Re-assessment and optimisation of an organic acid extraction method for automation

MM Phiri

25264834

Dissertation submitted in partial fulfilment of the requirements for the degree *Magister Scientiae* in Biochemistry at the Potchefstroom Campus of the North-West University

Supervisor: Prof BC Vorster

Graduation October 2017

ACKNOWLEDGEMENTS

I would like to thank the following people without whom this study would not have reached this point.

Firstly, my supervisor, Prof. Chris Vorster for the guidance, motivation and tolerance granted during the course of the study. I am deeply indebted to him.

I would like to thank Dr Zander Lindeque for all the analytical help and support given to me. His door was always open when I had questions or needed assistance.

Thanks to Mrs Clarina Vorster (Accredited member of the South African Translations' Institute: SATI) for editing the language and spelling of my dissertation.

Thanks to Mrs Lurinda Ras for all the technical help granted during the experimental phase of the study.

A special thanks to the staff of PLIEM, Dr. Marli Dercksen, Mr. Jano Jacobs and Ms. Elmarie Davoren, to mention but a few, for all the help rendered and experience shared during the course of my studies.

Great appreciation goes to the BOSS personnel, Mr. Peet Jansen van Rensburg and Cecil Cooke for their support, encouragement and assistance in allowing me to run my samples on their GC/MS machine.

I would like to acknowledge all the personnel of the Biochemistry Department for playing a role in one way or another, adding to the success of my studies.

I also want to thank the North-West University, for providing a safe place to study and the bursaries granted to help fund my studies.

I offer gratitude to all my colleagues, friends and family for the moral support rendered to me.

A special mention goes to my wife, Buya Ennie Phiri, for accompanying me on this journey, through the highs and lows and for all her prayers. Last but not the least, my heart is full of gratitude to the Lord for His grace that enabled me to study and make it this far.

ABSTRACT

Sample preparation is a necessary prerequisite for GC/MS analysis of urinary organic acids for clinical diagnosis of inborn errors of metabolism (IEM). The sample clean-up step in the analytical process poses a challenge. It involves the isolation of the analytes of interest by an extraction process from a complex matrix to one that is more suitable for the analytical platform. As opposed to a fully automated and high-throughput sample preparation protocol, the existing in-house urinary organic acid extraction method is still performed manually. It is a labour-intensive and time-consuming, requires multiple exhaustive pipetting steps and uses large amounts of toxic solvents that can be hazardous to health. Literature documents the progress made in miniaturising and automating the solvent extraction, but scarce literature is available on how this has been applied to the urinary extraction of organic acids. Thus, the development of a method that can be fully automated would improve the sample throughput and eliminate the intense labour and most of the other setbacks associated with manual extraction.

The aim of the study was to reassess the in-house organic acid extraction method and optimise it for automation. The experimental workflow involved the selection of an initial suitable miscible solvent for rapid extraction of organic acids and one that would enable better extraction of very polar organic acids. This was followed by the selection of a suitable immiscible solvent that would ensure good isolation of organic acids, quick evaporation and clear phase separation that would render centrifugation unnecessary. The solvent ratios and volumes were optimised and miniaturised to small volumes of solvents. The miniaturised organic acid protocol was translated into a fully automated extraction procedure on a liquid AutoSampler. The automated method was validated for linearity, imprecision, recovery and inaccuracy.

A two-phase extraction system using two optimal solvents, acetonitrile and ethyl acetate, were found to be efficient in the extraction of urinary organic acids. It enabled efficient and rapid extraction. The analytical range of the method for most of the analytes was established to be between 1 – 500 mg/l. The correlation coefficient (*r)* of all analytes was generally > 0.99, with two exceptions. The analytical ranges of the specific analytes showed that the test results within these ranges are reliable and can be reported. The repeatability was generally below 20%, but had higher within-laboratory precision. The automated method's overall imprecision was better than the

in-house method. The inaccuracy of the method was determined by a method comparison experiment with ERNDIM EQAS samples for quantitative organic acids. The mean of the test results was compared to the mean of all the laboratories. The proportional systematic error of the method ranged from -0.18 to 2.06. The constant systematic error for the analytes was -5.75 and 5.66. The total error of the method determined demonstrated a reduction in random and systematic errors when compared to the current in-house manual method. It was also noted that the correlation coefficient between the new method and the expected results was substantially better when compared to the current in-house method and by implication that the regression model fit was substantially better. This creates an opportunity for bias correction through the use of extraction factors, instrument response factors, the use of external calibration curves or reassignment of standard/calibrator concentrations for the new method as opposed to the current in-house method where this is not an option.

Based on the findings in this study, it was concluded that an automated procedure for LLE of urinary organic acids was successfully developed. The goal of having a method that could give consistent extraction and meet the criteria for automation was achieved. All the extraction steps were optimised and the method proved to have good extraction efficiencies for organic acids and to improve on the performance of the existing in-house method.

KEY WORDS

- Organic acids
- Organic aciduria
- Liquid-liquid extraction
- Miniaturization
- Automation
- Method validation
- Inaccuracy
- Imprecision
- Sigma metric
- Optimisation
- Extraction efficiency
- Recovery

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Chapter 2

Chapter 3

Chapter 5

ABBREVIATIONS

A

ACN	-	Acetonitrile
Aq	-	Aqueous phase

B

BSTFA	-	*N*, *O*-bis(trimethylsily)trifluoroacetamide

C

CV	-	Coefficient of Variation

D

D	-	Distribution ratio
DLLME	-	Dispersive liquid-liquid micro-extraction

E

E_A	-	Extracted fraction
EtAc	-	Ethyl acetate
EQA	-	External Quality Assurance
ERNDIM	-	**E**uropean **R**esearch **N**etwork for evaluation and improvement of screening, **D**iagnosis and treatment of **I**nherited disorders of **M**etabolism

G

GC	-	Gas Chromatography
GC/MS	-	Gas chromatography-mass spectrometer
GHB	-	γ-Hydroxybutyrate
GLUT1	-	Glucose Transporter Type 1

H

HCl	-	Hydrochloric acid
HF-LPME	-	Hallow-fiber liquid-phase micro-extraction

I

IEM	-	Inborn error of metabolism
IMD	-	Inherited Metabolic Disorders

IS	-	Internal standard
K		
K_D	-	Distribution coefficient
L		
LLE	-	Liquid-liquid extraction
LLME	-	Liquid-liquid micro-extraction
LOD	-	Limit of detection
LOQ	-	Limit of quantification
LPME	-	Liquid-phase micro-extraction
M		
MS	-	Mass Spectrometry
O		
OA	-	Organic acid
Org	-	Organic phase
P		
PLIEM	-	Potchefstroom Laboratory for Inborn Errors of Metabolism
S		
SBSE	-	Stir-Bar Sorptive Extraction
SD	-	Standard Deviation
SDME	-	Single-drop micro-extraction
SOP	-	Standard operating procedure
SPE	-	Solid-phase extraction
SPME	-	Solid-phase micro-extraction
T		
TCA	-	Tricarboxylic acid
TEa	-	Total Error Allowable
TIC	-	Total ion chromatogram
TMCS	-	Trimethylchlorosilane

CHAPTER 1: INTRODUCTION

1.1. Background

The analysis of urinary organic acids (OA) by the use of gas chromatography-mass spectrometer (GC/MS) is a widely-used method for the diagnosis of organic acidurias (Kumps *et al*., 1999). This has been the case since the 1960's when Dr. Tanaka and colleagues isolated and identified urinary *N*-Isovalerylglycine for the diagnosis of an inborn error of metabolism (IEM) (Tanaka & Isselbacher, 1967). The coupling of flame ionization to GC as the detector provided sufficiently high efficiency resolution for numerous organic acids; however, the detector lacked specificity and could not identify a number of compounds (Tanaka *et al*., 1980).

Hence the coming of the hyphenated technique of GC/MS resolved this problem. It did this by improving the detection and identification of many abnormal metabolites in human urine that were previously unknown. The advantages for the GC/MS over other techniques, such as liquid chromatography–mass spectrometry and tandem mass spectrometry, are well established. Some of these advantages are the availability of commercial spectra libraries, good chromatographic resolution and a long period of field experience, excellent selectivity and resolution (Kaluzina-Czaplinska, 2011).

Urinary organic acid analysis by GC/MS is an indispensable tool in the diagnosis of inborn errors of metabolism (IEM). Organic acidurias as a subgroup of IEMs are characterized biochemically by the accumulation of organic acids largely in urine and to a less extent in other body fluids (Blau *et al*., 2014). Many inborn errors of metabolism are as a result of loss of function of a specific enzyme (or gene product). This normally results in an alternative pathway leading to an accumulation of metabolites that are not present under physiological conditions (Blau *et al*., 2014, Kaluzina-Czaplinska, 2011) or alternatively, the accumulation of pathological amounts of normal metabolites. When an infant screens positive for one of the IEM, or has a family history with a high-risk for these disorders, urinary organic acid analysis by GC/MS is necessary to provide a wide range of metabolite profile in order to make an accurate and confirmatory diagnosis.

The major challenge of the urinary organic acid analysis by GC/MS is the requirement of good sample preparation and derivatization before analysis. Sample preparation is essential to the whole analytical process. It has major effects on the metabolite coverage and the quality of the results obtained (Raterink, 2014). The biological interpretation of the data depends on it as well. Sample preparation is the step that determines the rate of the

analytical process. It can be time-consuming, labour-intensive and error prone. And due to lack of conventional and standardized method of doing it, it is one of the major causes of inconsistencies between laboratories (Kaluzina-Czaplinska, 2011, Álvarez-Sánchez *et al*., 2010).

One of the essential components of this sample preparation is the extraction of organic acids from the subject's urine. There are various methods of extraction. Some of the methods include liquid-liquid extraction (LLE), solid-phase extraction (SPE), solid phase micro-extraction (SPME) and liquid-phase micro-extraction (LPME). The most used method for organic acids extraction from urine is by liquid-liquid extraction with the aid of organic solvents (Peters *et al*., 2008).

Optimizing and automation of the Liquid-Liquid Extraction of urinary organic acids would ameliorate many setbacks of the method. Little progress however has been reported on the automation of the sample preparation using LLE for urinary organic acids (Clement & Hao, 2012). There are a number of steps in the extraction protocol that pose as a challenge in the automation of the extraction protocol. But when these challenges are circumvented, a fully optimised automated extraction protocol for urinary organic acids will have an immerse number of benefits including high sample throughput, decreased labour costs, reduction of random errors caused by human sample handling, good precision and cost savings in the long term.

1.2. Problem Statement

Organic acid extraction is an important sample preparatory step in the analytical process. Its effectiveness directly affects the quality and interpretation of the final data obtained. The currently used in-house organic acid extraction method is performed manually. It is labour intensive. It largely involves exhaustive and time consuming pipetting steps, rotary shaking, centrifugations and the use of large solvent volumes during extraction. This affects the sample throughput and variability of the test results.

1.3. Justification

Thus, there is a need for the development of a LLE method for organic acids that can be automated. This will reduce the solvent volumes used during the extraction, increase the sample throughput and eliminate the multiple pipetting steps and centrifugations, reduce variability and random errors. Such methods have been developed for lipid extraction such as the BUME method by Lofgren and colleagues (Löfgren *et al*., 2012) and the simplified method for the chemical diagnosis of organic aciduria using GC/MS by Nakagawa *et al*

(Nakagawa *et al*., 2010). These methods were developed based on the physicochemical properties of the solvents and the analytes and by the application of sound chemical principles.

1.4. Structure of Dissertation

The dissertation is a compilation of chapters written in accordance with the North-West University, Potchefstroom Campus requirements for the completion of the Masters of Science in Biochemistry in dissertation format.

Chapter one gives a brief background to the study. The problem statement and substantiation for the study are highlighted here in some brief detail. A description of the layout of the dissertation is also included in this chapter. Chapter two discusses the nature of organic acids in man, their distribution in the physiological fluids and their endogenous and exogeneous origins. The definition, pathophysiology, types, clinical manifestation and diagnosis of organic acidurias are also summarised. An overview of the methods and theories of organic acid extractions is given and a brief discussion of the method validation steps and the experimental outline follows after that.

Chapter three states the aim for which the study was carried out and the objectives for the accomplishment of the aim are summarised. Chapter four contains the material and methods used in the study. This covers the methods utilized for the identification of organic acids and for the development and the validation of the method.

The results obtained in the method development and optimisation of the organic acid extraction method are shown and discussed in Chapter five. The chapter concludes with a summary of the developed and optimized method. In Chapter six the results obtained from a series of validation assessments are shown and discussed. Chapter seven provides a comprehensive conclusion of the results obtained in Chapter 5 and 6. Recommendations and future prospects for follow-up studies are also discussed in the chapter.

CHAPTER 2: LITERATURE REVIEW

2.1. Organic Acids in Man

2.1.1. Nature of organic acids

Organic acid is a term that refers to a wide range of compounds that are involved in the human metabolic processes (Kaluzina-Czaplinska, 2011). They are intermediary products of the metabolism of amino acids, sugars, biogenic amines, steroids, lipids and many other compounds, both endogenous and exogenous (Goodman & Markey, 1981). They are carboxylic acids that have a keto, hydroxyl, or other non-amino functional group (Chalmers & Lawson, 1982). They are distinguished from amino acids because they do not contain any amino groups. However, some nitrogen-containing compounds are considered as organic acids, such as pyroglutamate, shown in Figure 1.1b (Blau *et al*., 2014, Kaluzina-Czaplinska, 2011). They may also contain acidic phenolic groups. The general formula for organic acids is R-COOH; where R refers to the rest of any possible molecule (Blau *et al*., 2014). The structure of an organic acid is shown in Figure 1.1a.

Organic acids are low molecular weight, water-soluble organic compounds. They are characterized as weak acids; i.e. they do not fully dissociate to produce H^+ cations in a neutral water solution but at a pH of more than 4, they fully ionised for practical purposes (Blau *et al*., 2014). This makes the organic acids strongly hydrophilic and enables them to be excreted into urine. Organic acids in their physiological state are often present as their coenzyme A esters. Some good examples of this are propionyl-CoA and isovaleryl-CoA. Some acids, however, are always seen in their free form, e.g. pyruvic and 2-ketoglutaric acids.

Organic acids are widespread in nature and are often combined with other functional groups (William, 2013). Organic acids can be mono-, di- and tricarboxylic in nature. Figure 1.1c to d shows some examples of some of the organic acids. Simple acyl organic acids are typically composed of two to ten carbon atoms. They are liquids and have low melting points. Short-chain fatty acids are also included among the organic acids group. Long chain fatty acids with carbons greater than 8 are considered to be nonpolar organic acids. They are mostly bound to plasma proteins and are not excreted into the urine. Examples of these fatty acids are lauric, palmitic and stearic acids (Blau *et al*., 2014).

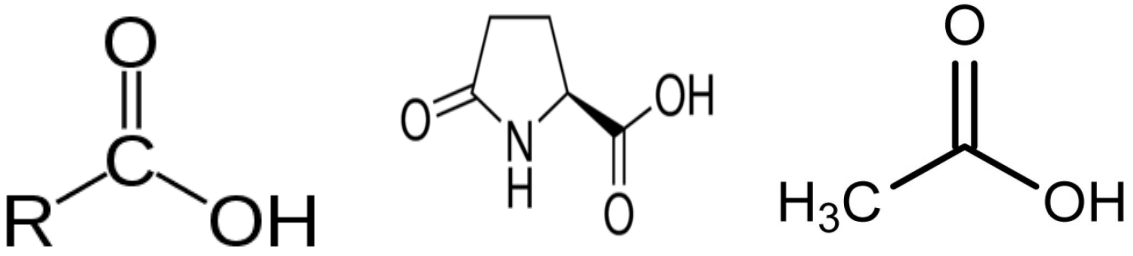
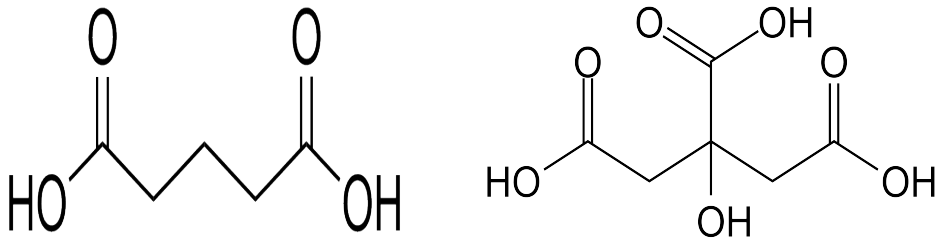


Figure 2.1 a. **b.** **c.**



d. e.

Figure 2-1 a. The general structure of an organic acid; b. pyroglutamate; c. acetic acid – a monocarboxylic acid; d. glutaric acid – a dicarboxylic acid and e. citric acid - a tricarboxylic acid

2.1.2 The distribution of organic acids in physiological fluids

Organic acids are found in the blood, cerebrospinal fluid, amniotic fluid, urine and saliva (Nordmann & Nordmann, 1961). Urine provides an averaged pattern of easily excreted polar metabolites discarded from the body as a result of catabolic processes that includes organic acids (Álvarez-Sánchez *et al*., 2010). In urine, more than 250 organic acids and glycine conjugates are typically present. Citric acid was one of the first organic acids to be reported in urine in 1917 (Chalmers & Lawson, 1982). Since then with the development of better analytical and detection techniques, many other organic acids have been reported.

Urine is the best fluid for the analysis of organic acids because their concentration in urine is much more than in blood. Urine is also favourable for this purpose due its lack of protein in it. This makes the analysis of the sample much easier. Lastly, it is easier to collect a urine sample because it is non-invasive and the sample is usually adequate for analysis (Baena *et al*., 2005).

2.1.3. Origin of organic acids

2.1.3.1. Endogenous and exogenous origin of organic acids

Organic acids are key components of virtually all pathways of intermediary metabolism (Figure 1.2). They are intermediates of metabolism pathways such as those of carbohydrates, fatty acids, amino acids, purines and pyrimidines, cholesterol and neurotransmitters. According to Kloos *et al*. (2014) endogenous organic acids can be sub-classed into four groups: the small organic acids that are crucial to aerobic respiration and energy metabolism; fatty acids that are fundamental to energy storage and membrane formation and involved in numerous physiological processes; eicosanoids and docosanoids that form a class of very important signalling molecules during several inflammatory and immunological events; and lastly bile acids that are the main metabolites of endogenous cholesterol.

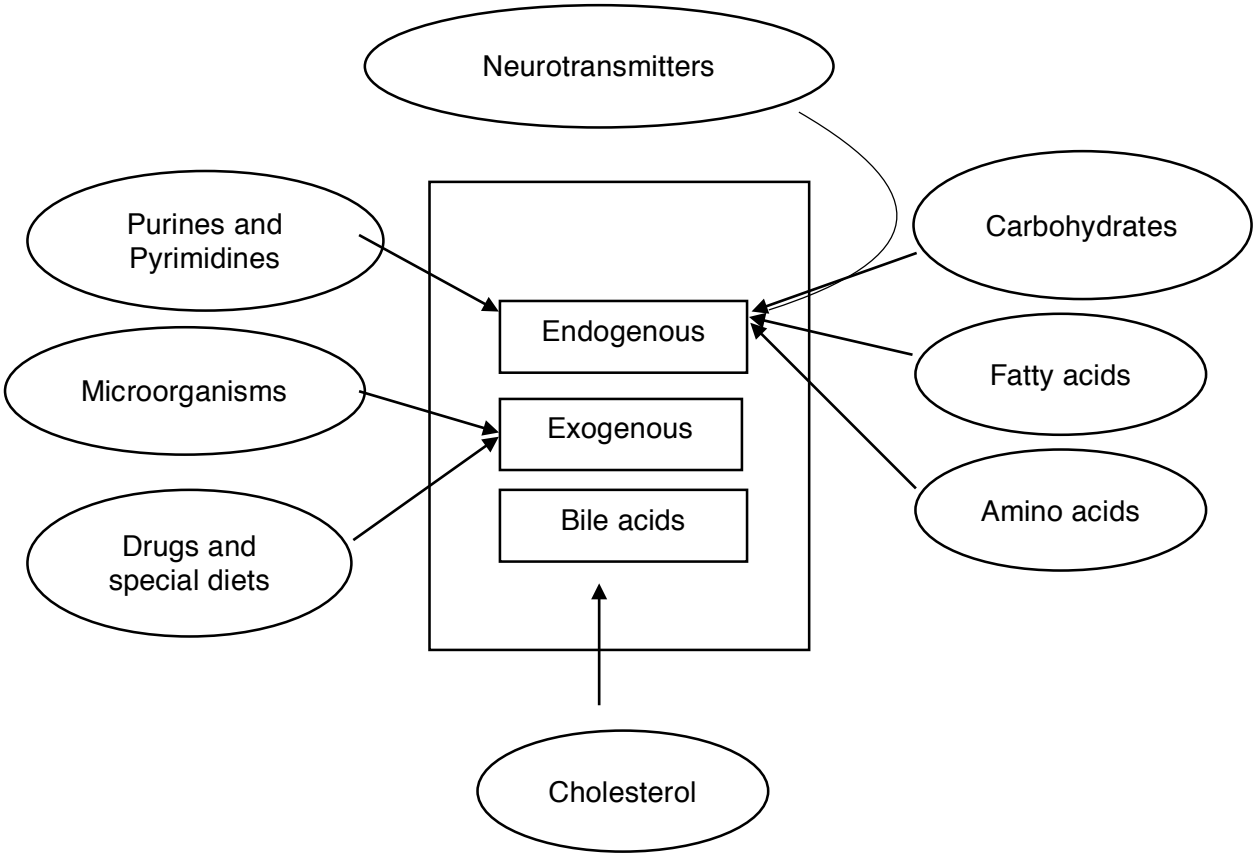


Figure 2-2 Organic acids in small molecule intermediary metabolism (Adapted for Blau *et al.,* 2003)

Organic acids are also key metabolites of exogenous compounds (Baena *et al*., 2005). Intestinal flora is a possible source of exogenous organic acids in the body. Examples of these organic acids are D-lactate, (which cannot be distinguished from L-lactate chromatographically), 3-hydroxypropionic acid, 4-hydroxyphenylacetic acid, 2-oxoglutaric acid, phenylpropionylglycine, succinic acid and uracil (Blau *et al*., 2014).

Medications are another source of exogenous organic acids. For example, intravenous solutions are a source of propanediol, L-DOPA of vanillinlactic acid, acetylsalicylic acid of 2-hydroxyhippuric acid and valproic acid is a source of numerous metabolites including dicarboxylic acids (Blau *et al*., 2014). Medium-chain triglycerides when taken orally increases saturated even-numbered dicarboxylic acids, mainly sebacate. Therefore, differential diagnosis in organic aciduria is critical for right diagnosis (Kumps *et al*., 2002). Adipic acids and a few others are sometimes from dietary origin (Blau *et al*., 2014).

2.2. Organic Acidurias

2.2.1. Definition of organic acidurias

Organic acidurias (or acidemias) are inherited metabolic disorders (IMD) that result in the accumulation and excretion of non-amino organic acids in urine (Vaidyanathan *et al*., 2011, Seashore, 2009). These disorders arise due to dysfunctions in specific enzymes in the intermediary metabolic pathways of amino acids, fatty acids oxidation and carbohydrates. Certain organic acids accumulate to toxic levels in body tissues that result in the pathology of the disorders. Some organic acids accumulate in blood shifting the pH to lower values causing metabolic acidosis (Blau *et al*., 2014).

2.2.2. Pathophysiology of organic acidurias

When there is severe deficiency of enzyme activity in a metabolic pathway, the body channels the excess intermediates through alternative pathways. This inevitably leads to abnormal metabolites accumulating in these pathways. The main biochemical mechanisms that lead to accumulated amount of organic acids in IMDs can be summarized into three. The first one is the accumulation of normal metabolites in a particular pathway before a blockage due to a dysfunctional enzyme. The second one is the accumulating of other normal metabolites from upstream pathways that fail to feed into the blocked pathway. The third and last one is the formation of abnormal metabolites when excess intermediates are channelled through metabolic pathways they don't normally use (Jones & Bennett, 2010).

The pathophysiology of these disorders therefore results either from an accumulation of precursors and deficiency of products of the affected pathways. Some of the accumulated metabolites are themselves toxic or are metabolized to produce toxic compounds. The toxicity of these compounds in the body organs like the brain, liver, pancreas and other organs is what brings about the pathophysiology of organic acidurias. Additionally, energy deficiency may add to the clinical syndrome due to defects in amino acid catabolism that provides energy for cells (Seashore, 2009).

2.2.3. Types and clinical manifestations of organic acidurias

Among the numerous types of organic acidurias that exist, methylmalonic aciduria, propionic acidemia and isovaleric acidemia are of the most prevalent forms (Organic Acidemia, 2016). These disorders have been categorized into five groups, which include branched chain organic acidemias, glutaric acidurias, fatty acid oxidation defects, disorders of energy metabolism and multiple carboxylase deficiencies (MCD). Most of the organic acidurias are inherited in an autosomal recessive manner, with a few X-linked forms that have been described (Organic Acidemia, 2016).

Leonard *et al*, (2011) state that although IEM have various ways and ages of presentation, there are five most common ways in which they present clinically: 1) neurological which includes acute encephalopathy, seizures, stroke-like illness and acute ataxia, 2) hypoglycaemia, 3) disorders of acid-base regulation, marked by ketosis or persistent acidosis after tissue perfusion is corrected, 4) cardiomyopathy and cardiac arrhythmias with hypertrophy and lastly 5) acute liver disease, which presents as hypo-albuminaemia, clotting abnormalities, conjugated hyperbilirubinaemia and other abnormalities caused by failure of absorption of fat soluble vitamins. The neonate may present with a metabolic disorder at birth or soon after that with a number of additional features such as ascites, dysmorphic syndromes, seizures and severe hypotonia (Leonard & Morris, 2011).

2.2.4. Diagnosis of organic acidurias

In addition to the major clinical presentations already discussed, urinary organic acid analysis by GC/MS is an indispensable tool in the diagnosis of inborn errors of metabolism (Vaidyanathan *et al*., 2011, Duez *et al*., 1996). When an infant that screens positive for one of the IEM during routine new-born screening, presents the above outlined clinical picture or has a family history with a high-risk for these disorders, urinary organic acid analysis by GC/MS is necessary to provide an accurate and confirmatory diagnosis (Blau *et al*., 2014). Good sample preparation is a required step in the analytical process of urinary organic acid analysis by GC/MS.

Freshly collected urine is the preferred sample for the analysis of organic acids profile. The organic acids must first be extracted from the urine in order to be analysed. This sample preparation process isolates the sample from its matrix and makes it suitable for GC analysis by making it more volatile through derivatization. Historically, the data was processed and abnormal peaks of organic acids that are many times more than their reference range gave indication of the defect present (Blau *et al*., 2014). But nowadays, there is need for quantitative organic acid analysis by GC/MS that recognises small increases of specific organic acids (Blau *et al*., 2014).

2.3. Organic Acid Extraction

There are a number of methods used for the isolation of organic acids from urine. Some of the methods include liquid-liquid extraction (LLE), solid-phase extraction (SPE), solid phase micro-extraction (SPME) and others besides (Kaluzina-Czaplinska, 2011).

2.3.1. Liquid-Liquid Extraction (LLE)

Due to the accumulation of organic acids in urine, extraction of urinary organic acids is most commonly done by LLE and SPE (Jones & Bennett, 2010). It follows that one of the most used methods for the extraction of organic acids from urine is LLE with organic solvents. LLE enables the extraction of polar and apolar metabolites into two separate fractions of aqueous and organic phases respectively. LLE is an extensively utilised technique in the laboratory. It is an extraction technique applied to liquids, liquid samples, or samples in solution, using organic solvents (Moldoveanu & David, 2014). In chromatographic sample preparation, LLE is used for analyte isolation by carrying out selective extraction of the analyte from the sample or components from the sample matrix that must be eliminated. It is also used for analyte concentration by extracting in small volumes the analyte that was initially in a large volume of liquids, e.g. organic acids in large volumes of urine (Moldoveanu & David, 2014).

2.3.1.1. Theory of Liquid-Liquid Extraction

The operational principal of LLE is the distribution of the sample between two immiscible liquids in which the compound and the matrix have different solubilities. One phase is usually aqueous and the other organic, the two phases having different densities. This enables the compounds to be extracted either from the top or bottom phase depending on the density of the organic solvent being used. Based on the principle that ‘like attracts like’, the more polar

hydrophilic compounds are drawn to the polar (aqueous) phase and the more non-polar hydrophobic compounds prefer the organic phase which is the top phase (Dean, 2009, Mitra, 2003).

It is essential for LLE to know how much of an analyte is transferred from one liquid to the other. Equilibrium of a substance is reached when it is equally distributed between the two phases, as shown in equation 1 (Dean, 2009) and the concentration in both phases is more or less constant.

$$A\ (aq) \longleftrightarrow A\ (org); \quad [1]$$

Where A is the analyte of interest, (aq) is the aqueous phase and (org) is the organic phase.

The two terms that describe the distribution of a compound between two immiscible solvents are Distribution Ratio (D) and Distribution Coefficient (K_D). Distribution ratio (D) is the total analytical concentration of a solute in the aqueous phase, regardless of its chemical form, in relation to its total analytical concentration in the organic phase. This relationship is shown in equation 2 (Berthod & Carda-Broch, 2014). Experimental conditions such as chemical reaction, ionization, precipitation and others influence the variation of the distribution ratio (Berthod & Carda-Broch, 2014).

$$D = \frac{\text{Concentration of A in all chemical forms in the organic phase}}{\text{Concentration of A in all chemical forms in the aqueous phase}} \quad [2]$$

Distribution Coefficient (K_D) can be defined as the ratio of the concentration of substance A in a single definite form in the organic phase to its concentration in the same form in the aqueous phase at equilibrium. K_D is best represented by equation 3 that indicates the activities of A being constant in the two solvents.

$$K_D = [A]_1 / [A]_2. \quad [3]$$

The $[A]_1$ in the equation being the molar concentration of substance A in organic phase and $[A]_2$ is the molar concentration of A in the aqueous phase (Dean, 2009). A more useful expression to determine the fraction of the analyte extracted is shown in equation 4 (Mitra, 2003), often expressed as a percentage.

$$E_A = A_O / A_{total} = C_o V_O / (C_o V_O + C_{aq} V_{aq})$$

Or

$$= K_D V / (1 + K_D V) = 1 - 1 / (1 + K_D V); \quad [4]$$

Where E_A is the extracted fraction, C_o is the concentration of the compound in the organic

phase and C_{aq} is the concentrations of the compound in the aqueous phase. V_o and V_{aq} are the volumes of the organic and aqueous phases, respectively and V is the phase ratio, V_o/ V_{aq}.

The extracted fraction (E_A) as expressed in equation 4 is equivalent to the analyte recovery. The variation of E_A as a function of K_D is such that E_A increases as K_D increases (Moldoveanu & David, 2014). The nature of the solute A and that of the two solvents, aqueous and organic, determine the value for K_D. For one-step liquid-liquid extractions, K_D must be large, i.e. greater than 10. This is to enable the quantitative recovery of an analyte in one of the two phases, since the phase-ratio V must be maintained within a relatively small practical range of values; e.g., 0.1<V<10 (Dean, 2009).

To achieve greater quantitative recovery, two or three extractions are required with fresh organic solvent, shown in equation 5 (Mitra, 2003). This enables a cumulative recovery that is greater than a single extraction (Berthod & Carda-Broch, 2014; Dean, 2009). The principle of cumulative repeated extraction is based on the following principles; the value of the distribution coefficient determines the net amount of analyte that is extracted. The ratio of the two volumes of the two phases either increases or decreases the amount of analyte extracted. Multiple portions of the same amount of extracting solvent extract more analyte than a single portion of the same volume of solvent. The original concentration of the aqueous sample does not affect the recovery of the analyte (Mitra, 2003).

$$R\,(n)_A = 1 - (1 / 1 + K_D\,V)^n \qquad [5];$$

Where *R* is the recovery of analyte *A and n* is the number of extractions done on the same sample with same volume of organic solvent (Moldoveanu & David, 2014). Some mathematical simulations examine the effect of concentration on recovery by single or repeated. The recovery factor of analyte *A* expressed as a percentage in equation 6 (Dean, 2009);

$$\%\ R_A = 100K_{D\,/}\,K_D + (V_O / V_E) \qquad [6]$$

where V_O is the volume of the original sample and V_E is the extraction solvent volume. It is important to note that the recovery is independent of the sample concentration. The recovery factor can also be expressed in the equivalent form to equation 4;

$$\% R_A = 100 [K_D (V_E / V_O) / 1 + K_D + (V_E / V_O)]$$

$$= 100 [K_D (V) / 1 + K_D (V)] \qquad [7]$$

where $V = V_O / V_E$.

Applying equation 6 to find the percentage recovery of a 100 ml aqueous sample containing 100 mg/l of a compound having a molecular weight of 250 g/mol is extracted once with 10 ml of organic extracting solvent and assuming K_D to be *3,* substitution yields;

$$R_A = \frac{100 X 3}{3 + (0.1L / 0.01L)} = 23.08\%$$

But when equation 5 is applied to the previous calculation, having three successive multiple extractions where K_D = 3, V_E = 100 ml, V_O = 10 ml and n = 3, the cumulative yield when substituted is;

$$R(3)_A = \{1 – (1 / 1 + 3 X 0.1)^3\} X 100$$

$$= 54.48\%$$

A number of other approaches can be used to increase the recovery of solvent extraction by the increase of the value of K_D. K_D can be increased by changing the organic solvent, or by suppressing the ionization of an ionisable sample to make it more soluble in the organic phase. As discussed in 2.2.1 organic acids are nearly fully ionized at physiological pH. Therefore, the addition of HCl to the solution reduces the pH and suppresses the ionisation of organic acids by protonation. This makes the organic acids more soluble in organic solvents (Mitra, 2003).

Concentration of the sample in the organic phase can be increased by the ‘salting out’ effect where the neutral salt is used to decrease an analyte’s solubility in the aqueous phase (Majors, 2014). Sodium chloride is added to the sample to generate a salt concentration of over 1M. The effect of this is the analyte in the sample becomes less soluble and more of it is extracted into the organic solvent (Mitra, 2003; Majors, 2014). Another thing that increases recovery of the analyte from aqueous sample is to increase surface contact of the two phases by sufficient mixing. This works more to enable the two phases to reach equilibrium

thereby recovering as much of the analyte (Majors, 2014).

Recoveries of an analyte can be assessed in two ways; through an absolute or relative recovery experiment. The absolute recovery is the amount of a substance recovered from a biological fluid matrix compared to the unextracted standard (Use, 2011). An absolute recovery checks for the efficiency of the overall efficiency of the extraction system (Hassan & Cooper, 2009). For organic acids, this is typically done by spiking the matrix with a known concentration of a pure standard of a compound of interest and extracting it. However, the internal standard used for quantification is spiked after extraction but before the drying step (Hassan & Cooper, 2009). Therefore the absolute recovery is determined by the ratio expressed in formula 8 .

$$R_A\% = \left(\frac{[A]_{Extracted}}{[IS]_{Unextracted}} \div \frac{[A]_{Unextracted}}{[IS]_{Unextracted}}\right) \times 100 \qquad [8];$$

Where R is the recovery of the compound, [A] is the concentration of the compound of interest and [IS] is the concentration of the internal standard.

