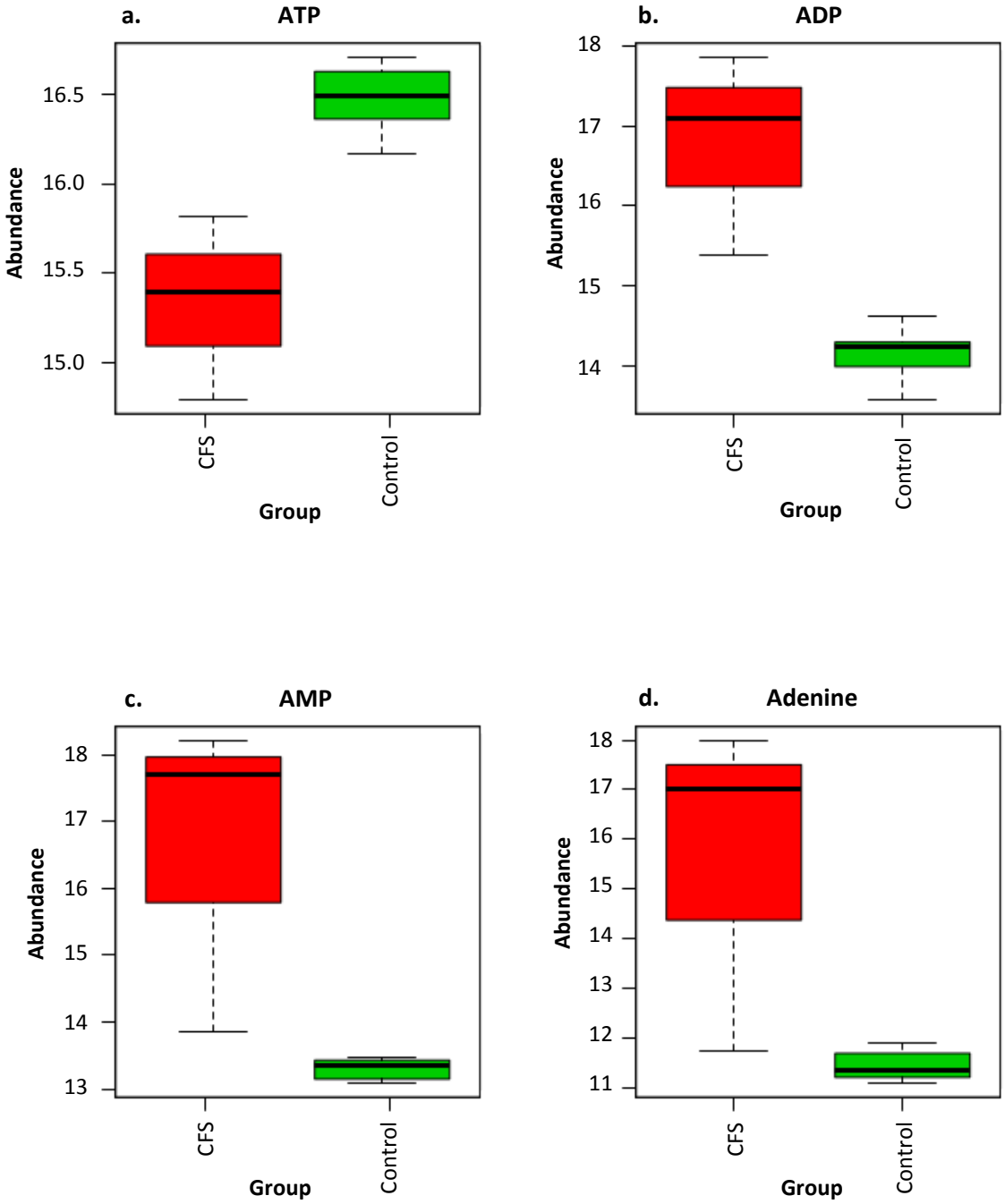
4.3. Method Application

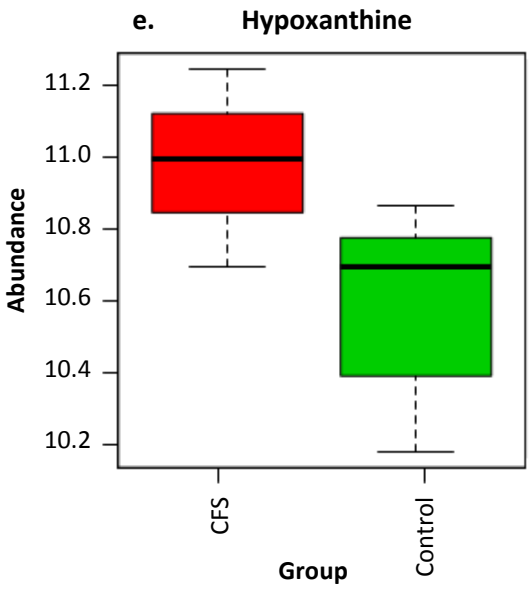
The proof of concept of the newly developed and validated method was tested by analysing and comparing the nucleotide levels of possible CFS patients and controls. As it was critical to prepare the blood samples immediately after collection in order to prevent nucleotide breakdown, the controls and possible CFS patients had to be nearby and available for blood collection. Due to lack of availability only a small number of possible CFS patient samples could be collected and used for this pilot study. The data obtained from the two groups were subjected to statistical analysis as described in section 3.6.2.

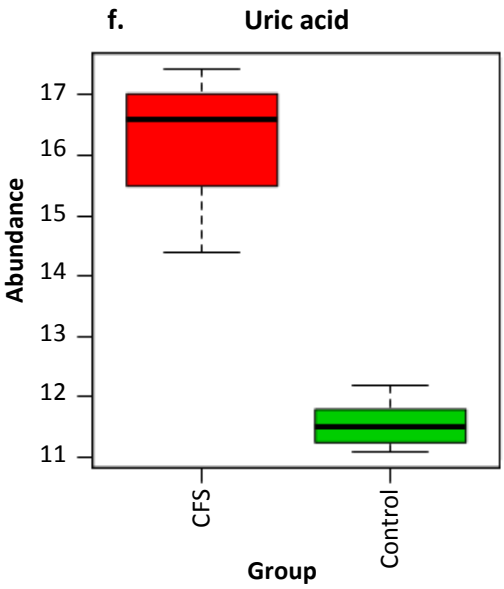
Comparing the nucleotide levels of the two groups, a general trend can be seen. The ATP (Fig. 4.9.a.) and oxidised pyridine nucleotide (Fig. 4.9. g.) levels were much lower