The extraction and chromatographic analysis of organic acids and inorganic anions found in milk and dairy produce.

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A Thesis submitted for the award of

Master of Science by Research

Waterford Institute of Technology

May 2018

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Acknowledgements

Firstly, I would like to express my sincere gratitude to my supervisors, Dr. Michael Breen, Dr. Mike Kinsella & Dr. Damian Connolly for their patience, time and guidance over the last few years and to Waterford Institute of Technology for their Scholarship - for without that, none of this research would have been possible. I'd also like to thank our extremely busy Instrumentation Specialist, Dr. Stephen Dowling for his immense knowledge of, chromatographic instrumentation. His support and guidance on technical issues is second to none (on a personal note, thanks for introducing me to Grimes pub and craft beer). Further to this I'd like to thank our Technicians: Aiden, Pat, Mari and Walter for all their help and chats, you all brighten the place immensely!

I'd like to thank my Gramma, Mother and Father for their support both emotional and financial. I've lost count of how many FaceTime sessions we've had where you've listened to me rant and rave about my experiments not working etc. Diolch o galon i chi gyd am bopeth da chi wedi wneud dros y blynyddoed i fi. Hêb teulu, hêb ddim.

A big shout out to all the postgrads and postdocs, old and new for also listening to me rant and rave in the lab and pub about issues from Brexit (down with that sort of thing) to rugby. Also thanks for the memories, we've had some good times and shared plenty of tasty beers, best of luck to you all in your endeavours.

A massive thank you to my partner, Kathy for supporting me throughout my postgraduate degree. I know it's not been easy looking after our baby boy on your own while I'm either at work, college or in my man cave writing this thesis, your continued support has been unwavering and I can never thank you enough.

And finally, in the words of Lady Galadriel, *"even the smallest person can change the course of the future"*, so following on from that, a big thanks to my baby boy, Logan, for smiling up at me every morning. That alone is motivation enough and certainly makes the world that much brighter – cariad mawr.

Iechyd da.

Abstract

Dispersive liquid-liquid microextraction (DLLME) is a ternary extraction system that consists of an extraction solvent, dispersing solvent and an aqueous sample containing the analyte of interest. DLLME is a powerful, miniaturised extraction technique that can increase an analytes concentration and achieve high enrichment factors (EFs) which makes it ideal for trace analysis. DLLME consists of injecting the binary mixture of extraction/dispersing solvent into the sample. This creates a very large surface area of fine extraction droplets. It is these droplets that analytes enrich, almost instantaneously into. Given the speed of the extraction, DLLME is a very attractive procedure that can increase sample throughput whilst lowering solvent consumption, waste and associated costs.

Several organic acids were identified as common constituents in milk and dairy produce. Organic acids appear in milk and dairy produce due to natural biochemical processes within the animal, bacterial activity, as preservatives and due to adulteration [1–3]. Levels of organic acids in milk and dairy produce vary drastically due to a number of factors such as: breed of animal, time of year, geography, diet, age, health, stage of lactation and starter culture type [4–9]. Organic acids are important in areas such as flavour studies, cheese ripening, human nutrition, monitoring the health of the animal and monitoring the quality of the product prior to sale to the consumer [10–12].

Milk and dairy produce consists of several components such as proteins, peptides, amino acids, carbohydrates, vitamins, minerals and lipids that require removal prior to analysis. These interferences were removed by mixing two solutions, carrez 1 (zinc acetate) and carrez 2 (potassium hexaferrocyanate) with the sample,

followed by centrifugation. This left the sample in an aqueous matrix, which is ideal for DLLME.

A number of chromatographic techniques were investigated during the course of the project, those being: high performance liquid chromatography (HPLC), gas chromatography (GC) and capillary zone electrophoresis (CE). The technique most appropriate to the use of DLLME and the separation of organic acids was found to be GC.

The optimised GC method consisted of injecting 1 µL of extraction solvent using a 10:1 split ratio then separating on a gradient method in 10 minutes using an Altech AT-100 polyethyleneglycol (PEG) column (15 m x 530 µm i.d. x 1.2 µm) . The method was validated for the analysis of six organic acids. Those acids were acetic, propionic, iso-butyric, n-butyric, iso-valeric and n-valeric acid. The method gave retention times of 8.79, 10.06, 10.67, 11.18, 11.68 and 12.49 minutes, respectively and %RSD of: < 0.06%. Peak area was also assessed and gave %RSD of < 0.0%. Coefficients of determination (R^2) were all ≥ 0.999. LODs for acetic, propionic, iso-butyric, n-butyric, iso-valeric and n-valeric acid were estimated to be: 21.88, 67.25, 8.04, 6.86, 39.38 and 21.68 µg/mL, respectively and LOQs were: 66.32, 203.79, 24.38, 20.82, 119.36 and 65.71 µg/mL, respectively.

The optimised extraction consisted of injecting a mixture of 100 µL chloroform (extraction solvent) and 700 µL acetone (dispersing solvent) into a 10-mL sample containing 20% w/v NaCl and pH adjusted to 2.50. This produced EFs up to ~ 45 times more concentrated than in the original sample. The optimised and validated method was then applied to real samples of milk and dairy produce, the following results were obtained: cow's milk: acetic: (NQ); n-butyric: 10.41 µg/mL.

Buttermilk: acetic: (NQ); n-butyric: 14.38 μg/mL; iso-valeric: 12.22 μg/mL; n-valeric: 12.78 μg/mL. Goat's milk: acetic acid (NQ); iso-butyric: 13.23 μg/mL; n-butyric: 16.46 μg/mL; iso-valeric: 13.12 μg/mL; n-valeric: 12.72 μg/mL. Cottage cheese: acetic acid (NQ); n-butyric: 4.04 μg/g. Brie cheese: acetic acid (NQ); n-butyric: 42.31 μg/g; iso-valeric: 0.39 μg/g; n-valeric: 0.72 μg/g. Probiotic yogurt: acetic acid (NQ); iso-butyric: 6.13 μg/g; n-butyric: 6.90 μg/g; iso-valeric: 5.91 μg/g; n-valeric: 6.06 μg/g. Greek yogurt: acetic acid (NQ); n-butyric: 11.00 μg/g.

Due to DLLME's ease of use, the quickness of the extraction procedure, large EFs, and the ability to carry out an extraction using readily available consumables within any laboratory (centrifuge tube, syringe and syringe needle), DLLME is the ideal extraction procedure for high throughput laboratories that are looking to minimise cost and labour while preserving the quality of results. Further to this, no method could be found at the time of writing that utilised DLLME to extract the highly volatile organic acids mentioned above from milk and dairy produce which lends to the novelty of this body of work.

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Index of abbreviations

AAS: Atomic absorption spectrophotometry

ACN: Acetonitrile

AU: Absorbance unit

BPA: Bisphenol A

BPB: Bisphenol B

CE: Capillary zone electrophoresis

CEC: capillary electrochromatography

CGE: Capillary gel electrophoresis

CIEF: Capillary isoelectric focusing

CITP: Chiral capillary isotachophoresis

CMC: Critical micelle concentration

CP: Cloud point

CP-DLLME: Cloud point – dispersive liquid-liquid microextraction

CPE: Cloud point extraction

CPSC: Cloud point sample clean-up

CTAB: Cetyltrimethylammonium borate

CTAH: Cetyltrimethylammonium hydroxide

CZE: Capillary zone electrophoresis

DAD: Diode array detector

DCM: Dichloromethane

DI: Deionised water

DLLME: Dispersive liquid-liquid microextraction

DLLME-GC-MS: Dispersive liquid-liquid microextraction-gas chromatography-mass spectrophotometry

EDTA: Ethylenediaminetetraacetic acid

EF: Enrichment factor

EOF: Electroosmotic flow

FIA: Flow injection analysis

FID: Flame ionisation detector

GC: Gas chromatography

GC-ECD: Gas chromatography-electron capture detector

GC-FID: Gas chromatography – flame ionisation detection

GC-MS: Gas chromatography-mass spectrophotometry

GC-NPD: Gas chromatography- nitrogen phosphorus detector

GC-QTOF-MS: Gas chromatography-quad time of flight-mass spectrophotometry

HETP: Height equivalent to a theoretical plate

HPLC: high performance liquid chromatography

HPLC-MS-MS: High performance liquid chromatography-mass spectrophotometry-mass spectrophotometry

HS: Head space

HS-GC: Head space- gas chromatography

IBCF: iso-butylchloroformate

IC: ion chromatography

ICH: International Conference on Harmonisation

IEC: Ion-exclusion chromatography

IP- Ion pair

IPA: Isopropyl alcohol

IP-DLLME: ion pair-dispersive liquid-liquid microextraction

IUPAC: International Union of Pure and Applied Chemistry

LC: liquid chromatography

LC-MS: Liquid chromatography-mass spectrophotometry

LLE: Liquid-liquid extraction

LOD: Limit of detection

LOQ: Limit of quantification

MAE: Microwave assisted extraction

MALDI-MS: Matrix assisted laser desorption ionisation-mass spectrophotometry

MALDI-TOF-MS: Matrix assisted laser desorption ionisation- time of flight-mass spectrophotometry

MASE: Microwave assisted solvent extraction

MEKC: Micellar electrokinetic chromatography

MeOH: Methanol

MS: Mass spectrophotometry

ND: Not detectable

NQ: Not quantifiable

PDAM: Pyrenyldiazomethane

PDC: Pyridinedicarboxylic acid

PEG: Polyethyleneglycol

PP: Pour-point

PTFE: Polytetrafluoroethylene

RP: Reversed phase

RP-LC: Reversed phase liquid chromatography

RSD: Relative standard deviation

SAX: Strong anion exchange

SAX-SPE: Strong anion exchange-solid phase extraction

SD: Standard deviation

SDL: Starter distillates

SLE: Solid-liquid extraction

SPE: Solid phase extraction

SPE-HPLC-MS: Solid phase extraction – high performance liquid chromatography-mass spectrophotometry

SPME: Solid phase microextraction

TOC: Total organic carbon

TX: Triton X surfactant

UAE: Ultrasound assisted extraction

UASE: Ultrasound assisted Soxhlet extraction

UHT: Ultra high temperature

UK: United Kingdom

UMP: Uridine monophosphate

UV: Ultraviolet

VWD: Variable wavelength detector

WCOT: Wall coated open tubular

TABLE OF CONTENTS

TABLE OF TABLES

TABLE OF FIGURES

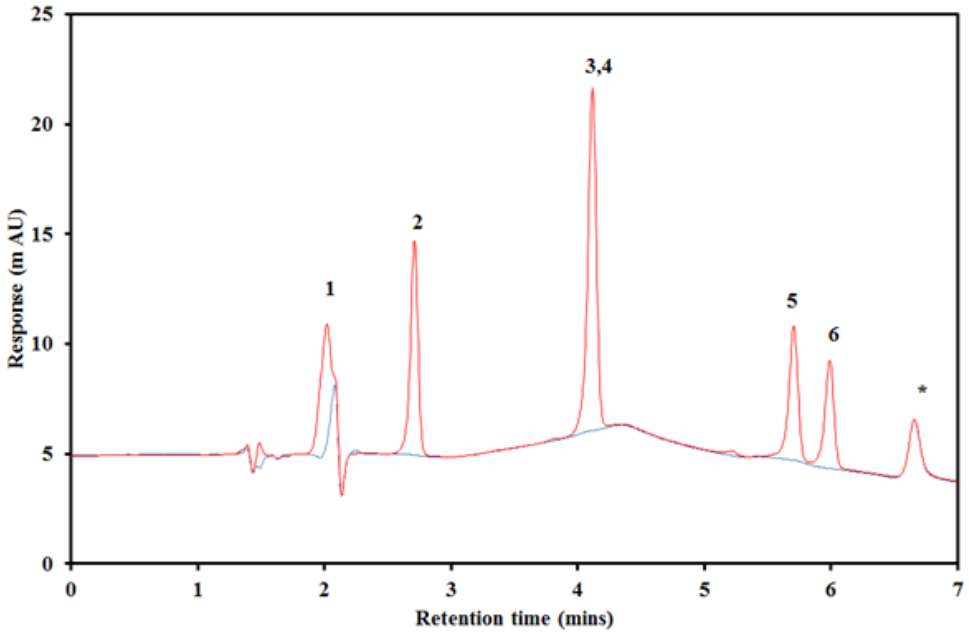


*Figure 4-10. Blue: blank (water), red: standard mix (0.25 mg/mL each in water). Peak I.D: 1: acetic acid; 2: propionic acid; 3/4: iso/n-butyric; 5: iso-valeric; 6: n-valeric acid; *: unknown. Acetic elutes on a system peak, iso/n-butyric could not be separated.....178*

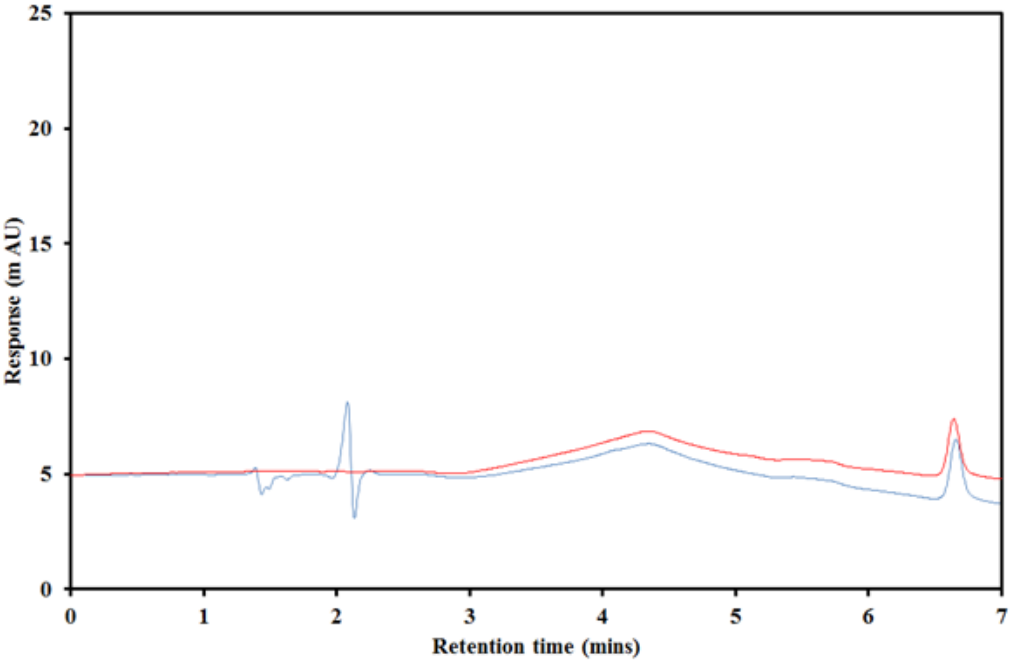
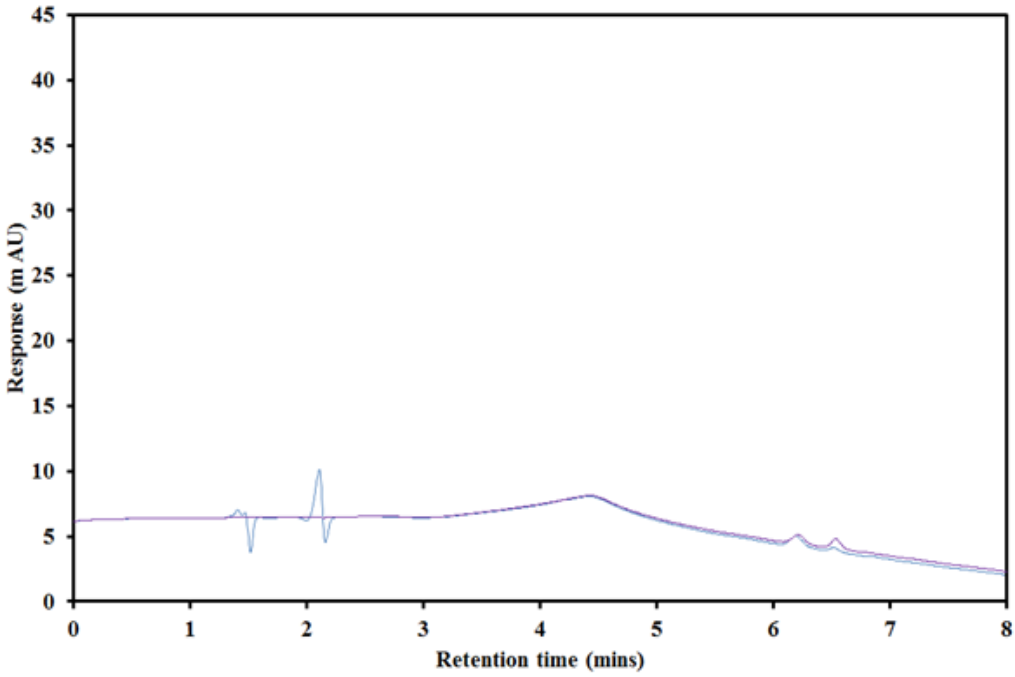


Figure 4-11. Blue: water blank, red: ghost run. Peaks could be



1. Introduction

1.1 Aim

The aim of this thesis was to identify the most common organic acids and/or inorganic anions found in milk and dairy produce. Find the most appropriate technique for the separation and detection of the identified organic acids and/or inorganic anions. Develop and validate a suite of methods capable of separating the identified organic acids and/or inorganic anions. Identify extraction techniques most suitable for the extraction of organic acids and/or inorganic anions from milk and dairy produce that comply with the principles of sustainable development and green chemistry [1]. Finally, develop and validate a method for the extraction of organic acids and/or inorganic anions from milk and dairy produce that is compatible with the mode of separation and apply it to real life samples of milk and dairy based produce.

1.2 Chromatographic objectives

Chromatographic objectives that must be met were to obtain a resolution of ≥ 1.5 between peaks of interest. The linear range shall cover 25, 50, 75, 100, 150 and 200 % of the expected analyte concentration, with an R^2 acceptance criteria of ≥ 0.99. The extraction should be accurate, demonstrating a recovery of between 90 – 110 % (unless sufficient justification can be made). Retention time precision was assessed via triplicate injections at three concentration levels (25, 100 and 200%, where 100% is the expected concentration obtained from literature sources [2–4]), results must be ≤ 2% RSD (unless sufficient justification can be made). Injector precision should be repeatable with ≤ 1% RSD (n=5). Limits of detection (LOD)

and limits of quantification (LOQ) estimations should have a signal to noise ratio of 3:1 & 10:1 respectively. Enrichment factors for the extraction must ≥1.

This section will establish the nature and chemistry of the sample matrix, with an emphasis on the role of organic acids in milk and dairy based produce. Following this will be a discussion of each organic acids' physiochemical properties, which will give some context and practical considerations on the most appropriate methods of separation and extraction. The more traditional extraction techniques available are liquid-liquid extraction (LLE), solid-liquid extraction (SLE) and distillation. A literature review will focus on the extraction of organic acids, with an emphasis on the more popular miniaturised extraction techniques such as, cloud point extraction (CP), solid phase extraction (SPE), solid phase microextraction (SPME) and dispersive liquid-liquid microextraction (DLLME). This identified a gap in the literature in the use of DLLME for the extraction of organic acids from milk and dairy produce.

One of the instruments best suited for the analysis of organic and inorganic anions, an ion chromatography system was not available. However, three powerful instrumental techniques remain in the analytical toolbox, those being: capillary electrophoresis (CE), high performance liquid chromatography (HPLC) and gas chromatography (GC); all of which have potential in the analysis of organic acids. CE is possibly the most powerful of the three as it can rapidly separate both organic acids and inorganic anions simultaneously, and with efficiency comparable to GC [5]. A fundamental understanding of these techniques has been assumed, therefore instrumental introductions in the relevant chapters will contain only a brief overview of the more important aspects studied, those being: manipulation of

selectivity, retention and resolution and the variables that affect them, such as: pH, temperature, buffer additives and stationary phase chemistry.

Foods and beverages are complex samples that contain several interferences such as proteins, lipids and carbohydrates that require removal prior to analysis. Sample preparation is often described as being the most important step in any analysis [6,7]. It is estimated that approximately 30% of analytical errors originate from the actual preparation of the sample, confirming the importance of a reliable and robust extraction [6,7].

There has been growing concern of late in relation to food safety and its effects on the human body [8]. These concerns are not only voiced by academics, governments and leaders of the food industry but also by an increasingly informed and curious consumer [8]. The modern consumer is conscious of the effects of food additives and their foods general composition such as protein, fat and carbohydrate concentrations [9].

In relation to food safety, numerous food and beverage scandals have been uncovered over the years that serve as reminders as to the need for quick, accurate and robust analytical assays [10–12]. Many chemicals such as vitamins, minerals, preservatives, sugars and antioxidants are added during the manufacturing process to stabilise the product, increase shelf life and improve its quality or taste. Unwanted chemicals may also find their way into the food chain; chemicals such allergens, mycotoxins, antibiotics and leachate from plastic food containers, all of which pose a significant risk to human and animal health. This puts extra pressure on laboratories regarding sample throughput and the cost of solvents and consumables. Due to the increase in demand for food analysis, there is clearly a

need for more rapid, robust, cheaper and ultimately greener extractions and analyses. As will be demonstrated, DLLME will prove to be quicker, cheaper and use far less organic solvent than any of the traditional, more established techniques.

1.3 Organic Acids

The organic acids introduced here were chosen as they were identified in the literature as common organic acids found in milk and dairy produce [2–4,13,14]; Table 1-1 shows the most commonly cited organic acids in milk serum and their average concentrations, with many of the short chain acids coming under the 'other' category. This section will discuss the properties and importance of the chosen organic acids. Some are used as additives to stabilise or increase the palatability of produce, while others are required for nutritional and biochemical processes [13,15–18]. Most of the organic acids studied during this research have been small, highly polar carboxylic acids ranging from one to five carbon atoms in length; except for orotic and hippuric acids which have an aromatic moiety and uric acid which is a purine. These smaller, aliphatic organic acids are often referred to in the literature as fatty acids, short chain fatty acids or volatile fatty acid (these terms are often used interchangeably).