The relative recovery is the amount of a compound that is recovered from the matrix with reference to the extracted standard that is spiked into the same sample (Hassan & Cooper, 2009). The recovery is relative to the recovery of the internal standard (Duez *et al*., 1996). The matrix effect is one of the things that is checked for here (Huber, 2010). Equation 9 expressed the formula used to determine the relative recovery of compound A.

$$R_A\% = \left(\frac{[A]_{Extracted}}{[IS]_{Extracted}} \div \frac{[A]_{Unextracted}}{[IS]_{Unextracted}}\right) \times 100 \qquad [9]$$

Other alternatives are to compare the extraction recoveries to that of an established reference method's results. The assumption of this approach is that the uncertainty of the reference method is known (Huber, 2010). Another approach is analysing a sample with known concentrations (a certified reference material, or spiked blank matrix with compound of interest) and comparing the measured value with the true value from the reference material.

2.3.1.3. Distribution coefficient for complex systems

A compound *x* in most practical cases is present as several different chemicals species in

equilibrium that are still identified as compound *x.* This is besides the simple systems where compound *x* is present as a sole species that is not involved in any chemical equilibrium. Carboxylic acid can be present as RCOOH, or as $RCOO^-$ ion and yet still identified as a unique acid (Moldoveanu & David, 2014). Equation 10 gives expression to the entire partition process that is described by a global parameter of the distribution coefficient (*Dx*) for cases when the analyte x participates in other equilibria represented by species, $x_2 \ldots x_n$, all still being identified as compound *x*.

$$Dx = \frac{Cx1,(org) + Cx2,(org) + \cdots + Cxn,(org)}{Cx1,(aq) + Cx2,(aq) + \cdots + Cxn,(aq)} \qquad [10]$$

where *Dx* is the *distribution coefficient, Cx* is the concentration of *x, org* is the organic phase and *aq* is the aqueous phase.

The constants Kx_1, Kx_2 and $K_{1,2}$ are given by the expressions:

$$Kx1 = \frac{Cx1,(org)}{Cx1,(aq)}, Kx2 = \frac{Cx2,(org)}{Cx2,(aq)}, K1,2 = \frac{Cx2,(aq)}{Cx1,(aq)} \qquad [11]$$

From equation 10, Dx can be written in the following expression as in equation 12 (Moldoveanu & David, 2014):

$$Dx = \frac{Cx1,(org) + Cx2,(org)}{Cx1,(aq) + Cx2,(aq)} = \frac{Kx1 + Kx2\ K1,2}{1 + K1,2} \qquad [12]$$

The parameter *Dx* describes the extraction equilibrium of many systems involving compounds with basic, acidic, or amphoteric character, when the use of *Kx* is not appropriate. In such cases, the value of K must be replaced with D in the equation of extracted fraction *E,* e.g. equation 4 (Moldoveanu & David, 2014).

2.3.1.4. Thermodynamic theory of LLE

As previously discussed about the equilibrium of analyte *A* in equation [1], in thermodynamics, the equilibrium for the distribution of *A* between the two immiscible liquids phases (organic and aqueous) is attained when the difference between the chemical potentials $\mu_{A,\ (org)}$ and $\mu_{A,\ (aq)}$ of the component of *A* in each of the two phases is zero (Berthod, A. 2004, Moldoveanu & David, 2014). This is represented by equation 13.

$$u^0_{A,(org)} + RT \ln a_{A,(org)} = u^0_{A,(aq)} + RT \ln a_{A,(aq)} \quad [13]$$

where $u^0_{A,(org)}$ and $u^0_{A,(aq)}$ are the standard chemical potentials of compound *A,* $a_{A,\,(org)}$ and $a_{A,\,aq)}$ are the mole fractions of compound *A* in the two phases; *R* is the gas constant; and *T* is the absolute temperature. If the chemical potential is not identical in the two phases, mass transfer of compound *A* takes place and the mole fraction a changes so that the chemical potential of *A* becomes equal in both phases thereby establishing equilibrium (equation 14).

$$u^0_{A,(org)} - u^0_{A,(aq)} = RT \ln \left[\frac{a_{A,(aq)}}{a_{A,(org)}}\right] \quad [14]$$

in which, when $\frac{a_{A,(aq)}}{a_{A,(org)}}$ is substituted for distribution constant, K_D, the expression is;

$$\frac{a_{A,(aq)}}{a_{A,(org)}} = K_D = \exp\left[\frac{u^0_{A,(org)} - u^0_{A,(aq)}}{RT}\right] \quad [15]$$

Partition coefficients are usually expressed as molarity ratio. There is a proportional relationship between molar solubilities [A] and mole fractions a as seen in equation [16];

$$[A]_1 = \frac{a_A}{V_1} \quad [16]$$

where V_1 is the molar volume (M^{-1}) of solvent 1.

Moldoveanu *et al*, 2015, state that it is energetically favourable to have nonpolar compounds extracted in nonpolar solvents and polar compounds in polar solvents.

2.3.1.5. The effect of temperature and chemical reactions on LLE

Equations [13] and [14] show that there is a relationship between the distribution constant and the temperature of compound A. K_D is sensitive to temperature in a directly proportional manner (Berthod, A. 2004). The free energy of transfer, $\Delta G_{2/1}$, equation 14 can be expressed as;

$$\Delta G_{2/1} = RT \ln K_{D2/1} \quad [17]$$

According to Berthod *et al*, 2004, based on the assumption that the standard molar enthalpy

is constant in a limited temperature range, the plot of $\ln K_{D2/1}$ against $1/T$ should produce a straight line in a classical Van 't Hoff plots, with slope $\Delta G_{2/1}/R$. Temperature also affects the mutual solubilities of the two solvents. The two-phase system of extraction becomes a one-phase at a certain critical solution temperature. When the solvents are not very miscible and the temperature change is not dramatic, it is possible to consider that the effect of temperature on the K_D value is not great (Berthod & Carda-Broch, 2014).

Chemical reactions affect the concentration of a particular analyte, the distribution ratio (*D*) but the distribution constant does not change. The implication of this is that the concentration of the species will change in the other phase to maintain the chemical potentials equal to the two phases. The distribution ratio of the analyte can change in a dramatic fashion (Berthod & Carda-Broch, 2014). An example of a carboxylic group is given to illustrate this point. The representation of this compound is;

$$AH \leftrightarrow A^- + H^+ \qquad [18]$$

AH is a hydrophobic compound. In a two-phase liquid system, when the pH changes there is also change in the distribution of the carboxylic acid. At low pH values, AH typically prefers the organic phase with very high D values. On the contrary, the ionised form of AH, A^-, is hydrophilic and with increase in pH values, most of the solutes is found in the aqueous phase. When the pH = pKa of the solute, the distribution ratio is halved (Berthod & Carda-Broch, 2014).

2.3.1.6. Selection of solvents

The choice of the solvents is of critical importance in LLE (Dean, 2009). The extraction selectivity and efficiency depend on it. Table 2.1 shows the physical properties of some solvents. There are a number of factors affecting the selection of solvents. i). the solvents to be selected must be immiscible. If this is not the case, immiscibility can be induced by the 'salting out' effect or addition of a buffer to the sample-solvent mixture (Wells, 2003). A clear phase separation between the two solvents is necessary for extraction to be accomplished. ii). The density of the extracting solvent must be considered. Solvents denser than water make up the lower phase and those less dense than water make up the top phase. The choice is based on what is more advantageous and expedient when separating the two solvents. It is also a question of which phase would be easier or preferable to aspirate (Mitra,

2003). iii). the solubility of water in a solvent is an important factor in the selection of a solvent. For use in GC/MS, the solvent must be dry and free of water. The effectiveness of solvent drying by anhydrous substances depends on how on how much water is dissolved in the solvent by extraction (Moldoveanu & David, 2014). Solvents are also to some degree soluble in each other, which eventually lead to mutual saturation when two are mixed. Data on the solubility of the solvent in water and vice versa provides critical information for the selection of a good solvent that will be fit for purpose (Majors, 2014, Wells, 2003; Dean, 2009).

Low boiling points is important for an extracting solvent for ease of sample concentration by evaporation (Moldoveanu & David, 2014). A mixed solvent system is sometimes used to get the desired characteristic of solvents. The partition coefficient of the solvent mixture is sometimes better than for a single solvent. This is due to the synergistic effect of the solvent mixture (Moldoveanu & David, 2014).

One parameter that is frequently used for the characterization of a compound regarding its polar or its hydrophobic character is the octanol/water partition coefficient log K_{ow}. This type of characterization can be applied to both solutes and solvents (Moldoveanu & David, 2014). K_{ow} is a dimensionless and operational definition of hydrophobicity based on the *n*-octanol reference system (Mitra, 2003). It is directly proportional to the partitioning of a solute between water and various other hydrophobic phases. The larger the value of K_{ow}, the greater is the tendency of a solute to move from the water phase to the organic solvent (Mitra, 2003). Equation [19] shows the linear dependence that exists between log K_{ow} for a solute *A* and the logarithm of K_D for the same solute in a different solvent system (aqueous / organic) (Moldoveanu & David, 2014).

$$\log K_{(aq.org),A} = a \log K_{ow,A} + b \qquad [19]$$

where a and b are constants specific for the solvent systems. Once parameters a and b are established based on the system of solvents, the equation can be used as a guide for selecting the best solvent system when other criteria are met. It can be concluded based on the observation that similar natures of solvents and solutes are needed for a favourable energetic interaction, that a more efficient extraction is achieved when one of the solvents and the solutes have similar values for log K_{ow} (Moldoveanu & David, 2014).

The capability of a solvent to dissolve a volatile solute is another parameter for the selection a solvent (Mitra, 2003, Moldoveanu & David, 2014). Again, the similarity in the nature of the solvent and solutes is needed for dissolvability. The polarity parameter (P') is useful in this case, although not always sufficient to characterise a solvent's properties (Moldoveanu &

David, 2014). Larger values for P' indicate a polar solvent (such as alcohols and water) and lower values close to zero show non-polarity, such as hexane (Moldoveanu & David, 2014). The more polar a solute is the more likely to dissolve in polar solvents and vice versa.

In a short concise way, the characteristics on which an organic solvent is chosen are the following; low solubility in water (<10), high volatility for easy and quick solvent evaporation in the concentration stage, compatibility with the choice of chromatographic analytical technique, polarity and hydrogen-bonding properties that enhance recovery of the analyte in the organic phase and high purity to minimize sample contamination (Majors, 2014; Dean, 2009). Ethyl acetate is the most used organic solvent in the extraction of organic acids (Kaluzina-Czaplinska, 2011, Peter *et al*., 2008) because it has a very low boiling point (34.5°C) and can dissolve a large number of organic compounds, both polar and nonpolar. The drawback of non-polar, water-immiscible organic solvents like ethyl acetate is their low dielectric constants. This makes them poor at extracting very polar or highly charged solutes. Acetonitrile is a water-miscible organic solvent used for the extraction of organic acids because it provides solubility for more polar compounds that the non-polar organic solvents, although it is water miscible and less used for LLE (Majors, 2014). The addition of an inorganic salt into a mixture of acetonitrile and water causes separation of the solvent from the aqueous phase, forming a two-phase system (Majors, 2014).

Table 3-1 Physical properties of Selected Solvents

Name	Structure and molecular weight	Boiling point °C	Melting point °C	Density g/ml	Solubility in 100g of water	Solubility of water in 100g of solvent	e - Dielectric constant	Dipole moment	Viscosity 10-3 Pa · s	Surface tension 10-3 J/m2
Acetone	(CH3)2C=O 58.08	56.3	-94.7	0.7850	miscible	miscible	20.7	2.7	0.3040	22.68
Acetonitrile	CH3CN 41.05	81.6	-43.8	0.7768	miscible	miscible	37.5	3.44	0.3409	28.45
n-Butanol, Butyl alcohol	CH3(CH2)3OH 74.12	117.7	-88.6	0.8057	8.3	19.7	17.5	1.66	2593	24.3
tert-Butanol,	(CH3)3C-OH 74.12	82.4	+25.8	0.7812	miscible	miscible	12.4	1.7	3.35	19.2
Chloroform	CHCl3 119.38	61.2	-63.5	1.4799	0.8	0.07	4.8	1.01	0.54	26.6
Cyclohexane	C6H12 84.16	80.7	+6.5	0.7739	< 0.1	< 0.1	2	0	898	24.4
Diethyl ether	(CH3CH2)2O 74.12	34.6	-116.3	0.7078	7.5	1.3	4.34	1.25	224	16.5
Dimethyl sulfoxide DMSO	(CH3)2S=O 78.13	189.0	+18.5	1.0958	miscible	miscible	46.7	3.9	1996	43
Ethanol	CH3CH2OH 46.07	78.3	-114.1	0.7851	miscible	miscible	24.55	1.7	1078	22
Ethyl acetate	CH3COOCH2CH3 88.10	77.1	-83.9	0.8945	8.3	3.3	6.02	1.78	426	23.2
Hexane	CH3(CH2)4CH3 86.18	68.7	-95.3	0.6548	< 0.1	< 0.1	1.88	?	0.2923	17.9
Isopropanol,	(CH3)2CHOH 60.10	82.3	-88	0.7810	miscible	miscible	19.9	1.66	2073	18.3
Methanol	CH3OH 32.04	64.7	-97.7	0.7866	miscible	miscible	32.6	1.6	0.5445	22.1
Pyridine	C5H5N 79.10	115.3	-41.6	0.9779	miscible	miscible	12.4	2.2	0.8826	36.5
Tetrahydrofurane, THF	C4H8O 72.11	66.0	-108.5	0.8844	miscible	miscible	7.58	1.6	461	26.4
Toluene	C6H5-CH3 92.14	110.6	-95.0	0.8623	52	< 0.1	2.38	0.36	552	27.8
Triethylamine	(CH3CH2)3N 101.19	89.5	-114.7	0.7235	13.3	?	2.44	0.82	341	20.1
Trimethylsilyl chloride	(CH3)3SiCl 108.64	57	-58	856	reacts violently	reacts violently	?	?	0.4	?
Water	H2O 18.02	100.0	0.0	997	-	-	78.39	1.84	0.8905	71.98

Adapted from TriMen Chemicals (http://www.trimen.pl)

2.3.1.7. Methodology of organic acid extraction by LLE

Tanaka and colleagues were among the first to use solvent extraction to extract organic acids from urine for gas chromatography-mass spectrometry analysis in 1967 (Tanaka & Isselbacher, 1967). The solvent extraction method they did followed the protocol of acidifying the urine to pH 1 with HCl and extracting with 3 ml of redistilled ethyl acetate. A known amount of an internal standard was added to the extract for quantitative analysis. The combined extract was dried over anhydrous sodium sulphate and evaporated to dryness under a nitrogen stream at 40^{o} for 1 hour. Acetone was added to re-dissolve the residue before injection into the GC column (Tanaka & Isselbacher, 1967).

Generally, the conventional as well as the in-house solvent extraction procedure for urinary organic acids that is followed by many laboratories begins with the determination of creatinine content of the urine sample. This is done to determine the volume of urine to use for the organic acid analysis. The calculation used is: 10/creatinine concentration (mg/dL) = volume of urine (mL). Sample volume must be within the range of 0.5 and 5 mL, not less or more than that respectively. An amount of internal standard is mixed with the urine. HCl is added to the urine sample to adjust the pH to 1. Two extractions are done with ethyl acetate and/or diethyl ether, either individually or in sequential combination of both solvents. The extraction is accomplished by rotary shaking of the mixture of the sample and the organic solvents. The extract is dried with anhydrous sodium sulphate to remove any water that may damage the GC column. After this the sample is pre-concentrated by evaporation of the extracting organic solvent under a gentle stream of nitrogen at 37^{o}C on a heat block. The residue is then derivatized by silylation with *N*, *O*-bis(trimethylsily)trifluoroacetamide-trimethylchlorosilane-pyridine (BSTFA TMS) in a stopped tube at 80^{o}C for at least 30 minutes. The derivatized sample is then injected in the GC/MS for analysis. Derivatization makes the sample more volatile and suitable for analysis by the GC/MS (Jones & Bennett, 2010, Kaluzina-Czaplinska, 2011). Appendix 1 contains the standard operating procedure (SOP) for the Potchefstroom laboratory for inborn errors of metabolism (PLIEM).

2.3.1.8. Choice of an internal standard in organic acid analysis

One of the essential things in organic acid analysis is the choice of an internal standard (Kaluzina-Czaplinska, 2011). The essential properties of an internal standard is its chemical properties and structure and its absence in normal and known physiological metabolic

processes (Duez *et al*., 1996, Kaluzina-Czaplinska, 2011). The internal standard must have a very close distribution coefficient with the analytes of interest, without which, there is likely to be proportional systematic errors when it is used for normalization (Moldoveanu & David, 2014). As such, the internal standard should have extraction efficiency similar to the compound of interest. This requirement cannot be adhered to when one internal standard is used in combination with a number of different compounds. In this scenario compounds with extraction efficiencies better than that of the internal standard will show extraction efficiencies greater than 100% unless a factor is applied to correct for different extraction efficiencies (Moldoveanu & David, 2014, Duez *et al*., 1996, Kaluzina-Czaplinska, 2011).

The most popular internal standards used for organic acids are tropic, undecanoic acid, hexadecandioic acid, pentadecanoic acid, malonic acid, 2-phenylbutyric acid, 2-ketocaproic acid and 2-hydroxyvaleric acid (Duez *et al*., 1996, Kaluzina-Czaplinska, 2011), to list but a few. The internal standard used in the in-house method is 3-phenylbutyric acid. The internal standard is important for normalization and relative quantification. According to Kaluzina-Czaplinska, (2011), whether its a quantitative or qualitative analysis, the internal standard must be added at the very beginning of the sample preparation. But for absolute recovery experiments, it is added at the end of the extraction (Hassan & Cooper, 2009) to give the extraction efficiency of the compounds.

2.3.1.9. Modifications and miniaturisation of solvent extraction of organic acids

Modern analytical chemistry has focused on the need to develop more efficient sample preparation techniques that reduce the cost, labour and time (Psillakis & Kalogerakis, 2003). Miniaturised sample preparation promises to overcome the shortfalls of the conventional extraction protocol. This is done by reduction in the amount of solvent volumes used and modifying some steps in the extraction protocol. It is for this reason that micro-extraction techniques are now posing as an alternative to the classical LLE and sample preparation procedures (Farajzadeh *et al*., 2014).

Developments in liquid-phase micro-extraction (LPME), which is a solvent-minimised sample preparation procedure, are single-drop micro-extraction (SDME), where the extractant phase is a drop of water-immiscible solvent suspended in the aqueous sample (Hu *et al*., 2013); dispersive liquid-liquid micro-extraction (DLLME), which is based on the tertiary component solvent system; and hallow-fiber liquid-phase micro-extraction (HF-LPME), a membrane-

based extraction where a porous membrane is used to support and protect the extraction solvent (Hu *et al*., 2013, Sarafraz-Yazdi & Amiri, 2010). These are fast, effective and minimal solvent approaches to performing LLE (Clement & Hao, 2012).

Liquid-liquid micro-extraction (LLME) is another modification of the conventional LLE that significantly uses smaller volumes of solvents (Clement & Hao, 2012). To obtain the required extraction efficiency in LLME, the ratios of the organic-aqueous ratios must be 0.001 to 0.01. The extracting solvents used have lower density than water for collection of the top phase. This technique had been proven to work well with various hydrophobic compounds, such as organochlorines (Clement & Hao, 2012).

The traditional LLE protocol of organic acid extraction was simplified by the use of smaller volumes of the organic solvents and shortening the extraction time and derivatization. Nakagawa *et al* modified the solvent extraction for organic acids for chemical diagnosis (Nakagawa *et al*., 2010). This involved the use of 200 µl of urine sample instead of 1 mL or more. The organic solvent volume used was reduced from 3 or 6 mL to 1.2 ml. Flash-heater derivatization was used in this method. Generally, the process was made shorter in time as well. This method was reported to enable simple, rapid and safer sample preparation for urinary organic acid GC/MS analysis (Nakagawa *et al*., 2010). Hassan *et al*, (2009) modified and optimised the analytical method for the analysis of γ-hydroxybutyrate (GHB) in post-mortem urine. A 100 ul of urine sample was used and 1 ml of ethyl acetate as the extracting solvent. They reported to having developed and validated a robust and sensitive method for the analysis of GHB in post-mortem urine as a marker of alcoholic ketoacidosis (Hassan & Cooper, 2009).

2.3.1.10. Automation of LLE for organic acids

Apart from miniaturization of solvent extraction, automation is one of the challenges and interests of analytical chemists (Kocúrová *et al*., 2013). The benefits of automation for LLE are myriad. Automating the LLE or urinary organic acids would increase sample throughput, eliminate random errors due to human operations, improve reducibility and repeatability, precision and accuracy in handling the sample would be better in comparison to human manipulation of the same (Lord & Pfannkoch, 2012). Automation would reduce the tedious labour associated with sample preparations, the cost is likely to reduce on staff salaries, less solvent volume would be used and the quality and consistence of the data after analysis is

likely to improve (Bengtsson, 1996). The more the steps that can be automated for the extraction procedure with good reproducibility, the less the amount of random error there will be in the final data (Clement & Hao, 2012, Lord and Pfannkoch, 2012).

Typically, there are three steps in sample preparation for GC analysis; sample clean-up, concentration and derivatization (Lord & Pfannkoch, 2012). Sample clean-up removes the sample from a matrix that is inappropriate for the analytical method. It involves the extraction of target analytes from the sample with the goal of having the sample in a more compatible state for GC analysis. The concentration step selectively concentrates the analyte in low levels to reach detection limit. The target analytes are usually in low concentration with reference to the bulk sample. Therefore, it is imperative to reduce the amount of matrix and still concentrate the sample. This is usually done by solvent evaporation and re-suspension. The last step is derivatization that makes the sample more volatile and increases the signal intensity of the analyte during GC analysis (Farajzadeh *et al*., 2014, Lord and Pfannkoch, 2012).

The challenges that have been highlighted in literature as being inherent in the automation process of LLE are the addition of acid to adjust the pH of the sample, the sufficient recovery of the organic solvent after extraction using an automated needle syringe which would need to be set some millimetres above the aqueous interface compared to manual manipulation, the removal of any remaining water in the organic solvent by the use of sodium sulphate, the evaporation of the solvent in the concentration step and the derivatization step before injection in the GC (Lord & Pfannkoch, 2012). Translating these manual steps to automated process possibly present a physical challenge of the workstation and a software challenge that will control these steps. With these possible challenges and limitations in view, it is, however still possible to automate liquid-liquid extraction on some workstation (Lord & Pfannkoch, 2012).

The advancement in the robotic hardware and computer software, coupled with reduction in their prices, have led to much progress in the automation of some steps in the sample preparation process (Lord & Pfannkoch, 2012). Automation of sample preparation procedure can have either all or one of these steps automated. Specific instrumentation such as autosamplers, workstations and robots are required for automated sample preparation. Autosamplers are designed to make sample preparation reproducible and reliable for GC analysis. The primary functions of autosamplers are to increase sample throughput by enabling the analysis to go on unceasingly, to improve accuracy and precision in comparison

to manual sample preparation and to avoid errors prone to manual sample handling (Lord & Pfannkoch, 2012). Workstations have more flexibility than autosamplers. They are intermediate class of instruments that can accommodate a wide range of vials and reagent carriers and a large number of accessories that can be interfaced to provide a wide array of possible solutions to automated sample preparation operations. Robotic equipment is usually larger and has a multiple component system, often with custom capabilities and software control that is dedicated to one type of analysis (Clement & Hao, 2012, Lord & Pfannkoch, 2012).

A good amount of progress has been made in the automation of LLE for industrial and environmental analytical applications (Clement & Hao, 2012). Application of automated LLE has been applied to the field of biomedicine. The sample and solvent volumes in these applications are often small (Clement & Hao, 2012). These devices have the potential to fully automate all the operations of LLE such as the addition of solvent to sample, thorough mixing of mixture and separation of solvent extract and raffinate. The software that operates these devices is capable of generally handling liquid extraction procedures. Both the hardware and software of this equipment have the ability to deal with liquid levels from as minute as 5 ul to 5000 ul with accuracy (Clement & Hao, 2012).

Lateef *et al*, 2013, did automate the LLE of forensic drugs from plasma using the Agilent 7696A Sample Prep WorkBench for bioanalytical workflow. They reported having good reproducibility from the 50 extracted samples with the automated protocol (Lateef. 2013). Most of the other efforts at LLE extraction have focused on LLME techniques such as DLLME, SDME and HF-LPME (Kocurova *et al*., 2013). Bengtsson *et al*, (1996) automated the sample preparation for organic acid analysis by GC/MS. They developed two different types of automated sample preparation. The one was based on the solvent extraction and the other on the solid phase extraction method. They used a robotic workstation to automate the extraction steps. Their findings were that the manual method was faster and more efficient than both the automated procedures. They concluded that there was need to improve the speed, reliability and design of the robotic workstation in order to get better sample preparation (Bengtsson & Lehotay, 1996).

Literature on the progress made in the automation of LLE of urinary organic acids for GC/MS analysis is scarce.

2.3.2. Other extraction techniques

2.3.2.1. Solid-Phase Extraction (SPE) of organic acids

Solid-phase extraction is a non-equilibrium method of selective adsorption of compounds from a mobile liquid sample onto a solid sorbent prior to elution (Mitra, 2003; Dean, 2009). It is one of the most widely used methods for sample preparation for liquid samples. Solid samples can also be isolated by this method but have to be dissolved or extracted into a liquid form (Majors, 2014). Careful selection of sorbent is crucial for the efficient recovery of semi volatile organics from liquids. There are different types of sorbents, the one used for the extraction of organic acids are strong anion-exchange sorbents (Liu *et al*., 2004). These sorbents contain ionisable functional groups such as carboxylic acids. The accumulation process used in organic acid isolation is the anion exchange. In this process, the sorbent contains a positively charged functional group and the organic acids in their ionized form in the liquid sample matrix have a negatively charged group which enables the exchange (Dean, 2009). The operational principle of ion exchange is the retention of ions of high ionic strength on the resin as the remaining constituents of a liquid matrix are eluted (Thompson & Markey, 1975).

From as early as the 1970s, investigations of the use of SPE with different sorbents such as weak anion exchange, strong anion exchange and some other disposable columns on urinary organic acids was already being done. Thompson *et al* (1975) reported anion exchange to be generally more effective than solvent extraction for isolating organic acid from urine. It was able to isolate polyhydroxy acids which are a challenge for solvent extraction. They reported good reproducibility as well. The setback was the time-consuming nature of the process. Fitch *et al* (1979) reported difficulty in obtaining reproducible results for the isolation of organic acids when they worked with anion exchange method by the procedure described by Thompson *et al* (1975). After modifying the method by increasing the volume of the eluting solvent from 18 ml to 40 ml, they reported being able to isolate carbohydrates such as glyceric acid. They were able also to obtain satisfactory reproducibility of aliphatic and aromatic acids. The disadvantages were the poor extraction and recovery of important organic acids such as of lactic acid, hippuric acid and citric acid and the difficulty of sufficiently drying extracts of the urine sample. Overnight lyophilisation proved to be time consuming as well. Sims *et al* (1981) reported the superiority of their modified method compared to the previously reported ones above. The modified procedure of anion exchange method was able to isolate the mono-, di- and tricarboxylic acids (Fitch *et al*., 1979, Sims *et al*., 1981, Thompson and Markey, 1975).

Liua and colleagues (2004) investigated the use the SPE for urinary organic acid analysis. The goal of their study was to develop a reliable sample isolation method with good sensitivities for a wide range of clinically significant metabolites and lower maintenance requirements on GC column and MS ionization. In order to obtain efficiencies for their extractions with SPE, they injected the same amount of the standard solution after derivatization on to the GC/MS and compared them to the analyte they extracted with SPE. They found extraction efficiencies of close to 100 for many of their analytes. They concluded that SPE using the strong anion exchange column Sep-Pak provided high extraction efficiencies for a wide range of pathological compounds. The drawbacks to this method were very low extraction recoveries were found for oxalic acid, malonic acid and mevalonic acid lactone, which are key metabolites in the diagnosis of IEM. They reported that this method could provide both an alternative and a complement to the solvent extraction for organic acid screening (Liu *et al*, 2004).

2.3.2.1.1 Advantages and disadvantages of SPE over LLE

Solid phase extraction has a number of important potential advantages over liquid-liquid extraction. SPE has the advantage of more complete extraction of the analyte, more efficient separation of interferences from sample, increased separation selectivity, reduced organic solvent utilization, easier collection of the total analyte fraction, good sample pre-concentration and more easily automated. It gives higher recoveries of the sample than LLE when the sorbent is appropriately selected. SPE meets many of the requirements of an ideal sample preparation technique; it is simple and inexpensive, selective, efficient and compatible with many separation methods (Theodoridis *et al*., 2000). Some of the disadvantages are mixed mechanisms in SPE can occur; irreversible adsorption of some analytes on SPE cartridges and more complex method development is required, it is also difficult to select an appropriate sorbent when dealing with a class of compounds with different chemical properties (Majors, 2014).

2.3.2.2. Solid Phase Micro-extraction (SPME)

Solid phase micro-extraction (SPME) is one of the new approaches that sought to address one of the setbacks of solvent extraction, i.e., the modification of the sample matrix by the organic solvent. Because SPME is a solvent-less method, the matrix of the sample remains unmodified. The method is reported to obtain a truer image of the system under investigation due to less interference with the sample matrix and better sample clean up. The separation principle of SPME is on the basis of the partitioning of the analyte between

the extraction phase and the matrix. There are generally two extraction modes; the one is direct sampling from the aqueous phase and the other is headspace (HS) extraction. The nature of the sample matrix, the analyte volatility and the affinity of the analyte to the matrix makes the basis selecting the mode one of the two to use. Direct-immersion is used in the extraction of medium volatile organic compounds, while the volatile compounds by headspace extraction (Theodoridis *et al*., 2000).

In 1998 Liebich and colleagues were the first to report of developing a new method of SPME for sample preparation of urinary organic acids. They described a method that took only about 40 minutes for the whole sample preparation. In this preparatory method, organic acids were derivatized directly in the aqueous urine using trimethyloxonium tetrafluoroborate as a methylation agent. Polyacrylate fiber was used in the extraction and after that the methyl esters were transferred and injected into a GC/MS. they reported having a well separated profile of urinary organic acids on the gas chromatogram (Liebich *et al*., 1998).

SPME was successfully applied in headspace (HS) extraction mode in combination with GC/MS to investigate the urinary metabolic profile for potential cancer biomarkers. Rocha *et al* (2012) carried out a comprehensive study of the urinary volatile metabolome by HS-SPME coupled to GCxGC-TOF-MS. 294 compounds were tentatively identified. Among those identified included organic acids, ketones, aldehydes, etc. The drawback of this method is that an SPME coat equilibrates with only the free fraction of the analytes that results in lower sensitivities but this is compensated for by the sensitivity of the MS instruments. The other limitation is lower coverage of the analytes compared to solvent extraction protocols (Bojko *et al*., 2014).

2.3.2.3. Other methods of micro-extraction

Other methods developed for micro-extraction of organic compounds are Liquid-Phase Micro-extraction (LPME) and Stir-Bar Sorptive Extraction (SBSE). In LPME a syringe is used to acquire 1 µL of organic solvent, which is allowed to exit the syringe but remain as a drop at the tip-end of the needle. The needle is then immersed in the aqueous sample that is agitated by the use of a magnetic stir bar. The drop is drawn back into the syringe after a definite time only to be injected directly into the gas chromatography. The SBSE pre-concentrates organic compounds using a magnetic stir bar coated with a sorbent. The organic compounds usually retain to the stir bar until they are desorbed in the organic solvent and injected into the gas chromatography (Dean, 2009). From the literature reviewed, data with regard the application to the analysis of organic acids by these methods

was hardly available. But they both pose as potential methods to be applied to organic acid extraction from biological fluids and to urine precisely.

2.4. The Relevance of LLE for Organic Acid Extraction

LLE of urinary organic acids is the most commonly used sample preparation method (Kaluzina-Czaplinska, 2011, Peters *et al*., 2008). Solvent extraction has a number of advantages that makes it relevant even at the present time: it can and is being used as a method for laboratory routine analysis of organic acids. It is an easy method that enables efficient extraction of aromatic carboxylic acid. It is associated with lower contamination by inorganic phosphates and sulphates (Niessen, 2001) and it has a good sample clean-up. It is capable of continuous multistage extraction that grants high separation factors and high purity of sample. It can be operated at different scales of volumes, from very small microliters to tons of litres. It is a rugged and relatively cheap (Clement & Hao, 2012).

The disadvantages of the method are the use of large volumes of solvents, extraction of neutral compounds such as urea and glycerol. It has poor extraction of polyhydroxycarboxylic acids (Kaluzina-Czaplinska, 2011). There is a potential to lose some organic acids during evaporation and drying. The process is also laborious and time consuming, rather difficult to be automated (Clement & Hao, 2012).

2.5. Method Validation

2.5.1. Method validation in general

Method validation is the process of confirming that the analytical method used for a specific test is fit for its intended purpose (Huber, 1997). The main purpose for validating a method is to assess the errors present in the test-results produced from a particular method (Westgard *et al*., 2008). Every analytical method is prone to have some errors. Validation helps to identify these errors and assesses whether the method is fit for purpose or not. There are three types of validations methods; full, partial and cross validations. Each method is necessitated by particular conditions. For example, a full validation would be necessary when developing and implementing an analytical method for the first time. It is also necessary when the metabolites being tested for have been changed or some have been included to them. A partial validation is necessary when there is a modification to a validated analytical method. Key parameters to this method are the determination of the precision and

accuracy in intra- and inter-assays. If need be, it can be a nearly full validation. The cross validation compares two analytical methods and is necessary when two or more analytical methods are used to generate data within the same study (Shah *et al*., 2000).

2.5.2. Method validation parameters

The selection of a method to be validated is the first most important task in this process. The method must be one that can be a routine analytical laboratory method having had demonstrated acceptable performance (Westgard *et al*., 2008). Some of the key parameters mostly considered in the validation GC/MS analysis of urinary organic acids methods are those related to the performance of the method. Such parameters as linearity, limit of detection (LOD), limit of quantification, accuracy or bias, precision and repeatability mostly included in many method validations (Nakagawa *et al*., 2010, Christou *et al*., 2014).

2.5.2.1. Linearity

According to the International Council for Harmonisation (ICH), linearity of an analytical procedure is defined as the ability to obtain test results that are directly proportional to the concentration of the analyte in the sample within a given range (Huber, 2010). A linearity experiment establishes the correlation between the measurement response and the concentration of the substance that is being measured by the test procedure (Westgard *et al*., 2008). This is displayed as a calibration curve. Because both physiological and pathological samples all contain analytes in varying concentrations, it is important to establish the accuracy of the test procedure response (Huber, 2010). It also enables the establishment of the reportable range of the method. It establishes and/or verifies the accuracy of the instruments or test system measurements response (Westgard *et al*., 2008).

2.5.2.2. Accuracy

Accuracy can be defined as the extent to which the test's measured value agree with the accepted, true, or reference value (Weitzel *et al*., 2007, Huber, 2010). The accuracy of an analytical method is established by the assessment of the inaccuracy or systematic error of the method. In order to estimate the inaccuracy a method comparison experiment is done. The test method is compared to a reference method and the systematic differences at critical decision concentrations are the errors of interest (Westgard *et al*., 2008).