than those of the controls. The degradation products of ATP (ADP, AMP and uric acid) as well as the reduced pyridine nucleotide levels appeared to be much higher in the CFS patients than in the control group (Fig. 4.9. b., c., f., h. and i.). Adenine and hypoxanthine (Fig. 4.9. d. and e.) levels were much the same for both groups. As illustrated by the principle component analysis (PCA) plot (Figure 4.10) there is a definite difference in the nucleotide levels of the control group and those of the possible CFS patient group.

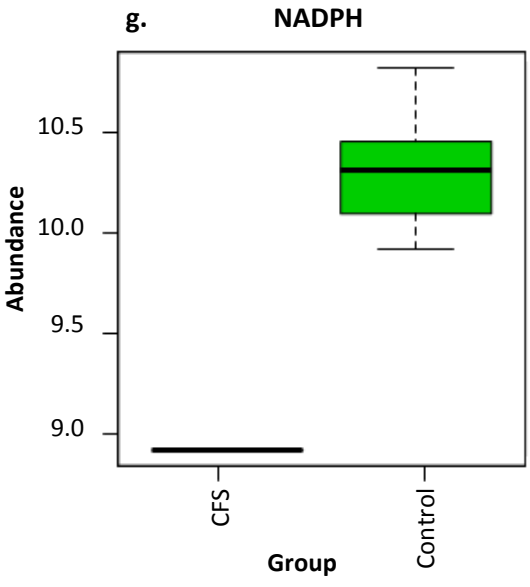
There is also significant variation in nucleotide levels within the CFS patient group as can be expected when patients are chosen based only on the results of a questionnaire. The possible CFS patient group was associated with higher levels of ADP, AMP, adenine, hypoxanthine, NADP and NAD while the control group was associated with higher levels of ATP.