At least four of the organic acids in question are not directly synthesised by our bodies and can only be gained through our diet with milk being one such source [19,20]. Milk is considered to be a good source as many of the acids are derived from both animal feed (silage, grain and pasture) and bovine biochemical synthesis [19]. The occurrence of most organic acids in dairy produce is from the metabolism of larger organic compounds such as lipids, proteins and carbohydrates [21,22]. However, some such as acetic and lactic acid are also added as preservatives.

Addition of such acids lowers the natural pH which hinders microbial growth [21,22].

Table 1-1. Quantity of common organic acids in milk serum; adapted from Walstra [23]

Organic acid	Average concentration (mg/kg)
Citrate	1600
Formate	40
Acetate	30
Lactate	20
Oxalate	20
Others	10

1.4 Properties of organic acids

Each organic acid has its own dissociation constant and logP value. Dissociation constants, otherwise known as pk_a values, describe the point at which 50% of a species is ionised. This can be manipulated to influence solubility during extractions; increasing the pH neutralises the acids and renders them less soluble in aqueous medium. Analyte ionisation is also manipulated in chromatographic separations; neutralised analytes have greater retention in reversed phase HPLC which can influence selectivity. These concepts will receive a more detailed discussion in the coming chapters.

LogP values describe the lipophilicity of a molecule. Positive integers refer to the hydrophobicity of a molecule, the larger the integer (and molecule) the more hydrophobic its character. However, analytes with small positive integers are

sparingly soluble in aqueous medium. Negative integers relate to the hydrophilicity of an analyte. They will be fully soluble in aqueous medium and sparingly soluble in organic solvent, depending on the logP value of that solvent. LogP values are also important when considering extraction protocols as these values can give an indication of whether an analyte will partition into the extraction solvent. These physiochemical properties have been summarised in *Table 1-2*.

Table 1-2. Table of physiochemical properties of the major organic acids evaluated.

Common name	Molar mass (g/mol)	LogP*	pk_a*
Lactic acid	90.0779	-0.47	3.73
Tartaric acid	150.086	-0.40	3.03, 4.37
Malic acid	96.9	-0.27	3.40, 5.10
n-butyric acid	88.11	0.79	4.82
Iso-butyric acid	88.11	1.02	4.60
n-valeric acid	102.13	1.37	4.81
Iso-valeric	102.13	1.21	4.78
Propanoic acid	74.08	1.21	4.87
Oxalic acid	90	-0.26	1.25, 3.67
Citric acid	294	-1.32	3.13, 4.76, 6.40
Pyruvic acid	88.06	0.07	2.39
Formic acid	46.03	-0.27	3.74
Acetic acid	60	-0.22	4.76
Succinic acid	118	-0.40	4.21, 5.72
Fumaric acid	116.07	-0.04	3.02, 4.38
Orotic acid	156.10	-1.23	2.40, 9.50, 13.00
Uric acid	168.11	-2.17	3.89, 5.40, 5.80, 11.30
Hippuric acid	179.17	0.23	3.59, 1.59

*LogP and pk_a values taken from [24]

1.4.1 *Lactic Acid*

Lactic acid is the common name for 2-hydroxypropanoic acid and is an important fuel source for the body. It is a chiral molecule, is a product of carbohydrate metabolism and a degradation product of lactose [22]. L-lactic acid forms through anaerobic glycolysis from its precursor, pyruvic acid. It is then catalysed by an enzyme, lactate dehydrogenase. D-lactic acid is not found in humans or most mammals and cannot be metabolised [25]. It has a logP value of -0.47, which means it has hydrophilic characteristics and its pk_a is 3.73.

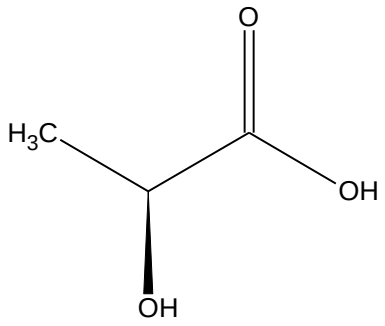


Figure 1-1. L-lactic acid

1.4.2 *Tartaric Acid*

L-tartaric acid is the common name for 2,3-dihydroxybutanedioic. It is found in nature while D and meso-tartaric acids are not found in nature, these are often used as food additives to enhance flavour [25]. It has a logP value of -0.40, which means it has hydrophilic characteristics, it has two pk_a values, 3.03 and 4.37.

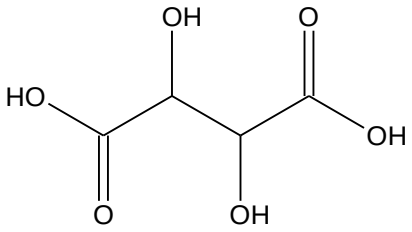


Figure 1-2. Tartaric acid

1.4.3 *Malic Acid*

Malic acid is the common name for 2-hydroxybutanedioic acid and comes in two forms D/L. Only L-malic is found in nature and often racemic mixtures of D & L are used as food additives to enhance flavour and as a preservative [25]. It has a logP value of -0.27, which means it has hydrophilic characteristics, it has two pk_a values, 3.40 and 5.10.

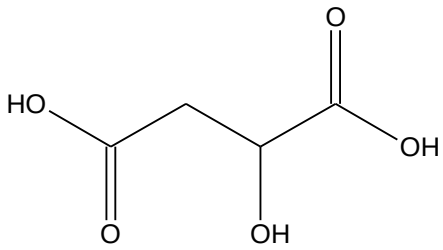


Figure 1-3. Malic acid

1.4.4 *Butyric Acid*

There are two forms of butyric acid studied throughout, those being iso-butyric (2-methylpropanoic acid) and n-butyric (butanoic acid). Both are volatile fatty acids that humans can not directly produce. Butyric (both forms) is a product of carbohydrate fermentation. Butyric, especially iso-butyric acid is a major energy

source for colonic epithelial cells, also known as colonocytes and play an important role in preventing disease and maintaining normal colon function [20,26,27]. As previously mentioned, one method that butyric enters our food chain is through dairy produce as silage. The silage that forms part of a cow's diet would naturally contain several volatile acids [28,29], as well as being produced through natural biochemical process. However, butyric acid is sometimes used as a food additive [30,31] and to enhance flavour [32]. n-butyric acid has a logP value of 0.79, which means it has hydrophobic characteristics, and has a pk_a value of 4.82. Iso-butyric acid has a logP value of 1.02 which means it has hydrophobic characteristics and a pk_a value of 4.60.

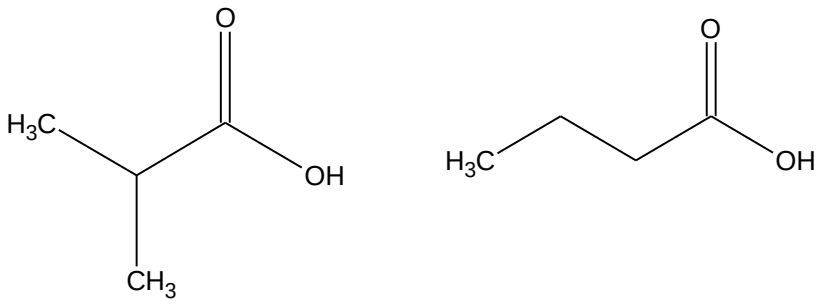


Figure 1-4. Left: iso-butyric acid, right: butyric acid

1.4.5 *Valeric Acid*

There are two isomers of valeric acid, iso-valeric (3-methylbutanoic acid) and n-valeric acid (pentanoic acid) and both are volatile. It is thought that valeric adds to the flavour of dairy produce and is also important for colonocyte health [32]. It is also an end product of carbohydrate fermentation and is not directly produced by the body [20]. n-valeric acid has a logP value of 1.37which means it has

hydrophobic characteristics and a pk_a value of 4.81; whereas Iso-valeric acid has a logP value of 1.21 which means it has hydrophobic characteristics and a pk_a value of 4.78.

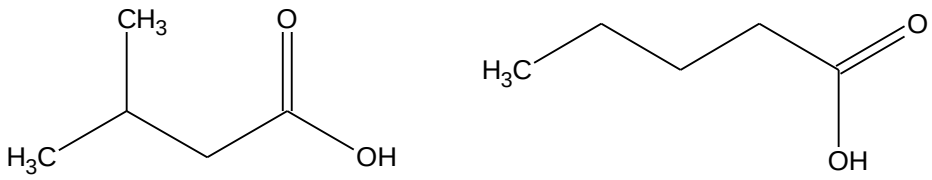


Figure 1-5. Left: iso-valeric acid; Right: valeric acid

1.4.6 *Propanoic Acid*

Propanoic acid is a product of bacterial growth in dairy produce [33]. It is also a product of carbohydrate fermentation by lactic acid bacteria and prevents spoilage in dairy products. Propanoic acid, along with butyric and acetic acid are thought to aid in a healthy colon [20]. Propanoic acid has a logP value of 1.21 which means it has hydrophobic characteristics and a pk_a value of 4.87.

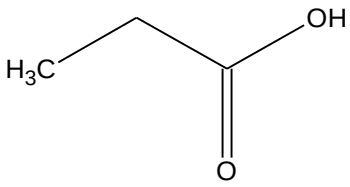


Figure 1-6. Propanoic acid

1.4.7 *Oxalic Acid*

Oxalic acid is the common name of ethanedioic acid and occurs in both insoluble and soluble form in foods. Research suggests that the water soluble form can hinder the absorption of milk calcium in the body [34]. It is also thought that the binding of calcium ions to oxalate can limit bacteriophage development in dairy and so addition can limit spoilage [35]. Oxalic acid has a logP value of -0.26 which means it has hydrophilic characteristics and two pk_a values 1.25 and 3.67.

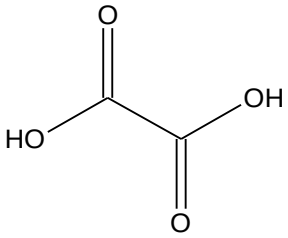


Figure 1-7. Oxalic acid

1.4.8 *Citric Acid*

The citric acid used came in the form of trisodiumcitrate-dihydrate (trisodium 2-hydroxypropane -1, 2, 3-tricarboxylic acid). Citric acid is present in milk in its ionised form, citrate and is the most predominant organic acid found in milk [22,23]. Citric acid degrades readily whilst in storage and many of the acids present are degradation products due to its hydrolysis, this is also true of lactose and lipids in dairy produce [22,23]. Citric acid has a logP value of -1.32 which means it has hydrophilic characteristics and three pk_a values, 3.13, 4.76 and 6.40.

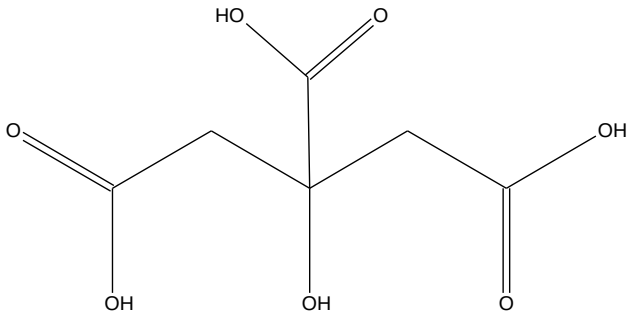


Figure 1-8. Citric acid

1.4.9 *Pyruvic Acid*

Pyruvic acid is the common name of 2-oxopropanoic acid and is an important metabolic intermediate in the citric acid cycle. Mammals lack a means of synthesising glucose from acetyl co-enzyme A and acetoacetyl co-enzyme A, however, pyruvate can be converted into phosphoenol-pyruvate and then into glucose [36,37]. Pyruvate can be obtained from the diet (cow's milk) or from de-aminated amino acids from metabolic processes within the body [36,37]. Pyruvic acid can be used as a marker to determine milk quality as it is not destroyed during pasteurization. It has a logP value of 0.07 which means it has more hydrophobic characteristics and a pk_a value of 2.39.

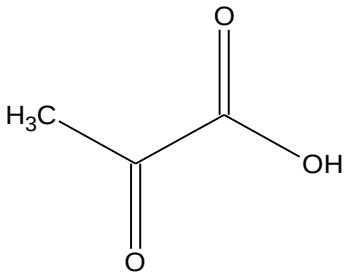


Figure 1-9. Pyruvic acid

1.4.10 *Formic Acid*

Formic acid is the common name for methanoic acid. It is one of the six volatile acids studied and is present in dairy as it is a degradation product of lactose. It is also added as an acidulant to control microbial growth by lowering the natural pH, thus acidifying the product. It also contributes to the flavour of milk [21,33]. Formic acid has a logP value of -0.27 which means it has more hydrophilic characteristics and a pk_a value of 3.74.

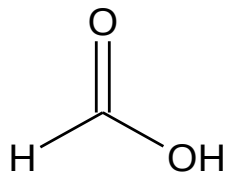


Figure 1-10. Formic acid

1.4.11 *Acetic Acid*

Acetic acid is the common name for ethanoic acid. It is also volatile and a degradation product of lactose. Acetic is used as an antimicrobial additive but is also believed to add to the flavour of dairy [22,33,38]. Acetic acid has a logP value of -0.22 which means it has more hydrophilic characteristics and a pk_a value of 4.76.

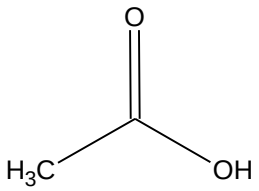


Figure 1-11. Acetic acid

1.4.12 *Succinic Acid*

Succinic acid is the common name for butanedioic acid and occurs in fermented dairy mainly due to the metabolic activity of the starter cultures used. Given this it can vary drastically depending on the type of starter culture [22,39]. Succinic acid has a logP value of -0.40 which means it has more hydrophilic characteristics; it has two pk_a values, 4.21 and 5.72.

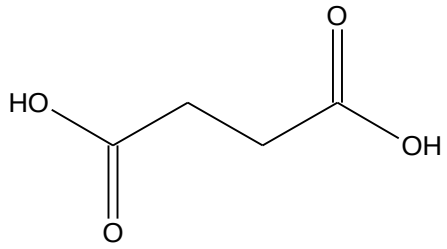


Figure 1-12.Succinic acid

1.4.13 *Fumaric Acid*

Fumaric acid is the common name for butenedioic acid and is also an important intermediate involved in the citric acid cycle and thus fuel for the body and occurs due to the breakdown of amino acids [40]. Fumaric acid has a logP value of -0.04 which means it has more hydrophilic characteristics and two pka values, 3.02 and 4.38.

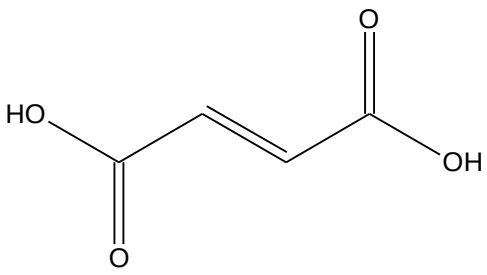


Figure 1-13. Fumaric acid

1.4.14 *Orotic Acid*

Orotic acid is the common name for 2, 4-dioxo-1H-pyrimidine-6-carboxylic acid. Orotic is an important molecule involved in nucleotide synthesis. It's major end product is uridine monophosphate, abbreviated to UMP [41]. It occurs in dairy as a result of normal bovine metabolic processes. It is used as a marker for bacterial activity and flavour studies and has nutritional significance [42]. The main source of orotic acid in the human diet is from milk [43]. Orotic acid has a logP value of -1.23 which means it has more hydrophilic characteristics; it has three pk_a values, 2.40, 9.50 and 13.00.

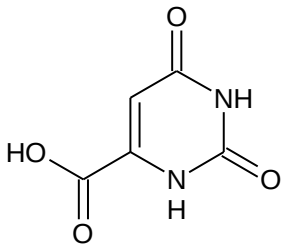


Figure 1-14. Orotic acid

1.4.15 *Uric Acid*

Uric acid is the common name for 7, 9-dihydro-1H-purine-2, 6, 8 (3H)-trione. It is a purine that has four ionisable hydrogens at positions 1, 3, 5 & 7 and thus, four ionisation constants. Uric acid is a purine degradation product that is insoluble in water and is a result of normal bovine metabolic processes. Like orotic, it is used in flavour studies and to monitor bacterial activity [42,44]. Uric acid has a logP value of -2.17 which means it has more hydrophilic characteristics. Uric acid also has four pk_a values, 3.89, 5.40, 5.80 and 11.30.

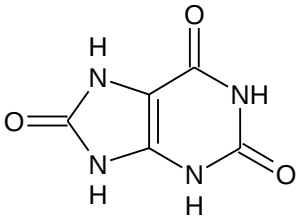


Figure 1-15. Uric acid

1.4.16 *Hippuric Acid*

Hippuric acid is the common name for benzoylaminoethanoic acid and appears as the non-protein nitrogen fraction of milk [45]. It is a common excretion product formed from benzoic acid and glycine that originates from odd chain fatty acids [46] and can be used to predict the presence of naturally occurring benzoic acid in milk that originate from plant based feeds [47]. Hippuric acid has a logP value of 0.23 which means it has more hydrophobic characteristics and two pk_a values, 1.59 and 3.59.

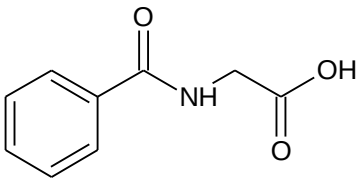


Figure 1-16. Hippuric acid

1.5 Milk and Fermented Dairy Produce

Milk is a complex biological fluid that consists of fat globules, casein micelles, leukocytes and lipoproteins held within an aqueous serum [23]. It is estimated that the average concentration of organic acids contained in bovine milk is 0.17% w/w

and that they are present in the serum, which is the aqueous portion of milk [23]. Though it should be noted that this figure can change drastically depending on physiological factors (breed of cow, age, stage of lactation and mastitis) as well as seasonal and husbandry factors [48]. A number of authors conclude that seasonal factors such as moving the cow from winter stall feeding (silage, grain, soy and corn fortified with vitamins and minerals) to pasture in spring and summer change not only the colour of milk but also its composition with a reduction in fat content in the summer [48–50]. Similar reports on seasonal changes to the concentrations of organic acids in milk (except for butyric acid) could not be found. Butler *et al.* [51] studied the effects of seasonal change and management systems, also known as husbandry factors (organic versus conventional farming on short, medium and long chain saturated and unsaturated fatty acids) [51]. The four short chain fatty acids studied were: C4, C6, C8 and C10, with C4 (butyric acid) being the only relevant fatty acid to this work [51]. The group found that fatty acid profiles were lower in the winter than in the summer, but could be increased by addition of, for example, oil seed to reduce seasonal differences at winter [51]. Husbandry factors that affect milk composition are therefore: feed nutrition, dietary supplements, organic farming and conventional farming. These affect the quality and composition of the diet having effects upon fatty acids, protein, lactose, mineral and citrate levels [23,48,51]. The composition of bovine milk has been summarised in *Table 1-3*. As can be seen the largest component is water, at ~ 87 % while the total of all organic acids is ~0.17 %. Fat content is ~ 3 % and protein & casein at 3.25 and 2.6, these components will require removal prior to extraction and analysis.

Table 1-3. Approximate Composition of Milk from Lowland Breeds. Adapted from Walstra et al. [8]

Component	Average content in milk (% w/w)	Range (% w/w)
Water	87.1	85.3-88.7
Non-fat solids	8.9	7.9-10.0
Fat in dry matter	31	22-38
Lactose	4.6	3.8-5.3
Fat	4.0	2.5-5.5
Protein	3.25	2.3-44
Casein	2.6	1.7-3.5
Mineral substances	0.7	0.57-0.83
Organic acids	0.17	0.12-0.21
Miscellaneous	0.15	N/A

Fermented dairy produce include items such as cheese, yogurt, buttermilk and kefir. They are typically made from pasteurised milks that have been treated with some strain of lactic acid bacteria [50]. There are also several non-bovine or dairy-free milk products on the market such as, goat's milk and cheeses, soy milk, coconut milk, hemp milk and almond milk.

1.6 Sample Pre-treatment

Sample pre-treatment depends on the physical nature of the sample; gaseous, liquid or solid, biological or environmental. Further considerations are the manner of sample storage or any derivatisation requirements.

Prior to the extraction of analytes from complex matrices, there is often need for a sample pre-treatment step. This serves several purposes, such as to remove unwanted interferences such as proteins and lipids, and to render the sample in a form that is more compatible with the method of extraction and separation.

To extract the organic acids that are contained within an aqueous biological sample matrix such as milk, semi-solids such as yogurts and solid samples such as cheeses one must first homogenise the sample. This can be achieved via mixing the sample with distilled water, solvent, acid or base and either passing it through a food processor or by using a stomacher. Cheeses must first be grated prior to homogenisation. This extract can then be filtered or centrifuged to aid in removal of interferences such as lipids and proteins from the sample matrix.

1.6.1 *Protein precipitation and lipid removal.*

Proteins and lipids can be precipitated with metals, acids and organic solvents. Each mode of precipitation produces different interactions on the proteins. Organic solvents typically used are ACN and MeOH, usually in a 3:1 ratio of solvent to sample [52]. These solvents lower the dielectric constant of the matrix, which increases the attraction between charged protein molecules. The solvent removes hydrated water molecules resulting in the aggregation of proteins via hydrophobic interactions. This action minimises interactions with the solvent, ultimately resulting in precipitation of the protein fraction [52]; the target analytes will then reside in a solvent matrix.

Adding acids lowers the pH, deprotonated acids bind to form insoluble salts with the proteins [52]; target analytes will then reside in an aqueous matrix. Addition of metals alters the isoelectric point of the protein creating an insoluble complex [52];

target analytes will again reside in an aqueous matrix. Often acids are used in conjunction with metals to maximise the effect of protein precipitation, one such technique is the carrez method [53]. The carrez method incorporates the addition of two solutions to the sample, one solution consists of potassium hexaferrocyanate and the other, zinc sulphate. The carrez method will be discussed in detail in **chapter 5**. The target analytes will again reside in an aqueous matrix.