Another way of assessing the accuracy of an analytical method is by analysing samples with known concentrations (usually matrix modified) and comparing the test result value and that supplied with the reference sample as the true value. The **E**uropean **R**esearch **N**etwork for evaluation and improvement of screening, **D**iagnosis and treatment of **I**nherited disorders of **M**etabolism (ERNDIM) is an example of an external quality assessment scheme that provides such reference samples to assess the accuracy of an analytical method. A comparison graph is plotted to illustrate the differences between the test method and the reference method (Fowler *et al*., 2008; Peters *et al*., 2008). Statistics of the regression from this comparison plot provides critical information with regard to the inaccuracy of the test method. Recovery and interference experiments can also be used to provide additional information on the accuracy of the method (Westgard *et al*., 2008).

2.5.2.3. Precision

The precision of an analytical method is the closeness of the agreement between a series of measurements obtained from multiple sampling of the same homogenous sample under the prescribed conditions (Huber, 2010, Weitzel *et al*., 2007). The error that is established in this experiment is imprecision or random error of the method. A replication experiment is done to estimate this. The purpose of this is to observe and establish the variation expected in a test result under normal operating conditions in a laboratory (Westgard *et al*., 2008).

The conditions of the replication experiment determine the type of precision estimate obtained (Magnusson & Örnemark, 2014). There are three such types of precision that are determined, repeatability, within-laboratory precision and reproducibility. Repeatability is the closeness of agreement of serial measurements of the same analyte carried out under the same conditions, e.g. same measurement procedure, analyst, laboratory and instrument. This is carried out over a short time interval and it is also termed intra-assay precision (Weitzel *et al*., 2007, Huber, 2010). Intermediate precision expresses within-laboratories variations. These variations include different analysts, different days, different instruments, etc. Reproducibility expresses between-laboratories variations, to be considered in the case of standardizing an analytical procedure (Huber, 2010).

Obtaining test results from 20 samples of the same material is the way of carrying out a repeatability experiment. The important statistics considered here are the mean, standard deviation and the coefficient of variation. In an ideal setting, the test result variations should be small; the tests results should all be nearly the same (Westgard *et al*., 2008). The minimum recommended studies to determine the method's precision is by selecting two different control samples that represent high and low critical medical decision levels and

analyse the materials 20 times. Alternatively, an Analysis of Variance (ANOVA) experiment can be used to estimate the repeatability and within-laboratory precision simultaneously (Westgard *et al*., 2008).

2.5.3. Method validation using ERNDIM samples

Method validation using an external quality assessment (EQA) is essential to provide reassurance and reliability of the biochemical tests. They are helpful in identifying analytical problems, improvement of accuracy and offer comparability of results among the participating laboratories (Fowler *et al*., 2008; Peters *et al*., 2008). Participating in these schemes is important for different laboratories that do routine diagnostic tests for inborn metabolic defects by the qualitative analysis of urinary organic acids. The assessments done by the EQA give confidence to the participating laboratories that their test results are reliable and can be used to make good clinical decisions.

ERNDIM foundation was established in 1994 after adopting an external quality assurance scheme from the UK, which targeted specialized laboratories (Fowler *et al*., 2008; Peters *et al*., 2008). The purpose of its founding was to raise the levels of accuracy, precision, reproducibility and harmonization of laboratory testing in this field. ERNDIM offers three types of schemes; quantitative, qualitative proficiency and diagnostic proficiency testing. The quantitative scheme aims to get the test accuracy, recovery, precision and linearity for each laboratory after supplying them with samples of variable metabolite quantities. The qualitative proficiency focuses mainly on organic acids and acyl-carnitines. Participating laboratories are given matrix-modified samples where variable quantities of a range of metabolites are added to the urine. The laboratories are to analyse and interpret the results to either make or exclude a diagnosis. The diagnostic proficiency testing aims to assess the selectivity of the test based on clinical details provided analytical performance and interpretation of results (Fowler *et al*., 2008).

The assessment targets mostly the methodology and interpretation of IEMs. Under the category of methodology, the equipment used, whether oximation and an internal standard are used, is queried. For the right interpretation of the test sample, marks are given. It is from these scores and the questionnaire answers that the assessment is done and validated (Peters *et al*., 2008).

2.6. Literature Summary

Organic acids play key roles as intermediates of virtually all pathways of intermediary metabolism. Although they are classified as weak acids, they almost fully ionise at a pH above 4 and become strongly hydrophilic, enabling them to be excreted mostly in urine and to a less extent in other body fluids. When a metabolic disorder is present, there is a profuse accumulation and excretion of organic acids in urine. Therefore, the profiling of organic acids in urine is essential and cannot be done without in the screening and diagnosis of IEM.

Urinary organic acids are often in a matrix that is incompatible for GC analysis. This necessitates sample preparation steps that involve extraction of organic acids from the complex urinary matrix, concentrating them and making them more volatile and increase their signal intensity in the GC/MS by derivatization. Urinary organic acid extraction is mostly done by LLE, a process that is rate limiting, manually operated, tedious, labour intensive, error prone, time consuming and a health hazard. A lot of effort by analytical chemists has been given to the miniaturisation and automation of the solvent extraction procedure. A number of review papers have been published showing recent advancement in the efforts to miniaturise and automate the extraction of these analytes. The benefits of automating the extraction procedure of OAs are myriad, especially in contrast to the manually operated procedure.

The need for developing a fully automated LLE of organic acid is apparent from the scarcity of literature studies on it. Added to that, is the other need for the development of a quantitative analytical method for organic acid analysis. Whereas previously, the diagnosis of organic aciduria was done qualitatively, by merely inspecting of abnormal chromatographic peaks that are many times above the reference ranges of the organic acids of interest, but that is not the case anymore. Small increments in specific organic acids can be pathognomonic; therefore, metabolic laboratories now need a detailed quantitative analytical system of organic acid by GC/MS for accurate diagnosis. It is for this reason that ERNDIM also facilitates an external quality assurance programme for both qualitative and quantitative analysis of organic acid. Therefore, full method validation for any such analytical procedure is necessary to judge the method's performance in this regard and ascertain its fitness for the purpose of both qualitative and quantitative urinary organic acid analysis by GC/MS.

CHAPTER 3: AIM AND OBJECTIVES

3.1 Introduction

Literature documents that urinary organic acid analysis is indispensable for the diagnosis of IEM by GC/MS. The sample clean-up step in the analytical process poses as the challenge. It involves the isolation of the analyte of interest by an extraction process from an inappropriate matrix to one compatible with the analytical platform. As opposed to a fully automated and high-throughput sample preparation protocol, the in-house urinary organic acid extraction is still performed manually. It is a labour-intensive and time-consuming step involving the use of large amounts of solvents which can be hazardous to health. Thus, the development of a method that can be fully automated would improve the sample throughput, eliminate the intense labour and the time spent on sample preparation.

3.2 Aim and Objectives

Therefore, the aim of this study was to reassess and optimize the conventional/in-house organic acid extraction method for automation.

The specific objectives were:

I. To choose suitable immiscible solvent for the following tasks:
    a. Good solubility of organic acids in the upper phase
    b. Upper phase extraction of organic acids
    c. Easy and fast drying of sample
    d. Clear phase separation making centrifugation unnecessary
II. To choose suitable miscible solvent that will enable;
    a. Faster extraction by mixing completely with the sample
    b. Better extraction of organic acids which are not efficiently extracted by immiscible solvent.
III. To optimize the combinational volume of the two solvents
IV. To minimize solvent extraction volumes by doing the whole extraction procedures in gas chromatography (GC) vials.
V. Increase the speed of extraction as consequence of the above
VI. Miniaturize the method
VII. Automate the method
VIII. Validate the method

3.3 Experimental Outline

Based on the aim and objectives outlined above for this study, the following experimental plan was constructed. In order to develop and optimize the method for automation, organic acid standards of high purity were used to test the extraction efficiency of the solvents. This led to the other steps of the experiments as outlined below and explained in the following chapter.

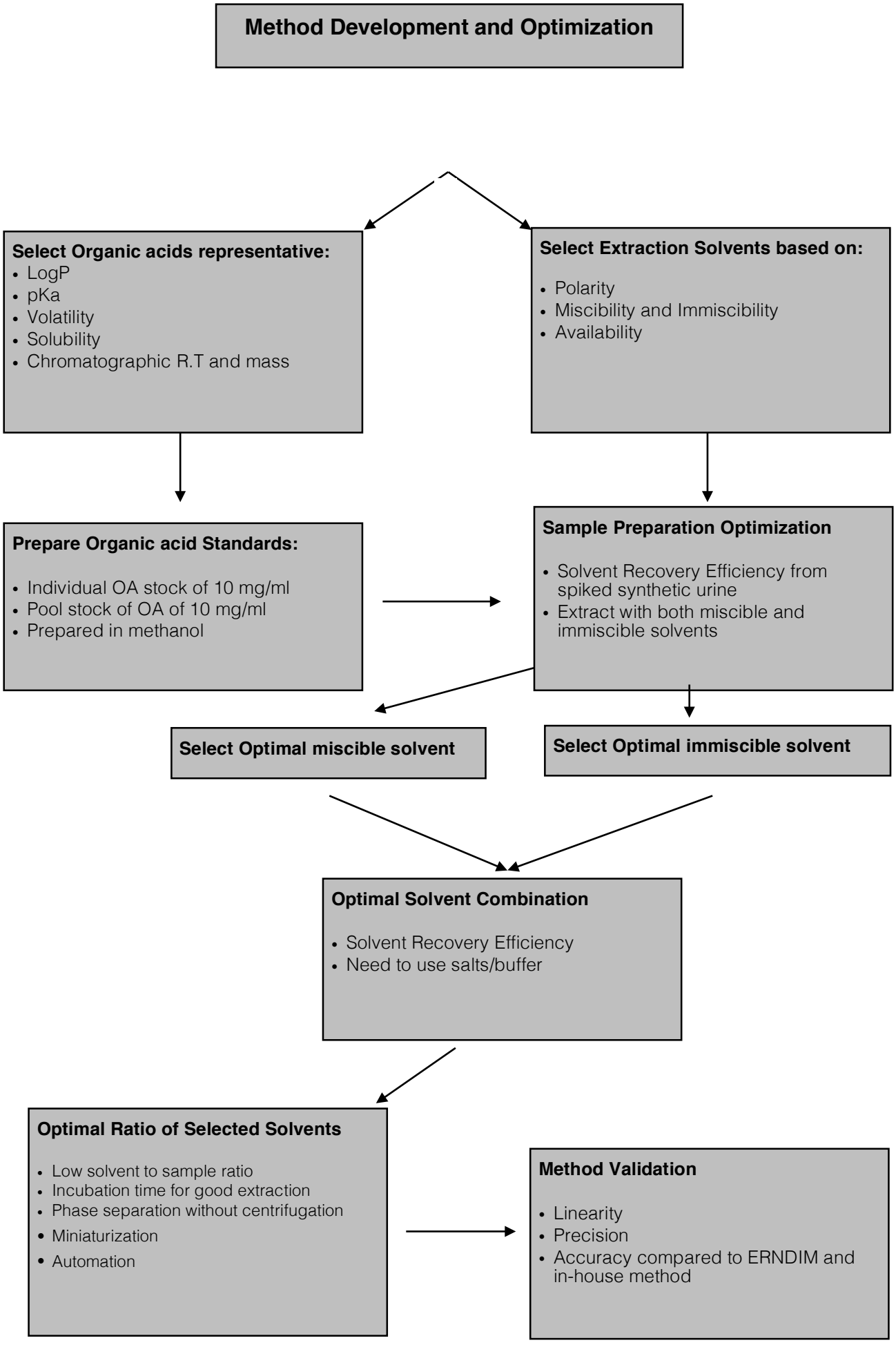


Figure 4-1 Experimental workflow

CHAPTER 4: MATERIALS AND METHODS

4.1 Introduction

The qualitative and quantitative profiling of urinary organic acids by GC/MS is an important tool for the diagnosis of inborn error of metabolism. In the analysis of organic acids at PLIEM, the extraction step using the in-house extraction method is the most labour-intensive and time consuming of this analytical process. This process is error prone due to the many analytical steps and sample spillages. The analysts' exposure to toxic reagents such as the extracting solvents and the derivatization reagent pose health harmful effects. Therefore, it was necessary to develop a method that could be miniaturized and automated. This involved developing and optimizing an organic acid extraction method that could be miniaturised and automated on a liquid AutoSampler. An initial one-phase solvent for rapid and simultaneous extraction of the analytes was selected among the miscible solvents. A secondary two-phase solvent was selected from immiscible solvents for the purpose of isolating the organic acids and the formation of a spontaneous two-phase system from the previous one-phase system after pipette mixing. The solvent volumes and the sample to solvent ratios were optimised and the method was miniaturised and automated. However, the derivatization and the GC/MS procedures were not part of the evaluation and optimisation process.

4.2 General Materials

4.2.1 Reagents, standards and solutions

The organic acid standards were selected and purchased based on their different physiochemical properties, namely; volatility, pKa, solubility and logP, for the purpose of developing and validating the method. Table 4.1 shows the properties of the organic acid standards and their selection criteria. All the organic acids were purchased from Sigma Aldrich Co., South Africa. The list included glycolic acid (cas # 79141), α-ketoglutaric acid (cas # 328507), succinic acid (cas # 110156), lactic acid (cas # 79334), malonic acid (cas # 141822), succinylacetone (cas # 15168), glutaconic acid (cas # 1724023), adipic acid (cas # 124049), methylmalonic acid (cas # 516052), fumaric acid (cas # 110178), ethylmalonic acid (cas # 601752), phenyllactic acid (cas # 103822), vanillymandelic acid (cas # 55107), 4-hydroxyphenylpyruvic acid (cas # 156398), sebacic acid (cas # 111206), 4-phenylbutyric acid (cas # 1821121), stearic acid (cas # 57114), 3-methylglutaconic acid (cas # 5746907),

phenylacetic acid (cas # 828013), glutaric acid (cas # 110941), citric acid (cas # 77929), pyruvic acid (cas # 127173).

Honeywell Burdick & Jackson, spectrometry grade, methanol (10071675), acetonitrile (10071618), water (10071715), acetone (10071616), isopropyl alcohol (10071758) and ethyl acetate (10071643) were purchased from Anatech, South Africa. High purity hexane (Sigma-Aldrich, cas # 296090), *N*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) with trimethylchlorosilane (TMCS) (Fluka, cas # 33155), diethyl ether (Merck, K44140221) pyridine (Sigma-Aldrich, cas # 270970), butanol (Sigma-Aldrich, cas # 71-36-3), sodium sulphate (Merck, 1.06649.0500) and magnesium sulphate (9909207) were used.

Table 5-1 List of Organic Acids, their Physiochemical properties and Selection criteria

Organic acid	Selection Criteria	CAS No	Volatility	pKa	Solubility	LogP
2-Ketoglutaric acid	Solubility	328-50-7	12,17	2,66	53,1	-0,11
3-Methylglutaconic acid	Volatility	5746-90-7	13,27	3,85	8,69	0,29
3-Phenylbutyric acid	IS	4593-90-2	17,52	4,79	0,54	2,47
4-Hydroxyphenylpyruvic acid	Solubility	156-39-8	16,75	2,91	1,49	1,6
4-Phenylbutyric acid	Solubility	1821-12-1	17.99	4,81	0,51	2,29
Adipic acid	LogP	124-04-9	14,24	3,92	32,2	0,13
Citric acid	pKa	77-92-9	15,54	3,05	106	-1,3
Ethylmalonic acid	Log P	601-75-2	11,98	2,5	94,1	0,66
Fumaric acid	LogP	110-17-8	9,35	3,55	24,1	-0,041
Glutaconic acid	Volatility	1724-02-3	11,36	3,69	17,6	0,05
Glutaric acid	Solubility	110-94-1	12,17	3,76	56	0,046
Glycolic acid	Volatility	79-14-1	6,2	3,53	608	-1
Lactic acid	LogP	79-33-4	8,06	3,78	562	-0,47
Malonic acid	pKa	141-82-2	8,13	2,43	197	-0,33
Methylmalonic acid	pKa	516-05-2	10,06	2,48	149	0,21
Phenylacetic acid	pKa	103-82-2	13,85	4,55	3,61	1,72

Organic acid	Selection Criteria	CAS No	Volatility	pKa	Solubility	LogP
Phenyllactic acid	pKa	828-01-3	16,7	4,02	9,8	1,18
Sebacic acid	Log P	111-20-6	22,61	4,72	0,91	1,93
Sorbitol	Selectivity	50-70-4	17,12	12,59	229	-2,7
Stearic acid	Solubility	57-11-4	38,64	4,95	6,61E-05	8,02
Succinic acid	LogP	110-15-6	10,14	3,55	211	-0,4

4.2.2 Organic acid preparation

Stock solutions of each organic acid standard at 1 mg/ml were prepared in methanol in a volumetric flask and stored refrigerated at - 20°C (Blau *et al*., 2008). A pre-mix stock solution of the organic acids was also prepared by separately weighing 100 mg of each organic acid standard and then dissolved in a small beaker with some methanol before decanting into a volumetric flask and filling it with 100 ml of methanol. The final concentration of the organic acid pre-mix solution was 1 mg/l.

4.2.3 Internal standard preparation

The internal standard 3-phenylbutyric acid (3-PBA), (Sigma-Aldrich, cas # 4593-90-2) was prepared according to the in-house protocol as shown in Appendix 1. 26.25 mg was weighed and dissolve in a few drops NaOH. 50 ml distilled H2O was added to it to have a final concentration of 0.52 mg/ml of internal standard. It was then stored in the refrigerator at 4°C/. 4-Phenylbutyric acid (4-PBA), prepared as in section 4.2.2 was also used as a second internal standard when needed.

4.2.4 Sorbitol

Sorbitol (cas # 50-70-4) was prepared as the organic acid standards (section 4.2.2) at the concentration of 1 mg/ml in methanol and stored at - 20ºC in the refrigerator. It was added to the organic acid standard mix to test for the selectivity of the method. Being a very polar compound, with logP value of -2.7 and high water solubility, sorbitol is typically not extracted

by some solvents used for extraction of organic acids. It was therefore used as an indicator of the selectivity of either the extracting organic solvent or the whole extraction system.

4.2.5 Synthetic urine

Synthetic urine (Surine, DTI, Lot 72110) was purchased and used for analysis. Ampath Pathologies determined the biochemical composition of the synthetic urine. The molar concentration (and reference range) of the synthetic urine was as follows: The creatinine concentration was 7.7 mmol/l (3.5 – 23.0), urine sodium was 205 mmol/l (20 – 230), potassium was < 2 mmol/l (17 – 80), chloride was 263 mmol/l (15 – 250), urea was 278 mmol/l (150 – 500), urine osmolality was 655 mmol/l (300 – 800), calcium was < 0.50 mmol/l, the calcium/creatinine ratio was < 0.06 (0.03 – 0.69), magnesium was < 0.40 mmol/l, the magnesium/creatinine ratio was 0.05 (0.08 – 0.51), urine phosphate was 1.20 mmol/l and the ratio between phosphate and creatinine was 0.2 (0.8 – 2.7). With the exceptions of a few electrolytes that were either elevated or low, the synthetic urine mimicked urine of a normal human subject in composition. The synthetic urine was tested for the presence of any organic acids in it using the in-house organic acid extraction method and none were present.

4.2.6 ERNDIM samples

ERNDIM EQAS 2016 Quantitative Organic Acids samples were used for the validation experiments. Seven lyophilised urine samples, all from the same basic urine but with various amounts of added analyte. The concentrations of these analytes varied between the physiological and typical pathological ranges (Martens & Weykamp, 2016). The test results were used to test accuracy and precision for the new method as well as to compare performance of the in-house method and the automated OA extraction method to the ERNDIM results.

4.3. Gas Chromatography-Mass Spectrometer

Two different GC/MS instruments were used in the course of the experiments. The first one was the Gas Chromatography Time of Flight Mass Spectrometer (GC-TOF-MS). This was used in the method development and optimisation phase of the study. The other was the Gas Chromatography Quadrupole Mass Spectrometer (GC-Q-MS). This instrument was

used in the method validation step. The reason for the shift of instruments to the GC-Q-MS was the unfortunate technical issues on the GC-TOF-MS that made it impossible to continue on that platform. Time constraints could not allow for a long wait to resume using the same instrument. The existing methods for organic acid analysis on these platforms were followed (described in section 4.3.1 and 4.3.2). The setback to this scenario was the limited ability to compare the experimental results from the two instruments, yet because of the distinction of the two phases of the study; the intra-instrument experimental results were good, reliable and effective for achieving the purposes of these phases.

4.3.1 GC/MS Parameters

The in-house GC-TOF-MS-system parameters consisted of an Agilent 7890 GC system coupled with a Leco Pegasus HT mass analyser. An Agilent 7693 auto sampler was used for sample introduction. Chromatography was performed on a Restek RXi®-5 column (10 m×0.2 mm×0.18 µm). A sample volume of 1 µl was injected (using a split/split less injector) per run with a 1:10 split ratio. The front inlet temperature was kept at 250 °C throughout the entire run. An initial oven temperature of 70 °C was maintained for 0.5 min and then increased at a rate of 40 °C per min to 110 °C where it was kept constant for 0.5 min. Temperature was then subsequently ramped as follows: 10 °C/min to 125 °C, 15 °C/min to 140 °C, 25 °C/min to 185, 12 °C/min to 200 and 30 °C/min to 300 °C, where it was maintained for 1min equating to a total run time of ∼12 min per sample. Nitrogen was used as carrier gas at a constant flow of 1 mL/ min. The transfer line was maintained at 225 °C and the ion source temperature at 200 °C for the entire run. Acquisition was delayed for the first 110 s which served as a solvent delay. Data were captured with an acquisition rate of 20 spectra (50–950 m/z) per second.

An in-house GC-Q-MS method was used. The following are the set parameters on the instrument. One microliter of sample is injected onto the GC using split injection at a split ratio of 10:1 and a constant pressure of 8.2317 psi. Injection port temperature was 280°C. Initial oven temperature was 50°C that was held for 1 minute then 20C/min to 60°C The temperature was then increased at 5°C per minute to 120°C then at 7°C per minute to 295°C which was held for a further 4 minutes. Run time was 42.5 min with 2 min post run at 300°C. The column used was a 30 metre VF1-MS/ DB1-MS UI with a constant flow of helium at1ml/minute at a pressure of 7.6522 psi. A solvent delay of 8 minutes is stipulated in the temperature program.

4.4 Sample Preparation

The in-house sample preparation procedure (appendix 1) was used for comparative purposes during the development of the new method. In brief the synthetic urine volume used was determined according to the creatinine values as shown in Table 4.2. The determined volume of the synthetic urine was added to the first kimax tube (10 ml) using a 1 ml pipette. Using a Pasteur pipette, 6 drops of 5N HCl was added to adjust the pH of the synthetic urine to 1. A 100 µl of internal standard (3-PBA) was added using a 100 µl pipette. 6 ml of ethyl acetate was added to the sample mixture and mixed on rotor for 30 minutes. The samples were centrifuged at 3000 revolutions per minute (rpm) for 3 mins to allow for separation of the organic and aqueous phases. The organic phase was carefully aspirated (so not to aspirate any of the lower aqueous phase) into a second large kimax tube using a second Pasteur pipette. 3 ml of diethyl ether was then added to the remaining aqueous phase in the first kimax tube and was mixed on the rotor for 10 minutes. The samples were centrifuged again as previously done for clear phase separation and the organic phase was aspirated using the second Pasteur pipette into the second kimax tube that already contained the ethyl acetate extract. Two spatulas of sodium sulphate were added to the second kimax tube to remove any water that may have been aspirated, as water damages the GC column. The mixture was vortexed and centrifuged for 3 minutes at 3000 rpm to allow the pelleting of sodium sulphate. The organic phase was separated from the sodium sulphate pellet by pouring it into a clean small kimax tube. The extracted organic phase was evaporated to dryness under nitrogen at 37ºC for about 45 minutes. The organic acid residue in the small tube was derivatized by adding 100 µl of BSTFA/TMCS (99:1) and 100 µl of pyridine using a Hamilton syringe and was left to incubate for 45 minutes at 60ºC. After allowing for 5 minutes for the extract to cool, it was transferred from the small kimax tube to a GC sample vial with insert using the Hamilton syringe and it was screwed tightly. It was loaded on the GC auto sample tray and 1 µl of the extract was injected into the GC.

These sample preparation steps were used as a guideline to achieve the set objectives of assessing the extraction efficiency of different extraction solvents, as well assessing the optimal extraction solvent volumes, sample-to-solvent ratios and the influence of sample mixing time.

In order to evaluate these parameters, absolute and relative recovery experiments were carried out. An absolute recovery experiment assessed the overall efficiency of the extraction system by determining the amount of analyte recovered from the urine versus the unextracted standards (Peters *et al*., 2007). This was done in two tubes, one having the

extracted organic acids and the other organic acids spiked post-extraction of blank urine, for similar matrix effect. Two 1 ml aliquots of synthetic urine samples were spiked with 20 µl of the organic acid standard mix containing 100 ppm of each organic acid analyte. A 100 µl of the internal standard was added to the synthetic urine organic acid mix. One synthetic urine sample was spiked before extraction and the other after extraction. The relative recovery is the amount of the analyte recovered from the matrix with reference to the extracted internal standard that is spiked into the same matrix (Hassan & Cooper, 2009).

Table 5-2 Determination of urine volume using creatinine concentration

Creatinine Concentration (mmol/l)	Volume of Urine to use (ml)
0.25 – 0.37	3.0
0.38 – 0.74	2.0
0.75 – 5.55	1.0
5.56 and above	0.5

4.5 Data Processing

Different in-house data processing software and methods were used, each relevant to the instrument used. For the GC-TOF-MS, an in-house protocol was used for data processing. ChromaTOF (Leco) was used to perform data extraction, in terms of baseline subtraction, peak detection and deconvolution. The “span” baseline tracking mode was selected with offset of 1. The program was allowed to automatically select smoothing parameters. Peaks were detected using an expected peak width of 3 s and signal-to-noise ratio of 20. Also, only masses between 100 and 800 m/z were used as model ions and any true peak had to contain five apexing masses. Compounds were identified via spectral matching to the NIST11 commercial library and an in-house created library (Reinecke *et al*. 2012). A spectral match of 80 % similarity (similar to 800 in ChromaTOF) was needed before a compound could be given an identity. Also, only identities that contain at least one Si element in its formula were allowed to eliminate spectral matching of non-derivatized compounds or incorrect derivatives (Venter *et al*., 2016).

The **A**utomated **M**ass **S**pectral **D**econvolution and **I**dentification **S**oftware (AMDIS; Stein 1999) was used to extract GC-Q-MS data according to the specifications of the program. The GC–MS data was analyzed in AMDIS in batch mode with the following deconvolution settings: component width of 20, adjacent peak subtraction of 1, resolution at low, peak

shape at low and sensitivity at low. The following masses were omitted as model ions to limit the detection of false positives: TIC, 73 and 147. The NIST08, an in-house created organic acid library (Reinecke *et al*. 2012) and an in-house created Organic Acids library were used to identify detected compounds based only on their mass spectral similarities. Compounds that were identified with low confidence were eliminated and those with more than one accurate hit were annotated with '?'.

Equation 21 was used to convert the intensity values to concentration (mg/L) using the internal standard.

$$[A] = \frac{R_A}{R_{IS}} . [IS] \qquad [21]$$

Where $[A]$ and [IS] are the concentrations of analyte A and the internal standard, R_A and R_{IS} are intensity values of analytes *A* and the internal standard respectively.

The extraction efficiency for each evaluation was determined firstly by calculating the concentration of each organic acid analyte from both the pre-extraction and post extraction spiked samples, then comparing the extracted organic acid analyte/IS ratio to that of the unextracted organic acid analyte/IS ratio. The recovery of each analyte was expressed in percentage form.

4.6 Method Development and Optimisation

In order to develop an optimised and automated LLE organic acid extraction procedure, a number of requirements were initially postulated. Some of the requirements were adapted from the BUME method: an automated chloroform-free 96-well method for total lipid extraction from blood plasma (Löfgren, 2012). Although the postulations were applied to the automation of a lipid extraction protocol, the principles could still be applied to the automation of an organic acid extraction protocol on a liquid sample handler. The following were the method requirements:

- Use a post creatinine corrected urine sample volume of 0.5 ml in a glass vial. According to Jones and Bennett, (2010), the sample volume must not be less than 0.5 ml for effective extraction. Since the aim was to carry out the whole extraction in a 2 ml GC vial, the maximum volume it could take without spillages during the extraction procedure was 1.8 ml. Consideration also had to be taken of the addition of internal standard and HCl acid before the addition of the extraction solvents. Yet the sample volume could not be less than the stated one.

- Have an initial one-phase extraction with a miscible solvent that would enable rapid dissolution of organic acids. This solvent would mix completely with water thereby enabling rapid extraction of organic acids from urine.
- Use a secondary immiscible solvent for a two-phase extraction that would give an extracted upper organic phase. It was desirable to work with the upper extracted phase because it is easier to aspirate and there is less contamination of sample during the aspiration steps, as opposed to working with the bottom phase. The optimal solvent was required to not only be a good extractant of urinary organic acids but to also be able to extract the initial miscible solvent 1 into it.
- Solvent to sample volume ratio of ≤ 4:1 in the total mixture. In order to have good extraction efficiency of urinary organic acids, it is required to have a large solvent to sample ratio. But owing to the volume capacity of a GC vial, such a large solvent to sample would not be attained without reducing the volume of the urine sample. This leaves little space for an increased volume of extraction solvents.
- Spontaneous phase separation without centrifugation. In order to increase the sample through put, having to remove the samples from the liquid autosampler to place them in a centrifuge would be tedious and rate-limiting, especially in the case of large sample batches. It was therefore necessary to have a method that would eliminate the need to centrifuge by obtaining rapid clear phase separation simply letting the samples to stand.
- A method that would enable the performance of three repeated cumulative extractions with solvent 2 for greater recovery of organic acids (Mitra, 2003).
- Fully automated extraction procedure up to solvent evaporation step. There is a possibility to even automate the evaporation step, as well as the derivatization one, but that was beyond the scope of this study.
- Less than 40 minutes' extraction time per sample and less than 4 hours for 24 samples. Due to the miniaturisation of the extraction, the extraction procedure could be quickened and shortened, hence the requirement.

4.6.1. Construction of a reference library

The compound identification experiment was performed for the purpose of accurately identifying the selected organic acids and to construct a reference library and a data processing method on the GC/MS-TOF. The experimental procedure followed was: 20 ul of each organic acid standard was dried down at 37 °C under nitrogen and the residue was derivatized with 100 µl of BSTFA + TMCS, 99:1 and 100 µl of pyridine. One microliter was injected into the GC-TOF-MS. Data was presented in the form of a total ion chromatogram

(TIC). Library search was used to identify the mass spectra and confirmation of these identifications was done by operator-comparison against chromatographic retention time, spectra match and chemical structure of the compound in some cases.

4.6.2 Selection of optimal solvents

A number of solvents have been cited in literature studies that have been used for urinary organic acid extraction. These solvents are mostly non-polar immiscible solvents because miscible solvents are unsuitable for conventional LLE. Among these solvents are ethyl acetate, diethyl ether, methyl acetate, etc. Some of these solvents have been used in combination with each other, e.g. ethyl acetate and diethyl ether. Yet polar miscible solvents are better extractants of more polar organic acids (Clement & Hao, 2012). The solvent for the initial one-phase extraction was selected from among the miscible solvents. The secondary optimal solvent for the two-phase extraction was selected from the immiscible solvents. The following were the experiments carried to achieve the study's objectives. In order to select the optimal miscible solvent for the initial one-phase extraction, the following solvents were selected and the procedure below was followed.

4.6.2.1 Selection of optimal solvent 1

The objective for selection of solvent 1 was to have a solvent that could give an initial one-phase extraction step. This solvent is important for rapid dissolution of organic acids in the organic solvent as it mixes completely with water thereby enabling rapid extraction of organic acids from urine. Methanol, ethanol, isopropanol, acetone and acetonitrile were among the solvents selected for the one-phase extraction step of urinary organic acids. The protocol for the extraction of organic acids from urine described in section 4.4 was followed for these experiments. A few modifications were made in accordance with experimental objective. In this experiment, two modifications were made; firstly, 6 ml and 3 ml of each of the selected solvent were used in the first and second extractions respectively. Secondly, sodium chloride and magnesium sulphate were added to induce phase separation between the miscible organic solvent and the sample. An absolute recovery experiment was conducted where the organic acids standard mixture was spiked before and after extraction. All samples were done in six replicates.

4.6.2.2 Selection of optimal solvent 2

After the selection of solvent 1 for the initial extraction step, solvent 2 was selected. The solvents that were investigated for selection as an optimal extracting solvent for urinary organic acids were ethyl acetate (EtAc), diethyl ether (D.E), methyl tert-butyl ether (MTBE) and the combination of ethyl acetate and diethyl ether. Butanol was also assessed in an initial experiment, but its solubility in water was high (20g of solvent in 100 g of water). This was clearly evidenced in the observation of the solvent's failure to give a clear phase separation between the phases. Initial attempts were made to extract with various non-polar solvents. Some of the solvents used in the earlier extractions were hexane, heptane, combinational mixtures of butanol and methanol and hexane with ethyl acetate. This was in an attempt to narrow down to a list of solvents that were later on investigated for optimal solvents for urinary organic acids extraction.

The sample preparation procedure in section 4.4 was followed. However, a few modifications were made. For ethyl acetate and diethyl ether, there was no modification to the protocol, but for each individual solvent such as MTBE, D.E and EtAc, the second extraction was done by the addition of 3 ml of the same solvent. Recovery experiments were done in triplicates.

4.6.3 Optimisation for the Two-Phase extraction

Following the selection of suitable extraction solvents from the previous steps, one being a miscible solvent and the other immiscible solvent, there was need to establish a two-phase extraction system between the aqueous phase and the two organic solvents. The method requirement for this was the addition of the secondary immiscible solvent to the previously formed one-phase system would give a spontaneous formation of a two-phase system after mixing, thereby having the first solvent move to the top organic phase.

To get the optimal ratio between the miscible and immiscible solvent for the two-phase extraction system, a number of ratios were investigated (Table 4.3). 10 ml of water was poured into each of the 4 100 ml measuring cylinders, after which 10 ml of the suitable miscible solvent was added to each one of them. The mixture was vortexed to mix completely. The volumes of the immiscible solvent added were 10, 20, 30 and 40 ml from the 1st measuring cylinder to the forth one respectively. The mixtures were again vortexed and left standing to allow for clear phase separation. Visual observation of the distribution of the phases was used to guide the selection of the optimal sample-to-solvent-solvent ratios.

The experiment was repeated, but using smaller volumes this time round. 1 ml of synthetic urine was acidified with six drops of 5N HCl acid. The volume level of the synthetic urine was marked on the kimax tube with a marker for the purposes of tracing its level, before the addition of 1 ml of the selected water-miscible solvent to it. The new volume level of the synthetic urine and the miscible was marked as well, followed by vortexing of the mixture for a minute. Similar to the previous step, 1, 2, 3 and 4 ml of the suitable secondary immiscible solvent were added to the sample mixture. The levels were marked again. This final mixture was vortexed for a minute and rotary shaken for 10 minutes, followed by centrifugation. Again, visual observation of the phases was used as a guide the selection of the optimal solvent-to-solvent ratio that met the objective of the experiment.

After establishing the two-phase system with the optimal ratios of the solvent 1 and 2, the extraction efficiency of this system was investigated using the sample preparation protocol in section 4.4. The experiment was done in triplicates. A salt buffer made of magnesium sulphate and sodium chloride (4:1 w/w). Two sets of sample preparations were carried out, both with the two solvents in the exact ratios. The only difference is that to one set the salt buffer was added and to the other it was not added. This was to assess the effect of the use of salts in getting a better phase separation where a miscible solvent is concerned as well as its effect on the extraction efficiency of the solvents.