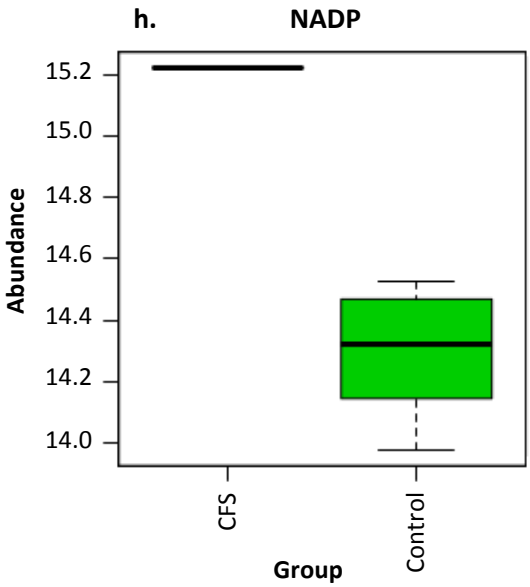
Thus by using the new LC-ESI-MS/MS method and the Piper fatigue scale it was possible to see a distinct difference between the controls and CFS patients, regardless of nucleotide instability and the changes in the nucleotide levels due to perchloric acid addition.




e.
Hypoxanthine
Abundance
11.2
11.0
10.8
10.6
10.4
10.2
CFS
Control
Group


f.
Uric acid
Abundance
17
16
15
14
13
12
11
CFS
Control
Group


g.
NADPH
Abundance
10.5
10.0
9.5
9.0
CFS
Control
Group


h.
NADP
Abundance
15.2
15.0
14.8
14.6
14.4
14.2
14.0
CFS
Control
Group

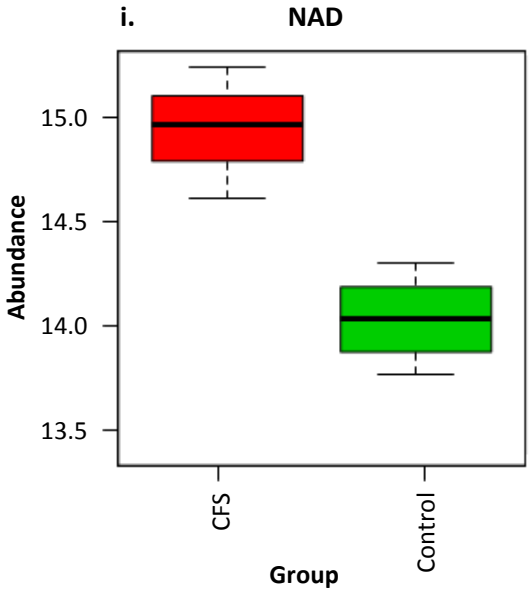


Figure 4.9: Comparison of nucleotide levels in possible CFS patients and controls. Blood nucleotide levels of 14 relatively healthy individuals and 3 possible CFS patients were analysed and compared. There is a general trend towards a decrease in ATP and oxidised pyridine nucleotide levels and an increase in ATP degradation products and reduced pyridine nucleotides.

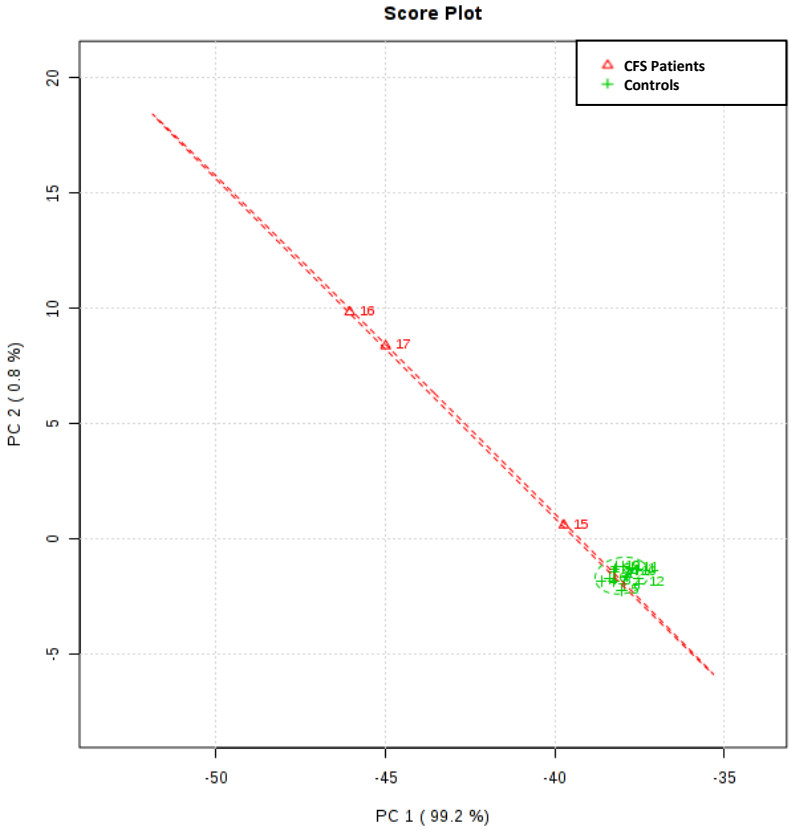


Figure 4.10: Principal components analysis (PCA) diagram illustrating the relationship between the control group and possible CFS patient group.