More novel approaches to protein precipitation are the use of surfactants in a technique called cloud point sample clean up (CPSC) [54]. This technique exploits surfactant properties; heating surfactants causes a phase separation that removes the proteins [54]. Surfactant properties are fully described in the cloud point extraction section of this introduction.

1.6.2 *QuEChERS method*

The QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) method is a relatively new sample clean technique that was developed by Anastassiades *et al* in 2004 [55]. The technique is typically used for the clean-up of food samples for the analysis of pesticides [56,57]. The sample is mixed with an organic solvent such as ACN and anhydrous MgSO4 and NaCl with the aim of promoting the partitioning of the aqueous phase from the solvent by hydrating the sample. This is followed by centrifugation, the analytes are then extracted from the organic solvent phase. Following this, analytes are typically extracted from the solvent using SPE [56] though as will be demonstrated in **Chapter 2**, QuEChERS is becoming popular as a pre-sample clean-up technique in DLLME where researchers have used the clean, analyte rich solvent obtained from the QuEChERS as DLLME solvents [58–62].

1.7 Traditional Extraction Techniques

The aim of any extraction technique is to isolate, purify and enrich the target compound/s by removing the analyte/s from the surrounding sample matrix. The compound/s must be isolated for several reasons, those being: removal of particles too large to pass through the analysis system, removal of compounds that have potential to precipitate out in the analytical system, remove unwanted compounds that may cause side reactions further on, remove unwanted compounds to produce a less convoluted chromatogram and to remove compounds that have the potential to co-elute with the target analyte/s. This action increases the signal to noise ratio, thus lowering limits of detection (LOD) and limits of quantification (LOQ), improve recoveries and repeatability of retention times giving a more robust analysis. There are three main extraction/purification techniques, those are liquid-liquid extraction (LLE), solid-liquid extraction (SLE) and distillation.

1.7.1 *Liquid-liquid extraction (LLE)*

One of the most common extraction techniques is liquid-liquid extraction (LLE), also known as solvent extraction. It has been used to extract a large number of compounds from a variety of matrices such as, the extraction of lipids from tissue [63] and isoflavones from foods [64] as well as inorganic species from foods [65].

LLE is a relatively simple extraction procedure that separates based on an analytes relative solubility between two immiscible liquids. One liquid carrying the compound of interest - usually water - is mixed with an immiscible organic solvent in a separating funnel. Mixing the liquids forms a dispersion of droplets that the analytes are extracted into. Two distinct phases are formed with the higher density solvent at the bottom. The phase containing the analyte is removed and kept. The

remaining phase is repeatedly washed to maximise mass transfer of all analytes from one phase to the other has been achieved. Mass transfer of analytes from the aqueous phase (C_{aq}) to the organic phase (C_{org}) is given by the partition coefficient (k_{α}), where acceptable values ≥ 1 and is gauged via addition of a known quantity of a pure standard of the same target analyte(s). The remaining, analyte rich solvent can then be evaporated, which enriches the product.

$$K_{\alpha} = \frac{C_{org}}{C_{aq}}$$

Equation 1-1. Partition coefficient

Advantages of LLE are:

- Extremely cheap apparatus.
- Operational simplicity.
- Efficient.
- Extract compatible with most instrumentation.
- Enriches analytes.

Disadvantages of LLE are:

- Time: repeated washing, waiting for complete phase separations and collection is time consuming and labour intensive.
- Toxic solvents pose risks to human, animal and environmental health.
- Large volumes of solvent used is costly.
- Large volumes of waste solvent generated; disposal is costly.
- Formation of emulsions.

1.7.2 *Solid-liquid extraction (SLE)*

Franz Soxhlet invented and described the first use of Soxhlet extraction for the extraction of lipids from dairy products in 1879 [66,67]. This is a very popular extraction technique and is used to extract from solids into a liquid phase. The basic principles involve continuously washing of the solid sample with a suitable hot solvent in a closed system. The sample is held in a porous thimble inside a glass Soxhlet extraction chamber. The chamber is connected to a heated round bottom flask containing a solvent. An extraction solvent is chosen based on the analytes solubility and the interferences insolubility. Heated vapour travel up into the condenser where it cools and falls into the extraction chamber. This action fills the chamber holding the sample with hot solvent, washing the analyte from the sample into the solvent. Once the chamber is filled it empties back to the round bottom flask, through the siphoning arm, taking the extracted analyte with it; this is counted as one complete cycle. Numerous cycles, sometimes over several hours, ensure maximum mass transfer from sample to solvent to flask.

The contents of the round bottom flask are then evaporated leaving the analytes in the form of a residue that can be re-constituted in a small amount of solvent thus enriching and increasing the analytes final concentration.

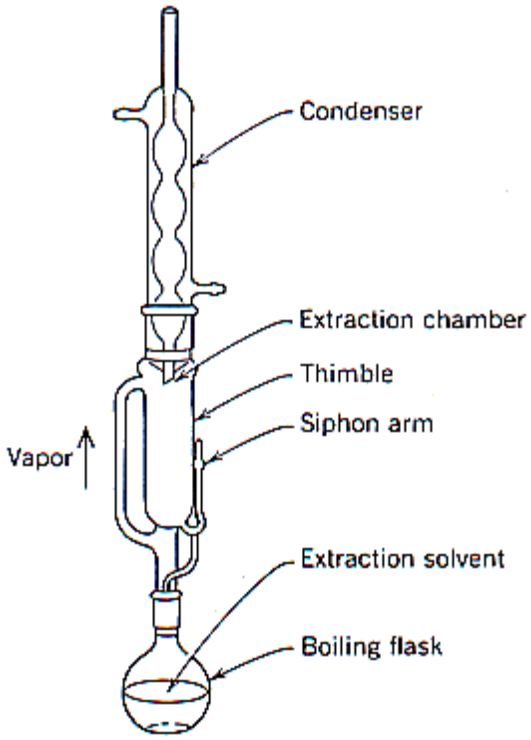


Figure 1-17. Schematic of Soxhlet extraction apparatus [68] as described above.

Advantages of Soxhlet extractions are:

- Less organic solvent than LLE
- Less labour intensive than LLE: the extraction can be left unattended, freeing up analyst time
- Fully automated extraction units known as Soxtec extraction units
- Enriches product

Disadvantages of Soxhlet extraction are:

- Cumbersome, expensive and delicate glassware
- Large volumes of flammable toxic, organic solvent
- Time: extractions can take several hours
- Sample loss through poorly sealed joints and solvent transfer
- Hot solvents are dangerous

Examples of solid-liquid extractions are: the extraction of lipids from dairy [66], chlorinated biphenyls from soil [69] and atmospheric polycyclic hydrocarbon particles [70].

These techniques have been modified over the years to address critical factors such as time, solvent use and recovery. Variations of Soxhlet extractions are: microwave assisted extraction (MAE), and ultrasound assisted extraction (UAE) and UAE combined with Soxhlet (UASE) [71].

1.7.3 *Microwave assisted solvent extraction (MASE)*

Microwave assisted solvent extraction (MASE), also known as microwave assisted extraction (MAE) was first experimented with in the 1980's using commercially available microwaves [72,73]. The basic principle of MAE is that non-ionising microwave radiation causes dipole rotation of analyte and solvent molecules resulting in movement from the solid sample into the solvent. The microwaves heat the entirety of the solvent at once resulting in the solvent rapidly reaching its boiling point which leads to much shorter extraction times and far less solvent usage [72]. MAE has been used for extraction from environmental matrices [72], food matrices [74] and natural product extractions [75].

1.7.4 *Ultrasound assisted solvent extraction (UAE)*

Ultrasound assisted solvent extraction (UAE) is a relatively simple technique that involves placing the solid sample in solvent and subjecting it to ultrasonication, which are very fast, high frequency pulses. UAE has seen applications in food analysis [76].

1.7.5 *Ultrasound assisted Soxhlet extraction (UASE)*

Ultrasound assisted Soxhlet extraction (UASE) combines ultrasound with Soxhlet. The Soxhlet chamber is placed in a modified ultrasound bath and ultrasound waves are applied to dramatically speed up the analysis. The first paper describing this by Luque-Garcia [77] and was used to extract fat from nuts. An example diagram of the modified apparatus is below and taken from the same paper [77].

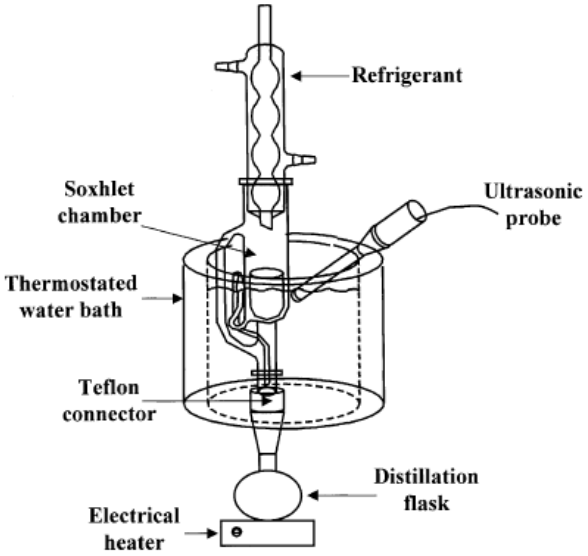


Figure 1-18. Luque-Garcia's modified UASE apparatus

1.7.6 *Distillation*

Distillation separates solvents based on boiling point. The most well-known is possibly the fractional distillation of hydrocarbons to produce a variety of fuel sources from crude oil. Though it has been used vastly in several industries ranging from petrochemical to pharmaceutical [78]. Examples of analyses are the determination of the rancidity of foods [79], mercury in environmental samples [80] and ethanol volume [81].

Figure 1-19. Distillation apparatus.

Advantages of distillation are: ability to purify complex mixtures, less labour intensive than LLE, enriches the final product and automation.

Disadvantages of distillation are: time consuming, still requires large volumes of solvents, and may require further purification, cumbersome and expensive glassware, flammable vapours and the use of toxic solvent.

1.8 Extraction of Organic Acids

Organic acids are a diverse group of organic compounds that contain one or more carboxylic acid (COOH) functional group. They appear in a wide variety of sample matrices including: foods and beverages. An ideal sample preparation technique will remove interferences, concentrate the analytes of interest and render analytes in a form that is compatible with the technique of choice. Further to this, analysts are now required to comply with the principles of sustainable development and green chemistry [1]. These issues have been addressed with the miniaturising of popular techniques, thereby reducing and/or eliminating the use of toxic solvents which will be discussed throughout this section.

There are several popular miniaturised extraction techniques available for the extraction of organic acids, those being:

- Dispersive liquid-liquid microextraction (DLLME)
- Solid phase extraction (SPE)
- Solid phase microextraction (SPME)
- Cloud point extraction (CP)

The following chapters will all involve the use of DLLME for the extraction of organic acids from milk and dairy produce. Given this there is a dedicated chapter devoted to the theory and method development of DLLME, a literature review describing the uses of DLLME as well as preliminary investigations and results in the use of DLLME.

1.9 Solid Phase Extraction (SPE)

Solid phase extraction (SPE), is an extraction and preconcentration technique designed for the extraction of a wide variety of analytes, including organic acids from a range of matrices. SPE involves the passing of a solubilised, aqueous sample through a cartridge containing a solid phase sorbent. Analytes from solution adsorb onto the sorbent while interferences pass through. This simultaneously concentrates and purifies the sample. Ideally, the solvent should be compatible with the analytical instrumentation used to separate the compounds. Most organic solvents used in GC can be used as extraction eluents due to their volatility. Most organic solvents used as eluents are also compatible with reversed phase HPLC using non-polar C_{18} column, but not with the resin based ion exclusion columns. Most organic solvents, with the exception of ACN and MeOH, are not compatible

with IEC columns as they swell and distort the resin resulting in a fouled column. In this instance, the analytes can be back-extracted to an aqueous medium.

Prior to the use of any SPE, the cartridge sorbent must be primed. The steps are termed, condition, load, wash and elute. The process begins with the conditioning of the sorbent. Its purpose is to wet the sorbent, solvate the functional groups and remove trapped air and residuals from the manufacturing process. The sample can then be loaded. Analytes will bind to the functional groups, while some of the interferences will be held, unbound in the sorbent. Interferences can then be washed off by passing a solvent through that is similar to the matrix, if the matrix is aqueous, then water can be used. Analytes can then be removed with an eluting solvent that is powerful enough to disrupt the bond.

Due to the wide variety of SPE bonded phases a variety of analytes can be extracted and enriched. Due to the nature of the solvents used, SPE is compatible with most analytical instrumentation. SPE is capable of extracting metals from earth [82], pharmaceuticals [82], antibacterial agents in wastewater [82], pesticides in plants and water [82] as well as for the extraction of organic acids from, foods and drinks [83–87], plant matter [88,89] and biological fluids [90].

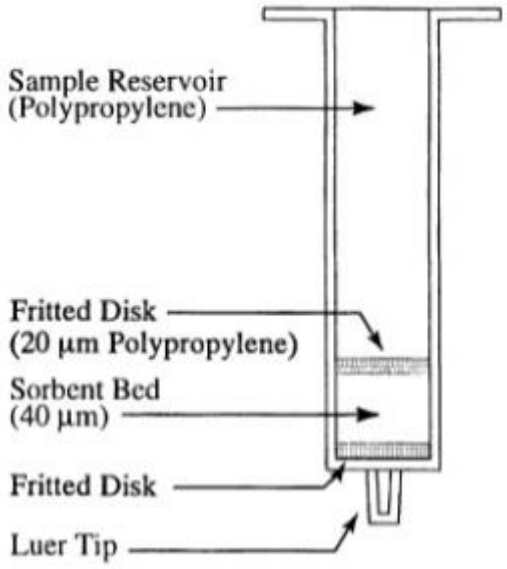


Figure 1-20. Schematic of an SPE cartridge [91]. Solvents are held in the reservoir; pressure is applied and the solvent is drawn through the sorbent bed where specific chemistries retain either the sample (retentive SPE) or the interferences (non-retentive SPE).

1.9.1 *Brief History and Notable Developments*

Solid phase extraction was first developed in the late 1970's to overcome some of the difficulties associated with LLE. Advantages of SPE over LLE are reduced consumption of toxic organic solvents, which lead to reduced cost in regards to solvents purchased and solvent disposal. Further to these, SPE is less labour intensive and more environmentally friendly than LLE due to less solvent usage [91,92].

1.9.2 *Modes of SPE*

Several retention mechanisms are available, those being: non-retentive, reversed phase, normal phase and ion-exchange. Non-retentive SPE can be used to trap interferences on the column, allowing the analyte(s) to pass straight through.

Reversed phase SPE is analogous to that of RP-LC whereby the support is functionalised with a hydrocarbon, typically C_{18}. This is often chosen when using aqueous samples. Retention is based on hydrophobic interactions such as non-polar Van der Waals dispersion forces. Analytes can be selectively desorbed using a variety of organic solvents. Normal phase is usually chosen when removing polar analytes from organic solvents. Retention is based on polar interactions (such as dipole-dipole, induced dipole, pi-pi and hydrogen bonding). Typical phases include aminopropyl and cyanopropyl. Elution is a function of eluotropic strength (solvent polarity).

The most commonly used mode of SPE for the analysis of organic acids is anion exchange, though larger, less polar organic acids (typically with more than seven carbons) can be retained on a C_{18} cartridge [84]. This type of extraction process involves the introduction of a polar, aqueous sample of ionised acids to a sorbent functionalised with basic species such as ternary amines. Retention is based on ionic interaction between the cationic solid phase and anionic analytes and can be controlled through manipulation of pH. Elution of analytes can be achieved by either increasing ionic strength thus competition for the solid phase, altering the pH – addition of excess protons neutralises the analyte or sorbent, or using an elution solvent containing a highly-charged anion; many methods described below employ the use of acids in conjunction with more polar solvents such as MeOH to greater effect.

1.9.3 *Literature Review on the Extraction of Organic Acids via SPE*

The presence of antibiotic residues in milk can cause several problems such as causing allergic reactions in hypersensitive people and inhibiting starting cultures

in producing milk products such as cheeses and yogurts [93]. Given this, Bruno *et al.* [93] developed an SPE protocol followed by LC-MS for the detection of β-lactams in milk. The group used a carboprep SPE cartridge that was conditioned with dilute HCl and water. Following the passing of the milk sample, the cartridges was washed with water and MeOH. Analytes were eluted with a dichloromethane/MeOH/formic acid mixture. Given that formic is an analyte, this method would not be appropriate.

Moors *et al.* [84] compared the use of SAX and C_{18} SPE sorbents for the extraction of the food preservatives, sorbic acid and benzoic acid and artificial sweeteners, aspartame and saccharin from foods. The SAX sorbent functionalised with quaternary amine ligands was conditioned with MeOH and water prior to loading of sample. The sample was then washed with aliquots of water. The authors found all but aspartame was retained and so the first washing aliquot containing aspartame was collected and passed through a C_{18} cartridge. The retained analytes were then removed with MeOH and sulphuric acid. The eluate was neutralised prior to chromatographic analysis via HPLC. The C_{18} cartridge was conditioned with MeOH modified with 1% phosphoric acid prior to the addition of the aspartame aliquot from the SAX cartridge. The sample was not washed and was eluted with MeOH. The authors found that due to the hydrophobic nature of the analytes, all could be separated on the C_{18} cartridge, and all but aspartame on the quaternary amine. They reported recoveries between 71 – 102% for the C_{18} and 96 – 99% on the SAX cartridge.

Perfluorinated organic acids are thought to lead to developmental, reproductive and systemic toxicity [94,95] which led to Kuklenyik *et al.* developing an automated SPE HPLC-MS protocol to measure their level in human serum and milk. Their

method gave LODs as low as 1 ng/mL. The conditioning of their Oasis HLP SPE cartridge consisted of passing MeOH and formic acid through the column to activate the ligands, followed by sample loading, washing with formic acid, a formic/MeOH mix and an ammonium hydroxide/water mix. Analytes were eluted with an ammonium/ACN mix prior to analysis [94].

Huopalahti *et al.* [88] used SPE for the extraction of anthocyanin and organic acids. Anthocyanin is responsible for some of the colours seen in fruits and flowers, it produces red, blue and purple hues, while the organic acids contribute to the flavour, especially the more volatile species. Anthocyanin was isolated on a non-polar C_{18} cartridge. Organic acids were retained on a SAX column that was primed with a KH_2PO_4 buffer solution prior to loading of sample. Organic acids were eluted with dilute sulphuric acid; both fractions were then chromatographically separated using HPLC. No LODs, recoveries or EFs were given as analytes were quantified using titration.

Verhaeghe *et al.* [96] developed a SPE method followed by the GC-MS analysis of urinary organic acids using a SAX SPE column. The SAX cartridge was activated using methanol followed by deionised water and 1 M acetic acid. Neutral and basic compounds were removed by washing with water. Acids were then eluted using n-butanol/formic acid/concentrated sulphuric acid (80/20/0.5), ethyl acetate/formic/sulphuric (80/20/0.5) and MeOH. Recoveries of the acids ranged from 34 – 109.9%.

Cherchi *et al.* [87] extracted organic acids from honey using a SAX cartridge and analysed the fraction using HPLC. The cartridge was conditioned with 1 M NaOH and washed with water and acetic acid prior to sample loading. The sample was

washed through with water and the cartridge left to dry. Acids were eluted using dilute sulphuric acid and directly injected into the LC system.

Schwnninger *et al.* [85] developed a SPE fractionation protocol for the analysis of organic acids produced by yeast that is thought to aid in tackling spoilage in dairy produce. A 10 g, C_{18} cartridge was conditioned with ACN and water. Pre-treated sample was passed through and washed with 5% ACN and eluted with 95% ACN. The group then used gel filtration to further clean up the sample.

As can be seen, a variety of bonded phases have been used. An even wider variety of solvents and solvent mixtures have been used to condition the bonded phase in the SPE cartridge, wash off non-retained interferences and then elute, sometimes selectively, the analytes under investigation.

1.10 Solid Phase Microextraction (SPME)

Solid phase microextraction (SPME) is a powerful extraction and preconcentration technique capable of high enrichment factors. SPME was designed specifically for automated use on GC systems. It consists of a fibre that adsorbs analytes from either gaseous or aqueous matrices then, often selectively, desorbs analytes into the GC system and onto the head of the column.

SPME consists of a hollow stainless steel needle that houses the solid phase fibre. The hollow needle is used to pierce the vial and the fibre is then extended beyond the tip and exposed to the sample matrix which can be a liquid or a gas. Samples then adsorb to the fibre until an equilibrium is reached. The fibre is then retracted back into the steel needle and introduced to the septum of an injector. The needle is again extended to allow the analytes to desorb in the injector. Analytes may then be focused using cold trapping or temperature programing at the head of the

column. Since the extraction procedure does not require the use of extra solvents to adsorb or desorb the analytes onto or from the fibre, the same fibre can be used multiple times, SPME can be considered as a truly green extraction procedure [1,97,98]. SPME has been the subject of a number of review papers that can be accessed for a fuller understanding of the technique [99–101].

SPME has been used in environmental analysis [82], biomedical analysis [102] and flavour studies [103] to extract analytes such as pesticides [104], hydrocarbons [82], phthalate esters [105] and fatty acids [82] as well as organic acids from slurry [106] and foods and drinks [107–109].