Table 5-3 Investigated ratios between the miscible and immiscible solvent

Ratios	Water	Miscible Solvent	Immiscible Solvent
1st Cylinder	1	1	1
2nd Cylinder	1	1	2
3rd Cylinder	1	1	3
4th Cylinder	1	1	4

4.6.4 Optimisation of solvent volume ratios

Being guided by the optimal ratios of the two-phase extraction system between solvents 1 and 2, the optimal volumes of the two extraction solvents were investigated. One of the postulated method requirements for automation was to ultimately carry out the whole extraction procedure in a 2 ml vial. This was to be accomplished by reducing the volumes of the solvents that were to be used in extraction. Therefore, the aim was to have the minimal

amount of the solvents that could still give rapid and good extraction efficiencies of the organic acids.

4.6.4.1 Optimal sample to acetonitrile ratio

The objective of this experiment was to get an optimal ratio of sample volume to the volume of acetonitrile to enable a rapid and efficient extraction of organic acids. The postulated ratio was 1:1 sample-to-acetonitrile that was then used as a reference to assess whether using less volume of acetonitrile could be used in the extraction procedure and still obtain good extraction efficiency of the system. A relative recovery experiment was carried out to achieve this objective. For the purpose of achieving the objective, a relative as opposed to an absolute recovery was deemed suitable because it would give comparative data based on the reference ratio of 1:1.

Table 4.4 shows the 4 different solvent ratios that were used in the protocol. The organic acid standards were extracted as described previously in the sample preparation section 4.4. The relative recoveries of were determined by comparing the extracted organic acids/IS percentage to that of the reference sample-to-acetonitrile ratio (1:1) recoveries percentages. The experiment was done in triplicates for each solvent ratio combination.

Table 5-4 Optimal volume ratios of sample to acetonitrile

	Sample (ml)	Acetonitrile (ml)	Ethyl acetate (ml)	Sample:ACN Ratios
A	1.00	0.25	5.00	1 : 0.25
B	1.00	0.50	5.00	1 : 0.50
C	1.00	1.00	5.00	1 : 1.00
D	1.00	2.00	5.00	1 : 2.00

4.6.4.2 Optimal acetonitrile to ethyl acetate volume ratio

The optimal volume ratio of ethyl acetate to acetonitrile in the extraction procedure that would give the most recovery of organic acids was investigated in this experiment. This was determined by keeping the sample and acetonitrile volumes constant, while varying the volumes of ethyl acetate, as shown in Table 4.5. The sample preparation in the previous section 4.6.4.1 was followed, the modification being the varying ethyl acetate volumes.

Table 5-5 Optimal volume ratios of acetonitrile to ethyl acetate

	Sample (ml)	Acetonitrile (ml)	Ethyl acetate (ml)	ACN:EtAc Ratios
A	1.00	0.50	1.00	1 : 2
B	1.00	0.50	1.50	1 : 3
C	1.00	0.50	2.00	1 : 4
D	1.00	0.50	2.50	1 : 5

4.6.5 Optimisation of sample: solvent ratio

After the establishment of the optimal volume ratios for sample-to-acetonitrile and acetonitrile-to-ethyl acetate, the sample-to-solvent ratios were investigated and optimised. The objective of this optimisation experiment was to find a low sample-to-solvent ratio of equal to or below 1:4 that could still secure a good extraction efficiency of the standard organic acids. In order to carry out this experiment, 4 different sample-to-solvent ratios as shown in Table 4.6 were employed in the recovery experiment. Note that the solvent ratio remained constant.

Table 5-6 Sample-to-Solvent ratios

Series (Ratios)	Sample (ml)	Solvent (ml)	
		Acetonitrile	Ethyl acetate
A (1 : 0.5)	1	125	375
B (1 : 1)	1	0.25	0.75
C (1 : 2)	1	0.5	1.5
D (1 : 4)	1	1	3

4.6.6 Optimisation of sample-solvent mixing time

After the selection of suitable solvents and their optimal volume ratios, the effect of sample-solvent mixing time on extraction efficiency was investigated in this experiment. If the extraction time was to be reduced and miniaturised, this time-consuming and limiting step needed to be investigated and optimised for the miniaturized and automated method.

The extraction protocol in section 4.4 was followed with the following modification. All the samples tubes were mixed on the rotor for 5 minutes after the addition of acetonitrile, keeping that constant. After the addition of ethyl acetate, one set of samples were left to mix on a rotor for 5 minutes, another for 10 minutes and the other two for 20 and 30 minutes respectively. The rest of the protocol was the same for each set of samples.

4.6.7 Miniaturisation of the extraction protocol

After selecting the optimal solvents for the two-phase extraction, optimizing the solvent volumes, sample-to-solvent ratio and time for mixing for the sample and solvent, the extraction was miniaturised for possible automation on the Hamilton Autosampler. The sample volume was reduced to 500 µl of surine into a 2 ml vial. This was spiked with 20 µl organic acids standard. 300 µl of acetonitrile was added after the addition of 100 µl of internal standard (concentration of 0.52 mg/l). Four drops 5N of HCl was added to acidify the sample to pH 1. Instead of vortexing the sample for sufficient mixing, repeated aspiration/dispensing steps (pipette mixing) was done without letting air-bubbles in as this affected the phases settling time. A volume of 600 ul of ethyl acetate was added to the mixture. The pipette mixing was repeated 10 times. The mixture was left for 5 min to settle and obtain clear phase separation. Six hundred microliters of top organic solvent phase were aspirated into a second clean vial. For the second extraction step, 600 µl of ethyl acetate was added to the first vial, pipette mixing was repeated and the mixture was allowed to obtain clear phase separation by standing for 5 min again. Another 600 µl of top phase was transferred into second vial. A final 600 µl of ethyl acetate was added to the first vial, then left to stand for phase separation without the aspiration and dispensing step. A last volume of 600 ul of the top phase was aspirated and added to the second vial. The collected sample was dried at 40 °C under gentle stream of nitrogen. The residue was derivatized with 100 µl of BSTFA+TMCS (99:1) and 100 µl of pyridine. It was left to react at 50^{o}C for 30 minutes (Christou *et al*., 2014). After cooling for about 5 min the derivatized sample was transferred into inserts and 1 µl was injected into the GC/MS.

To evaluate and determine the extraction efficiency of this miniaturized organic acid extraction protocol, an absolute recovery experiment was done as described previously.

4.6.8 The automated organic acid extraction method

After finalizing the protocol in terms of solvent selections, ratios, mixing times and miniaturisation, the method was set up on a MicroLab (ML) Star Autosampler (Hamilton Technologies) liquid handler. The miniaturised method was translated into an automated extraction procedure. Initially some steps in the extraction protocol were performed manually. The method was automated on the Hamilton ML Star Autosampler. Twenty microliters of the organic acid standard and a 100 µl of the internal standard were manually pipetted into GC vials. The vials were then loaded in the vial carrier on the Hamilton. The Hamilton was programmed to pipette 500 µL of the sample into each of the vials. The

sample was manually acidified with 3 drops of 5N of HCl. An amount of 200 µl of acetonitrile was added to the sample mixture. In order to mix the acetonitrile and the sample, the pipette-mixing circle was programmed on the Hamilton AutoSampler; it aspirated 300 µl of the top part of the sample mixture and dispensed it at the bottom of the vial at high pressure. This action was repeated twice for each sample. This enabled thorough mixing of the sample and acetonitrile. Six hundred microliters of ethyl acetate were automatically pipetted into the vials. Mixing as described earlier followed this, only it was done 4 times per sample for the ethyl acetate/ sample mix. The samples were then centrifuged for 1 minute at 3500 rpm by the operator and put back on the Hamilton. Six hundred of the top phase was aspirated and dispensed into the second vial on the other sample carrier. A second extraction step was carried out by an addition of 600 µl of ethyl acetate, followed by the mixing step and centrifugation. 600 µl of top phase was again aspirated and dispensed into the second vial. In order to get as much of the extracted organic acids, another 600 µl of ethyl acetate was pipetted into the first GC vial yet without the mixing step. The mixture was just centrifuged and 600 µl transferred to the second GC vial.

The final extracted solvent was 1.8 ml. This was dried at 37^{o}C under a gentle stream of nitrogen. The drying took approximately 15 minutes. The residue was then derivatized with a 100 µl of BSTFA+TMCS (99:1) and 100 µl of pyridine at 60^{o}C for 30 minutes. The sample was then transferred into GC inserts and 1 µl was injected into the GC/MS.

4.6.8.1 Determining the need for centrifugation and drying of sample using sodium sulphate

A recovery comparison experiment was conducted to determine the effect of using $NaSO_4$ with/without centrifugation after extraction with the automated protocol. Four sets of samples were investigated (see Table 4.7) using the automated extraction method. The same described protocol was run for all the samples except after the mixing step. The first and second sets of samples were centrifuged while the other two were left to stand for 5 min for the two phases to settle. After the extraction was done, the first and third sets of samples had $NaSO_4$ added to them. All the samples were then evaporated, derivatized and injected as described previously.

Table 5-7 Summary of sample sets

No.	Sample Set	Pre-extraction spiking	Post-extraction spiking
1	Centrifugation with Sodium sulphate	3	3
2	Centrifugation without Sodium sulphate	3	3
3	No Centrifugation but with Sodium sulphate	3	3
4	No Centrifugation and no Sodium sulphate	3	3

4.7 Method Validation

The automated sample preparation method performance was evaluated in a number of method validation parameters, such as linearity, imprecision and inaccuracy. The sigma metric for method decision was also determined.

4.7.1 Linearity

The linearity of the automated method for each of the standard organic acids was investigated. Calibration standards were prepared by spiking surine with the organic acid standard mixture at concentration ranging from 0 to 500 mg/L. A seven-point calibration range was done. All the calibrators were made up in methanol. The calibration standards were extracted with the automated organic acid protocol.

4.7.2 Imprecision study

The imprecision of the automated protocol was estimated was by analysing three samples per day for a period of five consecutive days. Using the synthetic ‘blank’ urine, three pools of varying concentrations were made up, namely lower reference range, upper reference range and a critical value. The spiked urine samples for each level were further aliquoted to have 15 samples for each level. Each level was analysed in triplicates per day of the five days. The within run and within-laboratory was calculated from the variance components obtained.

4.7.3 Inaccuracy study

The inaccuracy of the automated organic acid extraction protocol was estimated by analysing the external quality assessment (EQA) ERNDIM samples. Seven samples were provided and they were analysed in triplicates to determine the inaccuracy on the method. The method's results were compared to those of the reference method and that of the PLIEM. All the results were compared but of special interest are the organic acids whose standards were used during the method development.

From the regression statistics, the proportional error was calculated from the slope, the constant systematic error from the y-intercept and the random error from the standard deviation about the regression line. The systematic error (bias) was estimated at the critical medical decision concentration (MDC) point using the regression equation, with the exception of the organic acids where the MDC was not available. The method bias was calculated as the percentage bias from the ERNDIM assigned concentration. For the organic acids, whose MDCs were not available, the bias was calculated simply using the equation $SE = Y_{av} - X_{av}$; where av is the average of Y and X. The MDCs were adapted from Blau *et al*, (2008). The differences between the measurements of the test method and the reference method were presented in a comparison plot. Regression statistics were performed in Microsoft Office Excel 2010. These statistics provided information on the correlation of the two methods and estimated the systematic error of the new method.

CHAPTER 5: METHOD OPTIMISATION RESULTS AND DISCUSSION

5.1 Introduction

The aim of this study was to reassess, develop and optimise an organic acid extraction method for automation. This was in view of attempting to improve on the extraction of the in-house method with the automated extraction method. The first part of the study focussed on method development by selecting suitable solvents, solvent volumes sample-to-solvent ratios, miniaturisation parameters that met the automation method requirements. This chapter documents and discusses the results of the method development and optimisation experiments, while the next chapter discusses the method validation experimental results.

5.2 Organic Acids Identification

The Table 5.1 summarizes the most abundant quantitation ions (Q-ions) for each organic acid that was injected on the GC/MS-TOF. Each organic acid was injected separately and collectively from a standard mixture into the GC/MS. The organic acids were identified by their chromatographic retention time and the mass spectra from the TOF-MS. The individual peaks of the organic acids were used to construct a user library for peak identification. The user library had the name the common name of the organic acids, their quantitation ions, retention times and the areas. Appendix 2 shows the TIC of all the organic acids with the x-axis displaying the mass-to-charge (m/z) ratio and the y-axis the counts of each m/z.

Table 6-1 Summary of highest quantitation ions and their retention times for the organic acids

Organic Acid	Q-ion (m/z)	Retention Time (s)
Lactic acid	117	150
Glycolic acid	66	158
Malonic acid	171	247,95
Malonic acid	148	248,85
Methylmalonic acid	56	260,9
Phenylacetic acid	91	313
Ethylmalonic acid	55	317,35
Succinic acid	148	342,8
Fumaric acid	245	378,15
Glutaric acid	55	423,1
3-Phenylbutyric acid	118	439,6
4-Phenylbutyric acid	117	509,3
Adipic acid	111	510,95
Glutaconic acid	171	687,65
Citric acid	273	793,4
Sebacic acid	55	821,5
Sorbitol	103	893,95
Stearic acid	117	1025,3

5.3 Optimal Solvents for Extraction

5.3.1 Optimal miscible solvent

From the five miscible solvents that were assessed for extraction efficiencies of urinary organic acids, methanol, ethanol and isopropanol could not be used. The organic solvents could not be separated from the aqueous phase despite using the salting out effect to force phase separation. Isopropanol was observed to have phase separation, however the extracted organic solvent was observed to have high water content. This made it impractical to use the solvent because its selectivity was decreased due to the high water content. This also affected the solvent's rate of evaporation at 37ºC as the samples were observed to take a long time to dry.

Acetonitrile and acetone were the only two organic solvents that were successfully assessed. Table 5.2 summarises the recovery percentages of the organic acids extracted by the two solvents. Acetone was observed not to have as clear phase separation as acetonitrile. It was observed to only extract some of the spiked organic acids and failed to extract others. The organic acids that were extracted with acetone were fumaric acid, glutaric acid, glycolic acid, lactic acid and succinic acid, most of which had low logP values and more polar than most of the other organic acids used.

Acetonitrile had good extraction efficiencies for most of the spiked organic acids as opposed to acetone. The extraction efficiencies for acetonitrile ranged from 16% (citric acid) to 67% (4-Phenylbutyric acid). Acetonitrile was observed to display some selectivity in its extraction of the spiked analytes by small quantity of the spiked alcohol sorbitol was extracted from urine. Acetone, on the other hand, proved to favour polar compounds in extraction as was evidenced by the over extraction of sorbitol. Great variance was observed in the acetonitrile results. Despite its better extraction efficiency, the extracts from it were still rather "dirty", something that was also noted by Majors, (2014). This is the plausible cause for the great variance in the results. The lowest CV was 4% for glycolic acid and the highest was 72% for glutaconic acid. It was observed that the possible cause of the variation between the extracted analytes was because of the presence of water in acetonitrile which may influence the extraction efficiency of various organic acids differently, depending on the distribution of the organic acid between the two phases. The analytes extracted with acetone were not consistent in all the samples; averages from the triplicates could not be obtained and thereby CVs could not be calculated.

Figure 5.1 and Table 5.2 illustrate the extraction efficiencies and variation of the organic acids according to increasing logP values. There was no trend observed on the extraction efficiency of organic acids with increasing logP values. According to Majors, (2014), acetonitrile was the preferred solvent for the extraction of polar compounds, such as very polar pesticides and organic acids. In the Quick, Easy, Cheap, Effective, Rugged and Safe (QuEChERS) technique for the isolation of polar compounds, acetonitrile was the solvent of choice because it easily separated from water upon addition of salts and since it has a higher polarity than acetone, the medium to high polarity compounds have much solubility in acetonitrile than in acetone or even ethyl acetate (Majors, 2014). Therefore, based on the findings of the results and literature studies, acetonitrile was selected for further experimentation. It was selected as suitable for the phase-one solvent in method development. Acetonitrile fulfilled the requirement of being a solvent that mixes completely with the urine sample and has the polarity that enables it to extract organic acids from the aqueous phase into the organic phase.

Table 6-2 Mean recoveries and CVs for organic acids extracted with acetonitrile and acetone

Analytes	**Acetonitrile**		**Acetone**	
	Mean Recovery %	CV %	Mean Recovery %	CV %
Sorbitol	-	-	114	-
Citric acid	16	58	-	-
Glycolic acid	42	4	69	-
Lactic acid	57	7	124	-
Succinic acid	48	29	2	-
Malonic acid	56	26	-	-
Fumaric acid	54	19	6	-
Glutaric acid	66	19	17	-
Glutaconic acid	46	72	-	-
Adipic acid	55	51	-	-
Methylmalonic acid	55	27	-	-
Phenylacetic acid	64	13	-	-
4-Phenylbutyric acid	67	35	-	-
Stearic acid	49	22	61	-

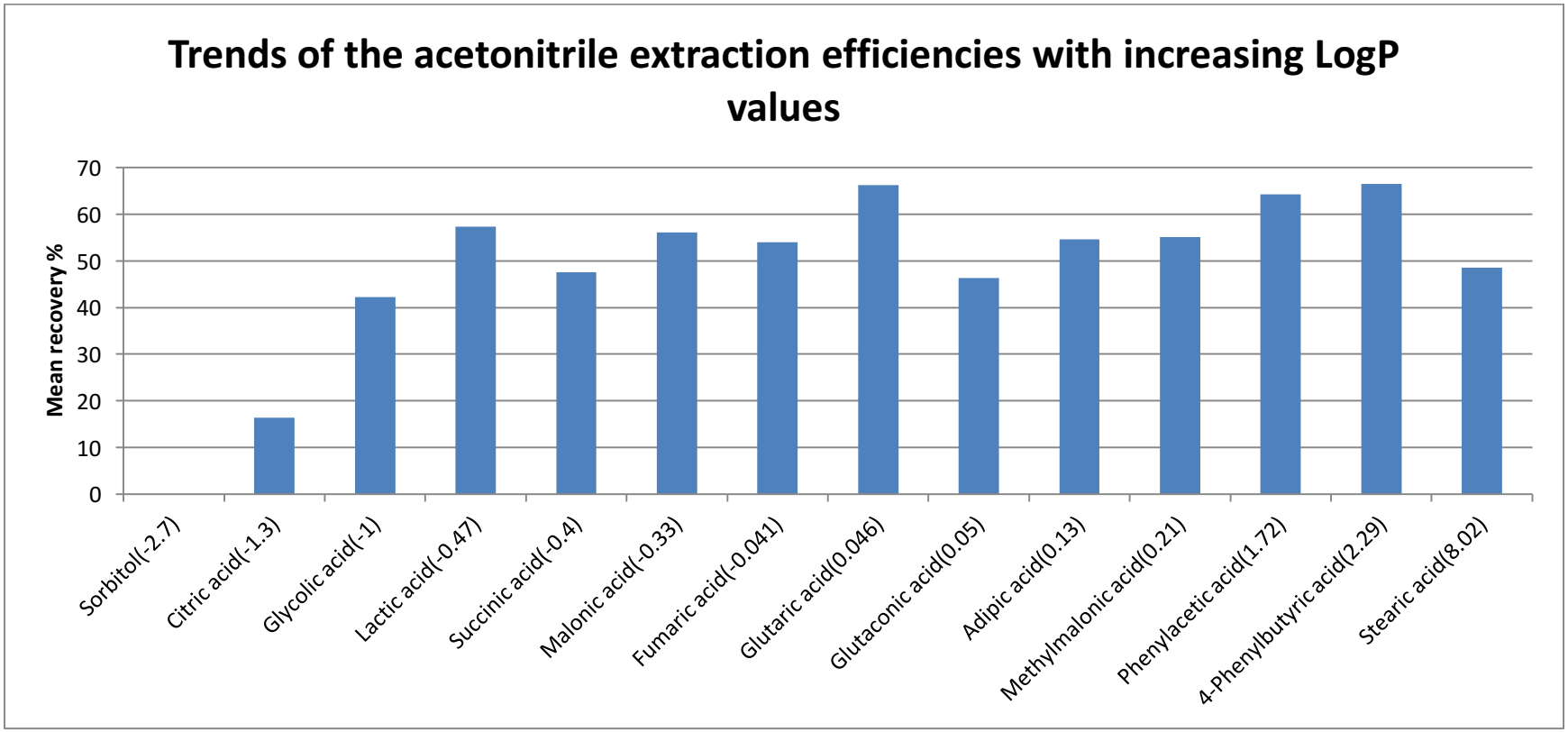


Figure 6-1 Extraction efficiencies of the organic acids increasing with logP values

5.3.2 Optimal immiscible solvent 2

A number of immiscible solvents were investigated for a suitable secondary organic solvent for urinary organic acid extraction. The postulated automation method requirement was to have a solvent that would ensure good extraction of organic acids in the upper phase, spontaneous phase separation and creation of a two-phase extraction system with easy and fast evaporation during the sample concentration step. The solvents assessed included hexane, heptane, combinational mixtures of butanol and methanol, hexane with ethyl acetate, diethyl ether, ethyl acetate and methyl tert-butyl ether (MTBE).

Observations from the initial extraction results showed hexane, heptane, combinational mixtures of butanol and methanol and hexane with ethyl acetate to be poor extractants of urinary organic acids. Hexane and heptane are non-polar solvents. Therefore even in combination with ethyl acetate, the solvents were observed to have very poor extractability of the organic acids. Butanol and methanol on the other hand are polar solvents and they completely mixed with the urine sample, thereby being unable to have phase separation, which is necessary for LLE of the sample. All these solvents were used in the BUME method for the extraction of lipids from plasma and they were observed to have good recoveries (Löfgren, 2012), however their physiochemical properties prevent them from being effective extraction solvents for urinary organic acid.

The results showed that different solvents had different extraction efficiencies for organic acids. Figure 5.2 illustrates graphically the recovery efficiency of each extracting solvent while Appendix 3 shows the table with percentages and CVs for each organic acid extracted by the different solvents. MTBE showed poor extraction of the spiked organic acids. The mean percentage recoveries ranged from 5 – 87%, with citric acid being the least extracted and malonic acid being the highest recovered. It also had a higher variance compared to the other solvents ranging from 18% (fumaric acid) to 66% (malonic acid). Diethyl ether in a similar manner was observed to have low recoveries for urinary organic acid. It had a minimum of 1% mean recovery for citric acid and the maximum of 61% for methylmalonic acid. The CVs for the organic acids ranged from 3% (citric acid) to 64% for malonic acid, although most of the organic acids had their CVs ≤ 10%, with 16% being the average CV.

Ethyl acetate had the best extraction efficiency of the solvents investigated. The recoveries ranged from 27% for citric acid to 186% for malonic acid. The organic acids with recoveries over 100% showed that they were extracted more relative to the internal standard. The majority of the organic acids had recoveries above 90% with the average percentage recovery for all the organic acids being 90%. Good repeatability as was observed as the CVs

of the majority of the organic acids was below 20%. The only exceptions to this were glutaconic acid (43%) and malonic acid (60%). The interesting observation was that when diethyl ether was combined with ethyl acetate in the second extraction step, it seemed to have better extraction efficiencies of the organic acids than on its own, as seen in Figure 5.3. There were small differences observed in the extraction efficiencies of ethyl acetate and the combination of ethyl acetate and diethyl ether. It was observed, however, that when the second extraction was done with diethyl ether, the extraction efficiency of glutaconic acid was reduced and that of glycolic and lactic acids improved. The average CVs for ethyl acetate and ethyl acetate/ diethyl ether was observed to be 16% for both. The general observation was that diethyl ether did not seem to necessarily improve the extraction efficiency of ethyl acetate.

It was generally observed that organic acids with larger logP values had better extractions, especially with ethyl acetate. According to Blau *et al*, (2014), the extraction recoveries of the organic acids depends on their polarity; the more the hydroxyl groups an acid has, the less the recovery (Blau *et al*., 2014). Based on the observations made, ethyl acetate was the solvent of choice for the secondary extraction solvent. According to Peters *et al*, (2008), 63% of all the laboratories that participated in the ERNDIM EQAS for qualitative urinary organic acid analysis used ethyl acetate as the extraction solvent. Blau *et al*, (2014) also state that ethyl acetate or diethyl ether is one of the most widely used solvents for organic acid extraction. Ethyl acetate proved to have good solubility of organic acids, good repeatability and high extraction efficiencies compared to the other solvents that were investigated. It was observed to have a fast evaporation rate due to its low boiling point.

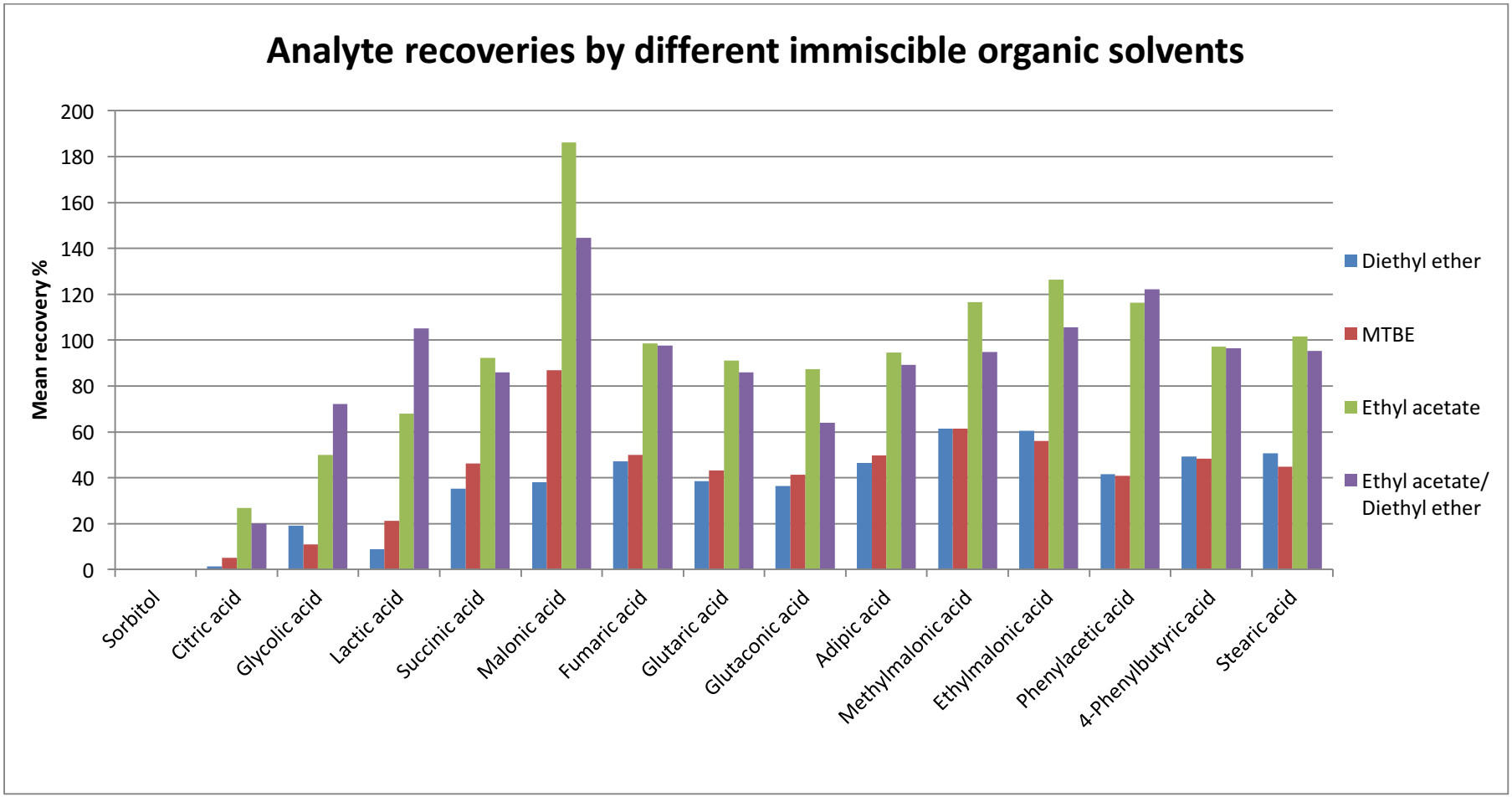


Figure 6-2 Illustration the extraction efficiencies of the organic acids (arranged according to logP values) using different extracting solvents

5.4 Secondary Two-phase Extraction Optimisation

After the selection of the optimal miscible and immiscible solvents, a third experiment was carried out to optimize the secondary two-phase extraction protocol. Acetonitrile the optimal water miscible solvent was assessed in combination with ethyl acetate. The water miscible solvent was needed for the one step fast extraction, which then would be extracted into the ethyl acetate in the second step extraction.

It was observed that at ratio ≥ 3:1 (ethyl acetate to acetonitrile), most of the acetonitrile moved from the aqueous phase into the organic phase of ethyl acetate. This meant that there was need to add at least three times as much ethyl acetate to acetonitrile to accomplish the two-phase extraction. At this stage the optimal ratio of ethyl acetate to acetonitrile was not known but the ratio 3:1 was selected for further experimentation based on the visual observations of phases from the experiment carried out in section 4.6.3. The establishment of the optimal solvent ratios for these was done in section 4.6.5 and reported in section 5.5. The acetonitrile/ethyl acetate combination was further assessed in the development of the two-phase urinary organic acid extraction method on whether the addition of salt improved the extraction efficiencies or not. The protocol described in section 4.6.3 was followed.

The results of the extraction efficiencies of the organic acids in relation to logP values are shown graphically in Figure 5.3. The average recoveries and CVs are shown in Appendix 4. The mean recovery for the acetonitrile/ethyl acetate with salts was overall higher than those of the combination without the addition of the salt buffer. The mean recovery percentages ranged from 41% (citric acid) to 106% (Phenylacetic acid). Most of the organic acids had recoveries above 80%. The CVs of all the organic acids gave an average of 7%, with all of them falling below 20%, except for glutaconic acid (25%). The extraction efficiencies of the organic acids with larger logP values were observed to be better than those with smaller values. In other words, those organic acids that higher logP values were observed to have higher recoveries such as phenylacetic acid (106) and 4-phenylbutyric acid (102%). The setback of the addition of salt is that some selectivity of the extraction system was relatively lost as observed in the increased extraction of alcohol sorbitol (8%).

On the other hand, the absolute recoveries of the no-salt addition solvents were comparatively. The mean recoveries ranged from 39% (citric acid) to 100% for phenylacetic acid. The overall average recovery for this solvent combination for all the organic acids was 69%. This set of samples demonstrated poorer repeatability compared to the other set. The average CV for all the organic acids was 16%. Most of the CVs for individual organic acids

fell well below 20% but there was an outlier, glutaconic acid with 85%. The upside of this solvent mixture is that it provided better selectivity by the observation in how much of the sorbitol (1%) was extracted.

From the data of the foregoing observations, the solvent with salt addition demonstrated better extraction efficiencies and good repeatability compared to the one without the salt added to it. There would be a trade-off between having a simpler sample matrix without salts in it or a more complex matrix yet with better extraction efficiencies and low variance. The advantage of a simpler matrix is less co-extraction of non-organic acids, which would lead to a decreased signal-to-noise (S/N) ratio. In line with the overall aim of simplifying and optimisation of the liquid-liquid extraction for automation, yet with good extraction efficiencies and repeatability, the solvent without salt addition was considered for further experimentation.

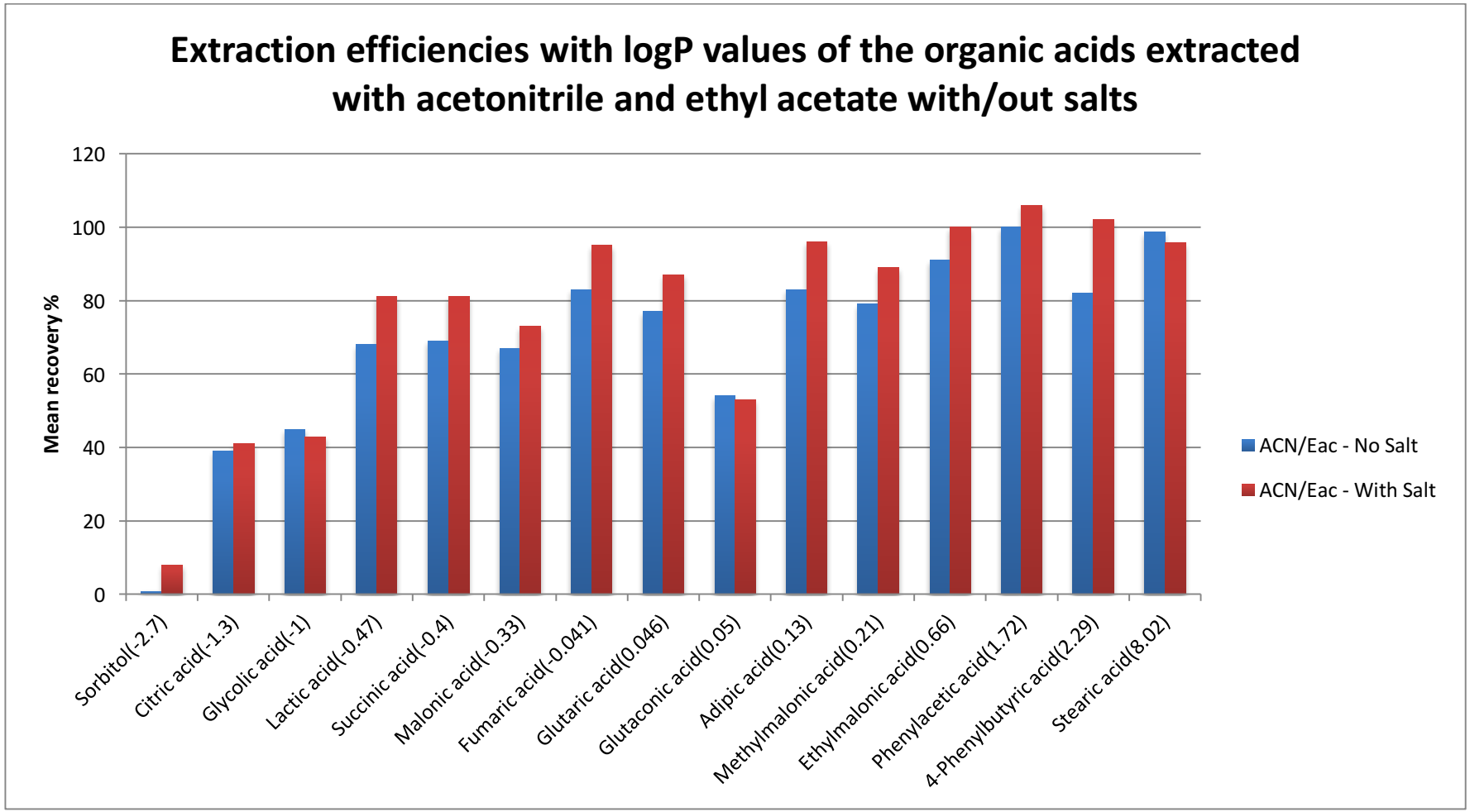


Figure 6-3 showing extraction efficiencies with logP values for the organic acids extracted with acetonitrile and ethyl acetate with/out salts. The extraction efficiencies with increasing logP values between for the organic acids extracted with the solvents with and without salt addition. Organic acids with higher logP values had higher recoveries compared to those with lower values like citric and glycolic acids

5.5 Optimisation of Solvent Volumes

5.5.1 Optimal sample-to-acetonitrile volume ratio

Section 4.6.4.1 describes the protocol followed to optimise sample-to-acetonitrile volume ratios. In the optimisation of the two-phase extraction system experiment (section 4.6.3), the 1:1 sample-to-acetonitrile ratio was taken as the optimal ratio. Therefore, the other ratios were tested and compared to the reference ratio of 1:1 to investigate if less volume of acetonitrile could be used and yet give good extraction efficiencies. It is important to note that the 1:1 ratio did not necessarily represent a 100% recovery of the organic acids, but was rather used only as a reference to the other ratios. Figure 5.4 shows that the 1:2 sample-to-acetonitrile ratio had better recoveries compared to other ratios that were investigated. Besides the poor recoveries for 2-ketoglutaric acid (8%), 4-hydroxyphenylpyruvic acid (9%), citric acid (8%) and glutaconic acid (14%), the solvent ratio had an average recovery of 84%. The same ratio was also observed to have good CVs with most of them being below 15%. The recoveries of the 1:1 (ACN: EtAc) were overall ranked second and they were very comparable to the 1:2 combination. The average recovery of the 1:1 ratio for all the organic acids was 82%. The last two solvent ratios, 1:0.5 and 1:0.25 also had good recoveries that were comparable with the other solvent ratios. They had 79% and 74% overall average recoveries respectively.