Chapter 5: Conclusion

5.1. Introduction

Adenine and pyridine nucleotides could be used as potential biomarkers for energy related disorders such as chronic fatigue syndrome because of their various functions in the energy and redox pathways. Due to the instability of nucleotides in biological matrices it is crucial to find a suitable extraction method in order to stabilise nucleotide levels prior to analytical analysis. A sensitive and reliable analytical method is then needed for accurate nucleotide quantification. The aim of this study was thus to develop and validate such an analytical method. As LC-ESI-MS/MS technology was available and is one of the more sensitive and accurate methods for nucleotide detection, it was used as a platform for method development. Thus the general objectives for this study included optimisation of LC-ESI-MS/MS conditions, optimisation of nucleotide extraction conditions and to test the proof of concept of the analytical method by comparing blood nucleotide levels of possible CFS patients and controls.

5.2. General discussion of results

5.2.1. Optimisation and validation of the LC-ESI-MS/MS method

The first part of the study focussed on optimising and validating a new LC-ESI-MS/MS method for detection of various nucleotides and nucleotide degradation products. Nucleotides presented a challenge for conventional reversed-phase HPLC due to their strong polarity. It was thus necessary to consider other alternatives more suitable for retaining and separating polar analytes. After various attempts, acceptable nucleotide separation was obtained on an Aqua-C18 column using 5 mM ammonium acetate (pH 7.2) and 10 mM ammonium acetate/ acetonitrile (pH 7.9) as mobile phases. Validation of the newly developed method demonstrated acceptable linearity ($r^2 \geq 0.995$) and precision (Intraday CV ≤ 5% and Interday CV ≤ 15%) for all nucleotides. The newly developed LC-ESI-MS/MS method was assessed to be sensitive and reliable for nucleotide quantification in blood samples.

5.2.2. Deproteinisation and nucleotide stability

Nucleotide instability in biological matrices is a well-known problem. Nucleotides are unstable due to the presence of nucleotidase enzymes that are responsible for nucleotide hydrolysis. It is thus necessary to precipitate the proteins from the biological matrix during

sample preparation in order to stop nucleotidase activity and nucleotide breakdown. Perchloric acid is frequently used for protein precipitation in a wide variety of matrices including whole blood samples as were used during this study. Due to the many contradictory results and conclusions given in the literature about the use of perchloric acid for nucleotide extraction, it was necessary to determine if the use of other protein precipitation solutions would result in less nucleotide breakdown and thus higher nucleotide abundances. Out of the two protein precipitation solutions used (perchloric acid and acetonitrile) during this study, perchloric acid extraction produced the highest nucleotide abundances. This could be due to possible nucleotide losses and degradation during the longer sample preparation process for acetonitrile extraction. Thus perchloric acid was used for all sample preparations during this investigation. In order to optimise the perchloric acid extraction method it was necessary to determine the best neutralisation agent to use for perchloric acid neutralisation as well as the optimal sample pH for nucleotide detection. KOH was found to be efficient for perchloric acid neutralisation without interfering during the LC-ESI-MS/MS analysis. Better peak shape and higher nucleotide abundances were observed when samples were neutralised to pH 6-7. Lower sample pH (pH 3) significantly worsened the peak shape and separation, and drastically decreased the nucleotide abundances.

After the extraction method was chosen and optimised, nucleotide stability was assessed. Nucleotide standards were found to be stable in water and in 6% perchloric acid for at least 24 hours. However, nucleotides were not stable in blood samples regardless of whether perchloric acid was added or not. Addition of perchloric acid to blood samples caused a decrease in ATP and NADPH levels, and an increase in ATP degradation products as well as oxidised pyridine nucleotide levels. NADH was extremely sensitive to the acidic conditions and could not be detected. The results obtained from this study contradict the results described in the literature (Caruso *et al.*, 2004; Coolen *et al.*, 2008; Klawitter *et al.*, 2007) and thus perchloric acid may not be the best choice for nucleotide extraction from whole blood samples.