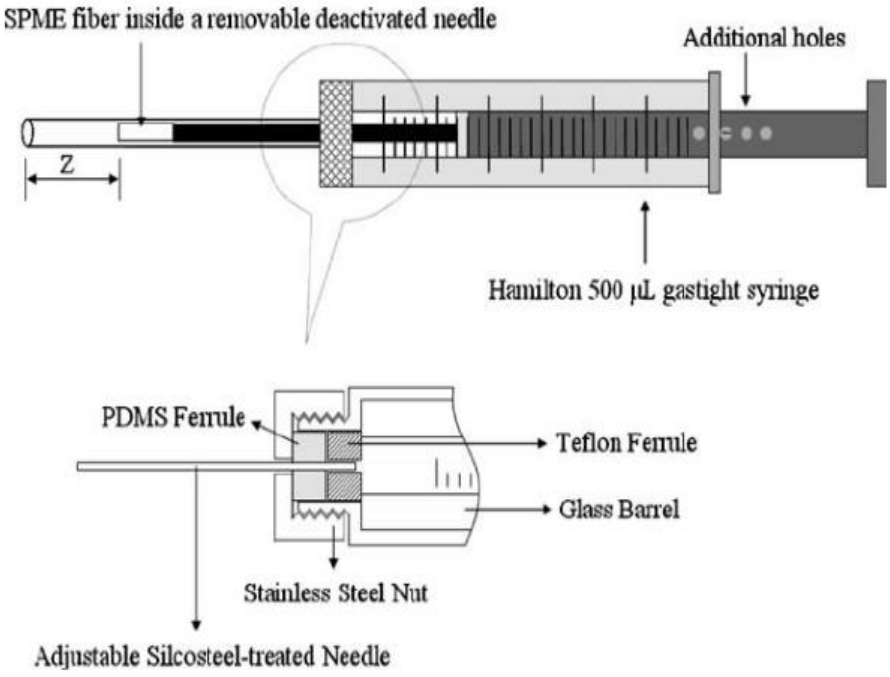


Figure 1-21. Schematic of a SPME device [98]. The septum houses and protects the SPME fibre while being introduced to the headspace of the sample vial. Once the lid has been perforated, the fibre is extended to allow interaction with the volatiles held in the headspace.

1.10.1 *Brief History and Notable Developments*

SPME was first introduced in 1990 by Arthur *et al.* [97] for the analysis of chlorinated hydrocarbons in water. The group used a Varian 3500 GC with a modified injector so that it could accept the SPME fibre and allow thermal desorption of the analytes directly onto the head of the column. Automation of SPME was also first described by Arthur *et al.* in 1992 [110]. This came before the release of the first commercially available SPME fibres [98]. The first commercially available GC with SPME auto sampler was a Varian 8200 in 1993 [98] although this had its limitations and was not temperature controlled which aids in the selective desorption of analytes from the SPME fibre. It was not until 1996 that agitation of the fibre was developed to improve desorption of analytes [98]; and in 1998 a fully automated SPME carousel became available with temperature control [98].

1.10.2 *Literature Review on the Extraction of Organic Acids via SPME*

Larreta *et al.* [106] used SPME to simultaneously extract and derivatised odorants. The group specifically screened for volatile fatty acids, phenols and indoles in animal slurry. Analytes were esterified on fibre; this was achieved by immersing the fibre in a 1-pyrenyldiazomethane (PDAM)/n-hexane solution at ambient temperature for 60 minutes. The SPME fibre was then removed and exposed to the head space (HS) gas of the slurry sample for 15 minutes at 35 ^{o}C. The fibre containing derivatised analytes was then inserted into a 300 ^{o}C injection port and held there for three minutes to desorb analytes. The SPME fibre was then baked at 300 ^{o}C for 15 minutes to clean the fibre. Important derivatisation variables that required optimisation were time of fibre immersion and fibre type. Other notable

variables for the extraction were: time the fibre spent in the HS, temperature of fibre in the HS for adsorption of analytes, temperature of fibre for desorption of analytes and time of fibre in injection port. The group reported LODs between 0.3 – 280 ng/L, no recoveries were given.

In lieu of the consumer perception of organic foods holding greater health benefits, Croissant *et al.* [107] studied the impact of cow diet on the composition and flavour of milk via GC-MS. They studied cows fed a TMR mix (corn silage, alfalfa hay, grain (soybean meal & ground corn) & minerals), whole cottonseed, soybean hulls, pelleted corn gluten fortified with vitamins and minerals) with no pasture and cows that were fed with 60% pasture, 30% ground corn and 10% cottonseed. The SPME of the volatile components of both raw and pasteurised milk was undertaken by placing the SPME fibre in the HS for 60 minutes to adsorb the volatile compounds. Desorption was achieved by placing the fibre in a 250 ^{o}C split less injector for five minutes. The study concluded that consumers could not differentiate between the feeding methods, thus consumer acceptance of organic milk versus 'non-organic' milk was not based on sensory differentiation. Cows fed with a larger pasture based diet did provide a healthier nutritional profile – higher conjugated linoleic acid and unsaturated fatty acids and lower saturated fatty acids. Though these nutritional profiles change drastically throughout the year as grass quality is dependent on time of year and weather.

Villeneuve *et al.* [108] also assessed the effects cow diet on the volatile organic acids in milk. The group identified and quantified 74 organic compounds, including organic acids that were extracted using SPME coupled with GC-MS. The fibre was exposed to the HS for 60 minutes. Analytes were thermally desorbed in a 255 ^{o}C injector for three minutes, the fibre was then cleaned by introducing it to a 270 ^{o}C

for 20 minutes. Their research found that different types of feeds such as hay, pasture or silage produced variations to the volatile compounds found in milk.

Rincon-Delgadillo *et al.* [109] analysed the organic acid profiles of starter distillates (SDLs) used to impart specific flavours and aromas to food and drink products. SDLs use alternatives to the potentially lethal diacetyl and so their volatile composition is of great importance. The SPME fibre was introduced to the HS where analytes were adsorbed at 40 ^{o}C for 20 minutes and desorbed at 200 ^{o}C for five minutes. The GC-MS analysis found a total of 40 compounds inclusive of organic acids such as acetic and butyric acid.

As can be seen throughout the SPME literature review of organic compounds in dairy products, extraction times vary with adsorption times of up to one hour; though thermal desorption is much quicker at around three minutes. Such high extraction times are clearly undesirable due to lower sample throughput. Temperature of the sample, agitation and fibre type are important parameter requiring optimisation in SPME. An important factor here is that sample preparation prior to extraction is minimal with no protein and lipid precipitation required, as well as on-fibre derivatisation. One drawback to SPME, is that it is limited to use with GC instrumentation only.

1.11 Cloud Point Extraction (CPE)

CPE is a powerful extraction and preconcentration technique that utilises surfactants as the main extraction solvent. Surfactants are amphiphilic compounds that can solubilise ionic and neutral compounds by forming micelles around the targeted analyte. This is achieved through alteration of temperature to induce formation of micelles.

Useful and important surfactant characteristics are cloud point (CP), pour point (PP) and critical micelle concentration (CMC). CP refers to the phenomena of surfactant clouding due to applied heat; each surfactant has its own specific temperature. Heat causes dehydration of the surfactant thus forcing the formation of micelles around the analyte, thus solubilising the analyte. Hydrophobic tails associate at the centre of the micelle to minimise its interaction with water leaving the more polar hydrophilic head exposed [111]. For this to occur, the surfactant must be present above its CMC.

CMC refers to the concentration at which surfactants spontaneously form micelles with each having its own, unique CMC [112]. Further heating causes a phase separation which consists of a surfactant, analyte rich micelle phase at the bottom and an aqueous micelle deficient phase above. Centrifugation then aids in further separation and increasing recoveries.

PP refers to the temperature at which a surfactant will flow, or pour under gravity. These are often above 0 ^{o}C which means the supernatant can be decanted leaving the bottom layer exposed and more easily accessible [113]. A number of review articles have been published describing surfactant phenomena in more detail than is available here [112,114–116].

For an analyte to successfully partition itself inside a micelle, it must have hydrophobic character. To enable partitioning of a polar ion, manipulation of pH to obtain the non-ionised form can be used or ion pairing (IP). Ion pairing is the pairing of an ion with another ion of opposite charge.

Unfortunately, surfactants are not readily compatible with several analytical techniques such as GC and IEC. In the case of GC, surfactants tend to stick to the

inlet and column causing spurious peaks and fouling of the inlet. In the case of IEC, surfactants tend to adsorb strongly to the polymer resins and foul the column. Methods are available to combat this and render the technique compatible with GC extending their use in the analytical laboratory. Froschl [117] first used a silica then a florosil column to remove Triton X-100 (TX-100) for the GC-ECD analysis of PCBs in water. Ohashi used cation exchange SPE [118] to remove surfactants for the analysis of phenothiazine tranquilisers in human serum. Takagai and Soares both derivatised excess surfactant [119,120] for the GC-MS analysis of PAHs, while a number of others used microwave and/or ultrasonic back extraction of the analyte into organic solvents [121–128] to marry surfactant extractions with GC based instrumentation.

1.11.1 *Brief History and Notable Developments*

The use of non-ionic surfactants as a novel extraction and preconcentration technique was conceived by Watanabe *et al.* in 1978 [129], who successfully exploited the properties of surfactants to extract and preconcentrate metal ions from tap water. CPE has since been used to extract and preconcentrate metals [130,131] and a variety of organic compounds from dairy based samples [132–138].

1.11.2 *Literature Review on the Extraction of Organic Acids via CPE*

Lopes *et al.* [139] used a multivariate approach to optimise their CPE of casein proteins from cow milk and subsequent analysis via MALDI-TOF-MS. Their optimum extraction used TX-114 and NaCl (salting out effect). The surfactant pellet was dissolved in acetone to make it compatible for analysis. Their analysis of the surfactant, analyte rich phase gave a concentration of 923 μg/mL. The author

also analysed the surfactant poor phase and gave a concentration of 67 μg /mL proving high extraction efficiency.

Kukusamude *et al.* [54] developed a CPE protocol for the extraction and CZE analysis of quaternary ammonium herbicides from milk. They found that a mixture of dilute phosphoric acid and TX-114 worked well. The upper phase was directly analysed via CZE. The group obtained LODs of 0.004 and 0.018 μg /mL for paraquat and diquat respectively.

Kukusamude also developed a method for the CPE of penicillin from milk samples [140]. Owing to the polarity of penicillin, they required a mixed micelle system, otherwise known as ion-pairing. This aided in neutralising the molecule and increasing solubility in the TX-114 surfactant. Proteins were precipitated form the sample using acetone:ACN (5:1). The residue was reconstituted in three mL of 10 m*M* phosphate buffer at pH 8.00 containing CTAB as the ion pairing agent. The aqueous phase was removed and with 1:1 MeOH:ACN prior to HPLC analysis. They found the critical parameters to be solution pH, CTAB reaction time, TX-114 concentration, type and concentration of salt, temperature of bath and heating time. The group reported LODs between 2 – 3 ng/mL, recoveries of 78 – 98% and EFs between, 15 and 40.

1.12 References

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2. Dispersive liquid-liquid microextraction

2.1 Introduction

Upon conclusion of a review of the literature, the most popular microextraction technique for the extraction of organic acids was solid phase extraction (SPE). SPE removes interferences from the samples matrices while also enriching the sample, offering high enrichment factors (EFs). EFs describe how much more concentrated the analyte is at the end of the extraction process in relation to its original concentration and can be calculated via *Equation 1-1* where C_{final} is the final concentration of the enriched analyte and $C_{original}$ is the concentration in the original sample.

$$\boldsymbol{EF = \frac{C_{final}}{C_{original}}}$$

Equation 2-1. Enrichment factor is calculated as the ratio between final concentration and initial concentration

SPE (as well as the other traditional and newer microextraction techniques) is laborious, expensive, time consuming and requires the use of specialised equipment (the cartridge and the sorbent). In the search for 'greener' extraction technologies neither technique can be considered green. LLE and SLE uses vast quantities of organic solvent because the extracted sample requires several washing steps to fully remove interferences. SPE addresses this issue and uses far less organic solvent, although a considerable amount is still used. SPE cartridges are designed for single use. They are then discarded and incinerated which is not environmentally friendly. Cartridges are also expensive, when laboratories assay

thousands of samples a week, vast amounts of solvents and SPE cartridges will be consumed. SPME addresses the hot-topic of solvent consumption in that extractions can be achieved without the use of solvents. Though well-known drawbacks to SPME are that fibres have a limited lifetime, are expensive, are exclusive to GC, and issues with carryover can cause problems.

The issues of laborious sample preparation, cost and environmental issues such as solvent use and waste have been addressed in a relatively new technique, dispersive liquid-liquid microextraction (DLLME). The technique was introduced by Rezaee *et al.* in 2006 [1]. Since then it has been developed to extract a large number of analytes from a wider variety of samples.

DLLME is compatible with a wide variety of instrumentation such as HPLC [2], GC [3], CE [4], atomic spectroscopy [4], matrix assisted laser desorption ionisation mass spectrometry (MALDI-MS) [5] and flow injection analysis (FIA) [6].

DLLME has been used to extract compounds such as metals [7,8], pesticides [9,10], polyaromatic hydrocarbons (PAHs) [11,12], phthalates [13,14], mycotoxins [15,16], phenols and phenol esters [17,18], antibiotics [19,20], organic acids [21–23], dyes [24,25] and amines [26,27] from a number of matrices. A comprehensive list has been compiled, and while the organic acids previously identified in the introduction have been chromatographically separated, at the time of writing, only hippuric acid had been extracted via DLLME which lends to the novelty of this body of work [28].

A common theme through all the articles reviewed is the importance of optimising the following parameters: type of extraction solvent, type of dispersing solvent,

volume of both extracting and dispersing solvent, sample pH, type and volume of salt, sonication and centrifugation time.

Volumes and concentrations all vary, which is what one would expect, though a commonality that was observed in the above cited papers, is that the lower the volume of extraction solvent, the higher the EF obtained.

2.2 Principles of DLLME

DLLME is a ternary solvent system consisting of an aqueous sample, a disperser solvent and an extraction solvent. The dispersing and extraction solvent must both be miscible with each other to create a binary solvent system which is rapidly injected into the aqueous sample. The role of the disperser solvent is to rapidly distribute the solubilised extracting solvent within the aqueous sample, therefore dispersing solvents must be miscible with water. The role of the extracting solvent is to quickly remove and enrich the analytes of interest, it must therefore not be miscible with water. Rapid injection of the binary solvent into the sample must generate a stable, turbid solution; often described as an emulsion. Turbidity occurs due to the presence of very fine, insoluble droplets of the extraction solvent, it is these droplets that analytes enrich, almost instantaneously into. The literature review has shown that in general, chlorinated solvents (tetrachloroethylene, carbon tetrachloride, chlorobenzene and chloroform) are the preferred extraction solvent, likely due to its higher density than water and ability to solubilise a wide variety of organic solvents; with methanol, acetone and acetonitrile being the preferred dispersing solvents due to their wide solubility in organic solvents (a breakdown of extraction solvents and disperser solvents can be found in *Table 2-1* - Table 2-3). This feature allows for easier recovery of solvent; removing the aqueous layer

above the organic solvent is much easier than removing a very small volume of organic solvent from the surface of the aqueous layer.

2.2.1 *Effect of pH*

Since pH controls the degree on acid/base ionisation it is an important factor to control as analytes must be in their neutralised form. This renders them less water-soluble which aids in their extraction into organic solvents and has a direct effect upon enrichment factors. A practical considerations here is the range of analyte pk_a values. Analyte pk_a values describe the pH at which 50% of the species is in its water-soluble, ionised form. To ensure maximum mass transfer of analyte into the extraction solvent, one requires the species to be in its neutralise form; to ensure this the pH of the solution should be at a minimum of 2 pH units below a species pk_a value for both acids and bases.

2.2.2 *Effect of Salt*

Salts such as NaCl can be used to alter the ionic strength and saturate the sample solution. This can be used to reduce the solubility of analytes in the aqueous phase and drive them into the extraction solvent. Salt concentration has a direct effect upon final enrichment factors. Use of salts should be avoided when using ion-pair reagents as they can initiate an ion-exchange reaction between salt and analyte, thus not extracting the compound.

2.2.3 *Effect of Extraction Solvent*

Selection of an appropriate extraction solvent is a critical parameter since this will contain the extracted analyte. It must:

- Be miscible with the dispersing solvent

- Be immiscible with the aqueous sample
- Be capable of extracting the compounds of interest
- Be compatible with the chosen chromatographic instrument

The ratio of extraction solvent to disperser solvent is of critical importance as it produces a more stable emulsion. A more stable emulsion allows for an increase of extraction of analyte from matrix into the extraction solvent.

The volume of extraction solvent has a critical effect upon enrichment factors. When using extracting solvents with a higher density than water, an organic phase appears at the bottom of the extraction vial as a sediment phase. Larger volumes of extracting solvent will yield lower enrichment factors due to dilution effects, therefore low volumes are always used, typically < 100 µL.

2.2.4 *Effect of Disperser Solvent*

The disperser solvent must be soluble in both the aqueous sample and the extraction solvent as its role is to distribute the extraction solvent throughout the sample. The volume and type of disperser solvent affects the turbidity of the solution and turbidity is often described in the literature as an emulsion. The emulsion that forms must be stable, by this, it is meant that the suspension should remain for a period of time. If the emulsion is not stable and the disperser/extraction solvent phase separates from the solution, analytes will partition to a far lesser degree. Typically, disperser solvent volumes vary from 500 – 1000 µL.

2.2.5 *Effect of Sonication and Centrifugation*

Sonication of the solution following DLLME can aid in increasing enrichment factors. By sonicating the ternary solution, much finer extracting solvent droplets are created. This can further increase surface area and enrichment factors.

Centrifugation creates a sediment phase by removing the fine droplets of extracting solvent from the bulk solution, thus also increasing both recoveries and enrichment factors.

2.2.6 *Needle Tip Diameter*

A further parameter that can be used to influence final EFs is the diameter of the needle tip. Narrow bore capillary tips will greatly enhance the final EF as it creates a finer spray to be distributed. A finer spray from the tip increases the number of droplets, thus surface area for analytes to enrich into.

2.2.7 *Advantages and Disadvantages*

DLLME is a powerful, miniaturised preconcentration technique that carries a number of advantages. DLLME is capable of very high EFs which makes it ideal for trace analysis. Since an equilibrium is established almost instantaneously following injection and due to the infinitely large surface area of the distributed extraction solvent droplets, the extraction is almost instantaneous [29–33]. This is clearly an attractive advantage over other extraction methods (SLE, LLE, SPE & SPME) as its speed will increase productivity and sample throughput. Very small quantities of organic solvents are used; in the hundreds of microliters range which significantly lowers solvent consumption, waste and cost. DLLME is compatible with most analytical techniques since the cleaned sample can be in either aqueous

or an organic solvent, depending on the original sample clean-up method used. And finally, DLLME is not laborious since it only consists of injecting a solvent mixture into the prepared sample, given this, it is extremely easy to use.

Disadvantages are that DLLME is not a fully green extraction technique since organic solvents (often chlorinated) are still required. However, the use of environmentally benign room temperature ionic liquids [34] and surfactants [28] in DLLME can ensure a truly green approach. DLLME is not directly compatible with ion exclusion columns as organic solvents and surfactants absorb and swell the polymer resin resulting in a fouled column. Finally it is non-selective. Any analytes not removed during the initial sample pre-treatment that are soluble in the extraction solvent will also be extracted and enriched.

2.3 Applications of DLLME in Food and Beverage Analysis

The following literature review will begin with highlighting the diversity of the DLLME technique by displaying the varied analytes that can be extracted and the variety of sample matrices that they have been extracted from. The literature review will then become more specific by exhibiting the variety of analytes extracted from milk and dairy produce. Finally, the review will display the only DLLME methods available for the extraction of organic acids from milk and dairy products, thereby highlighting the novelty and need for a method that can extract organic acids from milk and dairy produce.

2.3.1 *DLLME of Assorted Analytes Found in Foods and Beverages*

DLLME was first introduced by Rezaee *et al.* [1] in 2006 for the extraction and GC-FID analysis of 16 polyaromatic hydrocarbons (PAHs) from water. The optimised DLLME procedure consisted of injecting an acetone (disperser),

tetrachloroethylene (extractor) mixture into a 5-mL sample. This was then centrifuged to obtain a sedimented phase containing the enriched PAHs. The group obtained enrichment factors > 700, and between 60 – 106% recoveries. This seminal paper on DLLME identified important parameters that affect EFs as: type of extraction solvent, type of disperser solvent, volume of extraction solvent and volume of disperser solvent. PAHs have also been extracted from fish by Ghasemzadeh-Mohammadi *et al.* [12] and analysed via GC-MS. This group used a mixture of acetone (disperser) and chloroform (extractor) as their DLLME solvents to obtain EFs of 244 – 373 with recoveries between 82 – 105%.

Baliza *et al.* used DLLME for the analysis of cobalt complexes in water samples by atomic absorptions spectrometry (AAS) [7]. The dispersing solvent consisted of a mixture MeOH, a complexing agent, and chloroform as the extractor. The group achieved EFs of 16 for the Co-complex and recoveries between 94 – 104%. Aluminium has been extracted from water, fruit juices, wheat flour and milk via DLLME by Abdolmohammad-Zadeh and Sadeghi using an ionic liquid-based DLLME [8]. The extraction solvent consisted of an ionic liquid, 1-hexylpyridinium hexafluorophosphate and 8-hydroxyquinoline as a complexing agent, with ACN as the dispersing solvent. The extraction solvent mixture forms a fluorescent Al-complex which was then analysed via stopped-flow spectrofluorometry. This novel ionic liquid-based approach produced EFs of 100 with recoveries between 92 – 101%.

Organophosphorus pesticides have been extracted from foods such as watermelon and cucumber by Zhao and co-workers [9]. Following DLLME with chlorobenzene (extractor) and ACN (disperser) the pesticides were analysed via GC-nitrogen phosphorous detector (GC-NPD). The group recorded recoveries between 67 –

111% and extremely high enrichment factors, from 800 – 1000. Another group, Dashtobozorgi *et al.* extracted pesticides from cucumber and tomatoes using DLLME and analysis via HPLC-MS-MS [10]. The samples were first subjected to the QuEChERS (quick, easy, cheap, effective, rugged and safe) technique. The QuEChERS extract which contains and aqueous ACN solvent system was used as the dispersing solvent. The mixture was placed in a clean vial and carbon tetrachloride (extractor) was rapidly injected. Recoveries were in the range of 86 – 104%. No EFs were given.