It was interesting to note that with the increase in the volume of acetonitrile there was a corresponding decrease in the extraction efficiencies of 2-ketoglutaric and glutaconic acids. The overall conclusion from these observations was that even a small ratio of 1:0.25 of acetonitrile added to the sample was sufficient to guarantee good extraction efficiencies. The results demonstrated that the higher the ratio of acetonitrile to sample, such as in the case of the 1:2 ratio, the extraction efficiencies of a number of organic acids was better in comparison to the others volume ratios. The reference ratio of 1:1 was fairly comparable to the 1:2 ratios. The interesting observation was that the lowest sample-to-acetonitrile ratio (1:0.25) had better overall recoveries of organic acids compared to the ratio 1:0.5. Further experimentation was needed to investigate how much of the acetonitrile affected the extraction when the volumes of the ethyl acetate are varied. In meeting the objective of reducing the solvent volumes while maintaining good extraction efficiencies, the sample-to-acetonitrile volume ratios of 1:0.5 was selected for further method development and optimisation experiments.

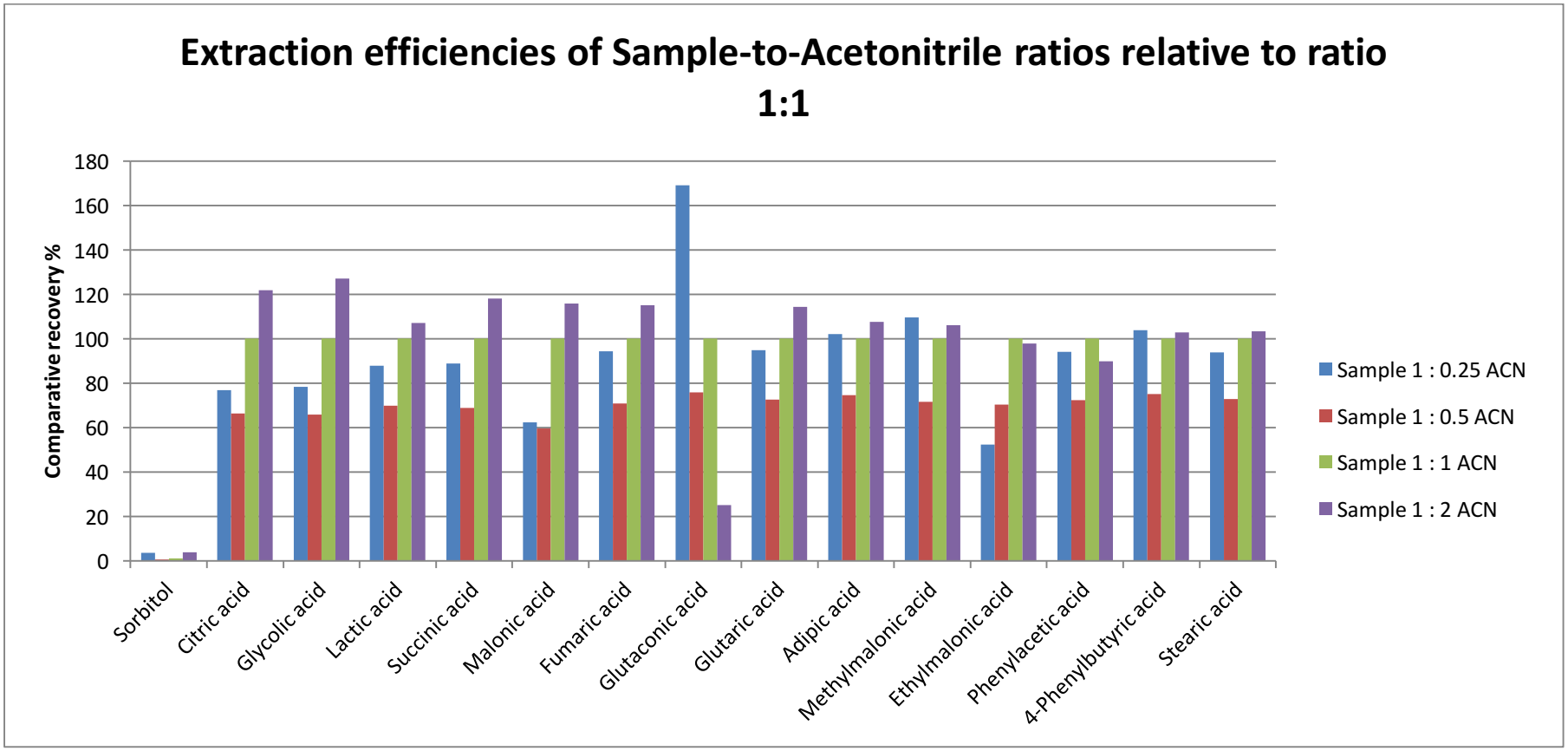


Figure 6-4 The effects of the different ratios of acetonitrile compared to the reference 1:1 sample-to-acetonitrile ratio.

5.5.2 Optimal acetonitrile to ethyl acetate volume ratio

The optimal volume ratio of ethyl acetate to acetonitrile was investigated (experiment described in section 4.6.4.2) with the goal to select a suitable volume for good extraction efficiency in a miniaturised extraction protocol. The other objective was to see the effect of varying the volume of ethyl acetate while keeping the volume of acetonitrile constant. Figure 5.5 showed that the extraction efficiencies were similar for all the ratios with the highest ratio (1:5) having a marginal overall better extraction efficiencies. This is in line with theory that a large K_D is influenced by the volume of the extractant, which leads to higher recoveries.

Building up on the observations of the previous results, it was clear that higher volumes of ethyl acetate was preferred for better phase separation and extraction of organic acids. Thus, the optimal volume of ethyl acetate was not necessarily dependent on the ratio of acetonitrile to ethyl acetate, but more on ethyl acetate itself as the main extraction solvent. As was concluded from the experimental results of the two-phase extraction system, there was need to have a higher ratio of greater than 3:1 for ethyl acetate and acetonitrile respectively, for effective extraction of acetonitrile into the ethyl acetate. Furthermore, for faster drying of the extracted organic solvents, a higher ratio of ethyl acetate to acetonitrile was preferred. The rationale for this is that acetonitrile is a water-soluble organic solvent and has a more water in it after extraction that can also affect the selectivity and extraction efficiencies of the organic acids.

Therefore, it was concluded that the highest ethyl acetate volume ratios (1:5) was optimal and was selected as suitable for further method development experiments. The objective was to have as much ethyl acetate as can be miniaturised and yet can extract the acetonitrile into it and all the other organic acids with greater extraction efficiencies.

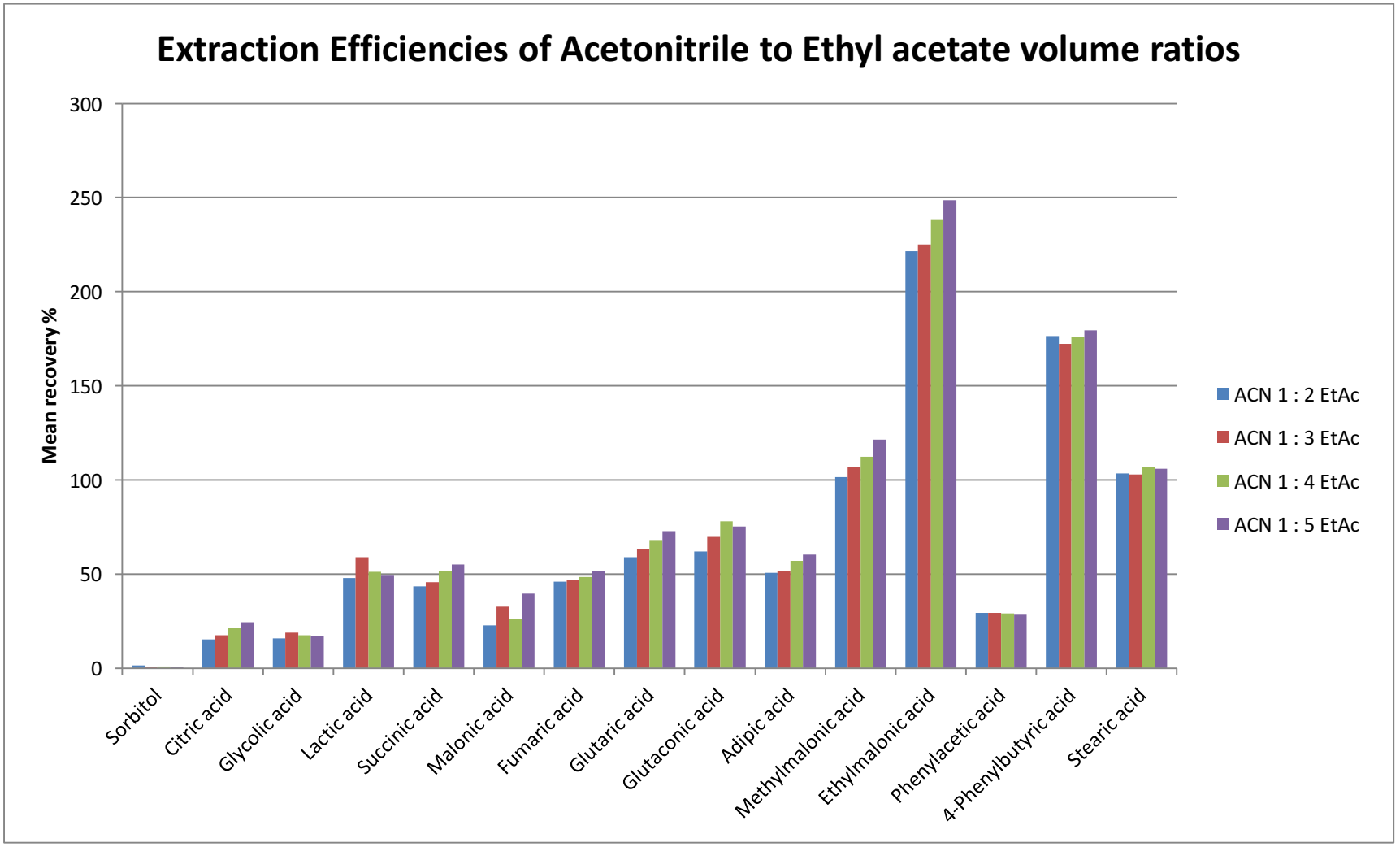


Figure 6-5 Recoveries of the analytes with different volumes of ethyl acetate

5.6 Optimisation of Sample-to-solvent ratio

Following the optimisation of solvent volume ratios, the optimal sample-to-solvent ratio for miniaturisation and automation was investigated, as described in section 4.3.5. Figure 5.6 shows, as was anticipated from previous experiments and theoretical principles from literature, the higher the solvent to sample ratio, the greater the recoveries of the organic acids were. The results showed that with increment in the solvent-to-sample ratios, there was a corresponding increase in organic acid recoveries. There were a few organic acids with an exception to this conclusion. One such example was stearic acid that showed its recovery to be independent of the variation to the solvent-to-solvent ratio. Another interesting observation from the graph is the less polar the compounds were, the less important the solvent to sample ratio became. From glutaconic acid to stearic acid the extraction efficiencies showed that they were not necessarily dependent on solvent-to-sample ratio. Although the highest ratio still had better extraction efficiencies in comparison to the others. The general conclusion, however, was that a larger ratio was necessary for good extraction efficiencies especially as far as polar organic acids are concerned.

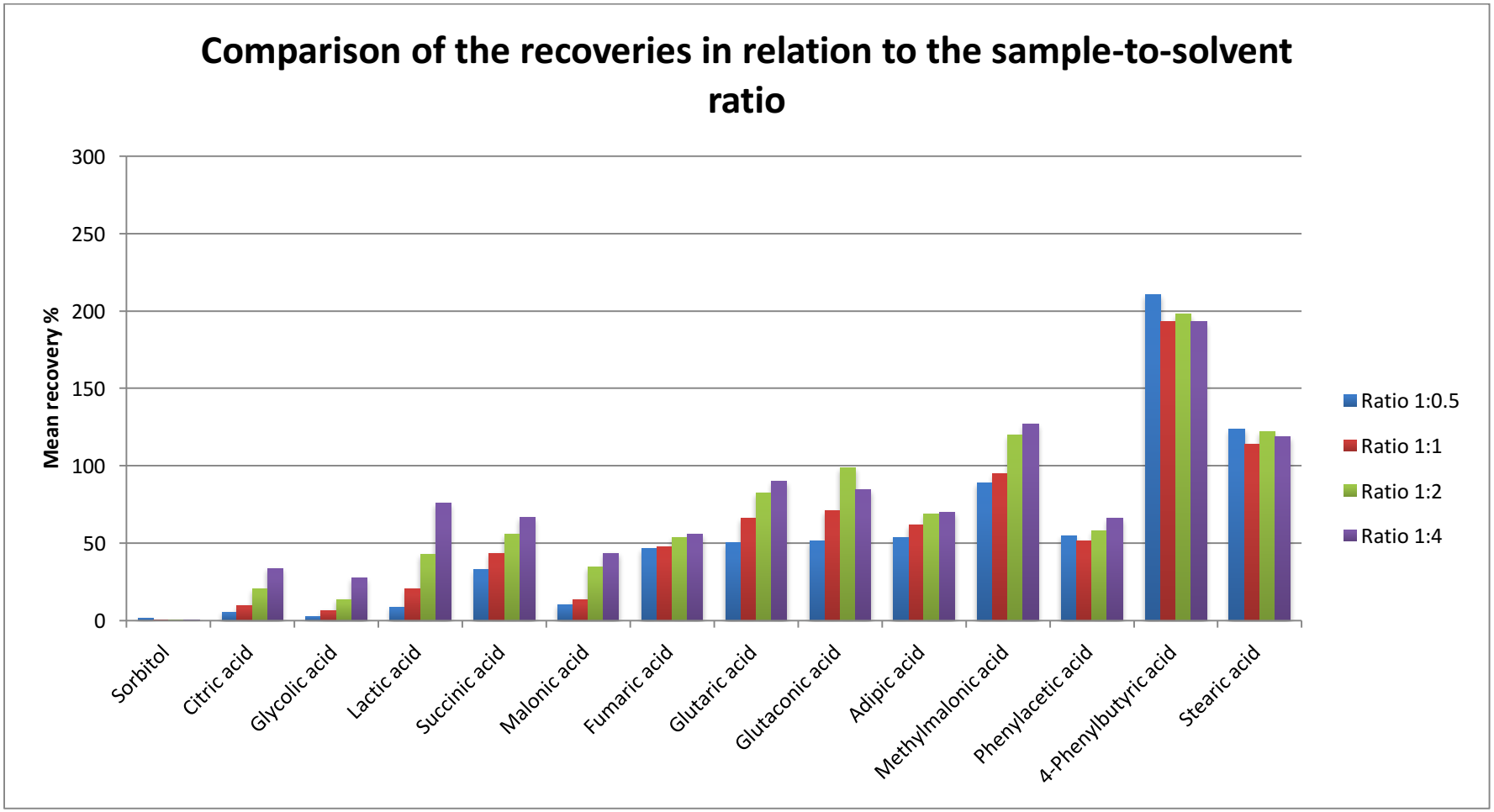


Figure 6-6 show the comparison between the organic acid recovery and the sample-to-solvent ratio. The organic acids were arranged in accordance with increasing logP values.

5.7 Optimisation of Sample-Solvent Mixing Time

An experiment to investigate the optimal sample mixing time is described in section 4.3.6. The results from the optimisation of the sample-solvent mixing time showed that the duration of the mixing time on a rotary wheel for the extraction step had little effect on the extraction yield. From Figure 5.7, the recoveries obtained for samples that had a mixing time of 30 minutes showed little differences with the sample that had an incubation time on the rotary wheel of 5 minutes. This observation was generally true for all the analytes. Neither were the CVs of the analytes affected by the amount of minutes the sample was left to mix for the extraction to occur. There was small decrease in the overall average of CVs from 10% in 5 minutes to 6% in 30 minutes. But this can be explained by the differences in the CVs of individual organic acids. For example, glutaconic acid had the lowest CV with an incubation time of 20 minutes while glycolic acid had its lowest when left to mix for a period of 5 minutes.

The mean recoveries of the organic acids were plotted against the sample mixing time to see the trends over mixing time periods. The general observation made was that there was no increment in recovery of an organic acid with increase in the mixing time period. The plausible reason for this observation is the two-solvent system of extraction was working to facilitate rapid extraction. The miscible solvent facilitated rapid extraction of organic acids from the aqueous phase into the immiscible organic solvent phase. Therefore, the least amount of time possible to obtain good extraction efficiency was selected for the miniaturisation protocol. For the manual miniaturised protocol, 5 minutes would therefore be used as the incubation time of the sample on the rotary wheel. For miniaturisation purposes, all samples would be vortexed for a period of about 30 seconds.

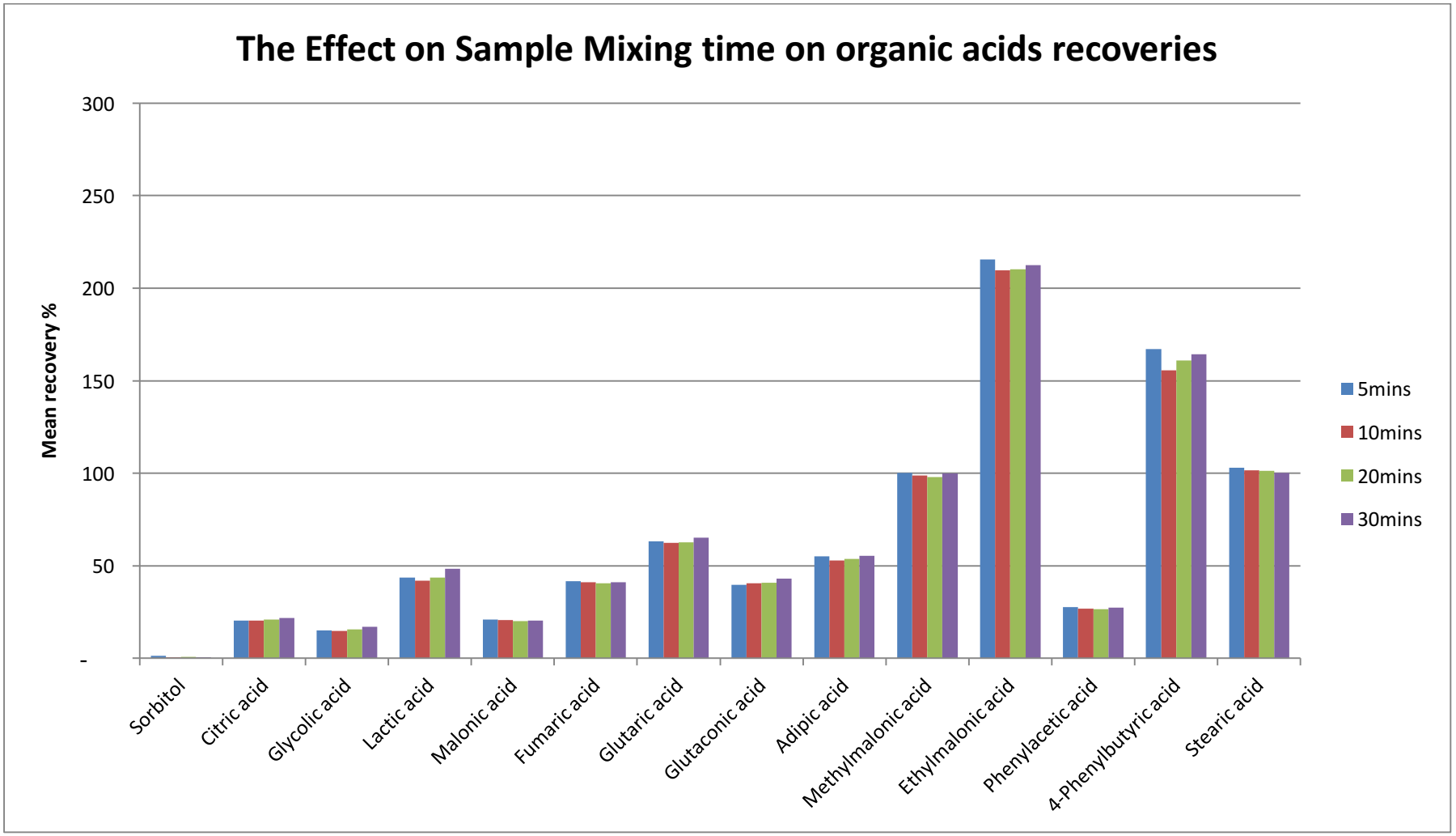


Figure 6-7 Illustration of the effect of sample mixing time on the yield of the analytes

5.8 Miniaturisation of the Extraction Protocol

A recovery experiment was done using the miniaturised extraction protocol as described in section 4.6.7. Figure 5.8 shows graphically the recoveries of the miniaturised extraction method in comparison to the in-house method. Appendix 8 has the table with a summary of the recovery percentages and the CVs. A number of the organic acids showed good recoveries and were comparable to those of the in-house method. With regard to certain organic acids such as glutaconic, glycolic and lactic acids, the miniaturised protocol had lower recoveries. These were some of the organic acids that demonstrated better extraction efficiencies with larger volumes of solvent and larger sample-to-solvent ratios (as observed in section 5.6). This perhaps explained the reason for the poor extraction of such organic acids such as citric acid, glycolic acid and lactic acid that all had recoveries less than 50% in the miniaturised method. Many other organic acids had good recoveries such as ethylmalonic, fumaric, methylmalonic, phenylacetic, stearic, succinic and adipic acids.

The miniaturised method generally had very comparable CVs with the in-house method. The differences between individual organic acids was observed not be significant. Examples are adipic acid (9% and 8%), ethylmalonic acid (6% and 8%) and glutaric acid (7% and 6%). Citric acid (89%) and glutaconic acid (28%) were the exception in terms of their CVs for the miniaturised method. The method overall showed better repeatability and was well comparable to the in-house method. The in-house method had a few higher CVs compared to the miniaturised method for glycolic acid (23% to 5%) and lactic acid (24% to 10%).

Taking into consideration that there less volumes of sample and solvents for the miniaturised method compared to the in-house one, the method good overall performance. One interesting observation was that the extraction efficiencies of the less polar organic acids were not affected by the amount of volume of solvent used in extraction. The opposite was the case for the more organic acids like citric acid. The extraction efficiencies for very polar organic acids is challenge as the acids prefer to be in the aqueous phase (Blau *et al*., 2014). From the graph, it was observed that from adipic acid to stearic acid were extracted better than the other ones which were less polar in the miniaturised method. This led to the conclusion that, for some organic acids, their extraction efficiencies are not so much dependent on the amount of solvents used but their own polarity

Therefore, it was concluded that the miniaturised method was a good and acceptable method to automate as was observed by its better CVs which allows the use of correction factors for more accurate quantification of organic acids. Further optimisation to improve the extraction efficiencies of the organic acids would be done in the automated method. .

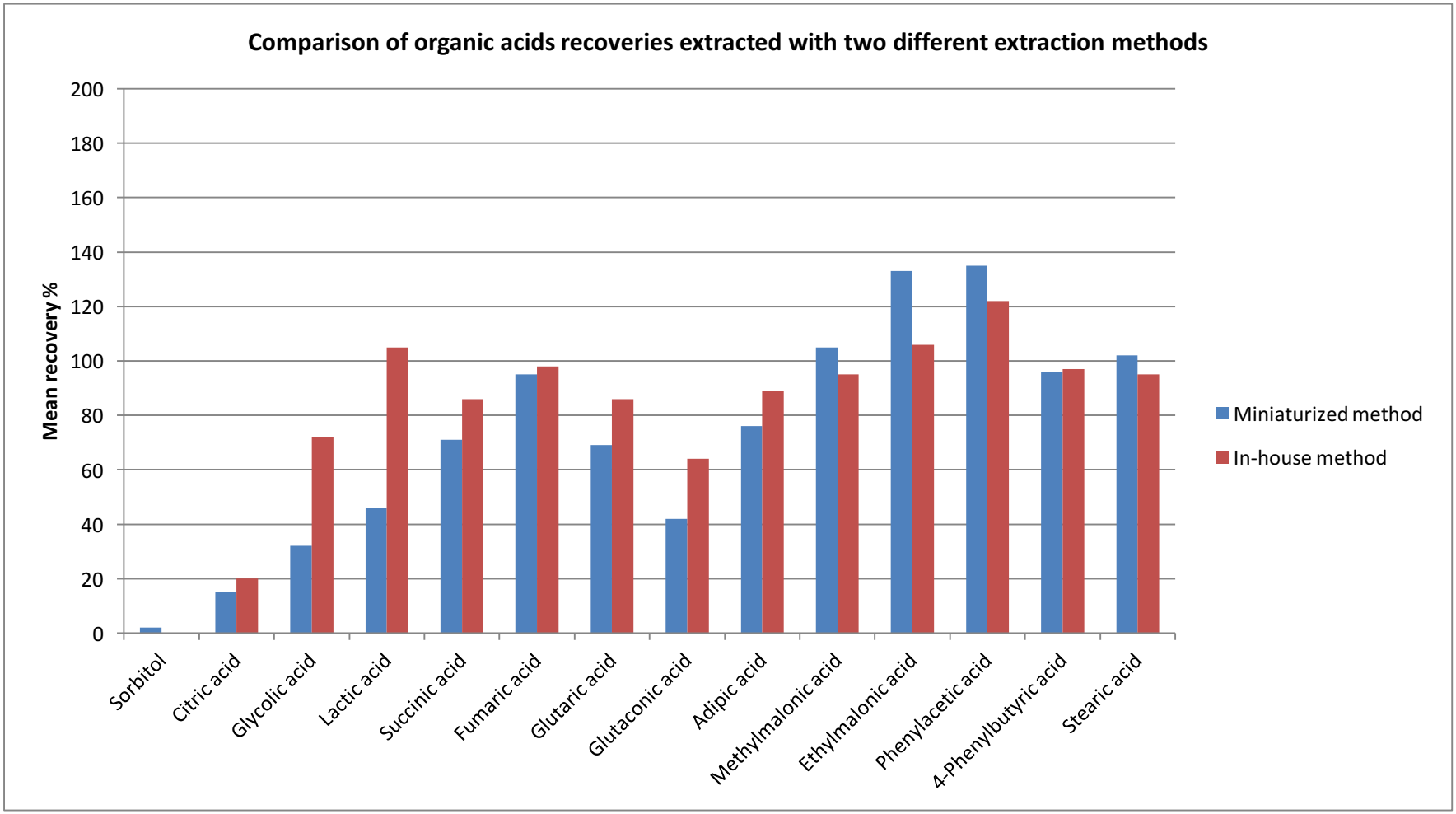


Figure 6-8 Mean recoveries of miniaturised method compared to the in-house method using larger amounts of solvents

5.9 The Automated Extraction Protocol

The miniaturised extraction protocol, having demonstrated its capability to extract urinary organic acids well with good efficiencies, it was translated into an automated liquid-liquid extraction for urinary organic acids. As described in section 4.6.8 the method was set up on a MicroLab (ML) Star Autosampler (Hamilton Technologies). The parameters for the workstations, such as speed and positions of the different aspiration and dispensing steps, were optimised to ensure high recoveries of organic acids. The parameters were also optimised to ensure a robust protocol capable of extracting a maximum of 24 samples in one run and work with volumes as low as 20 µl. Another parameter that was looked into in order to have a fully automated method was the need to centrifuge the sample and dry it with sodium sulphate. The liquid AutoSampler on which the method was set up did not have provision for online centrifugation of the samples. There was need to for evidence that eliminating centrifugation would not affect the efficiency of the automated method.

5.9.1 Optimisation of the automated extraction protocol

5.9.1.1 Determination of the need to centrifuge and addition of dehydrating reagent

The procedure described in section 4.6.8.1 was followed for this experiment. The results from Table 5.3 and Figure 5.9 revealed that the addition of sodium sulphate is critical to obtaining a good recovery and reduce variation between samples. The samples that did not have any sodium sulphate added to them had overall average CVs of 20% and 35%. Centrifugation without drying the sample with sodium sulphate did not help in reducing the variation in the extractions of organic acids. The two sets of samples that had the addition of sodium sulphate as a common factor had very similar CVs and recoveries despite one set of samples not having undergone centrifugation. From the observation made from this experiment, the centrifugation step proved to be something that could be done away with because it had small effects on the recoveries and repeatability in the extraction of organic acids. The drying step with sodium sulphate on the other hand, ensured good recoveries and little variation from sample to sample. Both the sample sets that had the addition of sodium sulphate to them had average CVs of ≤ 10%.

The amount of anhydrous sodium sulphate did not have to be exact from sample to sample. The purpose of this step was to remove any residue water after extraction. The presence of this water in sample seemed to increase variation in extraction of organic acids from sample

to sample. The selectivity of solvents with some amount of water in it was also reduced. Water present in solvent extract affected the drying of the extracted solvent by taking longer to dry. Another reason for the use of anhydrous sodium sulphate to dry the sample is the presence of water in samples damages the GC column.

From the findings of this experiment and in line with the postulated requirements for automation, the addition of sodium sulphate without centrifugation was chosen. The CVs for this method were consistently very low for all the samples, which showed the consistence needed in a method to be able to correct for systematic errors in an analytical method using extraction correction factors (Burtis *et al*., 2012, Duez *et al*., 1996). This enabled the method to be fully automated without having remove samples from the AutoSampler in order to put them in a centrifuge.

Table 6-3 Mean recoveries and CVs of the sample sets with/out centrifugation and sodium sulphate addition

Analytes	Centrif+NaSO4		Centrif-NaSO4		No Centrif+NaSO4		No Centrif-NaSO4	
	Mean recovery %	CV %	Mean recovery %	CV %	Mean recovery %	CV %	Mean recovery %	CV %
Sorbitol	0	7	0	12	0	4	101	15
Glycolic acid	10	9	2	8	10	5	98	28
Lactic acid	31	0	5	13	33	3	320	0
Succinic acid	32	4	5	4	31	3	157	28
Fumaric acid	84	2	11	41	88	9	47	45
Glutaric acid	83	7	10	15	87	5	126	24
Glutaconic acid	11	2	2	37	8	8	37	47
Adipic acid	48	4	7	33	51	5	86	43
Methylmalonic acid	119	1	11	16	110	5	144	19
Ethylmalonic acid	268	6	30	28	263	2	70	40
Phenylacetic acid	84	11	8	24	88	4		49
4-Phenylbutyric acid	69	30	9	13	75	13	62	41
Stearic acid	16	5	3	14	24	6	79	43
Average	66	7	8	20	67	6	105	32

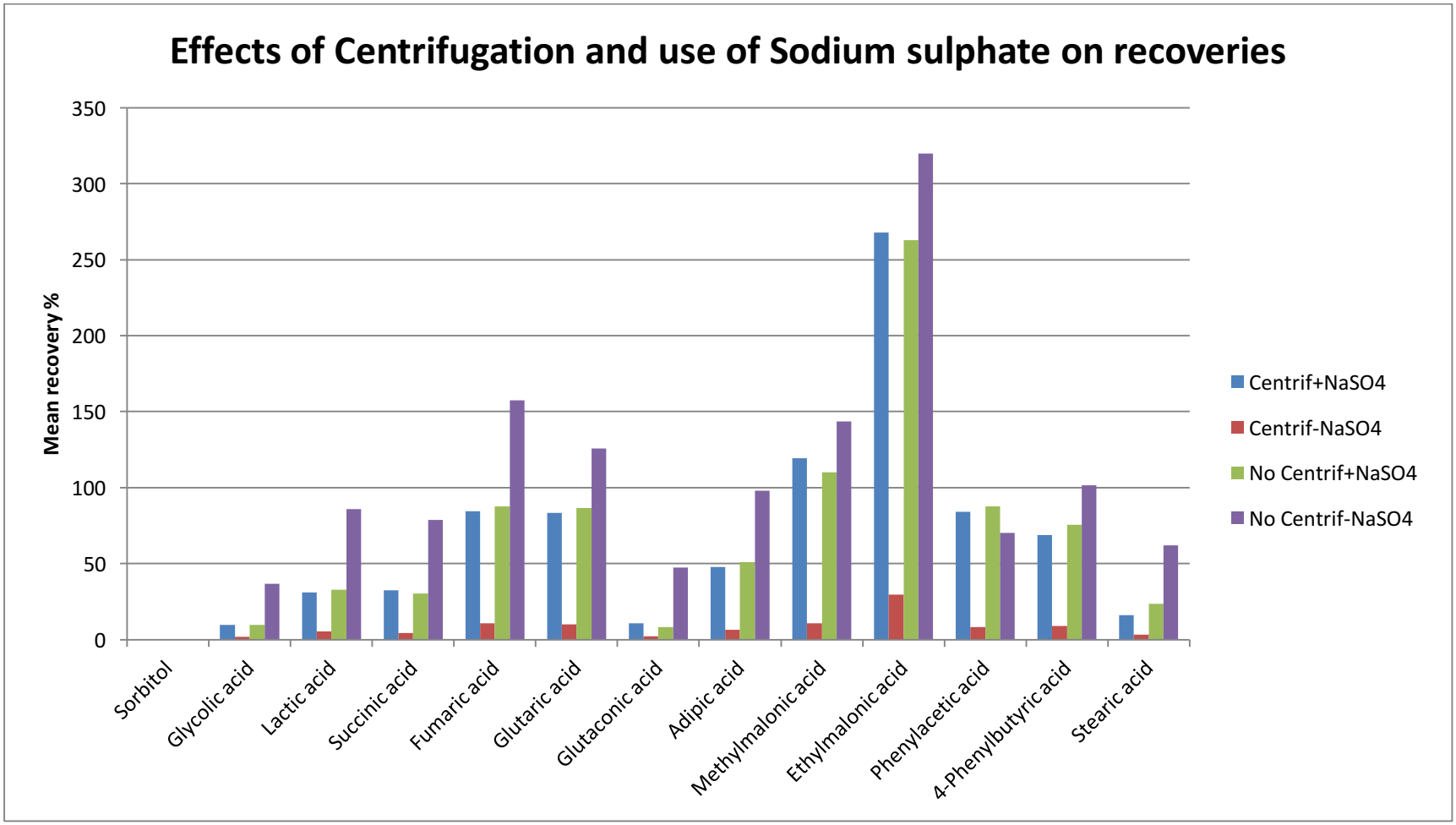


Figure 6-9 Influence of sodium sulphate to dry the sample, as well the effect of centrifugation on the overall extraction efficiency of a system

5.9.2 Summary of optimized automated extraction method

Prior to being loaded on the instrument, the urine sample is analysed for creatinine. The creatinine concentration is entered into a Microsoft Excel sheet together with the sample requisition number. The programmed software determined the volume of urine based on the creatinine concentration in mmol/l. The urine volume was mostly between 30 to 500 ul. All sample volumes were normalized for creatinine by dilution with normal saline to 500 ul. The calculated amount of normal saline aspirated and dispensed it into a 2 ml glass vial. The determined urine volume was then added to the saline. Based on the creatinine concentration, 23 ul of internal standard (3-phenylbutyric acid) was pipetted into the sample vial. The sample was acidified with 50 ul of 5N HCl acid. Two hundred microliters of acetonitrile were added to the sample, followed by a two times mixing step of the sample. The channel aspirating of 300 ul of the top phase and dispensing it at the bottom at high speed and pressure did the mixing. Ethyl acetate was added to the sample mixture by serial pipetting of 600 ul of it per sample. This was followed by a four times mixing step. A timer was built in that calculates that each sample must stand for 5 minutes before 600 ul of the top phase is transferred to the second vial. The extraction step with ethyl acetate was repeated, with 700 ul being transferred in the second time round. To ensure that most of the organic acids are extracted, a last volume of 300 ul of ethyl acetate was added to the sample and left to stand without mixing it. The same amount of the top phase was transferred to the second vial. The total volume of the extracted sample was 1600 ul. The sample was mixed again in the second vial (which had anhydrous sodium sulphate pre-added to it prior to the extraction protocol) in order to remove water from the sample. The dried extract was later transferred to a third rack with a clean glass vial. This was then ready for solvent evaporation under gentle stream of nitrogen. The automated extraction procedure was at this point completed. The residue sample after solvent evaporation are concentrated and derivatized with 40 ul of BSTFA/TMCS (99:1) and 10 ul of pyridine, having final volume of 50 ul after derivatization. One microliter is then injected into the GC/MS for chromatographic analysis.