Klawitter *et al.* (2007) suggested that the efficiency of protein precipitation may be matrix dependant as some matrices have higher nucleotidase enzyme activity. The temperature at which samples were stored, prepared and analysed is also a major contributing factor towards nucleotide instability. According to the literature (Caruso *et al.*, 2004; Coolen *et al.*, 2008; Klawitter *et al.*, 2007) nucleotides are more stable at 4°C as this slows down the enzymatic activity. As the samples for this study were left at 25°C in the auto-sampler, it could be a contributing factor to the nucleotide instability observed during the 24 hour period.

Another factor that caused significant variation in the nucleotide levels was the differences in volume between aliquots of the same blood sample due to pipetting error. Due to high viscosity and the difference in viscosity of the whole blood samples it was impossible to get the entire 500 µl sample out of the pipet tip, thus each aliquot had a different volume of blood. It was thus critical to gravimetrically aliquot each blood sample to be able to correct for the difference in volume when the data were processed.

5.2.3. Method application

The final part of this study tested the proof of concept of the newly developed LC-ESI-MS/MS method by measuring and comparing the nucleotide levels of controls and possible CFS patients. Due to a lack of a more quantifiable method, the Piper fatigue scale was used to assess the fatigue experienced by the two groups. As the Piper fatigue scale is an empirical questionnaire and thus based on the patient's feelings, opinion and experience, it was expected to see significant variation within the two groups. Not only was there variation within the two groups but there was also a significant difference between the possible CFS patients and the controls. The possible CFS patient group was associated with higher levels of ADP, AMP, adenine, hypoxanthine, NADP and NAD while the control group was associated with higher levels of ATP. Despite changes in the nucleotide levels due to perchloric acid and problems with nucleotide instability, it was still possible to distinguish between the two groups when the nucleotide levels were measured using the new LC-ESI-MS/MS method.

5.3. Future recommendations

- As perchloric acid causes significant degradation and does not stabilise nucleotide levels, other deproteinisation solvents should be considered. 5-Sulfosalicylic acid has been used for deproteinisation of plasma and whole blood for analysis of glutathione (Anderson *et al.*, 1980; Samiec *et al*., 1998), amino acids (Fekkes, 2012) and intermediary metabolites such as glucose, pyruvate and lactate (Khan *et al*., 1991). Sulfosalicylic acid could potentially be used for deproteinisation of whole blood samples prior to adenine and pyridine nucleotide analysis.

- As reduced pyridine nucleotides are highly sensitive to acidic conditions it is recommended that they are analysed separately from the adenine nucleotides. Caruso *et al.* (2004) reported successful alkaline extraction of NADPH and NADH with KOH.

- As temperature is a critical contributing factor to nucleotide stability, a temperature of 4°C should be maintained throughout sample preparation and analysis.

- It is necessary to find other ways to compensate for the differences in volume of blood aliquots due to pipetting error. One option would be to dilute a larger sample of blood with saline solution containing an internal standard before dividing into aliquots. This will make the blood less viscose and thus easier to aliquot, minimising pipetting error. Another option would be to use low retention pipette tips instead of normal pipette tips when dividing blood into aliquots.

- Another option for nucleotide extraction is to use gold nanoparticle technology. Huang *et al*. (2007) described successful analysis of glutathione and ATP in cell cultures with the use of aptamer-modified gold nanoparticles and laser desorption-ionisation mass spectrometry. According to Huang *et al.* (2007) this approach can be applied for the analysis of ATP and glutathione in more complex biological samples such as whole blood.

- Other possible markers such as acylcarnitines, lactic acid, reactive oxygen species (ROS), ferric reducing antioxidant power (FRAP) and glutathione levels for CFS patients and controls can also be determined and compared.

- It is also recommended that this method is applied to a larger group of possible CFS patients and controls to determine if the significant difference between the two groups is still present.

5.4. Conclusion

During this study an LC-ESI-MS/MS method for the quantification of selected nucleotides in whole blood samples, was successfully developed and validated. Based on the results obtained with the new LC-ESI-MS/MS method it was possible to distinguish between controls and possible CFS patients, despite problems with nucleotide extraction and stability. Thus the aims and objectives as set in chapter 1, was achieved. However, due the limitations caused by sample collection, preparation and nucleotide instability, it would not be practical to implement this method in routine analysis. Extensive further research is still required in order to find a way to stabilise nucleotide levels in blood and to prevent degradation.

Chapter 6: References

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