Yilmaz *et al.* designed a vortex-assisted DLLME of seven phthalic acid esters (PAEs) from beverages and subsequent quantification via HPLC [13]. The group found the optimum DLLME solvents to be a mix of MeOH (disperser) and chloroform (extractor) directly injected into the sample. Following the binary solvent injection, NaCl was added to further reduce the solubility of PAEs, thus forcing higher concentrations to partition into the chloroform; partitioning of the PAEs was further increased by placing the ternary system, inclusive of salt into an ultra-sonicator prior to centrifugation. Reported recoveries were > 90%, however no EFs were given. Perez-Outeiral *et al.* also devised a DLLME with floating solid organic droplet of phthalates from waters and wines followed by GC-FID characterisation [14]. Hexadecane (extractor) and ACN (disperser) were rapidly injected into a heated NaCl. Following centrifugation, the sample was cooled to 3 ^{o}C to obtain the floating solid organic droplet. They reported recoveries between 75 – 109% and extremely high EFs ranging from 854 – 1893.

Campone *et al.* devised a DLLME of aflatoxins from cereal products using chloroform as extracting solvent and MeOH:water as dispersing solvent. The group reported recoveries of 67 – 92% and very small EF of 2.5 via HPLC analysis [16].

Arroyo-Manzanares *et al.* compared the use of QuEChERS with ionic liquid – DLLME for the extraction and HPLC analysis of ochratoxins from wines [15]. The ionic liquid DLLME solvent consisted of $[C_6MIM][PF_6]$ as extractor, dissolved in the dispersing solvent: MeOH. The reported recovery and EFs for the ionic liquid DLLME were: 88 – 94% and 5 respectively. The QuEChERS method produced similar recoveries but gave a dilution factor of 0.3. The group concluded that the DLLME method was superior when considering LODs and enrichment since QuEChERS produced a net dilution. DLLME was also more favourable due to being less time consuming. Cost was similar, though QuEChERS excelled in terms of repeatability and intermediate precision which ranged from 3.8 – 4.2% RSD and 3.7 – 5.4% RSD respectively, compared to 6.5 – 8.1% RSD repeatability and 7.9 – 8.5% RSD for DLLME intermediate precision. This was an innovative use of QuEChERS as typically it is used as a form of sample pre-treatment prior to SPE [15].

Godoy-Caballero *et al.* extracted phenolic compounds from olive oil using a reversed phase – DLLME; 60:40 EtOH:water (extractor) and 1 mL of 1, 4 – dioxane (disperser) was rapidly injected into the olive oil, before quantitation via HPLC-MS[17].

Lai *et al.* developed a DLLME procedure for the detection of the antibiotic, oxytetracycline using europium-sensitized luminescence [19]. The DLLME procedure entailed homogenisation of the fish meat in the presence of Na_2EDTA, HCl and ACN. The filtered aqueous sample was then used as the dispersing solvent and injected directly into a mixture of DCM:hexane. The top layer obtained following centrifugation containing the oxytetracycline-EDTA complex was then analysed. This technique was designed as a screening technique for the

presence/absence of the antibiotic to determine whether further analysis is required, therefore, no LODs, recoveries of EFs were given. Amelin *et al.* developed a DLLME technique for the extraction of amphenicols in beef, pork and liver [20]. Initial purification and extraction was achieved by using the QuEChERS method. The ACN extract was then used as a dispersing agent and DCM as the extracting solvent in the DLLME procedure. The bottom layer obtained from centrifugation was then anlaysed via HPLC. Recoveries ranged from 64 – 85%, EFs were not given.

Ahmadvand *et al.* developed a DLLME process for the GC analysis of fatty acids in pomegranate seeds [21]. The esterified acids was mixed with chloroform (extractor) and injected into a NaCl solution. Moniruzzaman *et al.* optimised a DLLME procedure for the analysis of a wide variety of organic compounds such as, alcohols, organic acids, phenols and ketones found in honey via GC-QTOF-MS [23]. Prior to DLLME, the sample was cleaned up using LLE. The group used an ACN (disperser) and chloroform (extractor) DLLME mixture.

Yan *et al.* optimised a DLLME protocol for the analysis of Sudan dyes in eggs via HPLC [35]. The sample pre-treatment consisted of using molecularly imprinted SPE prior to the DLLME protocol. The group used tetrachloroethylene as an extraction solvent and a 95:5 solution of acetone:acetic acid as the dispersing solvent. The group reported recoveries between 86 – 107% and EFs of 18 – 20. He *et al.* also developed a DLLME with solidification of floating organic droplet for the HPLC analysis of Sudan dyes in foods [25]. The food samples (chili sauce, chili oil, chili powder and tomato sauce) were first subjected to QuEChERS method which rendered the sample in an ACN suspension. The reconstituted suspension was used as the dispersing solvent along with 1-dodecanol as the extracting solvent.

This group found that both sonication and centrifugation gave higher EFs than centrifugation alone. The group reported recoveries between 79 – 92% for Sudan 1, no EFs were reported.

Campillo *et al.* developed a DLLME procedure for the GC-MS analysis of nitrosamines in meats [26]. The samples were first subjected to microwave assisted extraction for initial sample clean-up. The optimum DLLME solvents were MeOH (disperser) mixed with carbon tetrachloride (extractor). The group reported EFs of 220 – 342. Almeida *et al.* also developed a DLLME-GC-MS application for the analysis for 18 nitrosamines in beer [27]. Their optimised DLLME solvents were a mixture of ACN as disperser and toluene as extractor, with iso-butyl chloroformate (IBCF) as a derivatising agent. They reported EFs from 30 – 70.

In 2014, Daneshfar *et al.* combined surfactant CP with DLLME [28]; since surfactants are classed as environmentally benign [36] CP-DLLME satisfies the principles of sustainable development and green chemistry, this means it can be classed as a 'green' extraction technique. Daneshfar's CP-DLLME used TOPEO7.5 as an extraction solvent in water (disperser) to extract the following organic acids: hippuric, salicylic, anthranilic and nicotinic acids from biological samples. A schematic describing how to perform such an extraction was given in the paper and has been reproduced in *Figure 2-1* [28]. It should be noted that not all DLLME procedures require temperature control, though extraction its self is identical.

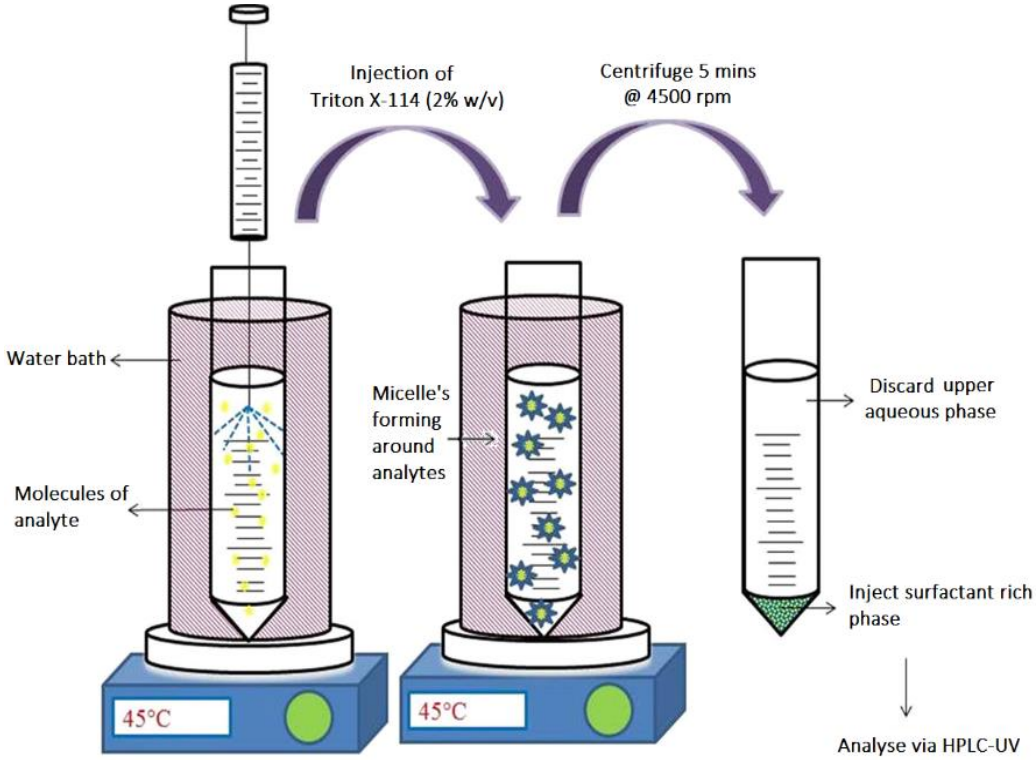

Figure 2-1.CP-DLLME procedure: 10 mL standard mix of nicotinic acid (600 µg/L), hippuric acid (600 µg/L), anthranilic acid (600 µg/L) and salicylic acid (250 µg/L) was placed in a 15 mL centrifuge tube, adjusted to pH 3.5 with 0.1 M HCl; 0.4 g NaCl was add and the mixture shaken until dissolved. Mixture incubated at 45 ^{o}C for 2 mins. 1 mL of TOPEO-7.5 at 2% was rapidly injected into solution using a 1000 µL syringe. Solution centrifuged for 5 mins at 2000 rpm. 120 µL of sedimented phase was taken and diluted with 400 µL ACN prior to HPLC-UV analysis [28].

Kamalabadi used microwave assisted DLLME to extract PAHs from coffee samples. Analytes were detected using GC-MS [37]. The extraction protocol favoured acetone and tetrachloroethylene as the dispersing and extracting solvents, respectively. The researchers obtained experimental EFs of between 155 and 248 for the PAHs found in coffee.

Khani used DLLME for the extraction of coumaric acid from vinegar, carrot juice and seeds [38]. They found that chloroform and ethanol were the optimum extraction and dispersing solvents (respectively). The group did not note any EFs from their UV-Vis results.

2.3.2 *DLLME of Analytes found in Milk and Dairy Produce*

Daneshfar designed a DLLME procedure for the extraction of cholesterol from a number of sample types, including milk [39]. The sample was treated with ACN to remove proteins and used EtOH as the dispersing solvent and carbon tetrachloride as the extraction solvent. The group obtained recoveries > 95% but gave no EFs.

Six Phthalate esters were extracted from bottled milk via DLLME and separated via GC by Yan [40]. Sample pre-treatment consisted of protein removal using trichloroacetic acid and lead acetate. The optimal ultrasound assisted DLLME extraction and dispersing solvents were tetrachloromethane and MeOH, respectively injected into a pH adjusted solution containing NaCl. The group reported high EFs between 220 and 270.

Gao developed a DLLME – HPLC method for the determination of sulphonamides in infant formula using ionic liquids as both disperser solvent ($[C_4MIM][BF_4]$) and extractor solvent ($[C_6MIM][PF_6]$) using NH_4PF_6 as an ion-pair reagent to increase recoveries [41]. The group evaluated the use of ultrasound time, pH and ion-pair concentration to optimise the extraction. The group recorded recoveries between 90 – 115%, again, no EFs were given.

Bisphenol A (BPA) and bisphenol B (BPB) were used in the manufacture of containers and leeching of these chemicals into food products can cause a number of health issues [42]. Cunha [42] devised a DLLME-GC analysis for their detection

in infant formula. The optimised procedure used a mixture of tetrachloroethylene as extractant and ACN as a disperser. High enrichment factors were obtained, ranging from 220 – 237.

Vinas [43] used DLLME to extract fat-soluble vitamins from infant formula. The optimised protocol used ACN to remove the proteins, the ACN supernatant was then used as the dispersing solvent and mixed with carbon tetrachloride (extraction solvent). This was directly injected into a vial of water; HPLC analysis gave EFs of 21 – 26.

Quigley used DLLME in conjunction with GC-FID for the extraction and analysis of fatty acids from milk [44]. This researcher found that chloroform was the optimum extraction solvent and methanol the optimum disperser solvent. LODs as low as 0.04 µg/mL were obtained and EFs of between 8 and 15.

2.3.3 *DLLME of Organic Acids in Milk and Dairy Produce*

Farajzadeh optimised an ion pair (IP) DLLME protocol for the extraction and HPLC identification of phthalic acids from a number of foods and drinks [45]. The protocol consisted of injecting a mixture of MeOH (disperser) with toluene (extractor) and tri-butyl amine as an ion pair reagent into a pH adjusted sample containing NaCl. The group reported EFs between 411 and 492.

Two common organic acids used as food preservatives, benzoic and sorbic acid were extracted from UHT milk and analysed via HPLC by Javanmardi [46]. Sample clean up consisted of protein and lipid removal via use of Carrez solutions (a mixture of aqueous metals and acids). The cleaned up sample was then subjected to their optimised DLLME procedure which used a mixture of acetone (disperser) and chloroform (extractor), injected into the salted sample solution. No EFs were

given. These same two organic acids were again extracted from milk and milk products such as yogurt by Abedi and quantified via GC [47]. Abedi found a mixture of octanol (extractor) and acetone (disperser) injected into a pH adjusted sample containing NaCl was the best combination of DLLME solvents. Proteins were again removed via use of the Carrez solutions. The group reported high enrichment factors of 143 and 170 for sorbic and benzoic respectively.

2.4 Conclusion

Considering the above review of the literature, the only organic acids extracted at the time of writing using DLLME were hippuric, salicylic, anthranilic and nicotinic acid from human serum and urine [28]; phthalic acid from foods [45]; benzoic, benzene-acetic, cinnamic, tetradecanoic and hexadecenoic acids from honey [23], benzoic and sorbic acid from milk [46,47] and coumaric acid from vinegar and carrots [38]. This apparent gap in the literature motivated the current work, which was to evaluate if DLLME can extract and concentrate the chosen organic acids identified in **chapter 1**, from milk and dairy based products.

The above authors have successfully used DLLME with many of the most popular analytical techniques. As listed in *Table 2-1*- Table 2-3, HPLC and GC was used numerous times to separate mixtures extracted via DLLME. Chlorinated solvents appear to be used widely with chloroform being used the most as an extraction solvent in DLLME; while methanol, acetone and acetonitrile have been used successfully as dispersing solvents due to their wide solubility in organic and aqueous solvents. Given this, the following work will investigate the use of DLLME with capillary electrophoresis (CE), high performance liquid chromatography (HPLC) and gas chromatography (GC).

*Table 2-1.Breakdown of solvents, analytes and analytical techniques discussed in the literature review (**section 2.3**). Chlorinated solvents have been used more extensively than any other solvent, with chloroform being the preferred solvent capable of solubilising a wide variety of organic compounds.*

Author	Analytes	Extraction solvent	Disperser solvent	Analytical Technique
Mohammadi [12]	PAHs	Chloroform	Acetone	GC-MS
Baliza [7]	Metal complexes	Chloroform	MeOH	AAS
Yilmaz [13]	Phthalic acid esters	Chloroform	MeOH	HPLC
Campone [16]	Aflatoxins	Chloroform	MeOH	HPLC
Ahmadvand [21]	Fatty acids	Chloroform	MeOH	GC
Moniruzzaman [23]	Organic acids and alcohols	Chloroform	MeOH	GC
Javanmardi [46]	Organic acids	Chloroform	Acetone	HPLC
Rezaee [1]	PAHs	Tetrachloroethylene	Acetone	GC
Yan [35]	Sudan dyes	Tetrachloroethylene	Acetone	HPLC
Cunha [42]	BPA, BPB	Tetrachloroethylene	ACN	GC

*Table 2-2. Breakdown of solvents, analytes and analytical techniques discussed in the literature review (**section 2.3**). Chlorinated solvents have been used more extensively than any other solvent.*

Author	Analytes	Extraction Solvent	Disperser Solvent	Analytical Technique
Perez-Outeiral [14]	Phthalates	Hexadecane	ACN	GC-FID
Arroyo-Manzanares [15]	Ochratoxins	Ionic liquid	MeOH	HPLC
Gao [41]	Sulphonamides	Ionic liquid	Ionic liquid	HPLC
Godoy-Caballero [17]	Phenolic acids	Ethanol	1, 4-dioxane	HPLC
He [25]	Sudan dyes	1-dodecanol	ACN	HPLC
Abedi [47]	Organic acids	Octanol	Acetone	HPLC
Farajzadeh [45]	Phthalic acids	Toluene	MeOH	HPLC
Daneshfar [28]	Organic acids	Surfactant	water	HPLC
Almeida [27]	Nitrosamines	Toluene	ACN	GC

*Table 2-3. Breakdown of solvents, analytes and analytical techniques discussed in the literature review (**section 2.3**). Chlorinated solvents have been used more extensively than any other solvent.*

Author	Analytes	Extraction Solvent	Disperser Solvent	Analytical Technique
Khani [38]	Coumaric acid	Chloroform	Ethanol	UV-Vis
Dashtobozorgi [10]	Pesticides	Carbontetrachloride	ACN	HPLC-MS-MS
Campillo [26]	Nitrosamines	Carbontetrachloride	MeOH	GC
Yan [40]	Phthalate esters	Carbontetrachloride	MeOH	GC
Vinas [43]	Vitamins	Carbontetrachloride	ACN	HPLC
Amelin [20]	Amphenicols	Dichloromethane	ACN	HPLC
Zhao [9]	Pesticides	Chlorobenzene	ACN	GC
Quigley [44]	Fatty acids	Chloroform	Methanol	GC
Kamalabadi [37]	PAHs	Tetrachloroethylene	Acetone	GC-MS

2.5 References

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3. The analysis of organic and inorganic anions in milk and dairy produce via capillary electrophoresis with indirect ultraviolet detection

3.1 Introduction

The use of electrophoresis as an analytical tool was first pioneered by Arne Tiselius as part of his doctoral thesis and, in 1948, he was awarded The Nobel Prize in Chemistry for his work in the field of electrophoresis and adsorption analysis [1,2].

Since then, several electrophoretic modes of analysis have been introduced, such as: micellar electrokinetic chromatography (MEKC), capillary isoelectric focusing (CIEF), capillary gel electrophoresis (CGE), chiral electrophoresis, capillary isotachophoresis (CITP), capillary electrochromatography (CEC) and the subject of this chapter: capillary zone electrophoresis (CZE, often further abbreviated to CE).

Electrophoresis is defined as the separation of ionic solutes based on differences in their rates of movement in an applied electric field [3]. The rate of movement of ionic species in solution in an applied electric field is called the electrophoretic mobility and is defined as the factor that determines the rate at which a given ionic solute moves via electrophoresis [3]. Electrophoretic mobility's can be expressed as:

$$v = \mu_e E$$

Equation 3-1. Describes that the migration velocity of an ion is due to the electrophoretic mobility and the strength of and electric field.

where v refers to the ions migration velocity (m s^{-1}), E refers to the strength of the electrical field (V m^{-1}) and μ_e refers to the electrophoretic mobility of the ion (m^2 V^{-1} s^{-1}). Further to this, the electrophoretic mobility of the ion can be expressed as:

$$\mu_e = \frac{q}{6\pi\eta r}$$

Equation 3-2. Describes that the electrophoretic mobility of an ion is dependent on the charge and radius of the ion, as well as the viscosity of the buffer.

where q refers to the charge carried by the ion; if the ion is an acid or a base it can be affected by pH and temperature. η refers to the viscosity of the solvent, this can also be affected by temperature and increased temperatures come not only from the thermostated cassette compartment holding the capillary, but also from the voltage applied to the capillary. Finally, r refers to the radius of the ion which can alter depending on the counter ion and complexing agents added to the buffer. Given this, differences in electrophoretic mobility occur due to the mass to charge ratio of an ion and the viscosity of the medium in which it migrates through.

Another factor that contributes to the migration velocity of ions in CE is the electroosmotic flow (EOF). EOF is defined as the bulk flow of liquid in CE; it is the motion of a liquid relative to a fixed charge surface caused by the electric field [3]. Therefore, EOF is largely affected by the viscosity of the buffer and the charge on the capillary wall. The charge on the capillary wall is termed the zeta potential and it is defined as the potential difference at any point within an electrical double layer [3].

Considering the only requirement of an analyte in CE is that it is ionisable, CE can separate a wide variety of anions ranging from inorganic anions such as chlorides, nitrates, nitrites and sulphates to organic anions such as organic acids, amino acids peptides and carbohydrates; all of which appear in milk and dairy produce [4–9]. Further to this, a paper published by Soga *et al.* had separated several species of anions in one analysis [10]. In view of this, CE was seen as a very powerful technique that could be exploited for the needs of this project.

3.1.1 *Capillary zone electrophoresis*

For CE separations to occur, both ends of a capillary are submerged into vials containing a buffer (which is analogous to a mobile phase). The capillary is then filled with the buffer via electroosmosis and a potential applied across the capillary. Ions migrate toward the electrode with the opposite charge and their velocity is partly governed by the strength of the electric field. Samples are introduced to the capillary from the anodic side and the detector is fixed in place at the cathodic side; this is the default configuration of all CE instrumentation and is referred to as traditional CE; this is depicted in *Figure 3-2*. Analytes present in their ionised form (anions), will not migrate toward the cathode as they have preference for the anode. However, some of the more weakly charged anions still reach the cathode due to the movement of the buffer and force of the EOF which is partly governed by the applied voltage (although this will take some time). As the detector is fixed in place, the polarity of the electrodes is reversed to allow the analysis of anions. Since the EOF moves toward the cathode, anions will remain in the source vial as they are migrating against the EOF. To counter this the EOF must be suppressed. This is achieved by adding modifiers such as quaternary amines to the buffer [11]. *Figure 3-1* serves as a visual representation of a separation in traditional CE. Note

that in *Figure 3-1* the small, highly charged cations migrate toward the cathode and past the detector first, followed by the larger, more weakly charged cations. Following this is the inseparable neutral band, followed by the larger, weakly charged anions and finally the small highly charged anions.

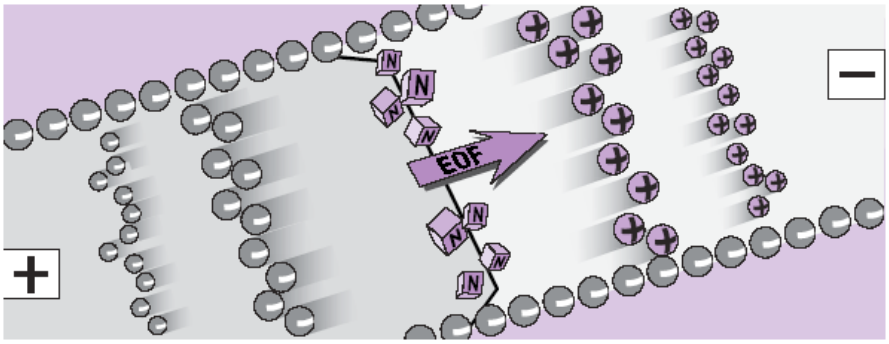

Figure 3-1. Representation of traditional CZE. Ions and neutral species are separating into 'zones' based on their mass to charge ratio. Smaller highly charged cations migrate more quickly than larger cations with a lesser charge.