For the purpose of evaluating the method to see its fitness for use in a clinical laboratory, a series of method validation experiments (as described in section 4.7) were conducted. The results of the validated assessment are described in Chapter 6.

CHAPTER 6: METHOD VALIDATION RESULTS AND DISCUSSION

6.1 Introduction

The optimised method described section 5.9.2 was validated in a number of parameters. The initial method development and optimisation study was done on a selected few organic acids, but in method validation the goal was to assess how the method could perform with regard to many other organic acids, especially the ones of interest in the ERNDIM EQAS for quantitative organic acid analysis. Therefore, only a number of method validation parameters were included, such as linearity, inaccuracy, imprecision and for method quality performance. The goal of the automated organic acid extraction method, like any analytical method, was to obtain consistent, reliable and accurate data. It was also assessed that the automated method was improving on the extraction to the in-house method, therefore the method was compared to it and the ERNDIM EQAS results. This method validation was used therefore as an important procedure in reaching these goals. The method was evaluated on for quality, consistence and reliability.

6.2 Linearity

The method's linearity (also called analytical range) is useful to determine the reportable range of an analytical method. Table 6.1 summarises the concentration ranges at which the method was investigated, the observed linear range and the correlation coefficient (*r*) for each analyte. Appendix (9) has the figures of the calibration curves of each organic acid and the r^2 values for each. The linear range for most of the analytes was observed to be between 0 to 300 mg/l, with some exceptions. Most of the analytes had the correlational coefficient (r) of >0.99 with the exception of glycolic acid (0.98). The method demonstrated that it had the capacity to analyse most of the organic at these concentrations without any need of dilution.

Table 7-1 Summary correlation coefficients and linear ranges for organic acids

Organic acids	Analysed Concentration range (mg/l)	Linear range (mg/l)	Correlation coefficient (r)
Lactic acid	0 – 500	0 – 400	0.996
Glycolic acid	0 – 500	0 – 500	0.981
Malonic acid	0 – 500	0 – 500	0.988
Methylmalonic acid	0 – 500	0 – 300	0.999
Glutaric acid	0 – 500	0 – 400	0.995
Phenylacetic acid	0 – 500	50 – 200	1.000
Ethylmalonic acid	0 – 500	0 – 300	0.999
Succinic acid	0 – 500	0 – 400	0.998
Fumaric acid	0 – 500	0 – 200	1.000
Glutaconic acid	0 – 500	0 – 300	0.998
Adipic acid	0 – 500	0 – 300	0.999
2-Ketoglutaric acid	0 – 500	0 – 300	0.996
Succinylacetone	0 – 500	0 – 300	0.990
Citric acid	0 – 500	0 – 300	0.995
Sebacic acid	0 – 500	50 – 200	1.000
4-Hydroxyphenylpyruvic acid	0 – 500	50 – 200	0.995
Stearic acid	0 – 500	0 – 300	1.000

6.3 Imprecision Study

The replication experiments were performed to estimate the imprecision of the automated organic acid method. Repeatability and within-laboratory precisions were determined from the data sets. Cochran C and Dixon Q tests detected some outliers in the data that were excluded. The CVs of all the analytes at the three concentration levels are tabulated in Table 6.2. The repeatability for 50 mg/l, measured by CV, ranged from 3% (ethylmalonic acid) to 29% (glutaric acid). All the organic acids had an average CV of 12%. The CVs for the concentration level of 100 mg/l were between 3 to 30%, with glutaconic acid being an outlier with 75%. A similar range of CVs is true for the 500 mg/l concentration level and glutaconic acid being an outlier again. The repeatability data demonstrated that there was a closeness of agreement between the results of successive measurements under the same conditions. Most of the organic acids had their CVs below 20%, which is acceptable (Blau, 2004, Hassan, 2009). The within-laboratory precision had larger variations in results compared to the repeatability results. The variations observed were due to variation in extractions and days of run. Only a few organic acids had their within-lab precision within the acceptable range of below 20%. It should be noted however that the mass spectrometer was set to scan

across the entire selected mass range. It is known that the method precision improves substantially when single iron monitoring / multiple reaction monitoring is selected which would have been more appropriate for a quantitative method. Since the aim of this study was to compare a miniaturized extraction method to the existing in-house method, the current instrument's settings were not modified. This enabled direct comparison of the extraction efficiency only between the new and the existing method.

Table 7-2 Method Imprecision calculated as Repeatability and Within-laboratory Precisions

Organic acids	Repeatability			Within-lab Precision		
	50 (mg/l)	100 (mg/l)	500 (mg/l)	50 (mg/l)	100 (mg/l)	500 (mg/l)
Citric acid	15%	12%	7%	49%	41%	62%
Glycolic acid	23%	30%	8%	52%	42%	52%
Lactic acid	12%	8%	8%	29%	25%	32%
Succinic acid	3%	3%	6%	23%	14%	19%
Malonic acid	20%	7%	5%	42%	24%	54%
Fumaric acid	3%	7%	31%	18%	13%	29%
Glutaric acid	29%	18%	6%	29%	28%	21%
Glutaconic acid	14%	75%	85%	38%	86%	96%
Adipic acid	9%	14%	5%	26%	21%	19%
Methylmalonic acid	10%	8%	3%	27%	15%	32%
Ethylmalonic acid	3%	5%	28%	20%	13%	40%
4-Phenylbutyric acid	9%	4%	5%	26%	6%	6%
Stearic acid	11%	6%	12%	31%	19%	18%
Average	12%	15%	16%	32%	27%	37%

6.4 Inaccuracy Study

ERNDIM samples as described in 4.2.6 were used to compare the automated and in-house method to each other and the reference method. Table 6.3 shows the results of the regression statistics and the amount of systematic errors in the method's results. The test method was plotted on the y-axis and the comparative method on the x-axis.

As seen from Table 6.3, the correlation coefficient (r) for all the analytes were all above 0.99 with the exception of 4-Hydroxybutyric acid (0.96), 3-hydroxyglutaric acid (0.378), 2-hydroxyglutaric acid (0.98), methylcitric acid (0.98), N-hexanoylglycine (0.97), mevalonic acid (0.97) and pyroglutamic acid (0.98). According to Westgard (2008), r is sensitive only to random error and is useful as a measure of the reliability of the regression statistics. The r values obtained for the automated method showed that the method had goodness of fit of the mathematical model between the observed results and the ERNDIM ones. The automated method's r values showed that relative amount of dispersion from the regression line of ERNDIM reference materials results was less compared to the existing in-house method. Since r depends on the analytical range of the data and how evenly spread it is, the method showed a measure of agreement between the ERNDIM reference material results. From the r values obtained in the automated method, it shows that the determined slope (derivation of proportional error) and the intercept (constant error) are reliable. The automated test method showed that the regression statistics were reliable and determination of other errors could be done with a great level of confidence in the data.

Constant error, on the one hand, was estimated from the y-intercept of the regression statistics. The regression statistics showed the constant error for the method ranged from -5.75 to 5.66, most of which were negative values. This showed that the automated method mostly had a constant error that is below the mean of the reference method. This provided an overall estimate of how the test method's results differed from the comparative method's results (Westgard *et al*., 2008; Johnson *et al*., 2008). Proportional error was estimated from the slope. It is the difference between the slope and its ideal value of 1.00. The highest proportional error for the optimised automated method was 2.062 and the lowest being 0,001. The proportional error is the greater contributor to the bias of the method. But the proportional error could be corrected by use of extraction efficiencies of the compounds when the correlation coefficient is greater than 0.975 or 0.99 (Burtis *et al*., 2012).

The bias (systematic error) of the method was estimated using the regression statistics at MDC. The test method had an average bias of 51%. Some organic acids had biases below 10% and these included adipic acid, ethylmalonic acid, fumaric acid, glutaric acid, glycolic

acid and 3-methylglutaric acid. Most of the other organic acids showed a large amount of bias at MDC. When compared to the existing in-house method, the automated method had less bias. This showed that the automated method was improving on the accuracy of the results obtained than using the manual method. According to Blau *et al* (2014), organic acid quantitative results should be within 20% accuracy. However, values between laboratories are often not comparable because the organic acid analysis methods vary with respect to extraction, derivatization, identification and sensitivity. This phenomenon is also evidenced in literature by the differences in specified concentrations (Blau *et al*., 2008, Scriver *et al*., 2012).

Table 7-3 Inaccuracy statistics from the method comparison plots for ERNDIM samples

Organic acids	Linear regression. r		Coefficient of the Slope (PE)		Y-Intercept (CE)		MDC Method Bias [d]	
	Automated OA Method	In-house Method	Automated OA Method	In-house Method	Automated OA Method	In-house Method	Automated OA Method	In-house Method
Methylcitric acid	0.991	0.426	0.324	0.059	-0.178	0.261	70%	91%
2-OH-glutaric acid	0.990	0.635	0.506	0.286	-3.012	36.141	51%	53%
3-Methylglutaconic acid	0.997	0.519	1.229	0.778	-1.272	19.777	22%	13%
3-Methylglutaric acid*	1.000	0.903	1.117	0.898	-0.732	5.392	3%	8%
3-OH-3-Methylglutaric acid	0.994	0.856	0.334	0.592	-5.716	9.049	69%	37%
3-OH-Glutaric acid	0.614	0.614	-0.179	-0.152	0.966	0.820	117%	115%
3-OH-Isovaleric acid	0.997	0.349	0.779	0.033	-0.864	1.682	22%	97%
4-Hydroxybutyric acid	0.982	0.941	0.311	0.228	-1.982	-0.878	73%	79%
Adipic acid	0.995	0.913	1.037	1.847	-0.212	-22.759	4%	80%
Glyceric acid	0.999	0.950597675	0.090	0.077	-0.522	0.852	91%	92%
Ethylmalonic acid	0.999	0.798	1.052	1.490	-0.787	4.596	5%	51%
Fumaric acid	0.997	0.918	1.074	1.821	-1.011	-16.530	5%	35%
Glutaric acid	1.000	0.956	0.933	0.806	-1.176	10.916	8%	10%

Organic acids	Linear regression. r		Coefficient of the Slope (PE)		Y-Intercept (CE)		MDC Method Bias [d]	
	Automated OA Method	In-house Method	Automated OA Method	In-house Method	Automated OA Method	In-house Method	Automated OA Method	In-house Method
Glycolic acid	0.999	0.854	0.233	0.129	-2.849	-9.467	78%	90%
N-Hexanoylglycine*	0.985	0.735	0.542	0.624	0.231	-1.501	45%	41%
Methylmalonic acid	0.998	0.997	0.598	0.795	5.660	-3.018	8%	38%
Mevalonic acid	0.987	0.493	0.001	0.034	-0.021	2.030	100%	74%
N-Acetylaspartic acid	0.998	0.757	0.176	0.095	-0.275	-0.898	82%	91%
Pyroglutamic acid	0.989	0.766	0.173	0.273	-5.755	-16.759	87%	85%
Sebacic acid	1.000	0.945	1.502	0.875	-0.528	3.831	44%	31%
Suberic acid	0.999	0.870	1.352	0.913	-1.617	10.531	32%	15%
N-Tiglylglycine*	0.998	0.838	0.500	0.550	-0.283	-1.217	51%	48%
Vanillactic acid*	0.994	0.711	2.062	1.886	-0.732	-8.868	105%	79%

* MDC Not used in determining the bias

The total error for the automated method compared to the in-house method is shown in Table 6.4. The total error combined the effect of the bias and the CVs of the method. The data that is shown in Table 6.4 is for the organic acids whose within-lab precisions were available among the ones tested for in the ERNDIM profile. The comparison for the total error for the two methods was done to show that there is great potential for the automated method to reduce both the random and systematic errors that maybe caused by human errors. The data clearly shows that the automated method had less total error. The anticipated total error was determined by correcting for the bias based on the r value. If the r for the organic acid was above 0.98, meaning that the linear regression gave the confidence that the systematic error could be corrected for and with this theoretically done, the new bias was assumed to be zero. Therefore, the anticipated total error for most of the acids was fairly better than the experimentally determined ones.

According to Westgard *et al*, (2008), the total error of a method provides information on its test performance and is the basis for judging the acceptability of the analytical errors. This is because total error shows how far wrong a test result might be due to random and systematic errors. The lowest total error was observed for fumaric and ethylmalonic acids. From these observations, it showed that the automated method could indeed improve on the extraction performance of the manually operated in-house method.

The overall judgement of the method is it provides quality analysis of organic acids provided uncertainties and correction factors are determined and used for accurate quantification. There is definite need for continual efforts to improve the method's performance. The use of the quality control samples to monitor the patients test results is an essential for better reliable results.

The results from the EQA scheme of quantitative organic acids showed great variation because of variability in practice and lack of international standardisation in the reference ranges used by different laboratories. It must be notes that the clinical significance of the great variation of the results mostly depends on the clinical context and as Blau *et al* (2008) stated it: "[the variation in organic acid analysis] may be less problematic than the results would suggest."

Table 7-4 Calculated and Anticipated Total Error of Automated and In-house Methods

Organic acids	Precision (CV %)		Total Error [a] (%)		Correction for Bias based on r^2?		Anticipated Total Error [b] (%)	
	Automated OA Method	In-house Method	Automated OA Method	In-house Method	Automated OA Method	In-house Method	Automated OA Method	In-house Method
Adipic acid	21	54	38.32	168.42	Yes (r^2=0.995)	No (r^2=0.913)	34.65	168.42
Ethylmalonic acid	13	101	26.30	218.03	Yes (r^2=0.999)	No (r^2=0.798)	21.45	218.03
Fumaric acid	13	50	25.98	117.22	Yes (r^2=0.997)	No (r^2=0.918)	21.45	117.22
Glutaric acid	28	46	53.93	86.49	Yes (r^2=1.000)	No (r^2=0.956)	46.20	86.49
Glycolic acid	42	56	121.30	182.39	Yes (r^2=0.999)	No (r^2=0.854)	69.30	182.39
Methylmalonic acid	15	91	32.97	187.04	Yes (r^2=0.998)	Yes (r^2=0.997)	24.75	149.49
Sebacic acid	29	66	92.12	140.36	Yes (r^2=1.000)	No (r^2=0.945)	47.85	140.36

a. Total Error (TE) was calculated using the formula: TE = Bias + 1.65 * CV

b. Anticipated TE (ATE) was calculated by the formula; ATE = 1.65 * CV when r was >0.98

CHAPTER 7: GENERAL CONCLUSION

In this study, a urinary organic acid extraction method was optimised and fully automated on the Hamilton ML Star AutoSampler. The goal of the study was to have a method that could give stable and consistent extractions, could meet all the postulated specification for automation and improve on the existing in-house organic acid method. The goal was not to optimise the whole organic acid analytical process, but just to improve the extraction step by automating it and thereby potentially reducing the random and systematic errors of the method.

The automated method is a solvent-based extraction protocol of organic acids from urine that has a run time of approximately 30 minutes per sample and about 3 hours per 24 sample batch. This was achieved using a single channel pipetting system. A multiple channel system or a 96-channel system could still dramatically lower the required extraction time. Although it is based on the in-house manually operated extraction method, it has many advantages such as quick sample throughput, non-labour intensive, less exposure to toxic solvents, no exhaustive pipetting steps, quick sample drying time and the ability to prepare a large number of samples within a run. This allows the analyst to spend more time on analysis and data processing as opposed to sample preparation.

One of the most noted drawbacks about liquid-liquid extraction is its apparent difficulty of automation (Clement, 2012). Much of the progress in automation of organic acid extraction for GC/MS analysis has been in solid phase extraction and less in LLE. The attempts made to automate the method by Bengtsson, *et al.* (1996) proved rather unsatisfactory. In this study, the inherent challenges in automation of the solvent protocol, such as the addition of HCl, internal standard, centrifugation and mixing were circumvented, enabling the full automation of the extraction step in the whole preparation process.

7.2 Method Development and Optimisation

A number of postulates were made for the automation of the extraction protocol. The automated method requirements were to use sample volume of 500 ul or less, perform the extraction in a 2.0 ml glass vial, have an initial one-phase extraction with a polar miscible solvent that would be extracted into the secondary two-phase extracting solvent, both solvents being good extractants of organic acids. Further postulates were that the sample-to-solvent ratio should not be more 1:4 for the purpose of having a total volume of less than 1.8 ml in the glass vial, elimination of centrifugation and have good extraction efficiency by

multiple cumulative extractions. The goal was to fully automate the sample clean up step before solvent evaporation in the sample concentration step. These postulates were translated into objectives to achieve the aim of full automation of the extraction protocol.

The initial objective of the method was to select two optimal solvents, one miscible and the other immiscible. Based on literature and confirmed by the experimental data, acetonitrile was selected as the optimal initial one-phase extractant. Acetonitrile being a polar solvent, miscible with water and having higher dielectric constants compared to immiscible, was observed to extract the more polar compounds better. An experiment was conducted to confirm this (see Appendix 10) and acetonitrile was observed to improve the extraction efficiencies of the organic acids, especially those that are more polar organic acids such as adipic acid, glutaconic acid, glutaric acid, glycolic acid, lactic acid and succinic acid, when used together with ethyl acetate.

Ethyl acetate was selected as the solvent of choice from the immiscible organic solvents. This was based on experimental data and confirmed by literature studies. Ethyl acetate proved to have good extraction efficiencies of organic acids and repeatability of extractions compared to other solvents that were investigated. It had the advantages of readiness to use, good viscosity thereby giving a clear phase separation with urine, relative low solubility index with water enabling rapid solvent evaporation and lower density than water giving upper phase extraction. It was also able to extract the acetonitrile from the aqueous phase when more than three times of it was added to the mixture of sample and acetonitrile. These two solvents met the requirements of initial one-phase and secondary two-phase extracting solvents.

After the sample-to-solvent ratio was optimised, a number of other steps were assessed and optimised. An aliquot of urine equivalent to 0.25 mmol creatinine, diluted with normal saline to a final volume of 500 ul was used. Creatinine normalisation was based on the in-house organic acid urine volume calculator, albeit miniaturised. Other miniaturised solvent extraction protocols used a total urine volume of 200 ul (Hassan, 2009, Nakagawa, 2010). The solvent volumes were reduced to 200 ul of acetonitrile and 600 ul of ethyl acetate based on the ratios that was experimentally proven to be able to extract the acids efficiently well. This satisfied the object of having an initial sample-to-solvent ratio of less than 1:4 and total volume of 1.8 ml in a 2.0 ml glass vial. Going beyond this volume led to spillages of sample and solvents. In order to increase the K_D and thereby achieve greater quantitative recoveries of the organic acids, three cumulative sequential extractions with fresh organic solvent each time was done.

Increased surface contact of urine and the organic solvents to increase the recovery of the organic acids was achieved by pipette mixing. In the miniaturised manual extraction protocol, a bench-top vortex mixer accomplished the mixing, something that could not be done by the AutoSampler. To circumvent this challenge in automation, the ML Star AutoSampler's 1000 ul tip head that is fully integrated in the hardware and software was used for the mixing of the urine and the organic solvents. The mixing was accomplished through pipetting mixing by repeated aspiring/dispensing steps (at high pressure). Dual heights were used for pipetting mixing, aspirating 300 ul of the top phase and dispensing it just above the surface of the bottom phase. Four mixing circles were done for the first two extractions and only two for the last extraction.

Clear phase separations were achieved by allowing the samples to settle for a period of 5 minutes. Drying the 3-pooled top phase organic solvents with sodium sulphate was more effective in removing whatever amount of water was in the organic solvents after extraction that might have been there due to the elimination of the centrifugation step. This also facilitated quick evaporation of the organic solvents for a period of ± 15 minutes. This met the objective of shortening the time of the whole preparation step because solvent evaporation is one of the rate-limiting steps in the whole protocol.

The aim in the method development and optimisation phase was not have the parameters that necessarily gave the best extraction efficiencies all the time, as important as that is, rather it was to meet requirements that would give stable extractions and lend itself to automation. Being able to reduce the total error of the method was necessary for accurate quantitative analysis of organic acids. Therefore, the automated method was optimised to give consistent extractions that could correct for proportional errors in the method, leading to low biases.

7.3 Method Validation

Before routine use of the automated organic acid extraction method for analysis by GC/MS, a number of method validation parameters were evaluated. These parameters were purposefully chosen to assess the method's performance compared to the in-house method, as well as to ERNDIM. The analytical range of the method for most of the analytes was established to be between 0 – 300 mg/l. The *r* of all analytes was generally greater than 0.99, with two exceptions, showing great agreement between the reference method's results and the automated test method. The analytical ranges of the specific analytes showed that the test results within these ranges are reliable and can be reported with a great amount of

confidence. The limitation to this is that the linear ranges were influenced by the internal standard used to normalise the analytes, meaning that their quantification and calibration model was relative and not absolute. The use of isotope labelled internal standards would improve the reported ranges. The repeatability was generally quite acceptable and had most of the CVs below 20%. The within-laboratory precision was lower than the in-house method. Running the method in SIM mode as opposed to scan is likely to substantially improve these within-lab precisions. Therefore, the automated method demonstrated that with further optimisation of some other aspects of the whole analytical process of organic acids analysis, such as using SIM mode, optimising the splits on the GC/MS , temperatures, derivatization, etc., (parameters which were beyond the scope of the study), the method would even give better imprecisions and accuracy in quantifications.

The inaccuracy of the method was determined by a method comparison experiment. The ERNDIM EQAS samples for quantitative organic acids were analysed with the test method. The advantage of using the ERNDIM samples is that they are authentic urine samples that are spiked with varied amounts of analytes between physiological and pathological ranges. The reports for these samples are valuable for use in method validation because they give information on other laboratory results that allows for various comparisons with all the laboratories. The mean of the automated method results was compared to the mean of all the laboratories. The proportional systematic error of the method ranged from -0.18 to 2.06. The constant systematic error for the analytes was -5.75 and 5.66. The method's bias was calculated at medical decision concentration for those that were available. Differences between extraction efficiencies of the internal standard and the compounds of interest and lack of correction factors would have been the cause of the observed bias in the method. This would suggest that the addition of more than one internal standard with properties that are similar to the compounds of interest would substantially improve the bias of the method. The total error for some organic acids were determined and compared to the in-house method for the purpose of showing that the automated method can improve on the extraction system by reducing some of the errors associated with manually operating the method. The total error for the automated method was less than the in-house method.

Among the urinary organic acids tested, both in the method development and validation, some were observed to be problematic to analyse. Citric acid proved problematic to extract. Low recovery percentages relative to the internal standard of the organic acid were consistent. This was regardless of the solvent or method of extraction used. The plausible reason for this is that citric acid is a polar compound that is so soluble in water that extracting it presents difficulties. Methylcitric acid, a structural derivative of citric acid was

one of the organic acids evaluated for in the ERNDIM EQAS. It is one of the organic acids used in the diagnosis of defects of propionate metabolism. The assigned concentrations for it in most of the reference samples were mostly low (below 7.50 umol/l). Methylcitric acid was either undetected or values much lower than the assigned concentrations were observed. It is one of the organic aicds that are essentially undetected under physiological conditions (Blau *et al*, 2008). Malonic acid was observed to give two peaks. This was the reason for most of its poor repeatability as seen by high CV values. Glycolic acid, a secondary marker of 4-hydroxybutyric aciduria (Blau, 2014), also showed poor recoveries (averaging 50%), when extracted with the combination of ethyl acetate and diethyl ether when it was 98%.

Some of the organic acids tested for in the method compare experiment using the ERNDIM EQAS samples included 4-hydroxybutyric acid, tiglyglycine, 3-hydroxyglutaric acid and hexanoyglycine. These organic acids are considered significant when present in urine in any quantity. Regardless of the relative quantity of some of these organic acids, their presence is considered pathognomonic (Jones, 2010). 4-Hydroxybutyric acid is present in succinic semialdehyde deficiency (Jones, 2010), but is rather problematic in that it may coelute with urea and has a similar spectrum as 3-hydroxybutyric acid. This may have affected the quantification of this organic acid during the analysis. Tiglyglycine is present in propionic acidemia and disorders of mitochondria oxidative phosphorylation (Jones, 2010). The automated method was able to extract this organic acid despite its low concentrations. 3-Hydroxyglutaric acid is present in 3-hydroxyacyl-CoA dehydrogenase deficiency and glutaric acidemia type 1 (Jones, 2010). Small quantities of it present in urine are pathognomonic It may coelute with 2-ketoglutaric acid and maybe the reason it was not identified in some of the samples. Despite the use of substantially low sample and organic solvent volumes, the automated method was able to detect these acids. The use of SIM mode and isotope labelled internal standard could improve their detection and quantification.

In conclusion, the automated organic acid method showed to be good method that could be used for quantitative and qualitative analysis of organic acids. It proved to improve on the extraction efficiencies of the existing method, giving better repeatabilities and bias. It also simplified the extraction process by automating it and thereby increasing the sample throughput. It substantially reduced the labour-intensiveness associated with the manual method, reduced the analyst's exposure to toxic solvents and the amounts of sample and solvents used. The total errors associated with the existing method were reduced with the new method. The method comparison with the ERNDIM samples also showed that the method performed better than the existing in-house method. Great variability was observed

in the ERNDIM results. A similar observation was made in the ERNDIM Annual Report for 2015 for Quantitative Organic Acids for all the participating laboratories (Martens & Weykamp, 2016). The reason for this is attributed to differences in practice and lack of international standardisation in the analysis of organic acids. The clinical context is the great determinant of how significant this variability is and is often less problematic than is suggested by the results (Blau, 2003).

7.4 Study Limitations and Future Recommendations

The goal and focus of the study was only to assess and optimise the extraction process and automate it and not to look at the whole analytical process of organic acid analysis. And only a select few organic acid standards were used in the optimisation phase. Much as the method demonstrated that extraction can be done with less volumes of sample and solvents and can be automated, there is need to relook at the whole process. For example, there is need to optimise the run in SIM mode, use more than one appropriate internal standard with similar extraction efficiencies to that of compounds of interests, obtain correction factors to be able to quantify the organic acids more accurately, etc. Therefore, a full and complete method assessment and optimisation is recommended. It will be important to follow the internationally recommended procedures for standardisation of the analysis of organic acids to reduce variabilities between laboratories.

Keeping the aim of the study of having stable extraction and meeting the criteria for automating the method and comparing it to the existing in-house extraction method, only purposefully selected parameters of method validation were carried out as opposed to a full validation. The objective was to show the inherent potential of the automated method to have better linearity or analytical range of the organic acids, improved imprecision and bias. Therefore, after a complete optimisation of the whole organic acid analytical process using the automated method, a full validation including all the necessary parameters is recommended for future consideration.

BIBIOLOGRAPHY

Álvarez-Sánchez, B., Priego-Capote, F. & De Castro, M. L. 2010. Metabolomics analysis I. Selection of biological samples and practical aspects preceding sample preparation. *TrAC Trends in Analytical Chemistry,* 29**,** 111-119.

Álvarez-Sánchez, B., Priego-Capote, F. & De Castro, M. L., 2010. Metabolomics analysis II. Preparation of biological samples prior to detection. *Trends in Analytical Chemistry,* 29**,** 120-127.

Baena, B., Cifuentes, A. & Barbas, C. 2005. Analysis of carboxylic acids in biological fluids by capillary electrophoresis. *Electrophoresis,* 26**,** 2622-2636.

Bengtsson, I. M. & Lehotay, D. C. 1996. Sample preparation with an automated robotic workstation for organic acid analysis by gas chromatography-mass spectrometry. *Journal of Chromatography B: Biomedical Sciences and Applications,* 685**,** 1-7.

Berthod, A. & Carda-Broch, S. 2014. Determination of liquid-liquid partition coefficients by separation methods. *Journal of Chromatography A,* 1037**,** 3-14.

BIPM, I., IFCC, I. & IUPAC, I. 1995. OIML. Guide to the Expression of Uncertainty in Measurement, 1995. *International Organization for Standardization (Geneva, Switzerland)*.

Blau, N., Duran, M. & Gibson, K. M. 2008. *Laboratory guide to the methods in biochemical genetics*, Springer.

Blau, N., Duran, M., Gibson, K. M., Dionisi-Vici, C. & Blaskovics, M. E. 2014. *Physician's Guide to the Diagnosis, Treatment and Follow-Up of Inherited Metabolic Diseases*, Springer.

Bojko, B., Reyes-Garcés, N., Bessonneau, V., Goryński, K., Mousavi, F., Silva, E. & Pawliszyn, J. 2014. Solid-phase microextraction in metabolomics. *TrAC Trends in Analytical Chemistry,* 61**,** 168-180.

Boujet, C., Drouet, C., Decoux, G. & Favier, A. 1982. Study of the extraction of organic acids from urine. Preliminary step to their gas chromatographic separation (author's transl). *Journal of chromatography,* 228**,** 67-74.

Burtis, C. A., Ashwood, E. R. & Bruns, D. E. 2012. *Tietz textbook of clinical chemistry and molecular diagnostics*, Elsevier Health Sciences.

Chalmers, R. 2012. *Organic acids in man: analytical chemistry, biochemistry and diagnosis of the organic acidurias*, Springer Science & Business Media.

Chalmers, R. & Lawson, A. M. 1982. *Organic acids in man*, Methuen.

Christou, C., Gika, H. G., Raikos, N. & Theodoridis, G. 2014. GC/MS analysis of organic acids in human urine in clinical settings: a study of derivatization and other analytical parameters. *Journal of Chromatography B,* 964**,** 195-201.

Clement, R. E. & Hao, C. 2012. *Liquid–Liquid Extraction: Basic Principles and Automation,* Ontario, Canada, Elselver Inc.

Cui, M., Fang, L., Zhou, H. & Yang, H. 2014. Effects of amino acids on the physiochemical properties of potato starch. *Food Chemistry,* 151**,** 162-167.

Cui, M., Fang, L., Zhou, H. & Yang, H. 2014. Effects of amino acids on the physiochemical properties of potato starch. *Food Chemistry,* 151**,** 162-167.

David, S., Sanjay, B. & Martin, M. 2011. Health Care Capacity and Allocations Among South Africa's Provinces: Infrastructure–Inequality Traps After the End of Apartheid. *American Journal of Public Health,* 101**,** 165-172.

Dean, 2009. *Extraction Techniques in Analytical Sciences,* West Sussex, United Kingdom, A John Wiley and Sons, Ltd., Publication.

Duez, P., Kumps, A. & Mardens, Y. 1996. GC/MS profiling of urinary organic acids evaluated as a quantitative method. *Clinical Chemistry,* 42**,** 1609-1615.

Dutta, S., Saikia, G. P., Sarma, D. J., Gupta, K., Das, P. & Nath, P. 2016. Protein, enzyme and carbohydrate quantification using smartphone through colorimetric digitization technique. *Journal of biophotonics*.

Erasmus, C., Minnie, L. J., Reinecke, C. J. & Wadman, S. K. 1985. Organic aciduria in late-onset biotin-responsive multiple carboxylase deficiency. *Journal of Inherited Metabolic Diseases,* 8**,** 105-106.

Farajzadeh, M. A., Nouri, N. & Khorram, P. 2014. Derivatization and microextraction methods for determination of organic compounds by gas chromatography. *TrAC Trends in Analytical Chemistry,* 55**,** 14-23.

Fetterman, J. L., Zelickson, B. R., Johnson, L. W., Moellering, D. R., Westbrook, D. G., Pompilius, M., Sammy, M. J., Johnson, M., Dunham-Snary, K. J., Cao, X., Bradley, W. E., Zhang, J., Wei, C. C., Chacko, B., Schurr, T. G., Kesterson, R. A., Dell'italia, L. J., Darley-Usmar, V. M., Welch, D. R. & Ballinger, S. W. 2013. Mitochondrial genetic background

modulates bioenergetics and susceptibility to acute cardiac volume overload. *Biochemical Journal,* 455**,** 157-167.

Fitch, W. L. Anderson, P. J. & Smith, D. H. 1979. Isolation, identification and quantitation of urinary organic acids. *Journal of Chromatography B: Biomedical Sciences and Applications,* 162**,** 249-259.

Fowler, B., Burlina, A., Kozich, V. & Vianey-Saban, C. 2008. Quality of analytical performance in inherited metabolic disorders: the role of ERNDIM. *Journal of inherited metabolic disease,* 31**,** 680-689.

Goodman, S. I. & Markey, S. P. 1981. *Diagnosis of organic acidemias by gas chromatography-mass spectrometry*, AR Liss.

Gong, Z., Ambrosone, C. B., Mccann, S. E., Zirpoli, G., Chandran, U., Hong, C. C., Bovbjerg, D. H., Jandorf, L., Ciupak, G., Pawlish, K., Lu, Q., Hwang, H., Khoury, T., Wiam, B. & Bandera, E. V. 2014. Associations of dietary folate, Vitamins B6 and B12 and methionine intake with risk of breast cancer among African American and European American women. *International Journal of Cancer,* 134**,** 1422-1435.

Hassan, H. M. & Cooper, G. A. 2009. Determination of beta-hydroxybutyrate in blood and urine using gas chromatography- mass spectrometry. *Journal of analytical toxicology,* 33**,** 502-507.

Hoffmann, G. F. & Feyh, P. 2003. Organic acid analysis. *Physician's guide to the laboratory diagnosis of metabolic diseases.* Springer.

Hoffmann, G. F., Meier-Augenstein, W., Stockler, S., Surtees, R., Rating, D. & Nyhan, W. L. 1993. Physiology and pathophysiology of Organic Acids in Cerebrospinal Fluid. *Journal of Inherited Metabolic Diseases,* 16**,** 648-669.

Hu, B., He, M., Chen, B. & Xia, L. 2013. Liquid phase microextraction for the analysis of trace elements and their speciation. *Spectrochimica Acta Part B: Atomic Spectroscopy,* 86**,** 14-30.

Huang, X. & El-Sayed, M. A. 2010. Gold nanoparticles: optical properties and implementations in cancer diagnosis and photothermal therapy. *Journal of Advanced Research,* 1**,** 13-28.

Huber, L. 1997. Validation of analytical methods: Review and Strategy. *LC/GC Magazine***,** 1-17.

Huber, 2010. Validation of analytical methods. *Agilent technologies*, 13-28.

Issachar, D., Holland, J. F. & Sweeley, C. C. 1982. Metabolic profiles of organic acids from human plasma. *Analytical Chemistry,* 54, 29-32.

Jankowski, J., Kubinska, M. & Zdunczyk, Z. 2014. Nutritional and immunomodulatory function of methionine in poultry diets - A review. *Annals of Animal Science,* 14, 17-31.

Johnson, R. 2008. Assessment of bias with emphasis on method comparison. *Clin Biochem Rev,* 29, S37-42.

Jones, P. M. & Bennett, M. J. 2010. Urine organic acid analysis for inherited metabolic disease using gas chromatography-mass spectrometry. *Clinical Applications of Mass Spectrometry: Methods and Protocols*, 423-431.

Kaluzina-Czaplinska, J. 2011. Current Applications of Gas Chromatography/Mass Spectrometry in the Study of Organic Acids in Urine. *Critical Reviews in Analytical Chemistry,* 41, 114-123.

Kloos, D., Lingeman, H., Mayboroda, O., Deelder, A., Niessen, W. & Giera, M. 2014. Analysis of biologically-active, endogenous carboxylic acids based on chromatography-mass spectrometry. *TrAC Trends in Analytical Chemistry,* 61, 17-28.

Kocúrová, L., Balogh, I. S. & Andruch, V. 2013. Solvent microextraction: A review of recent efforts at automation. *Microchemical Journal,* 110, 599-607.

Kumps, A., Duez, P., Genin, J. & Mardens, Y. 1999. Gas Chromatography-Mass Spectrometry Analysis for Organic Acids: Altered Quantitative Response for Aqueous Calibrators and Dilute Urine Specimens. *Clinical Chemistry,* 45, 1297-1300.

Kumps, A., Duez, P. & Mardens, Y. 2002. Metabolic, nutritional, iatrogenic and artefactual sources of urinary organic acids: a comprehensive table. *Clinical chemistry,* 48, 708-717.

Leonard, J. & Morris, A. 2011. The investigation and the initial management of children with suspected metabolic disease presenting acutely. *Paediatrics and Child Health,* 21, 51-55.

Li, H. & Xu, D. 2014. Silver nanoparticles as labels for applications in bioassays. *TrAC Trends in Analytical Chemistry,* 61, 67-73.

Liebich, H. M. 1990. Sample preparation for organic acids in biological fluids. *Analytica Chimica Acta,* 236, 121-130.

Liebich, H. M., Gesele, E. & Wöll, J. 1998. Urinary organic acid screening by solid-phase microextraction of the methyl esters. *Journal of Chromatography B: Biomedical Sciences and Applications,* 713**,** 427-432.

Liu, A., Kushnir, M. M., Roberts, W. L. & Pasquali, M. 2004. Solid phase extraction procedure for urinary organic acid analysis by gas chromatography mass spectrometry. *Journal of Chromatography B,* 806**,** 283-287.

Löfgren, L., Ståhlman, M., Forsberg, G.-B., Saarinen, S., Nilsson, R. & Hansson, G. I. 2012. The BUME method: a novel automated chloroform-free 96-well total lipid extraction method for blood plasma. *Journal of lipid research,* 53**,** 1690-1700.

Lord, H. L. & Pfannkoch, E. A. 2012. Sample Preparation Automation for GC Injection. *Theory of Extraction Techniques* Elsevier Inc.

Lvarez-Sa ´Nchez, B. A., Priego-Capote, F. & Luque De Castro, M. D. 2010. Metabolomics analysis analysis I. Selection of biological samples and practical aspects preceding sample preparation B. *Trends in Analytical Chemistry,* 29**,** 111-119.

Magnusson, B. & Örnemark, U. 2014. *Eurachem Guide: The Fitness for Purpose of Analytical Methods – A Laboratory Guide to Method Validation and Related Topics,* Available from www.eurachem.org.", Eurachem.

Majors, 2014. Sample Pre-treatment for Liquid Samples. *In:* MAJORS, R. E. (ed.) *Sample Preparation Fundamentals for Chromatography.* Agilent Technologies.

Marais, J. 2011. *Optimisation of the organic acid extraction method and analysis.* BSc Honors, North-West University.

Martens, G. & Weykamp, C. W. 2016. Erndim - Quantitative Schemes, Quantitative Organic Acids, Annual Report ERNDIM-EQAS 2015. Brussels/Winterwijk: ERNDIM.

Meyer, U., Heerdegen, K., Schenkel, H., Dänicke, S. & Flachowsky, G. 2014. Influence of various selenium sources on selenium concentration in the milk of dairy cows. *Journal fur Verbraucherschutz und Lebensmittelsicherheit***,** 1-9.

Mitra, 2003. Principles of Extraction and the Extraction of Semivolatile Organics from Liquids. *In:* WINEFORDNER, J. D. (ed.) *Sample Preparation Techniques in Analytical Chemistry.* Wiley-Interscience.

Moldoveanu, S. C. & David, V. 2014. *Modern Sample Preparation for Chromatography*, Elsevier.

Nakagawa, K., Kawana, S., Hasegawa, Y. & Yamaguchi, S. 2010. Simplified method for the chemical diagnosis of organic aciduria using GC/MS. *Journal if Chromatography B,* 878**,** 942-948.

Newsholme, E. & Hardy, G. I. L. 1999. Application of methionine-free nutrition as an anticancer treatment. *Nutrition,* 15**,** 422-424.

Niessen, W. M. 2001. *Current Practise of Gas Chromatography-Mass Spectrometry,* New York, Dekker.

Nordmann, J. & Nordmann, R. 1961. Organic acids in blood and urine. *Advances in clinical chemistry,* 4**,** 53-120.

Ohie, T., Fu, X.-W., Iga, M., Kimura, M. & Yamaguchi, S. 2000. Gas chromatography–mass spectrometry with tert.-butyldimethylsilyl derivatization: use of the simplified sample preparations and the automated data system to screen for organic acidemias. *Journal of Chromatography B: Biomedical Sciences and Applications,* 746**,** 63-73.

Oosterhuis, W. P. 2011. Gross overestimation of total allowable error based on biological variation. *Clinical chemistry,* 57**,** 1334-1336.

Organic Acidemia Association. 2016. *RE: Organic Acidemias.*

Palazzo, G., Facchini, L. & Mallardi, A. 2012. Colorimetric detection of sugars based on gold nanoparticle formation. *Sensors and Actuators B: Chemical,* 161**,** 366-371.

Peroni, D., Van Egmond, W., Kok, W. T. & Janssen, H.-G. 2012. Advancing liquid/liquid extraction through a novel microfluidic device: theory, instrumentation and applications in gas chromatography. *Journal of Chromatography A,* 1226**,** 77-86.

Peters, F. T., Drummer, O. H. & Musshoff, F. 2007. Validation of new methods. *Forensic science international,* 165**,** 216-224.

Peters, V., Garbade, S. F., Langhans, C. D., Hoffmann, G. F., Pollitt, R. J., Downing, M. & Bonham, J. R. 2008. Qualitative urinary organic acid analysis: methodological approaches and performance. *Journal of inherited metabolic disease,* 31**,** 690-696.

Psillakis, E. & Kalogerakis, N. 2003. Developments in liquid-phase microextraction. *TrAC Trends in Analytical Chemistry,* 22**,** 565-574.

Robert-Jan Raterink, P. L. R. J. V. R. R. T. H. 2014. Recent developments in sample-pretreatment techniques for mass spectrometry-basedmetabolomics. *Trends in Analytical Chemistry,* 61**,** 157-167.

Roszek, B., De Jong, W. H. & Geertsma, R. E. 2005. Nanotechnology in medical applications: state-of-the-art in materials and devices.

Sarafraz-Yazdi, A. & Amiri, A. 2010. Liquid-phase microextraction. *TrAC Trends in Analytical Chemistry,* 29**,** 1-14.

Saudubray, J., Desguerre, I., Sedel, F. & Charpentier, C. 2006. A Clinical Approach to Inherited Metabolic Diseases. (eds.) *Inborn Metabolic Diseases.* Springer.

Scriver, C. R., Blau, N., Duran, M., Blaskovics, M. E. & Gibson, K. M. 2012. *Physician's guide to the laboratory diagnosis of metabolic diseases*, Springer Science & Business Media.

Seashore, M. R. 2009. The Organic Acidemias: An Overview. http://www.ncbi.nlm.nih.gov/books/NBK1134/?report=reader.

Shah, P., Midha, K., Findlay, J. & Hill, H. 2000. Bioanalytical Method Validation—A Revisit with a Decade of Progress. *Pharmaceutical Research,* 17**,** 1551-1557.

Shea, T. B. & Rogers, E. 2014. Lifetime requirement of the methionine cycle for neuronal development and maintenance. *Current Opinion in Psychiatry,* 27**,** 138-142.

Sims, P., Truscott, R. & Halpern, B. 1981. Improved procedure for the anion-exchange isolation of urinary organic acids. *Journal of Chromatography B: Biomedical Sciences and Applications,* 222**,** 337-344.

Singh, B., Goswami, B., Gupta, V. K., Chawla, R. & Mallika, V. 2011. Application of sigma metrics for the assessment of quality assurance in clinical biochemistry laboratory in India: A pilot study. *Indian Journal of Clinical Biochemistry,* 26**,** 131-135.

Stöckl, D., Sluss, P. M. & Thienpont, L. M. 2009. Specifications for trueness and precision of a reference measurement system for serum/plasma 25-hydroxyvitamin D analysis. *Clinica Chimica Acta,* 408**,** 8-13.

Suh, J. W., Lee, S. H. & Chung, B. C. 1997. GC/MS determination of organic acids with solvent extraction after cation-exchange chromatography. *Clinical chemistry,* 43**,** 2256-2261.

Tanaka, K., Hine, D. G., West-Dull, A. & Lynn, T. B. 1980. Gas-chromatographic method of analysis for urinary organic acids. I. Retention indices of 155 metabolically important compounds. *Clinical Chemistry,* 26**,** 1839-1846.

Tanaka, K. & Isselbacher, K. J. 1967. The Isolation and Identification of N-Isovalerylglycine from Urine of Patients with Isovaleric Acidemia. *The Journal of Biological Chemistry,* 242**,** 2966-2972.

Theodoridis, G., Koster, E. H. M. D. & De Jong, G. J. 2000. Solid-phase microextraction for the analysis of biological samples. *Journal of Chromatography B: Biomedical Sciences and Applications,* 745**,** 49-82.

Thompson, J. A. & Markey, S. P. 1975. Quantitative metabolic profiling of urinary organic acids by gas chromatography-mass spectrometry. Comparison of isolation methods. *Analytical Chemistry,* 47**,** 1313-1321.

Vaidyanathan, K., Narayanan, M. P. & Vasudevan, D. M. 2011. Organic Acidurias: An Updated Review. *Indian Journal of Clinical Biochemistry,* 26**,** 319-325.

Venter, L., Van Rensburg, P. J., Vosloo, A. & Lindeque, J. Z. 2016. Untargeted Metabolite Profiling of Abalone Using Gas Chromatography Mass Spectrometry. *Food Analytical Methods,* 9**,** 1254-1261.

Weitzel, M. L., Lee, S., Smoot, M., Viafara, N. & Brodsky, M. 2007. *How to meet ISO 17025 requirements for method verification,* www.aoac.org, AOAC International.

Wells, 2003. Principles of Extraction and the extraction of semivolatile organics from liquids. *In:* SOMENATH, M. (ed.) *Sample Preparation Techniques in Analytical Chemistry.*

Westgard, J. O., Barry, P. L., Quam, E. F. & Ehrmeyer, S. S. 2008. *Basic Method Validation: Training in Analytical Quality Management for Healthcare Laboratories,* Madison Wisconsin, Westgard Quality Corporation.

Westgard, S. & Westgard, Q. C. 2009. Six Sigma metric analysis for analytical testing processes. *Abbott MS,* 9**,** 04.

Weyne, J. & Leusen, I. 1975. *Lactate in CSF in relation to brain and blood,* New York, Academic Press.

William, R. 2013. *RE: Carboxylic acids.*

APPENDICES

APPENDIX 1

ORGANIC ACID SOP PLIEM

INTRODUCTION AND OVERVIEW OF PROCEDURES

Organic acidemias are disorders of intermediary metabolism with characteristic accumulation of carboxylic acids, identified by GC/MS analysis of urine. Organic acid disorders comprise defects in which an abnormal acid or abnormal amounts of an acid are excreted. They include defects in the metabolism of deaminated amino acids, fatty acids and carbohydrates. Traditionally these compounds have been assessed as components in a urinary organic acid profile. To obtain this, the acids are extracted from acidified urine, concentrated to dryness and converted to trimethylsilyl esters (and to trimethylsiliylyl- ethers if they also contain a hydroxyl group) so that they may become volatile at the temperature of the injection port of a capillary gas chromatograph. They are usually separated on a relatively non-polar column (5% phenyl). Detection and identification of these compounds are not generally performed using bench top mass selective detectors.

The analysis of organic acids by gas chromatography (GC) and gas chromatography mass spectrometry (GC/MS) has become well established as an important procedure for the diagnosis of inherited disorders of amino acid and organic acid metabolism. The important steps of the procedure are isolation of the organic acids form physiological fluids, formation of volatile derivatives and GC analysis or, preferably, GC/MS analysis.

OVERVIEW OF PROCEDURES

The analysis of organic acids by gas chromatography mass spectrometry (GC/MS) has become well established as an important procedure for the diagnosis of inherited disorders of amino acid and organic acid metabolism (Goodman & Marker, 1981; Chalmers & Lawson, 1982). The important steps for the procedure are

1. Isolation of the organic acids from physiological fluids
2. Formation of volatile derivates and
3. GC/MS analysis

The value of the analysis is enhanced if quantitative as well as qualitative results are obtained using international units of mmol/mol creat for urine and µmol/L for plasma, cerebrospinal fluid and amniotic fluid. The acceptable error in quantitative results can be as much as 40-50% for diagnostic purposes, but for clinical management of patients, the errors for the critical metabolites should be less than 20%.

Volatile trimethylsilyl (TMS) derivates of the extracted organic acids are formed by heating with N,O-bis-(trimethylsilyl)trifluoraceteamine (BSTFA). The TMS derivates are less than ideal derivates for some classes of compounds such as acyl glycine, which form mono and d-TMS derivates, they are still the most useful and versatile derivates for the wide range of chemical functional groups in organic acids.

Organic acids are isolated from physiological fluids with ethyl acetate and diethyl ether extractions. In order to compensate for the large differences between urine concentrations and in order to obtain organic acid profile that appear similar regardless of this, a variable amount of urine, determined by urinary creatinine concentration is used. The organic acid extract is evaporated to dryness under nitrogen and the dried extract derivatized and sylation with BSTFA and TMCS and the derivatives are analysed on GC/MS. Abnormal metabolites on the organic acid profile must be verified by GC/MS (mass spectrometry).

III SAMPLE HANDLING, EXTRACTION, DERIVATISATION AND CHROMATOGRAPHY.

Reagents

Reagent preparation

Equipment

Handling of samples

Procedure:

- Extraction
- Derivatization
- Transferring the extract
- Loading vials on the auto sampler

Use of GC/MS

Chromatographic conditions

Data File Management

SAMPLE HANDLING EXTRACTION, DERIVATISATION AND CHROMATOGRAPHY

REAGENT

Definitions

GC – Gas chromatography

GC/MS – Gas chromatography mass spectrometry

BSTFA – Bis(trimethylsislyl)-trifluoracetamide

TMCS - Trimethylchlorosilane

IS – Internal standard

Product information

Ethyl acetate HPLC grade/distilled (Sigma: Cat # 494518)

Diethyl ether HPLC grade/distilled (Sigma: (Cat #: 309966)

3-Phenyl butyric acid (mw 164.21) (Fluka: Cat n#: 78243)

5 M Hydrochloric acid (Merck: Cat #: 100319)

Hexane (Sigma: Cat # 52767)

NasSO4 anhydrous (Merck: Cat # 1.06649)

BSTFA Bis(trimethylsilyl)-trifluoracetamid [Supelco: Cat no: 33027)

TMCS Trimethylchlorosilane [Sigma: Cat H T 4252]

Pyridine (Merck: Cat no: 5124060LC)

REAGENT PREPERATION

INTERNAL STD;

3-Phenyl butyric acid (3,1973mM)

26.25 mg, dissolve in a few drops NaOH and then to 50 ml distilled H2O

Store in fridge (4°C)

5M HCl;

50 ml 32% conc. HCl diluted to 100 ml H2O. Store in fridge

Storage and stability of chemicals

- Internal standard and 5 M HCl stored in fridge
- BSTFA & TMCS

Store in fridge after new container is opened, but must be at room temperature before opening to dispense and must be at room temperature for use. Use glass pipette to dispense.

- Na2SO4 must be desiccated during moist weather
- Store organic acid standard solutions in air tight container in fridge

EQUIPMENT

Distiller Pasteur pipettes (Merck: Cat #: 612-1702)

Kimax culture tubes (Large): 16 x 125mm (Lasec: Cat # GIMK 45066A16125)

Kimax culture tubes (Large): 13 x 100mm (Lasec: Cat # GIMK 45066A13100)

Graduated pipettes – 10-100ul and 100-1000ul (Merck: 3111000149 and 3111000165)

Roto-torque (Rotator) (Labotech: Cat #: 67003)

Heating block and Evaporating adaptor with Nitrogen (Pierce: Cat # 18840, 18817 and 18785)

Centrifuge (Lasec: Hermle, Z 206A)

Hamilton syringes 10 µl & 100 µl (Separations: Cat # 80391 and 80366)

Sample Vials, inserts and Caps (Separations: Cat # 11090500, 09151819 and 06090357)

Capillary GC/MS column: VF1-MS (30m x 0.25 x 0.25ID): (SMM-Instruments: Cat # CP8924 or Chemetrix: DB-1MS UI: Cat #: 122-0132UI)

Gas chromatograph Hewlett Packard 7890A (Chemetrix)

Mass Spectrometer system Hewlett Packard 5975C (Chemetrix)

Autosampler, 7693 (Chemetrix)

HANDELING SAMPLES

SPECIMEN:

Patient Preparation: Non-Specific

Type: Random urine sample. Serum when requested

Handling Conditions:

All handling conditions are documented in WI-ML-001. Work with latex gloves at all times.

- An extraction work list is obtained by the Q & A data basis
- Go to C:\report\Worksheet Templates\Organic Acid Volume Calculator and enter the creatinine values shown on the OA work list in the column headed Creatinine. Save the Volume Calculator then print it. (See attached example of work list)
- Check entries in Volume Calculator against the Q & A work list.
- Trim print out from Volume Calculator, file in organic acid work list file.
- Obtain urine samples from freezer and follow the defrost protocol stipulated in WI-ML-001.
- Use a marker pen and label 2 large culture tubes and 1 small culture tube for each accession. Label each tube with the lab number.

PROCEDURE

Preparation for extraction and calculation of volumes required

Stepwise extraction procedure:

A: URINE

Volume urine used according to creatinine values;

Creatinine	<	100	mg%	use 1 ml urine
Creatinine	>	100	mg%	use 0.5 ml urine
Creatinine	<	5	mg%	use 2 ml urine
Creatinine	<	2	mg%	use 3 ml urine

Add 6 drops 5M HCl to adjust the pH to 1.

Add Internal std. (IS) 5X creatinine mg% = volume in µl

B: SERUM

1 ml Serum

Add 100 µl Internal standard

Add 6 drops 5M HCl to adjust the pH to 1.

Procedure continues for urine and serum

1. Add 6 ml Ethyl acetate
2. Shake 30 min (Roto-torque)
3. Centrifuge ± 3 min
4. Aspirate the organic phase into a clean tube
5. Add 3 ml Diethyl ether to the aqueous (lower) phase
6. Shake 10 min
7. Centrifuge ± 3 min
8. Aspirate the organic phase & add to the ethyl acetate phase
9. Add two spatulas (Pasteur pipette) Ns2SO4
10. Vortex
11. Note: The Na2SO4 must now be powder & not flakes???. More Na2SO4 can be added if necessary.
12. Centrifuge

13. Pour the organic phase into a clean smaller kimax tube

14. Evaporate to dryness under Nitrogen at 37°C ± 1 hour

Derivatization

Hamilton syringes may be rinsed with Hexane, Pyridine, Acetone or sonicated if dirty.

Hamilton syringes are well rinsed with Hexane between and after use (5X), pull plunger out when not in use.

- Add BSTFA A: (2X creat mg% = volume in µl) for urine
- B: 40 µl for serum
- Add TMCS A: (0.4X creat mg% = volume in µl) for urine
- B: 8 µl for serum
- Add pyridine (same amount as TMCS)
- Incubate at 60°C for 1 hour (45 min - 70°C)
  - Transferring the Extract
- Transferring is done in the fume hood.
- Label an autosampler vial with the lab number of the sample.
- Allow derivatized samples to cool then transfer 40 µL to the corresponding labelled auto- sampler vial using a 100 µL Hamilton syringe.
- Loading the Vials on the Auto sampler
- Place the autosampler vials in the next and following empty positions of GC autosampler tray.
  - USE OF THE GC/MS
  - Full details on use of GC/MS equipment can be found in QP-ML-010.
  - Briefly, the method is as follows:
- All samples are acquired using the ORG-J&W method.
- Under the Sequence menu choose “Load Sequence” and select the previous day’s organic acid sequence.
- Under the Sequence menu choose “Edit Sample Log Table”. Delete previous day’s patient samples and starting with the reference urine, update vial position to reflect current run. Add new samples by clicking on the “Repeat” button which will update vial positions and data file names by one. Continue adding samples and changing data file names and Sample Names until all samples are entered into the sample log table. Click “OK”
- From the Sequence menu choose “Save sequence as” and rename the sequence with current date. E.g.020610A (if it is the first sequence of the day) of 020610B (If the

second sequence of the day. Also, create a folder to save all data of the day in c:\msdchem\1\data\Organic acid\sample list

- From the Sequence menu choose 'Load and Run' and update data file path to save files under a new directory with the current date e.g. c:\msdchem\1\data\Organic acid\sample list. Click "OK".
- From Sequence menu on "Run", then "run sequence". The software will process the sequence line by line until completed.

CHROMATOGRAPHIC CONDITION

- Briefly, these are as follows:
- Sample, 1 µl is injected onto the GC using split injection at a split ratio of 12:1 and a constant pressure of 8.2317 psi
- Injection port temperature is 280℃.
- Initial oven temperature is 50℃ that is held for 1 minute then 20C/min to 60°C for 0 min.
- The temperature is then increased at 5℃ per minute to 120°C then at 7℃ per minute to 295℃ which is held for a further 4 minutes.
- Run time: 42.5 min with 1 min post run at 300°C
- Column used is a 30 metre VF1-MS/ DB1-MS UI with a constant flow of helium at1ml/minute at a pressure of 7.6522 psi.
- A solvent delay of 8 minutes is stipulated in the temperature program

APPENDIX 2

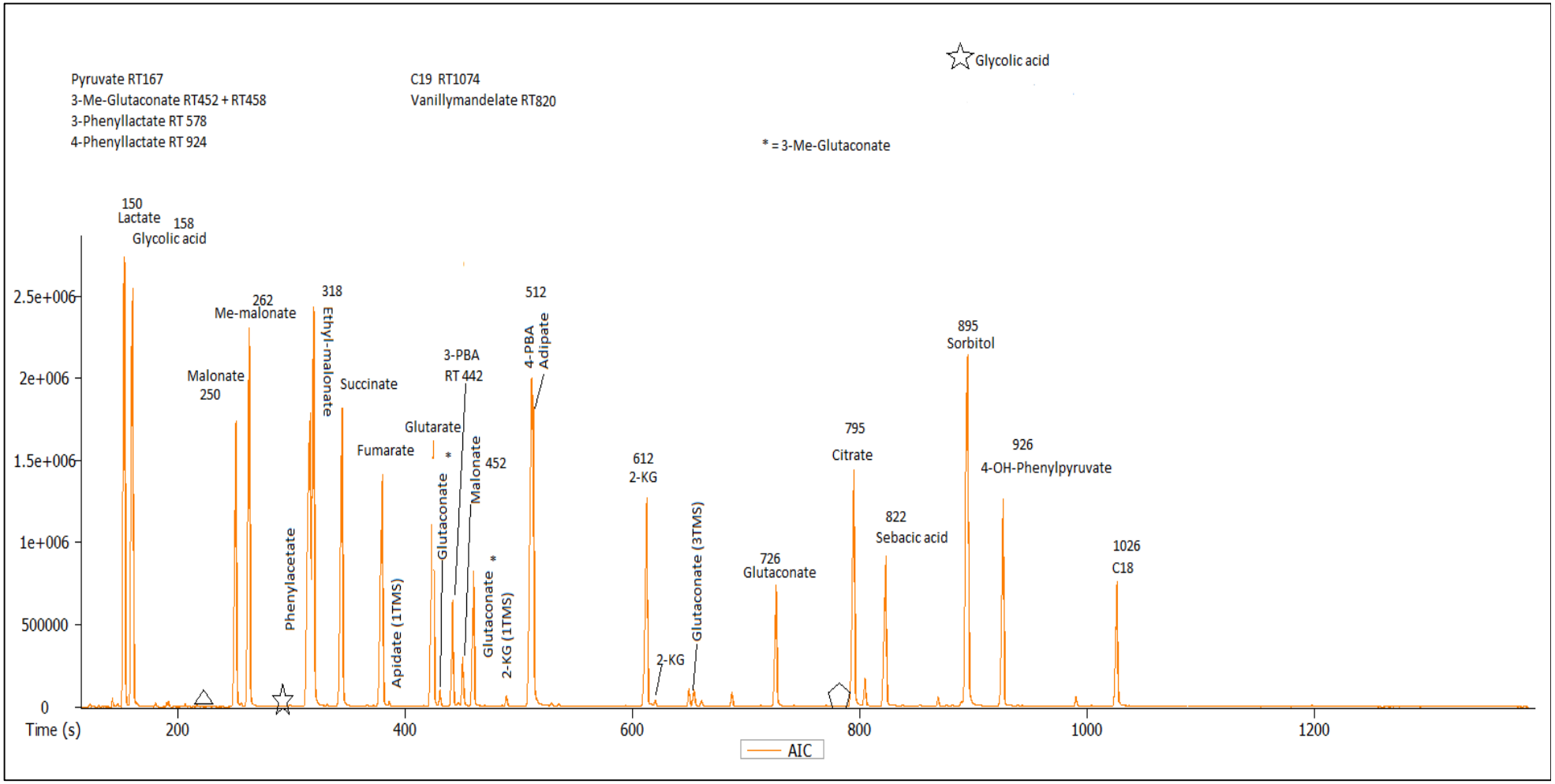


Figure showing the total ion chromatogram of organic acids

APPENDIX 3

Table showing Analyte recovery efficiency and coefficient of variation with different extractants

Analyte	Diethyl ether		MTBE		Ethyl acetate		Ethyl acetate/Diethyl ether	
	Average recovery %	CV %	Average recovery %	CV %	Average recovery %	CV %	Average recovery %	CV %
3-Methylglutaconic acid	44	9	43	23	94	11	100	9
3-Phenyllactic acid	47	5	49	19	100	11	96	7
4-Phenylbutyric acid	49	5	48	21	97	5	97	4
Adipic acid	46	6	50	20	95	11	89	8
Citric acid	1	3	5	23	27	13	20	8
Ethylmalonic acid	60	8	56	22	126	7	106	8
Fumaric acid	47	10	50	18	99	10	98	10
Glutaconic acid	36	30	41	20	87	43	64	60
Glutaric acid	39	6	43	23	91	10	86	6
Glycolic acid	19	31	11	42	50	7	72	23
Lactic acid	9	24	21	47	68	11	105	24
Malonic acid	38	64	87	66	186	60	145	39
Methylmalonic acid	61	23	61	28	117	16	95	5
Phenylacetic acid	41	7	41	22	116	11	122	9
Sorbitol								
Stearic acid	51	6	45	21	102	9	95	8
Succinic acid	35	6	46	18	92	9	86	7
Succinylacetone	31	50	32	26	45	34	51	28
Vanillylmandelic acid	20	77	89	110	96	6	88	8
Average	38	21	45	32	94	16	90	15

APPENDIX 4

Table showing the effect of salt addition on the extraction efficiencies of organic acids

Analyte	ACN/EA_No Salts		ACN/Eac_With Salts	
	Mean recovery %	CV %	Mean recovery %	CV %
4-Phenylbutyric acid	82	4	102	2
Adipic acid	83	13	96	5
Citric acid	39	11	41	7
Ethylmalonic acid	91	11	100	2
Fumaric acid	83	14	95	1
Glutaconic acid	54	85	53	25
Glutaric acid	77	9	87	2
Glycolic acid	45	10	43	11
Lactic acid	68	11	81	7
Malonic acid	67	6	73	2
Methylmalonic acid	79	11	89	3
Phenylacetic acid	100	2	106	4
Sorbitol	1	53	8	85
Succinic acid	69	10	81	1
Average	**69**	**18**	**78**	**11**

APPENDIX 5

Table showing the recoveries of different ratios of acetonitrile: ethyl acetate

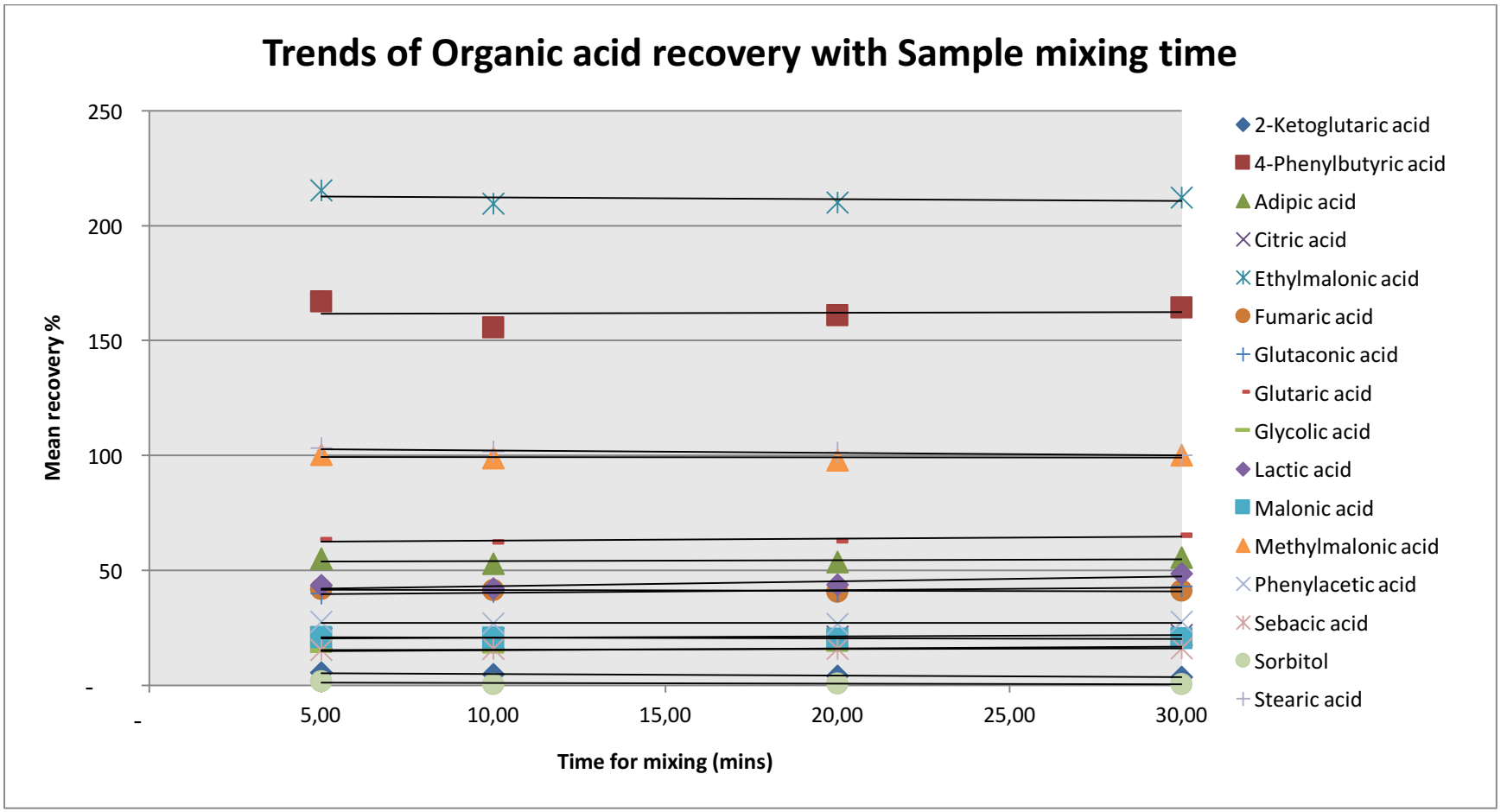
Analytes	ACN 1:20 EtAc		ACN 1:10 EtAc		ACN 1:5 EtAc		ACN 1:3 EtAc	
	Average %	CV %	Average %	CV %	Average %	CV %	Average %	CV %
4-Phenylbutyric acid	212	3	204	1	204	3	210	6
Adipic acid	81	5	79	2	80	4	86	5
Citric acid	5	4	6	3	7	3	8	13
Ethylmalonic acid	154		276	2	294	5	288	8
Fumaric acid	58	1	58	1	62	5	71	7
Glutaconic acid	91	15	55	74	54	42	14	70
Glutaric acid	97	3	99	1	102	5	116	7
Glycolic acid	30	6	33	3	38	1	48	16
Lactic acid	84	5	89	4	95	1	102	7
Malonic acid	45	16	58	32	70	11	80	5
Methylmalonic acid	154	5	134	3	140	5	149	3
Phenylacetic acid	65	3	67	1	69	1	62	6
Sorbitol	3	77	1	13	1	35	3	128
Stearic acid	118	3	117	1	121	6	122	3
Succinic acid	73	2	76	1	83	6	98	1
Average	74	11	79	10	82	11	84	19

APPENDIX 6

Table showing Sample-to-Solvent ratios and their corresponding recoveries

Sample to Solvent ratio/ Compounds	1 to 0.5		1 to 1		1 to 2		1 to 4	
	Average (%)	CV	Average (%)	CV	Average (%)	CV	Average (%)	CV
4-Phenylbutyric acid	211	4	193	5	198	2	193	2
Adipic acid	54	5	62	6	69	4	70	3
Citric acid	6	22	10	8	21	3	34	4
Ethylmalonic acid	0	52	0	39	1	8	1	10
Fumaric acid	47	4	48	8	54	3	56	4
Glutaconic acid	52	16	71	9	99	10	85	23
Glutaric acid	51	6	66	8	83	3	90	4
Glycolic acid	3	26	7	5	14	17	28	2
Lactic acid	9	22	21	8	43	20	76	0
Malonic acid	11	76	14	57	35	10	44	25
Methylmalonic acid	89	8	95	6	120	3	127	2
Phenylacetic acid	55	7	52	4	58	4	66	4
Sorbitol	2	64	1	5	1	17	1	32
Stearic acid	124	6	114	9	122	3	119	4
Succinic acid	33	7	44	7	56	3	67	7
Average	44	25	47	14	57	7	62	10