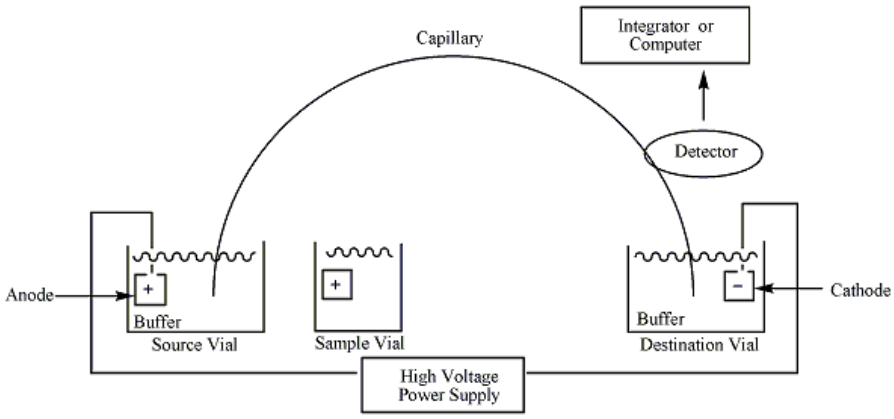

Figure 3-2. A schematic representation of a CE instrument operating in the traditional mode. Direction of flow is from the anodic side, past the detector that is fixed in place at the cathodic end.

3.1.2 *Reversal of EOF for anion detection*

Fused silica capillaries are naturally brittle, they are coated with a polyimide coating which imparts flexibility to the capillary (Figure 3-3). The capillary interior naturally carries a negative charge due to the $Si\text{-}O^-$ groups at the capillary surface. To counter this and suppress the EOF, quaternary amines are added to the buffer. The positively charged moiety of quaternary amines bind electrostatically with the negatively charged surface on the capillary wall. The hydrophobic tail of another quaternary amine then associates with that of the amine bound to the capillary wall via electrostatic interactions. The results is a bilayer on the capillary wall creating a net positive charge. However, recent research suggests that single tailed surfactants may actually form spherical micelles at the surface and only double tailed surfactants form a bilayer [12]. Anions from solution are then attracted to the surface and solvated anions migrate toward the anode under an applied voltage. This migration drags the bulk solution with it, resulting in a reversed EOF and a much shortened analysis time [13]. Incidentally, since EOF is generated, in part due to the charge on the capillary wall and not by a pump there is no drop in pressure. This produces a flat flow profile as opposed to a parabolic flow profile as seen in HPLC. This is an important feature of CZE as band broadening is eliminated which leads to much higher separation efficiency [3,14].

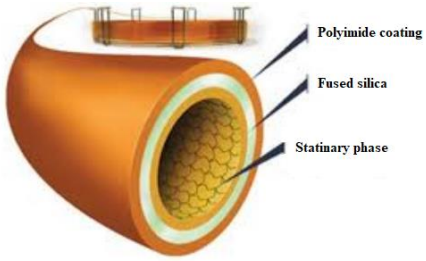

Figure 3-3. Schematic of a fused silica capillary column

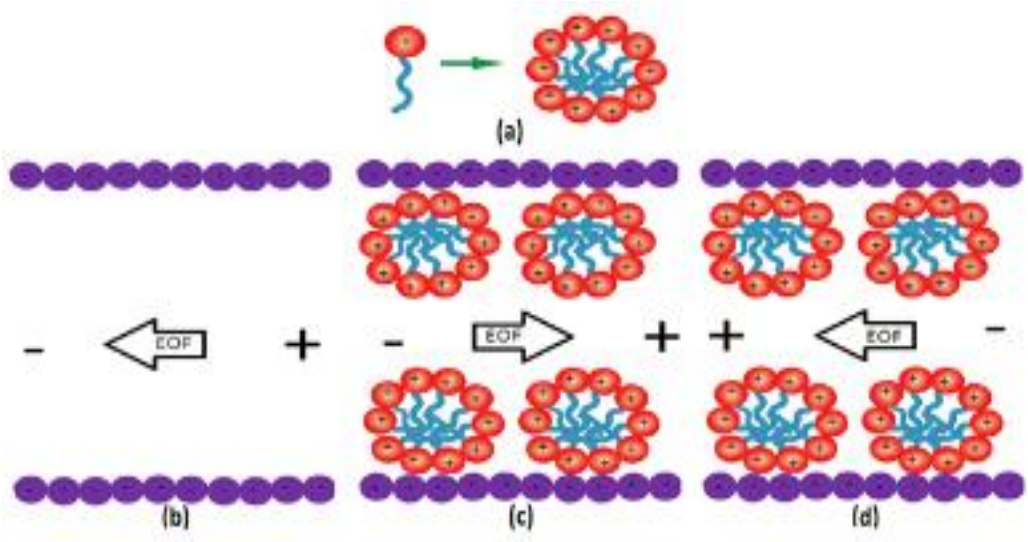

Figure 3-4. Depiction of EOF generation and reversal for anion detection. (a): micelle formation; (b): Default direction of EOF. (c): Reversal of EOF due micelle formation at capillary walls. (d): Reversal of EOF via switching of instrument polarity.

Above, (*Figure 3-4*) is an illustrative schematic of the theory of EOF and its suppression to detect anions. Section (a) is a depiction of a single tailed surfactant forming a micelle; hydrophobic carbon tails associate at the centre of the micelle, maximising interaction with each other and minimising interactions with water. Section (b) shows that the direction of the EOF is always toward the electrode with the same sign as the charge on the capillary wall, given this, the direction of EOF in traditional CE is toward the cathode and can be used to separate cations. Section (c) illustrates that surfactant micelles aggregate at the wall, thus balancing the charge. The direction of the EOF is then flipped toward the anode which is at the opposite side to the detector by reversing the polarity. Section (d) shows that when the polarity of the instrument is flipped, the direction of the EOF is reversed and the capillary contents then flow back toward the cathode and past the detector; this allows the separation of anions.

3.1.3 *Indirect UV Detection*

Detection of organic acids and inorganic anions can be problematic as some, such as chloride have no chromophore and others, such as short chain organic acids only weakly absorb ultraviolet (UV) radiation. UV detection is achieved indirectly via the addition of what is often termed as a probe. A probe refers to a highly absorbing compound that is added to the buffer. Pyridinedicarboxylic acid (PDC) has been used extensively [15–18] and so was used in the following CE work. As the highly absorbing probe continuously passes the detector its absorbance is constantly registered. When a non, or weakly absorbing analyte then passes the detection window, the detector records this as a drop in absorbance which is registered as a negative peak. Software then reverses this output and registers the peak as a positive peak that makes the electropherogram more familiar and easier to handle.

3.1.4 *CZE Method Development Parameters*

Typical parameters optimised during CE method validation are buffer pH, concentration of EOF modifier, concentration of probe in the buffer, capillary temperature, voltage, injection type, capillary length and detector response time [3,13,14]. Further methods are then available to enhance resolution or separate co-migrating compounds by using complexing agents [19].

CE has been used for the detection of organic acids and inorganic anions in a variety of samples such as foods, drinks and biological samples [10,20–25]. A method published in 1999 by Soga *et al.* [10], which was later adopted as an Agilent application note [26] showed that it was possible to separate a mixture of 43 organic acids, amino acids, inorganic anions and carbohydrates in less than 40 minutes using CE. Given the ability of CE and this method to separate several class of compounds found in milk and dairy produce in one simple analysis, CE was evaluated for its potential to separate and quantify organic acids from dairy products in this work.

3.2 Experimental

3.2.1 *Buffers*

All buffers were composed of 5 m*M* pyridinedicarboxylic acid (PDC) as the probe for indirect UV detection, and 0.5 m*M* cetyltrimethylammonium hydroxide (CTAH) to suppress the EOF which is essential for anion detection. Buffer pH was adjusted to pH 3.00. Each pair of buffer vials must be filled to the same volume (1.5 mL) to eliminate osmotic flow from one vial to another. Inter-run capillary conditioning was achieved by using a maximum of four injections from each buffer

pair. This reduces migration time shifting through the action of buffer depletion from continuously applied high voltages to the capillary.

3.2.2 *Instrumental*

An Agilent 7100 CE instrument was fitted with a one meter (91.5 cm effective length) fused silica capillary of 75 µm i.d. and was thermostated at 20 ^{o}C. Sample plugs were injected under 50 mbar of pressure for two seconds. Detection was achieved indirectly using a diode array detector (DAD) with a signal wavelength of 350/20 nm and a reference wavelength of 200/10 nm. The response time was set at 0.2 seconds and voltage -20 kV.

3.2.3 *Capillary conditioning*

A 30-minute pre-conditioning buffer flush was performed at the beginning of each analysis followed by a two-minute water flush. A four-minute inter-run buffer flush followed by a two-minute water flush was also implemented between each injection.

3.2.4 *Standards*

All standards were purchased from Sigma and prepared in deionised water. De-ionised water was collected from a Whitewater, Dublin DI unit fed by an ASTD Type 2 unit. It had a resistivity at 25 ^{o}C of ~ 18.6 MΩ/cm; conductivity: < 0.02 µS/cm and TOC: 0 ppb. Aqueous stock standards of malic acid, sodium chloride, potassium nitrate, zinc sulphate, oxalic acid, sodium hydrogen phosphate, tartaric acid, lactic acid, formic acid, trisodium citrate dihydrate, succinic acid and acetic acid at 1000 µg/mL were prepared. Stocks were diluted to 50 µg/mL for working standards.

3.3 Results and discussion

3.3.1 *Pre-conditioning*

The purpose of the 30-minute buffer flush was to ensure that the silanol groups that populate the surface were primed to the relevant pH and that the CTAH in the buffer had enough time to aggregate at the surface enabling suppression of the EOF. Pre-equilibration of the capillary prior to analysis is extremely important to replenish micelle layers at the capillary wall. Failure to do this will result in migration time shifts. The purpose of the water flush after this was to remove any unbound or weakly bound CTAH micelles that may be populating the capillary wall and free CTAH micelles present in the capillary. A four-minute buffer flush and two-minute water flush was implemented as a preconditioning step between each injection (inter-run) to rid the capillary of any excess analyte that may be present.

3.3.2 *Optimization of pH*

Buffer pH determines the degree of ionisation, and hence charge of the analytes and has a marked impact on electrophoretic separations. It is usually quoted that buffers should be at least 2.5 pH units away from the analytes pk_a. This ensures either full ionization or full neutralisation of analytes. The buffer pH was varied from pH 3.00 to pH 10.00 to assess its effects on selectivity and resolution between organic acids in the standard mixture. All other conditions were as reported in the instrumental section.

From the graph in *Figure 3-5*, pH 3.00 gives the greatest resolution between analyte bands. The largest selectivity effects were seen on lactate and phosphate between pH 3.80 and pH 5.20 as their migration time increases from 4.3 mins to 5.4 minutes

and 3.8 minutes to 4.1 minutes respectively. The greatest decrease in migration time was seen by succinate and acetate between pH 3.60 and pH 6.20. Acetate drops from 5.5 to 3.4 minutes while succinate dropped from 4.9 to 3.9 minutes.

The migration times of chloride, nitrate and sulphate all gradually increased from pH 3.00 to pH 6.00. The most variation in migration times due to pH occurred between pH 3.00 and pH 6.00. This is due to each analytes relative pk_a value. All pk_a values fall below 5.5 which means as the pH drops below this value, analytes become more ionised which lowers their migration times due to their attraction for the electrode and velocity of the EOF.

As can be seen in *Figure 3-6*, peaks one and two, (the inorganic anions) chloride and nitrate, have excessive peak fronting. This is due to a mismatch between the mobility or velocity of the buffer and the electrophoretic mobility of the analytes. In this instance the mobility of the analyte was greater than that of the buffer as chloride and nitrate are very small and highly polar ions; in CE, this manifests as peak fronting in early migrating peaks. It is unclear what the unknown peak in the blank at 5 minutes is, since it did not impact the analysis it was not investigated. This issue should have been resolved before proceeding.

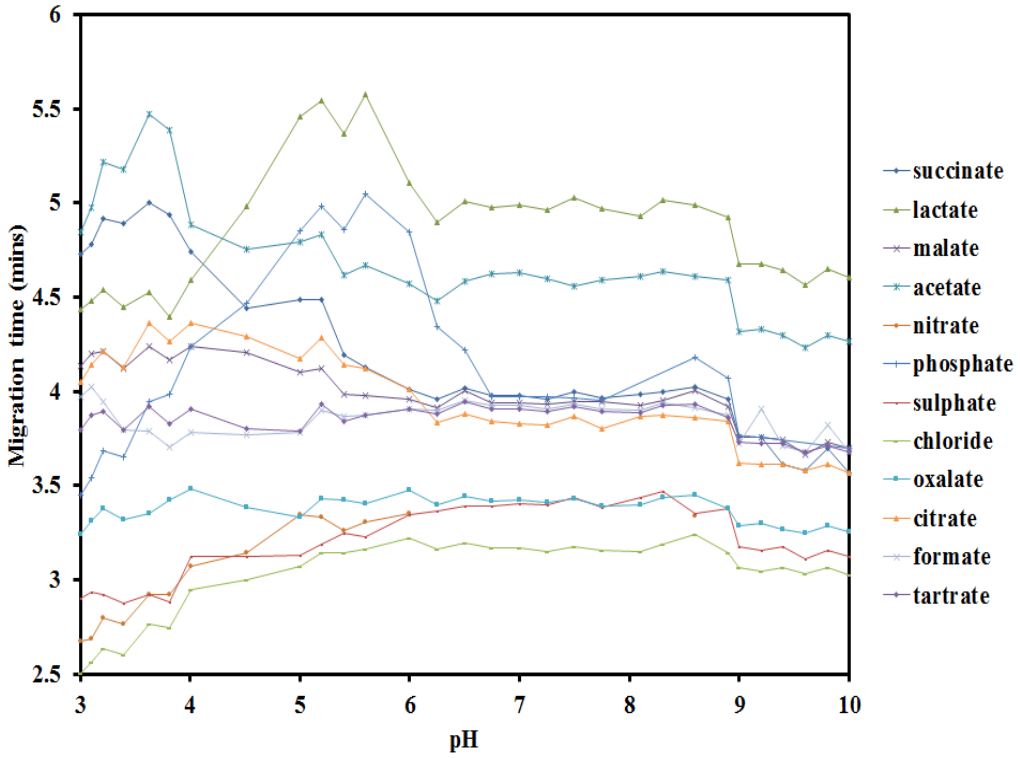

Figure 3-5. Graph of migration time (mins) against pH. This graph demonstrates that pH has a significant effect on selectivity of analytes such as lactate, phosphate and succinate. From the graph pH 3.00 appears to yield the greatest resolution. Instrumental: A 5 mM PDC buffer (used as indirect UV probe) with 0.5 mM CTAH as EOF modifier was used, along with a one meter (91.5 cm effective length) fused silica capillary of 75 µm i.d. that was thermostated at 20 ^{o}C. Sample plugs were injected under 50 mbar of pressure for two seconds. Detection was achieved indirectly using a DAD (350/20 nm, reference: 200/10 nm). The response time was set at 0.2 seconds and voltage -20 kV.

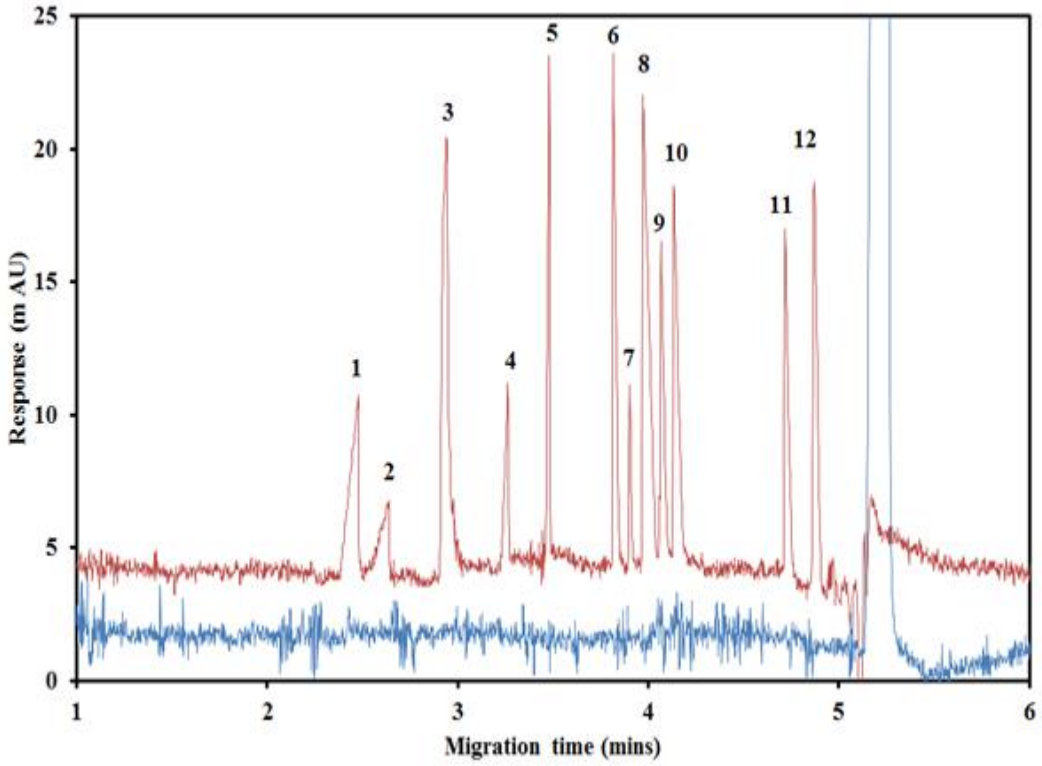

Figure 3-6. Electropherogram of a standard mix separation at pH 3.00. Blank: blue; standard mix: red. Analyte I.D: (1) Cl^-; (2) NO_3^-; (3) SO_4^{2-}; (4) oxalate; (5) PO_4^{3-}; (6) tartrate; (7) lactate; (8) formate; (9) citrate; (10) malate; (11) succinate; (12) acetate, each at 50 µg/mL. Instrumental: A 5 mM PDC buffer (pH 3.0) was used as the indirect-UV probe with 0.5 mM CTAH. Capillary length: one meter (91.5 cm effective length) x 75 µm i.d., thermostated at 20 °C. Sample plugs were injected under 50 mbar of pressure for two seconds. Detection was achieved indirectly using a DAD (350/20 nm, reference: 200/10 nm). The response time was set at 0.2 seconds and voltage -20 kV.

3.3.3 *Effect of temperature*

Since pH is temperature dependent, altering the temperature alters the degree of analyte ionisation. This affects parameters such as migration time, selectivity and resolution. Effects of temperature were assessed from 15 – 55 ^{o}C using a pH 3.00 buffer, whilst all other parameters held constant and as described in the instrumental section.

Altering temperature in this instance produced very little effect upon selectivity for most analytes. Only slight selectivity effects were seen with formate and citrate. At 35 ^{o}C formate and citrate began to co-migrate, and remain like this for the remainder of the study. Increased temperature also increased baseline noise as well as significantly decreasing migration times. There are several factors that may give rise to the reduced migration times. As temperature increases the medium becomes less viscous allowing the EOF to increase which allows analyte bands to travel more freely toward the detector [3,13]. Since temperature affects pH, the analytes may have also become more ionised, which in turn increases their affinity for the electrode, thus reducing migration time.

Given that no significant affect was had on selectivity, the temperature was kept at 20 ^{o}C to produce the best signal to noise ratio. It was also easier for the instrument to regulate the temperature at 20 ^{o}C, which is close to room temperature. As can be seen in Figure 3-7, temperature significantly degreased migration time, though this came at the expense of increased baseline noise which reduces signal to noise. The increase of temperature also did not have the desired effect of altering selectivity between analytes which would manifest as the crossing of plot lines in Figure 3-7.

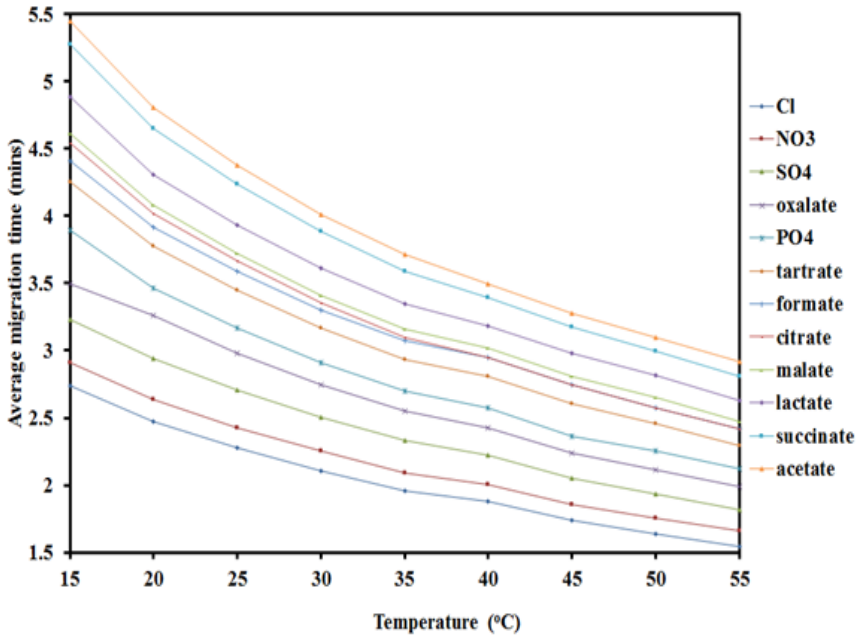

Figure 3-7. Plot highlighting the effect of temperature on migration time. Formate and citrate which begin to co-migrate at 35 °C. Instrumental: A 5 mM PDC buffer (pH 3.0) was used as the indirect-UV probe with 0.5 mM CTAH as EOF modifier. Capillary length: one meter (91.5 cm effective length) x 75 µm i.d., capillary temperature varied from 15 55 °C. Sample plugs were injected under 50 mbar of pressure for two seconds. Detection was achieved indirectly using a DAD (350/20 nm, reference: 200/10 nm). The response time was set at 0.2 seconds and voltage -20 kV.

3.3.4 *Effect of voltage*

Increasing the voltage applied across the capillary increases the velocity of the EOF which leads to lower migration times (Figure 3-7) and higher efficiencies (Figure 3-8) [14]. However, high voltages will also increase Joule heating which leads to broader peaks, migration time shifting or boiling of the buffer within the capillary [13]. Maximum voltage is highly dependent on the length of the capillary used as the length of the capillary influences how quickly temperature generated by the

applied voltage dissipates across the capillary. Voltage effects were assessed from -15 to -30 kV using a pH 3.00 buffer at 20 ^{o}C.

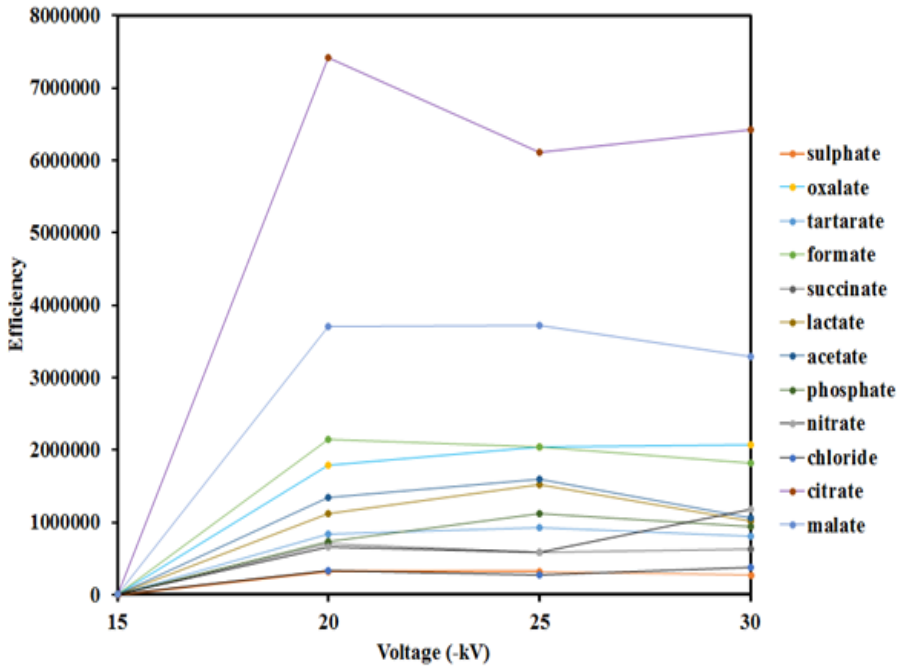

Figure 3-8. Effect of voltage on efficiency. Peak efficiency increases dramatically from 15 - 20 -kV for all analytes then the general trend is no increase in peak efficiency from 20 – 25 -kV, which then leads to a decrease in efficiency from 25 - 30 -kV. Instrumental: A 5 mM PDC buffer (pH 3.0) was used as the indirect-UV probe with 0.5 mM CTAH as EOF modifier. Capillary length: one meter (91.5 cm effective length) x 75 µm i.d., capillary temperature varied from 15 55 ^{o}C. Sample plugs were injected under 50 mbar of pressure for two seconds. Detection was achieved indirectly using a DAD (350/20 nm, reference: 200/10 nm). The response time was set at 0.2 seconds and voltage varied from 15 to 30 – kV.

Efficiency was calculated by dividing the number of plates by the length of the column. This was then plotted against voltage to define the optimum voltage that produced the highest efficiencies. As shown in Figure 3-9, -20 kV gave the highest efficiencies for citrate, malate, formate, tartrate, chloride, sulphate and succinate.

At voltages greater than -20 kV, the trend is toward lower efficiency. Phosphate, acetate, lactate and oxalate had the highest efficiencies at – 25 kV while nitrate was at its most efficient at -30 kV. Given the efficiency was highest for the most analytes at -20 kV coupled with relatively quick migration time, that was chosen as the optimum voltage. No selectivity effects were seen at any voltage with the exception of phosphate and acetate which began to co-migrate at voltages greater than -25 kV.

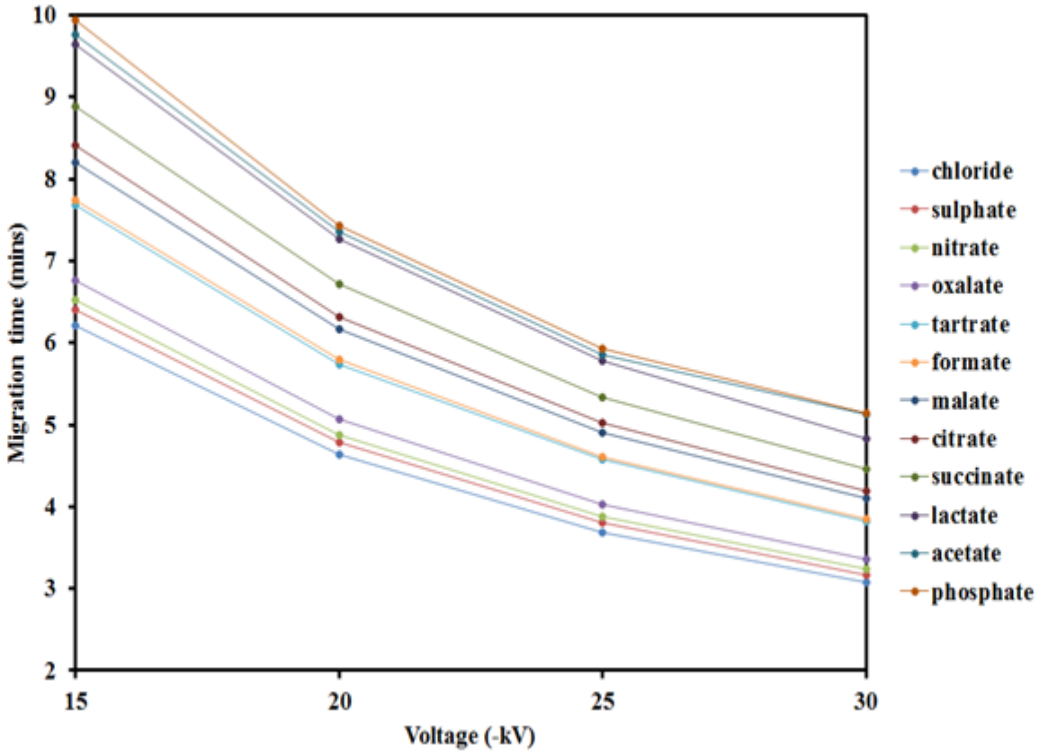

Figure 3-9. Effect of voltage on migration time. As can be seen increasing voltage decreases migration time for all analytes due to a higher EOF. No significant selectivity effects were observed. Instrumental: A 5 mM PDC buffer (pH 3.0) was used as the indirect-UV probe with 0.5 mM CTAH as EOF modifier. Capillary length: one meter (91.5 cm effective length) x 75 µm i.d., capillary temperature varied from 15 55 ^{o}C. Sample plugs were injected under 50 mbar of pressure for two seconds. Detection was achieved indirectly using a DAD (350/20 nm, reference: 200/10 nm). The response time was set at 0.2 seconds and voltage varied from 15 to 30 –kV.

3.4 Troubleshooting

Following the above work a large step began to appear in the baseline which masked all the analytical signals. Several steps were taken to resolve this issue. At first it was thought that the step may be due to buffer depletion, though the protocol had not changed. That protocol stated that no more than four injections per pair of buffer vials were to be used to limit buffer depletion. This conservative figure was decided upon to mitigate any potential issues associated with buffer depletion before any issues arose. It should be noted that most sources quote no more than 10 injections per buffer pair [13,14,20]. Following this, the instrument method was assessed for any amendments that may have been made - all parameters matched the SOP.

To eliminate contamination from water supplies, water from different purification systems in the college were injected and checked against bottled HPLC grade water purchased from Sigma. The step appeared in each injection and its size increased with injection. Since the step increased over time, carry over as a potential source of contamination was also eliminated.

Dirty electrodes can lead to alteration in current which could account for the step. The instrument was stripped and the electrodes sonicated in water and IPA to remove any particulates, this action did not resolve the issue. Current and pressure profiles were overlaid with the blank injection to determine if there was a spike in voltage or pressure that coincide with the step, though both were flat.

Several buffers were then made from different sources of de-ionised water, ensuring all were at the correct concentration (high buffer concentrations can lead to excess current, thus an increase in baseline). The purpose of this was to

investigate errors from the analyst in the making of the buffer, this also did not resolve the issue.

Capillary pre-treatment and inter-run conditioning protocols were re-visited and scrutinised for accidental amendments. Fresh washing solvents were made and injected, still the step remained. The capillary ends were checked for correct alignment and cracks or breakages under a microscope. No cracks could be found, though the capillary was still discarded and a fresh one cut, checked for faults, then equilibrated with fresh solvents. All buffers and standards were subjected to filtration through 0.45 μm PTFE filters as a matter of good lab practice; this action also degasses the buffer – neither improved the chromatography.

Finally, as previously mentioned, the method above is based on the work of Soga [20] and uses the same buffer in all but pH (this work: pH 3.00, Soga's: pH 12.00). Soga's method was published by Agilent as an application note [26] and Agilent sell the same buffer that was made in the laboratory at pH 12.00 under the name of 'Agilent Basic Anion Buffer'. This buffer was purchased and tested against the buffer made in the laboratory (pH 3.00). Despite the difference in pH, it was thought that the Agilent buffer could serve as a baseline to work from since all of its basic components were the same. As can be seen in Figure 3-10 a similar, increasing baseline was obtained for two blank injections and a ghost run. The laboratory made, pH 3.00 buffer (blue) had a large step with an absorbance of ~ 10 m AU. The Agilent buffer, pH 12.00 (red) had a larger step of ~ 20 m AU, finally the ghost run (purple) also had a step of ~ 20 m AU. Since the step still appeared in the ghost injection, this would imply that contamination from the buffer caused the step, but since the buffer that was purchased gave comparable chromatography, it could not be the buffer.

Given that this could not be resolved and time a conscious issue it was decided to leave the CE work and move on to other chromatographic techniques such as liquid and gas chromatography as both are suitable for the separation of organic acids. Further to this, both techniques are suitable to separate samples that have undergone dispersive liquid-liquid microextraction (DLLME). No DLLME was undertaken using CE.

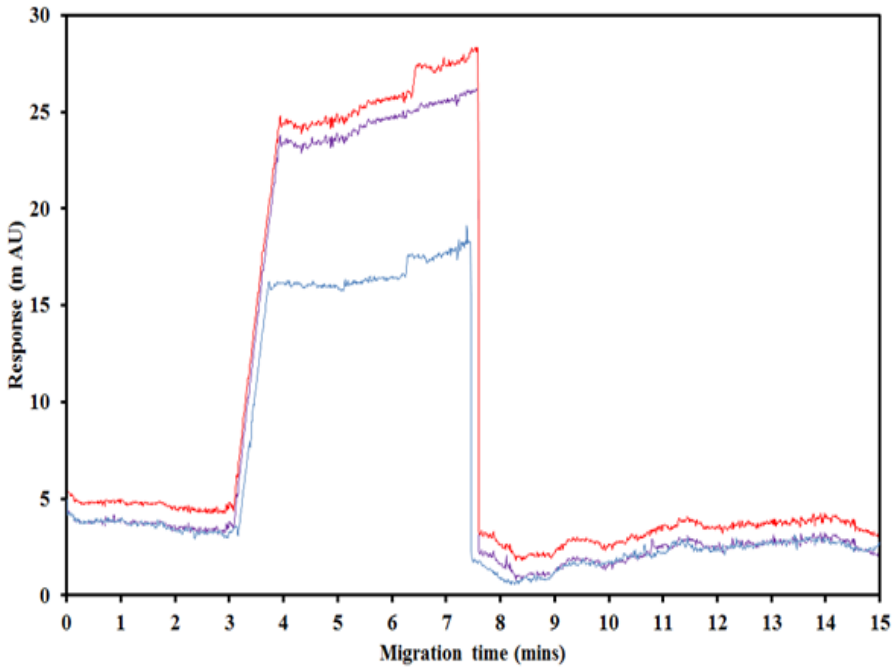

Figure 3-10. Blue: buffer made in the lab (pH 3.00 5, mM PDC buffer as indirect-UV probe with 0.5 mM CTAH as EOF modifier); red: buffer purchased from Agilent (5 mM PDC buffer as indirect-UV probe with 0.5 mM CTAB as EOF modifier pH 12.00); purple: ghost run. Capillary length: one meter (91.5 cm effective length) x 75 µm i.d., capillary temperature varied from 20 °C. Sample plugs were injected under 50 mbar of pressure for two seconds. Detection was achieved indirectly using a DAD (350/20 nm, reference: 200/10 nm). The response time was set at 0.2 seconds and voltage varied from 15 to 30 – kV.

3.5 Conclusion

A CE method for the separation of organic and inorganic anions was trialled using indirect UV as the mode of detection, this allowed for the detection of weekly absorbing anions and those that have no chromophore, such as inorganic anions. Parameters optimised include, pH, temperature and voltage, though ultimately the work could not be completed due to baseline issues that could not be resolved. Given that time was a constant issue, it was decided to move forward with using gas and liquid chromatography as the mode of separation. Since inorganic anions such as those used above will not retain on C_{18} columns or on a GC PEG stationary phase, only organic acids were studied herein. No DLLME was trialled on the CE method since it could not be validated. Despite DLLME not being used with this CE work, this section of work was included as it was important to show the sequence of events and rationale of future work.

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4. Dispersive liquid-liquid microextraction and chromatographic identification of organic acids in milk and dairy produce via high performance liquid chromatography with ultra violet detection

4.1 Introduction

Milk and dairy produce contain several organic acids with varying pk_a values. 18 of the most commonly cited organic acids found in milk and dairy produce [1–4] have been identified for detection. Analytes such as tartaric, malic and formic acids were chosen as they are often used as additives that enhance shelf life [5] or markers in flavour studies [6]. Analytes such as pyruvic, lactic and acetic can be used to determine milk quality [7–10], while analytes such as uric acid can be used to assess the health of the cow [11,12]. Given this, an extraction and separation of the 18 chosen analytes has the potential to assess several factors ranging from quality of produce and flavour studies to cow health in one simple analysis.

Organic acids are more commonly separated via ion chromatography (IC) [13,14]. The next most popular technique for their separation is high performance liquid chromatography (HPLC). HPLC is concerned with the separation of analytes soluble in aqueous medium. Irrespective of the mode of chromatography used, HPLC consists of two phases; a mobile phase and a stationary phase. These phases are developed to exploit physiochemical properties of analytes. The most common mode is reversed phase liquid chromatography (RPLC), which entails the use of a non-polar stationary phase and a polar mobile phase. RPLC has been used numerous times for the separation of organic acids [15–18]. No separation could be found that quantifies all of the organic acids identified in this body of work.

Given this, the aim of this chapter was to develop a separation of the 18 most commonly cited organic acids found in milk and dairy produce.

4.1.1 *Basic Principles of HPLC*

The goal of any separation is to attain optimum peak resolution in the minimum amount of time. The fundamental resolution equation (Equation 4-1*)* states that resolution (R_s) is affected by efficiency (*N*), selectivity (α) and retention factor (*k*). Efficiency refers to the number of theoretical plates achieved on a given column. It is a function of peak shape, width and height; narrower peaks increases resolution. Selectivity refers to the ability of a stationary phase to retain eluting analytes and other sample components; while retention factor is a measure of time and refers to the ratio between the solvent front and eluting peaks (values for *k* should be between 2 and 10).

$$R_s = \frac{1}{4}\sqrt{N}\ x\ \frac{\alpha - 1}{\alpha}\ x\ \frac{k}{k+1}$$

Equation 4-1. Fundamental resolution equation describes the effects of efficiency, selectivity and retention factor on the resolution of two adjacent peaks.

The most popular column used in RPLC is a C_{18} column which has a C_{18} ligand bound to porous silica beads of uniform size and shape. Uniformity and size of the beads affect the packing of the column. This is important as larger, non-uniformly shaped beads produce a less well packed column. When the former is coupled with inhomogeneity of size and shape, this leads to band broadening due to an increasing in the path of the travelling analyte; this is termed Eddy diffusion, multiple path effect or simply packing term and is referred to in Equation 4-2 as *A*. Eddy diffusion

lowers resolution (broader peak width shortens the distance between the apex of two adjacent peaks) and efficiency (broader, thus shorter peaks). A more accurate measure of efficiency is the height equivalent to a theoretical plate (HETP). Two other factors that affect HETP are longitudinal diffusion (*B/u*) and mass transfer (*Cu*). Longitudinal diffusion refers to diffusion of an analyte band due to the concentration gradient, this 'stretches' the band producing wider peaks at the base and can be minimised by using higher flow rates as well as shorter and narrower tubing. Finally, mass transfer refers to how quickly an analyte diffuses from the mobile phase into the pores of the column packing then back into the mobile phase. One action equates to one theoretical plate. Smaller particles mean that analytes diffuse to lesser extent into particles due to a smaller depth to the pores, this increases HETP. These terms are described in the Van Deemter equation (Equation 4-2) and can be used to produce a composite graph that relates the effect of linear velocity, or flow rate on Eddy diffusion, longitudinal diffusion and mass transfer and how it impacts on HETP.

$$HETP = A + \frac{B}{u} + Cu$$

Equation 4-2. Van Deemter equation describes the effect of Eddy diffusion, longitudinal diffusion and mass transfer on the efficiency of a peak.

4.1.2 *Traditional Reversed Phase Chromatography*

There are two modes of RPLC, namely isocratic and gradient. Isocratic involves using a single mobile phase composition, while a gradient involves the altering of %B; where %B refers to a component of the mobile phase, which is usually an

organic solvent that changes with time. Gradients are useful when separating chemicals with a wide range of polarities as increasing the strength of the organic solvent reduces the retention of highly non-polar analytes.

Chain length in RP columns influences retention; longer hydrocarbon chains such as the popular C_{18} have greater retention of non-polar analytes than their shorter chain counter parts. This is due to increased surface area, thus increased hydrophobicity. Therefore, retention is based predominantly on non-polar, hydrophobic interactions. Other 'secondary' mechanisms include polar interactions and ion-exchange. Polar interactions such hydrogen bonding and dipole-dipole interactions occur between polar functional groups of the analyte and residual silanol groups or polar end-capped groups, in this case, interactions are weak in comparison to non-polar interactions. Ion exchange occurs between deprotonated silanol groups. These groups can be end-capped in the column manufacturing process and entails neutralising the silanol groups with a counter ion or ligand [19]. These groups carry a negative charge and so only interfere with basic analytes. Elution is governed by solubility in water and length of molecular carbon chain; the shorter the acid chain the more water soluble, the less retention. Therefore, charged analytes, such as the organic acids studied tend to elute with the dead volume. Ion suppression of the acids can be used to increase retention by acidifying the mobile phase. Alternatively, ion pairing/ion interaction chromatography can be used to greater effect [20–22]. Both methods decrease solubility in water and increase retention. Acidifying neutralises the ion by addition of excess protons to the mobile phase while ion-pairing utilises the addition of, for example a quaternary amine that interacts with the anion. This neutralises the charge and increases hydrophobicity which allows for a stronger interaction with

the non-polar stationary phase. This increases retention time and can alter selectivity. Although this is highly dependent on the formation constant as some anions, especially dicarboxylic acids form weak ion-pairs or remain as neutral polar molecules [14].

4.1.3 *Ion Exclusion Chromatography*

In IEC, the functional group bound to the column carries the same charge as that of the analytes under investigation. They typically consist of divinylbenzene polymers functionalised with sulfonic acid groups. Sulfonic acid is a strong acid and therefore is completely ionised across the pH range, this is ideal for IEC since the like charge carried by the organic anions will be repelled by the sulfonic groups to differing extents and can-not enter the resin. Therefore, separations are based on exclusion rather than retention and can be loosely predicted by their dissociation constants [23]. Neutral and polar neutral molecules can enter the resin and so some reversed phase characteristics will be observed. This is especially true for long chain aliphatic acids and aromatic acids which are retained via reversed phase mechanisms such as pi-pi interaction between the aromatic moiety of the acid and the divinylbenzene support of the stationary phase resin [23]. This significantly increases retention times, though organic acids may be used sparingly to lower run times. Typically < 30 ACN and <5% MeOH are used as they tend to swell the polymer packing; ion pair reagents, especially metals and surfactants are strongly discouraged [23,24]. Metals, being cations will bind to sulphonic groups and form complexes, while surfactants will adsorb onto the polymer divinylbenzene resin. Both ultimately foul the column.

From the research carried out, the most popular mode of RPLC used for the separation of small, aliphatic and highly polar organic acids was ion exclusion chromatography (IEC). IEC has been used to separate organic acids found in dairy products [4,25,26], fruit juices [27], musts & wines [28], vinegars [29], berries [30] and uridine broths [31]. Typical mobile phases used to separate organic acids are sulphuric or phosphoric acid and optimised concentrations tend to be between 1 and 20 m*M*. These mobile phases may or may not contain organic solvents such as acetonitrile or methanol which can give some selectivity changes and shorter run times.

Few methods exist for the separation of short chain, highly polar organic acids using a non-modified, traditional RP C_{18} column or underivatised analytes. This is likely due to the mechanisms of retention on a C_{18} column which are largely driven by non-polar, hydrophobic interactions.