APPENDIX 7


Trends of Organic acid recovery with Sample mixing time
Mean recovery %
250
200
150
100
50
-
-
5,00
10,00
15,00
20,00
25,00
30,00
Time for mixing (mins)
2-Ketoglutaric acid
4-Phenylbutyric acid
Adipic acid
Citric acid
Ethylmalonic acid
Fumaric acid
Glutaconic acid
Glutaric acid
Glycolic acid
Lactic acid
Malonic acid
Methylmalonic acid
Phenylacetic acid
Sebacic acid
Sorbitol
Stearic acid

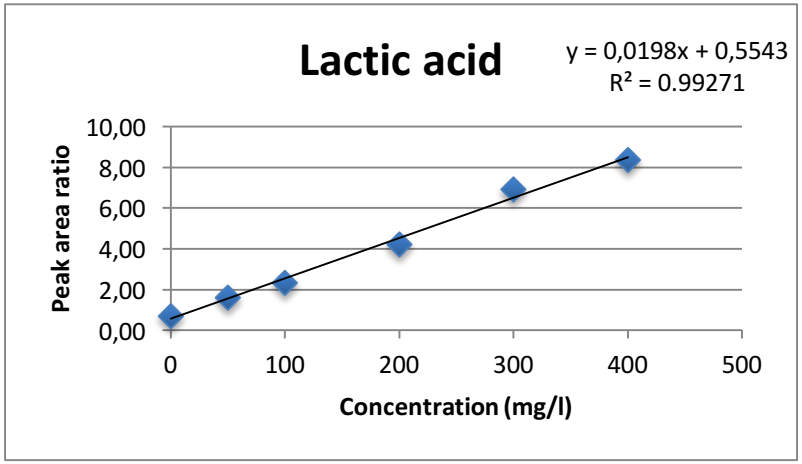
APPENDIX 8

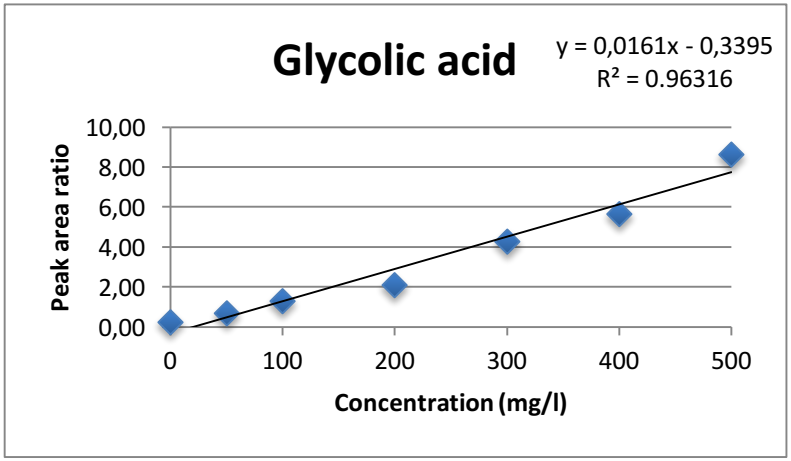
Table 5.7 Comparison of extraction protocols with the Miniaturised one

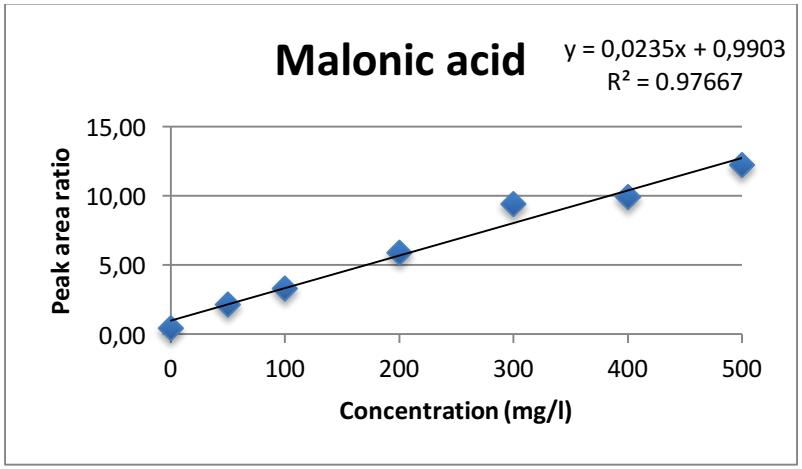
Analytes	Miniaturised Method		In-house Method	
	Mean recovery %	CV %	Mean recovery %	CV %
4-Phenylbutyric acid	96	4	97	4
Adipic acid	76	9	89	8
Citric acid	15	89	20	8
Ethylmalonic acid	133	6	106	8
Fumaric acid	95	7	98	10
Glutaconic acid	42	28	64	60
Glutaric acid	69	7	86	6
Glycolic acid	32	5	72	23
Lactic acid	46	10	105	24
Methylmalonic acid	105	13	95	5
Phenylacetic acid	135	4	122	9
Sorbitol	2			
Stearic acid	102	3	95	8
Succinic acid	71	19	86	7

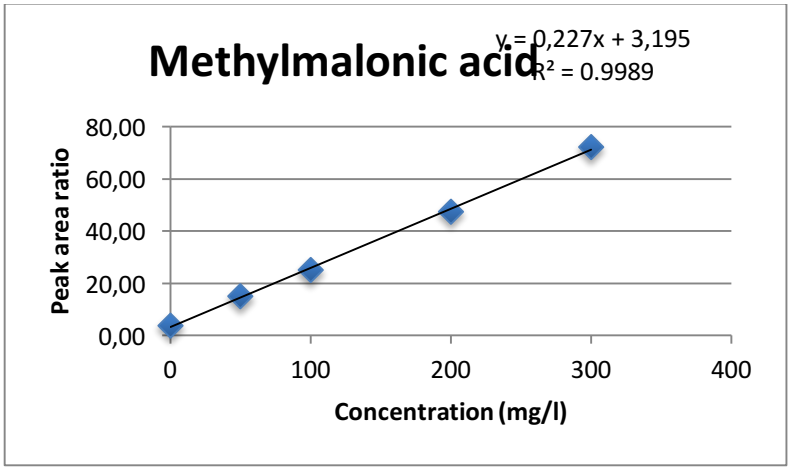
APPENDIX 9

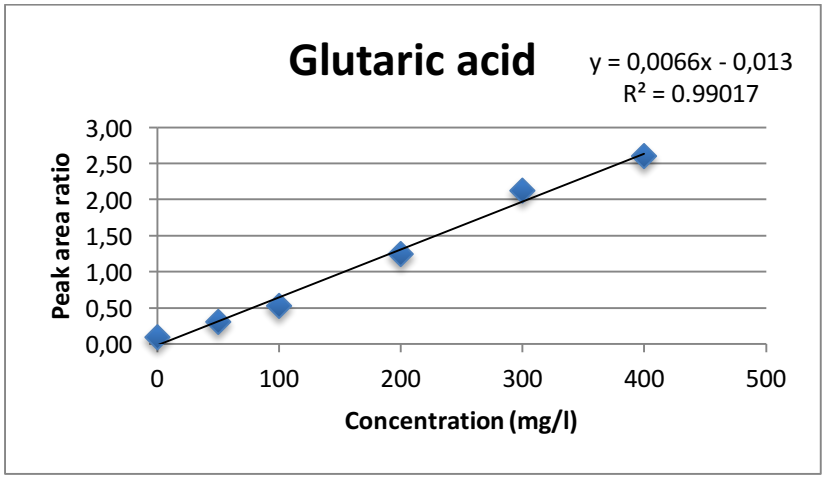
Organic acid linear curves

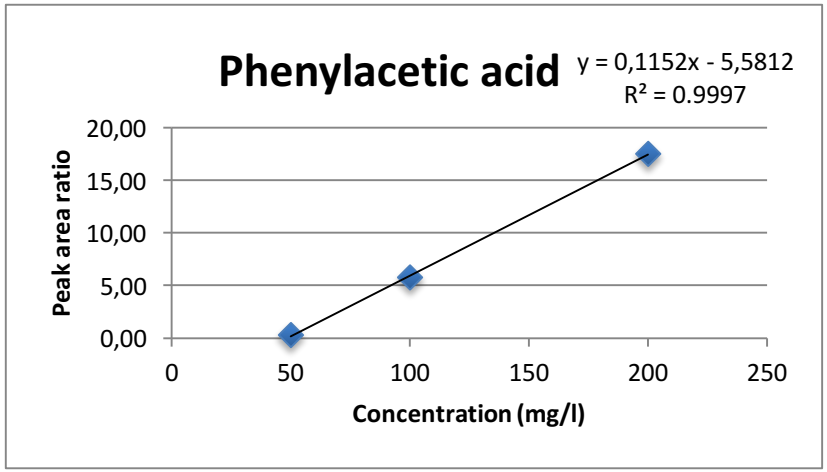


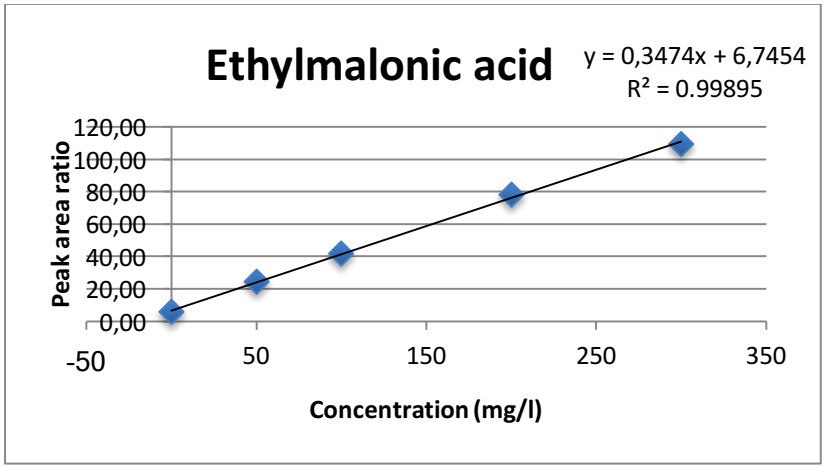


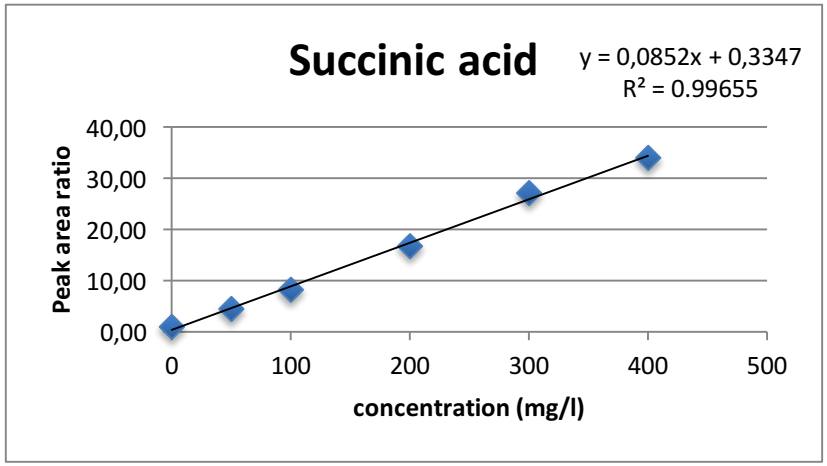


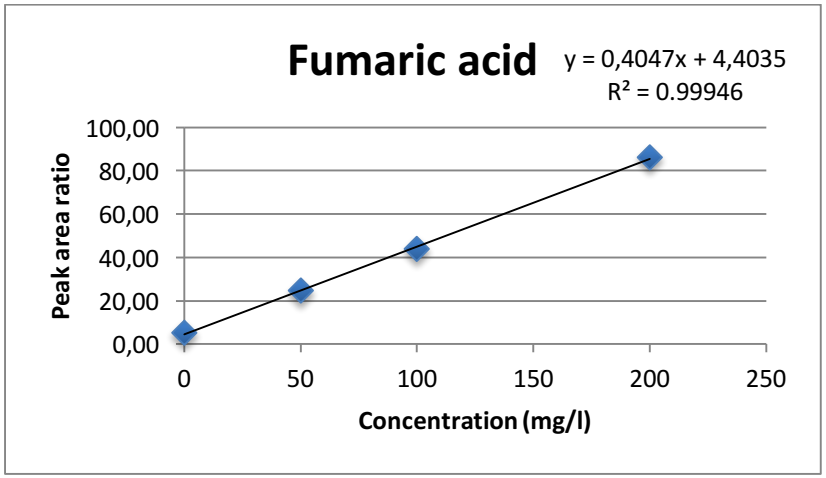


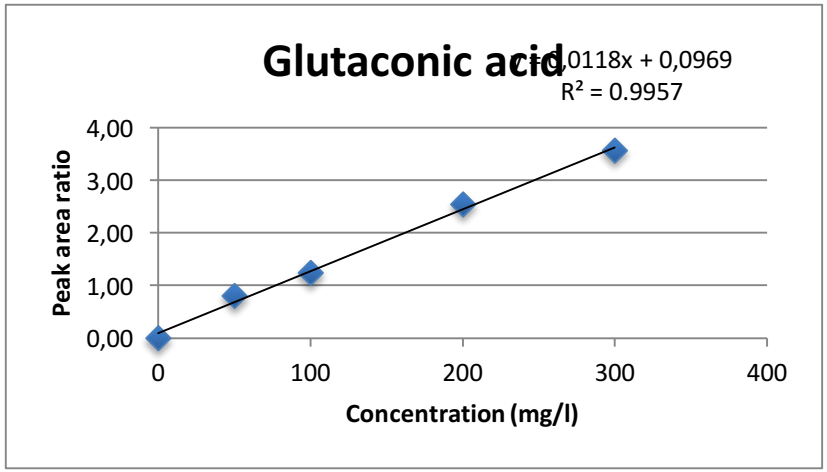

Glutaric acid
y = 0,0066x - 0,013
R² = 0.99017
Peak area ratio
3,00
2,50
2,00
1,50
1,00
0,50
0,00
0
100
200
300
400
500
Concentration (mg/l)

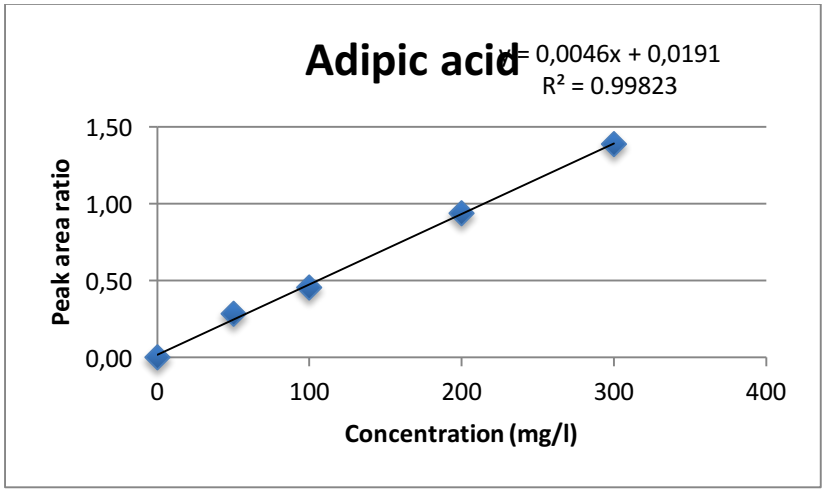

Phenylacetic acid
y = 0,1152x - 5,5812
R² = 0.9997
Peak area ratio
20,00
15,00
10,00
5,00
0,00
0
50
100
150
200
250
Concentration (mg/l)

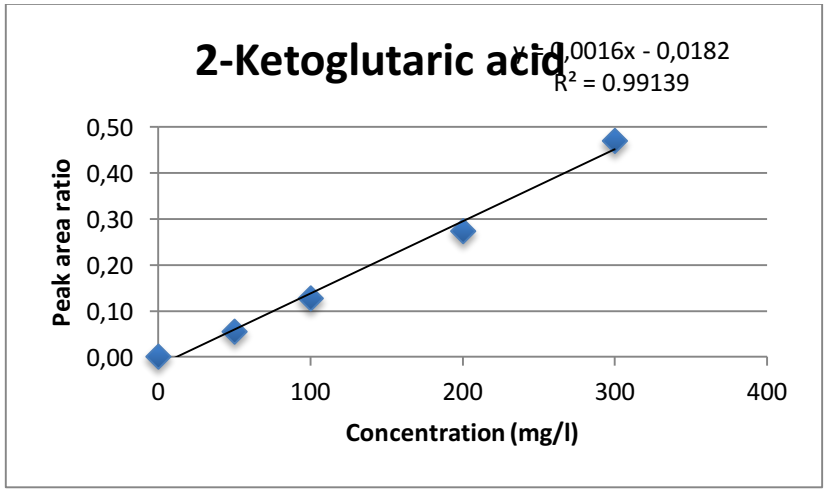

Ethylmalonic acid
y = 0,3474x + 6,7454
R² = 0.99895
Peak area ratio
120,00
100,00
80,00
60,00
40,00
20,00
0,00
-50
50
150
250
350
Concentration (mg/l)

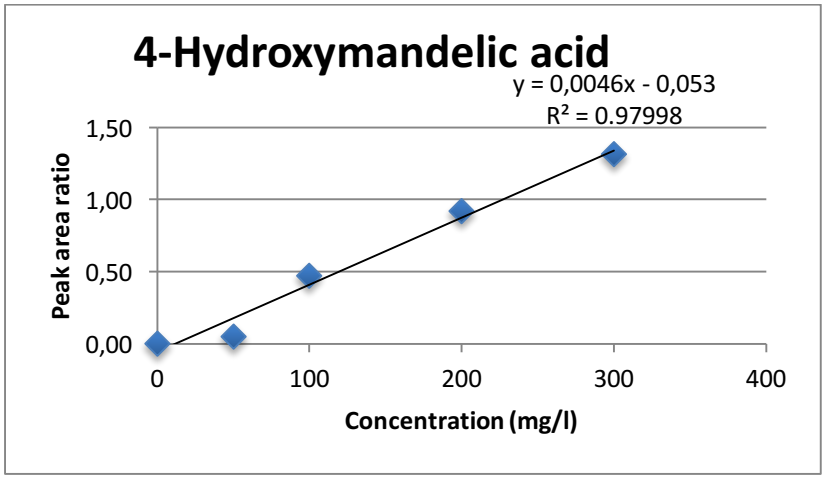

Succinic acid
y = 0,0852x + 0,3347
R² = 0.99655
Peak area ratio
40,00
30,00
20,00
10,00
0,00
0
100
200
300
400
500
concentration (mg/l)

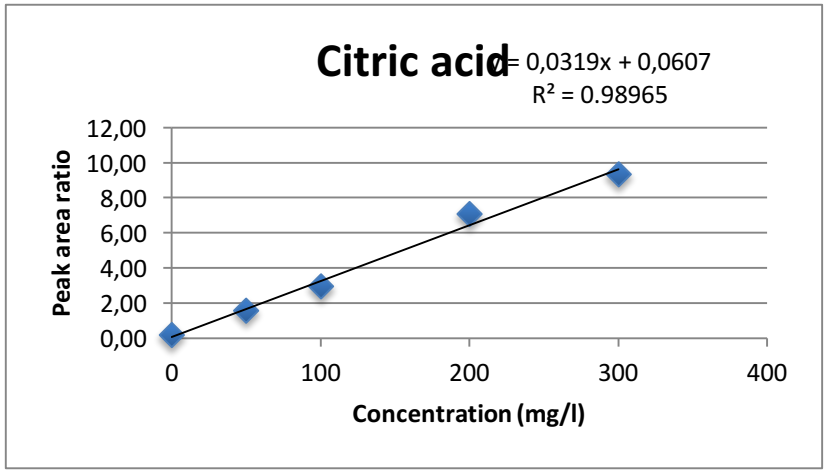

Fumaric acid
y = 0,4047x + 4,4035
R² = 0.99946
Peak area ratio
100,00
80,00
60,00
40,00
20,00
0,00
0
50
100
150
200
250
Concentration (mg/l)

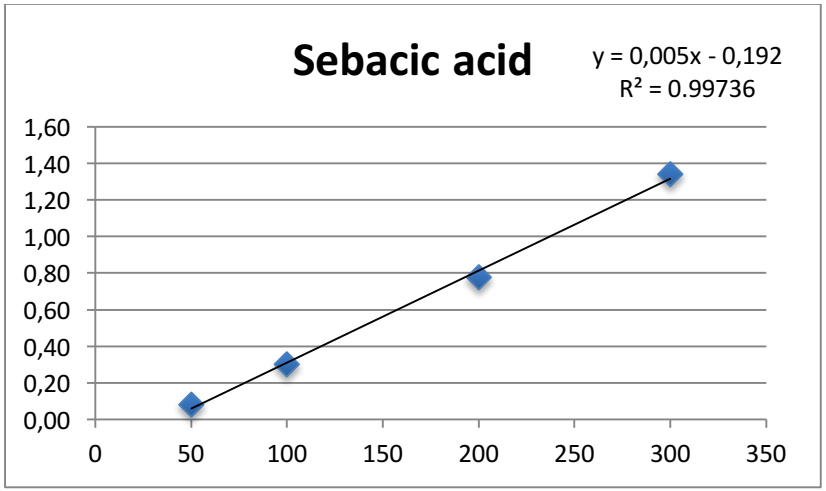

Glutaconic acid
y = 0,0118x + 0,0969
R² = 0.9957
Peak area ratio
4,00
3,00
2,00
1,00
0,00
0
100
200
300
400
Concentration (mg/l)

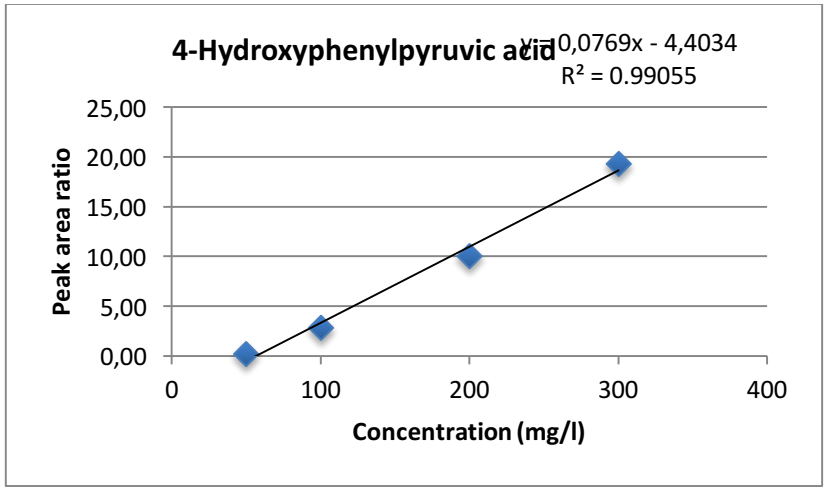

Adipic acid
y = 0,0046x + 0,0191
R² = 0.99823
Peak area ratio
1,50
1,00
0,50
0,00
0
100
200
300
400
Concentration (mg/l)

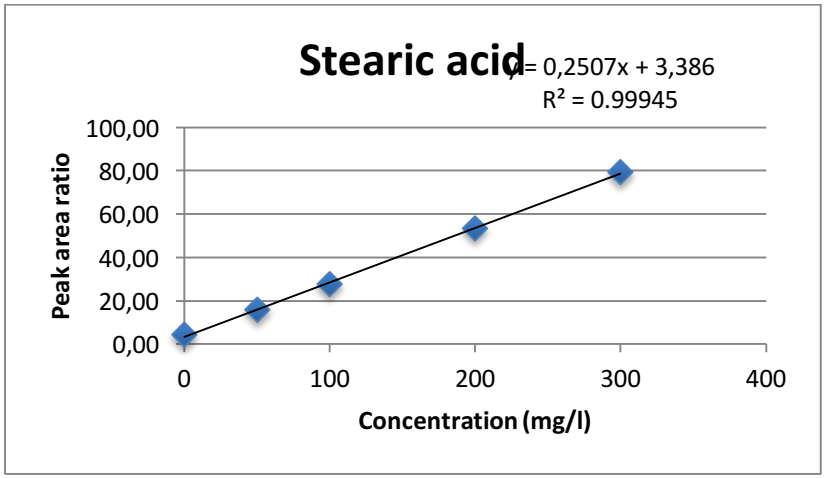

2-Ketoglutaric acid
y = 0,0016x - 0,0182
R² = 0.99139
Peak area ratio
0,50
0,40
0,30
0,20
0,10
0,00
0
100
200
300
400
Concentration (mg/l)


4-Hydroxymandelic acid
y = 0,0046x - 0,053
R² = 0.97998
Peak area ratio
1,50
1,00
0,50
0,00
0
100
200
300
400
Concentration (mg/l)


Citric acid
y = 0,0319x + 0,0607
R² = 0.98965
Peak area ratio
12,00
10,00
8,00
6,00
4,00
2,00
0,00
0
100
200
300
400
Concentration (mg/l)


Sebacic acid
y = 0,005x - 0,192
R² = 0.99736
1,60
1,40
1,20
1,00
0,80
0,60
0,40
0,20
0,00
0
50
100
150
200
250
300
350


4-Hydroxyphenylpyruvic acid
y = 0,0769x - 4,4034
R² = 0.99055
Peak area ratio
25,00
20,00
15,00
10,00
5,00
0,00
0
100
200
300
400
Concentration (mg/l)


Stearic acid
y = 0,2507x + 3,386
R² = 0.99945
100,00
80,00
60,00
40,00
20,00
0,00
Peak area ratio
0
100
200
300
400
Concentration (mg/l)

APPENDIX 10

The Influence of acetonitrile on extraction efficiencies of organic acids

Background

The research question for this experiment was; does acetonitrile have any significant effect on the extraction efficiencies when used together with ethyl acetate? Therefore, the objective of the experiment was to investigate the influence of the use of acetonitrile with ethyl acetate in the miniaturised organic acid extraction.

Methodology

An absolute recovery experiment was conducted according to protocol 5.8. Two sets of glass vials were used. The one set had the extraction carried on by extracting with the method as is, using both acetonitrile and ethyl acetate. The second set only had ethyl acetate added to it without adding acetonitrile. The same protocol was followed for both besides the explained modification. The experiment was done in hexaplicates.

Results and Discussion

Figures 10.1 and 10.2 show the comparisons of the mean recoveries and CVs respectively of the ethyl acetate extraction with and without acetonitrile. The extraction was demonstrated to increase by the addition of acetonitrile to ethyl acetate. Figure 10.1 shows that with the exception of malonic acid (which was over extracted by both methods), acetonitrile addition improved the extraction efficiencies of all the organic acids. The CVs however were better for the ethyl acetate-only extraction, but not significantly. As seen in Figure 10.2 all the CVs for both methods were very good and well below 20% with the exception of malonic acid for the acetonitrile/ethyl acetate method.

Conclusion

The addition of acetonitrile was observed to improve the extraction efficiencies of the organic acids in the miniaturised method. Therefore, it was concluded to have a beneficial influence when added to ethyl acetate in the miniaturised and automated organic acid extraction method.

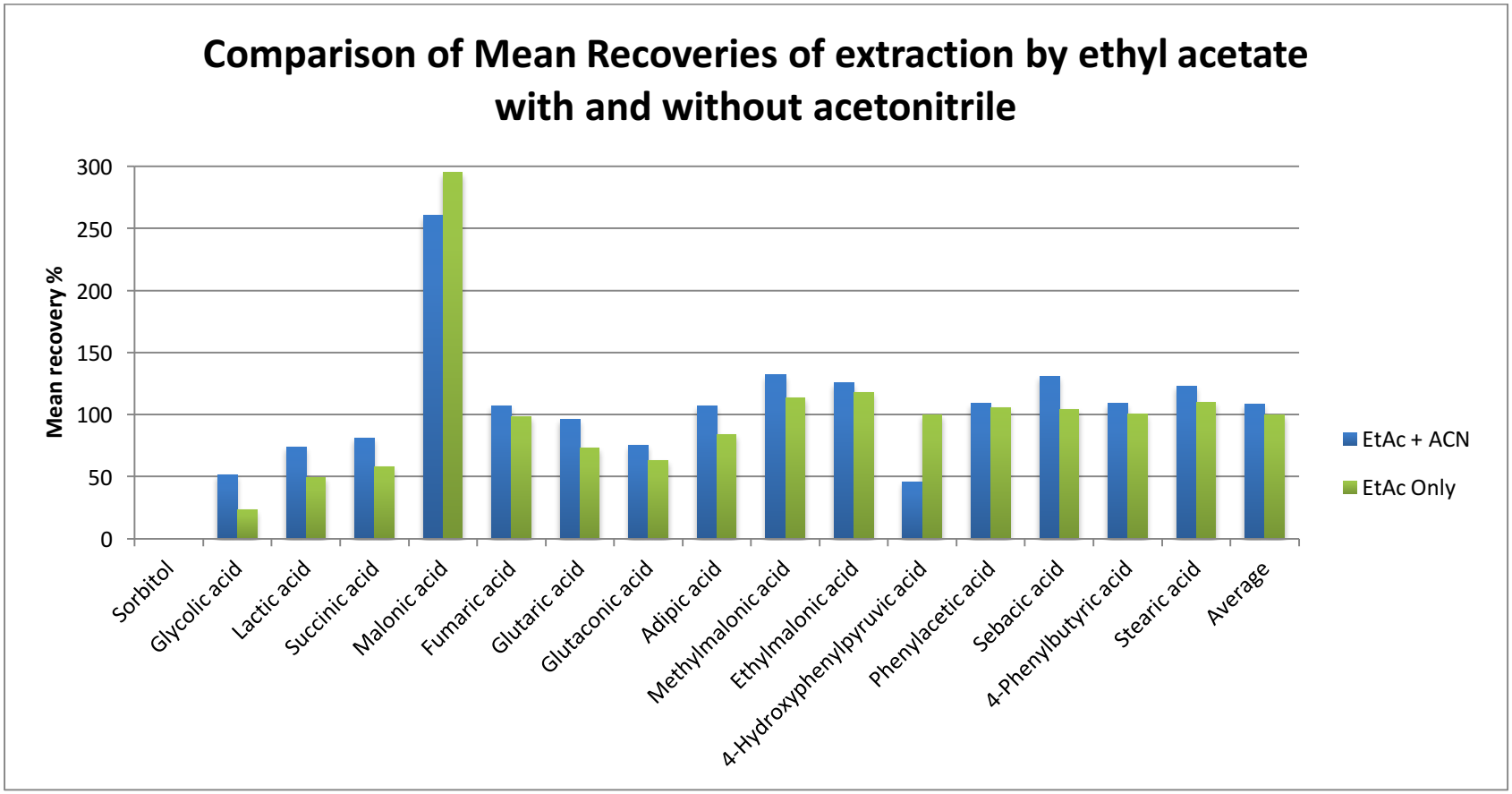


Figure 10.1 Comparison of extraction efficiencies between the ethyl acetate-only and acetonitrile/ethyl acetate miniaturised extraction method. The organic acids were arranged according to their polarity.

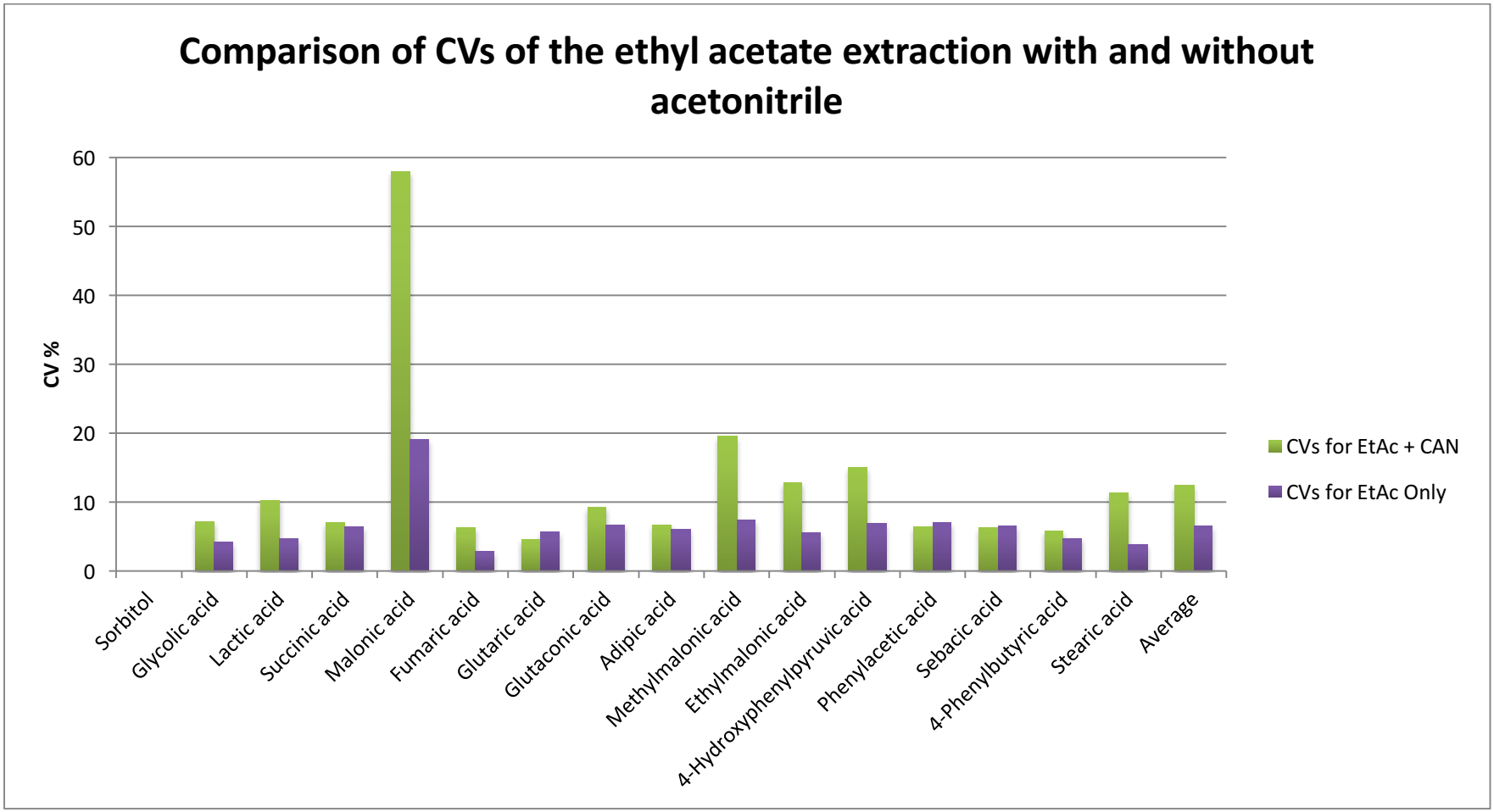


Figure 10.2 Comparison of the CVs for the two extractions done with ethyl acetate-only and ethyl acetate with acetonitrile