Gradient methods have been successfully developed by some authors to separate some of these analytes on a traditional C_{18} column with no modifications or additives (excluding solvents) [32]. Other authors have used ion-pairing/ion-interaction to separate acids on a C_{18} column [14]. Some carboxylic acids have a high affinity for metal cations and form complexes, thus increasing their retention and sensitivity. For example, dissolving an excess of copper sulphate in the mobile phase [14]. Other forms of ion pairing involve the use of quaternary amines [33].

Interestingly, a number of authors have dynamically modified traditional C_{18} columns by coating them with surfactants to create their own IEC columns [34–37], which shows that IEC is clearly the superior method of separating these particular analytes using a HPLC. Given the large body of work that has

implemented IEC to separate organic acids, this chapter will investigate the use of IEC columns as well as assessing the applicability of C_{18} for the separation of organic acids.

4.2 Materials and Methods

The first IEC experiment performed was based on a method previously published by Marsili *et al.* [25]. Following this, a separation of the 18 organic acids was attempted as no methods could be found in the literature at the time to separate the full suite of acids.

A gradient RP method for the separation of a smaller number of organic acids using a C_{18} column was also investigated. This method was intended to show orthogonality between a C_{18} and IEC separation of organic acids as well as a gas chromatographic separation of organic acids (**chapter 5**). The method was obtained as an application note form Perkin Elmer [32].

Further to this, an isocratic RPLC separation published by Nojavan *et al*. [38] was used to determine if the ion-pair dispersive liquid-liquid microextraction (IP-DLLME) of organic acids published in the same paper can be adapted to suit the needs of this project. Details of the IP-DLLME method can be found in the relevant section of the materials and methods, along with the details of a cloud point dispersive liquid-liquid microextraction (CP-DLLME) and its corresponding instrumental details, published by Daneshfar *et al.* [39].

The following chromatographic conditions have been separated into distinct sections to allow easier cross referencing between instrumental conditions, extraction protocols and extraction results for the analysis of organic acids.

4.2.1 *Instrumental*

4.2.1.1 Exploratory IEC Separation

The preliminary isocratic analysis was performed on a HP 1050 series HPLC fitted with a UV lamp operating at 220 nm. The mobile phase consisted of 9 m*M* sulphuric acid and had a flow rate of 0.7 mL/min. An Aminex HPX-87H (300 mm x 7.8 mm i.d. with 9 µm) strong cation exchange column was used and held at 60 ^{o}C with an injection volume of 25 µL.

4.2.1.2 Optimised IEC Separation

The optimised separation was carried out on an Agilent 1100 series HPLC instrument fitted with a G1322A degasser (serial number: JP63205744); G1312A binary pump (serial number: DE83102626); G1313A ALS auto sampler (serial number: DE54901170); G1316A heated column unit (serial number: DE53400716) and a G1314A VWD (serial number: JP55100842). Again, the Aminex HPX-87H column was used, it was thermostated at 60 ^{o}C for the main separation. The optimised mobile phase was 2 m*M* sulphuric acid with 10% ACN with a flow rate of 0.4 mL/min. Detection was achieved at 205 nm using an injection volume of 38 µL. De-ionised water was sourced from a Whitewater, Dublin DI unit fed by an ASTD Type 2 unit. It had a resistivity at 25 ^{o}C of ~ 18.2 MΩ/cm; conductivity: < 0.02 µS/cm and TOC: 0 ppb.

4.2.1.3 RP Gradient Separation

The optimised separation was carried out on an Agilent 1100 series HPLC instrument fitted with a G1322A degasser (serial number: JP63205744); G1312A binary pump (serial number: DE83102626); G1313A ALS auto sampler (serial

number: DE54901170); G1316A heated column unit (serial number: DE53400716) and a G1314A VWD (serial number: JP55100842). Mobile phase A consisted of 10 m*M* KH_2PO_4 adjusted to pH 2.40 with phosphoric acid. Mobile phase B consisted of ACN at 15%. The mobile phase was pumped through a HiChrom RPB, C_{18} (250 x 4.0 mm x 5 µm) at 1.5 mL/min. The gradient timetable is as follows: initial %B was 15% up to 60% B over 10 minutes. This was held at 60% B for 2.5 minutes then dropped down to 15% B over 12.5 minutes. This was then held for ten minutes to re-equilibrate the column. The column oven was thermostated at 30 ^oC with detection at 220 nm. An injection volume of 10 µL was used.

4.2.1.4 HPLC Instrumental Procedure used for IP-DLLME Investigation

Separation of organic acids was carried out on an Agilent 1100 series HPLC instrument fitted with a G1322A degasser (serial number: JP63205744); G1312A binary pump (serial number: DE83102626); G1313A ALS auto sampler (serial number: DE54901170); G1316A heated column unit (serial number: DE53400716) and a G1314A VWD (serial number: JP55100842). A 12 m*M* KH_2PO_4 buffer at pH 3.30 with 10% ACN was pumped through a Supelco Discovery C_{18} (150 x 4.6 mm x 5 µm) column at 1 mL/min. Detection was achieved at 280 nm with an injection volume of 20 µL.

4.2.1.5 HPLC Instrumental Procedure used for CP-DLLME Investigation

Separation of organic acids was carried out on an Agilent 1100 series HPLC instrument fitted with a G1322A degasser (serial number: JP63205744); G1312A binary pump (serial number: DE83102626); G1313A ALS auto sampler (serial number: DE54901170); G1316A heated column unit (serial number: DE53400716) and a G1314A VWD (serial number: JP55100842). A 12 m*M* KH_2PO_4 mobile

phase, adjusted to pH 4.25 containing 15% ACN was pumped through a Supelco Discovery C_{18} (150 x 4.6 mm x 5 µm) column that was thermostated at 25^{o}C at 1 mL/min. Detection achieved at 254 nm with an injection volume of 10 µL.

4.2.2 *Chemicals & Reagents*

4.2.2.1 IEC

Stock solutions for the exploratory investigation were made to 10 mg/mL in deionised water. Concentrations for individual standards and a standard mixture for exploratory work were as follows: citric acid: 1 mg/mL; lactic acid: 1.68 mg/mL; acetic acid: 0.88 mg/mL; propionic acid: 0.925 mg/mL and n-butyric acid: 1.23 mg/mL. All chemicals were purchased from Sigma-Aldrich, UK.

The optimisation process used stock reagents of malic acid, formic acid, acetic acid, lactic acid (85 % in H_2O), iso-butyric acid (all from Sigma) and pyruvic acid (TCI) made to 100 mg/mL by dissolving in deionised water. Stocks of hippuric, uric & orotic acid (TCI) and fumaric acid (Sigma) were made to 10 mg/mL by dissolving appropriate quantities in warm 500 m*M* NaOH due to their poor solubility in water. Stocks of trisodium citrate, oxalic, succinic, tartaric, propionic, n-butyric, n-valeric, and iso-valeric acid (Sigma) were also made to 10 mg/mL by dissolving appropriate quantities in deionised water. A 1M sulphuric acid (Sigma) solution was made from concentrate then diluted to make a 50 m*M* stock by adjusting to pH 1.30. This was then used to make all mobile phases at the appropriate concentrations. All stocks were kept in a 4 ^{o}C fridge for up to 2 months, a standard mixture and individual markers were then diluted to appropriate concentrations for analysis.

4.2.2.2 Gradient C_{18}

Stocks of acetic, iso/n-butyric, propionic and iso/n-valeric acids were made to a concentration of 10 mg/mL in water. These were diluted to the relevant concentrations found in later sections of this chapter.

4.2.2.3 CP-DLLME

For the CP-DLLME preliminary investigation stocks of hippuric and salicylic acid were made at 1000 µg /mL. Working standards and standard mixture were made to 0.5 µg /mL. A solution of Triton X-100 was made in deionised water to a concentration of 2%.

4.2.2.4 IP-DLLME

The IP-DLLME preliminary investigation stock of folic acid was made at 200 mg/mL and diluted down to a 2 mg/mL working standard. Cetyltrimethylammonium bromate (CTAB) was used as the IP due to its opposite charge. It was made at a concentration of 0.0015% and adjusted to pH 11.00 with 100 m*M* NaOH.

4.2.3 *Extraction protocols*

The extraction protocols were based on the work of Daneshfar [39] and Nojavan [38]. The work published by Daneshfar was based on the cloud point (CP) of surfactants and amalgamated CP with DLLME. Another was based on IP formation with polar analytes to increase hydrophobicity prior to DLLME and published by Nojavan.

Several parameters and reagents quoted in the original works were amended during experimentation. Reasons for these changes and their subsequent effects are

discussed in detail in the results section of this chapter. The purpose of these preliminary investigations was to check the performance of the extraction methods; to investigate the usefulness of these techniques for the extraction of the identified organic acids in milk and dairy produce; and to identify any factors that would require re-development to suit the needs of this project.

4.2.3.1 CP-DLLME via HPLC

10 mL of a 0.5 µg /mL standard mixture was placed in a 15-mL centrifuge tube and the pH was adjusted to pH 3.50 with 100 m*M* HCl. A mass of 0.4 g of NaCl was then added to the tube, the tube was then shaken until salt was dissolved. The centrifuge tube was then placed into a 45^{o}C water bath for 2 minutes, upon the 2-minute mark, 1 mL of a 2% TX-100 solution was rapidly injected into the centrifuge tube creating a turbid solution. This was then centrifuged at 3500 rpm for 5 minutes to further phase separation. The top aqueous phase was discarded. The bottom phase was removed and directly injected into the HPLC system [39].

4.2.3.2 IP-DLLME via HPLC

1 mL of a 2 mg/mL standard mixture was placed in a 15-mL centrifuge tube along with 9 mL of deionised water containing 0.015% CTAB at pH 11.00. A mixture of 100 µL octanol (extraction solvent) and 450 µL MeOH (disperser) was rapidly injected causing a cloudy solution to form. The tube was then centrifuged at 3500 rpm for five minutes and the organic phase take for HPLC analysis [38].

4.3 Results and Discussion

4.3.1 *IEC of organic acids*

The work began with an exploratory separation using the same resin based IEC column and work published by Marsili [25], that being an Aminex HPX-87H cation exchange column. The column had been standing for some time and required cleaning and regeneration. This consisted of flushing 5% ACN in 5 m*M* sulphuric acid for 4 hours, 30% ACN in 5 m*M* sulphuric acid for 12 hours followed by a regeneration fluid that consisted of 25 m*M* sulphuric acid for 16 hours through a reversed column at 0.2 mL/min and 65 ^{o}C [40]. Then ten injections of the standard mixture and markers were assessed for reproducibility. Tabulated results can be seen in Table 4-1, which show the %RSD for all analytes. ICH Guidelines states that %RSD must be < 2 %, the values obtained are < 1 %RSD which means that the results are acceptable and reproducible [41].

Table 4-1. Exploratory retention times for five organic acids with % RSD where n = 10. Conditions: 9 mM sulphuric acid mobile phase pumped through as Aminex HPX-87H column at 0.7 mL/min and thermostated to 60 ^{o}C with an injection volume of 25 µL and detections at 220 nm. All figures < 2%RSD as required by ICH Guidelines.

Injection number	***1***	***2***	***3***	***4***	***5***	***6***	***7***	***8***	***9***	***10***	**AVG (mins)**	**S.D (mins)**	**% RSD**
Citric acid	7.47	7.48	7.48	7.48	7.47	7.47	7.47	7.47	7.46	7.46	7.472	0.006	0.078
Lactic acid1	10.09	10.10	10.09	10.10	10.10	10.10	10.09	10.09	10.08	10.08	10.092	0.008	0.075
Lactic acid 2	10.56	10.58	10.	10.57	10.57	10.57	10.6	10.56	10.	10.	10.563	0.08	0.077
Lactic acid 3	11.30	11.31	1.31	11.31	11.31	11.31	11.30	11.30	11.29	11.30	11.303	0.006	0.053
Acetic acid	13.26	13.27	13.28	13.26	13.26	13.27	13.29	13.28	13.27	13.26	13.269	0.009	0.071
Propionic acid	15.56	15.57	15.56	15.58	15.58	15.56	15.58	15.57	15.56	15.56	15.567	0.010	0.062
n-butyric	19.02	19.02	19.05	19.04	19.02	19.03	19.05	19.03	19.01	19.01	19.029	0.16	0.086

All analytes were fully resolved and retention times were comparable to those in the Marsili paper. Lactic acid produced three peaks as in Marsili's paper, these were shown to be optical isomers of lactic acid [25], none of which interfered with the analysis. Based on these results a more in depth study was undertaken using extra organic acids.

4.3.2 *Optimisation of an IEC separation for organic acids - effect of mobile phase composition on selectivity, resolution and retention.*

Analyte retention in IEC is largely affected by column temperature and mobile phase pH. Both parameters alter the pk_a values of each analyte [23]. Optimisation of the sulphuric acid concentration in the mobile phase was the first logical step followed by temperature. Some analytes have more hydrophobic properties, therefore type and concentration of organic solvent in the mobile phase was used to enhance resolution and lower retention times. Organic solvents also lower the viscosity of the mobile phase which will lower the back pressure of the system. Since pH is temperature dependent this will have a large effect upon the degree of ionisation of some of the analytes, this will also aid in increasing or decreasing retention due the alteration of mobile phase viscosity. Given this, mobile phase concentration, column temperature and organic solvent were optimised.

4.3.3 *Sulphuric acid concentration*

The mobile phase composition was assessed by using the following parameters: 2 – 10 m*M* sulphuric acid with a flow rate of 0.7 mL/min, thermostated to 60 ^{o}C with an injection volume of 20 µL, results of which can be found in Figure 4-1. Since pH is related to concentration through, Equation 4-3 sulphuric acid concentration in the mobile phase can be used to manipulate the analytes ionisation state and

hence retention or the degree to which an ion is excluded from the stationary phase. Increasing pH values promotes ionization of analytes, while decreasing pH values will promote ion-suppression; which in turn allows the hydrophobic moiety of neutralised analytes to diffuse past the membrane thereby promoting an increase in retention via hydrophobic mechanisms.

$$pH = - \log [H^+]$$

Equation 4-3. Equation relating log of hydrogen ion concentration to pH

Later eluting analytes were separated at pH 2.00, though co-elution was seen between uric and acetic. At pH 4.00 most of the analytes began to slightly increase in retention and major selectivity effects are seen for orotic, malic, uric and fumaric acid, showing that they are most sensitive to the change in pH; this can be visualised in Figure 4-1 by the criss-crossing of data points for these analytes. Here there are several co-elution's between orotic/pyruvic/tartaric, and between fumaric/formic. At pH 6.00 there are further selectivity effects due the sensitivity of orotic, pyruvic, succinic, fumaric, acetic and uric to pH; co-elution was seen between pyruvic and uric. At pH 8.00 to pH 10.00 no more increase in retention was seen and co-elution between uric and fumaric was still evident.

Of the later eluting analytes, from propionic to valeric, no significant alteration in retention time was seen and no selectivity effects. Hippuric acid was the only late eluting acid to increase its retention. This can be attributed to the analytes much higher pk_a values which range from 4.60 to 4.87. Small changes in pH from 2 – 10 m*M* range from pH 2.00 - 2.70 and had no effect on their ionisation state as their pk_a is too high to be affected. Citric (pk_a 3.13, 4.76 & 6.40), tartaric (pk_a 3.03, 4.37),

lactic (pk_a 3.73) and formic (pk_a 3.74) are also unaffected by the changes in pH and retention remains constant.

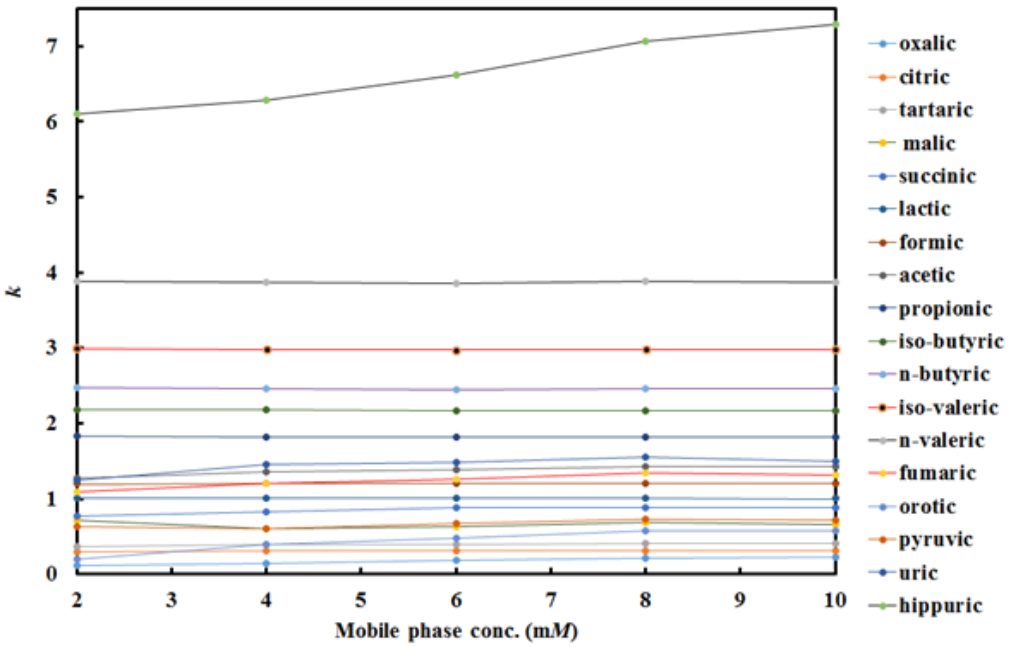

Figure 4-1. Retention factor (k) versus sulphuric acid concentration (mM) in the mobile phase. Later eluting analytes affected the least by changes in pH, while selectivity effects due to pH for early eluting analytes is significant. Conditions: sulphuric acid concentration varied from 2 – 10 mM and pumped through an Aminex HPX-87H column at 0.7 mL/min. Column thermostated at 60 °C, injection volume, 20 µL.

4.3.4 *Effect of organic solvent*

Manufacturer guidelines for this column state that a maximum of 30% acetonitrile and 5% methanol can be used before swelling the resin [40]. Therefore, the concentration range of 5, 10, 15, 20, 25 & 30% acetonitrile in a 2-m*M* sulphuric acid buffer was trialled at a flow rate of 0.7 mL/min and at 60 °C; the resulting chromatogram can be found in Figure 4-2. A plot of retention factor against ACN can be found in Figure 4-3, this serves as a visual aid to determine selectivity effects

associated with the alteration of solvent. The addition of 5% acetonitrile did not produce an improved chromatograph though the addition of 10% gave improved resolution between some of the co-eluting peaks along with a reduction in retention time. The addition of more than 10% increased the elution strength, which decreased reduced retention and decreased resolution. Any increase in acetonitrile after this had no benefit on resolution as many more analytes began co-eluting due to its elution strength. A dramatic decrease in retention time was observed for all analytes. Methanol was also trialled at 5%, this had no benefit to resolution. Following experimentation with organic solvents, the optimum concentration (10% ACN) still produced a number of co-eluting peaks.

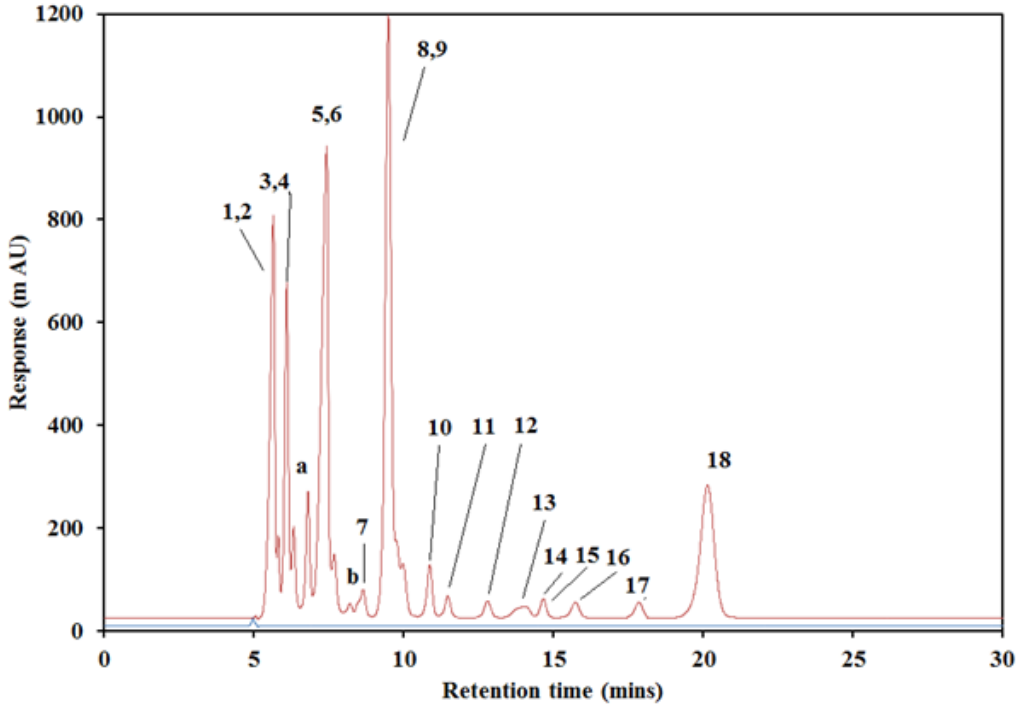

Figure 4-2. Blue: blank; red: standard mix. Conditions: 2 mM H_2SO_4, 10% ACN mobile phase with flow rate of 0.7 mL/min at 60 ^oC. Analyte identification: (1) uric; (2) oxalic; (3) orotic; (4) citric; (5) pyruvic; (6) malic; (7) succinic; (8) fumaric; (9) lactic; (10) formic; (11) acetic; (12) propionic; (13) iso-butyric; (14) n-butyric; (15) tartaric; (16) iso-valeric; (17) n-valeric; (18) hippuric acid; (a): contaminant from pyruvic (85% purity); (b): lactic isomer. The early eluting acids show unsatisfactory peak resolution. The reason for poor peak shape for iso-butyric acid is unknown.

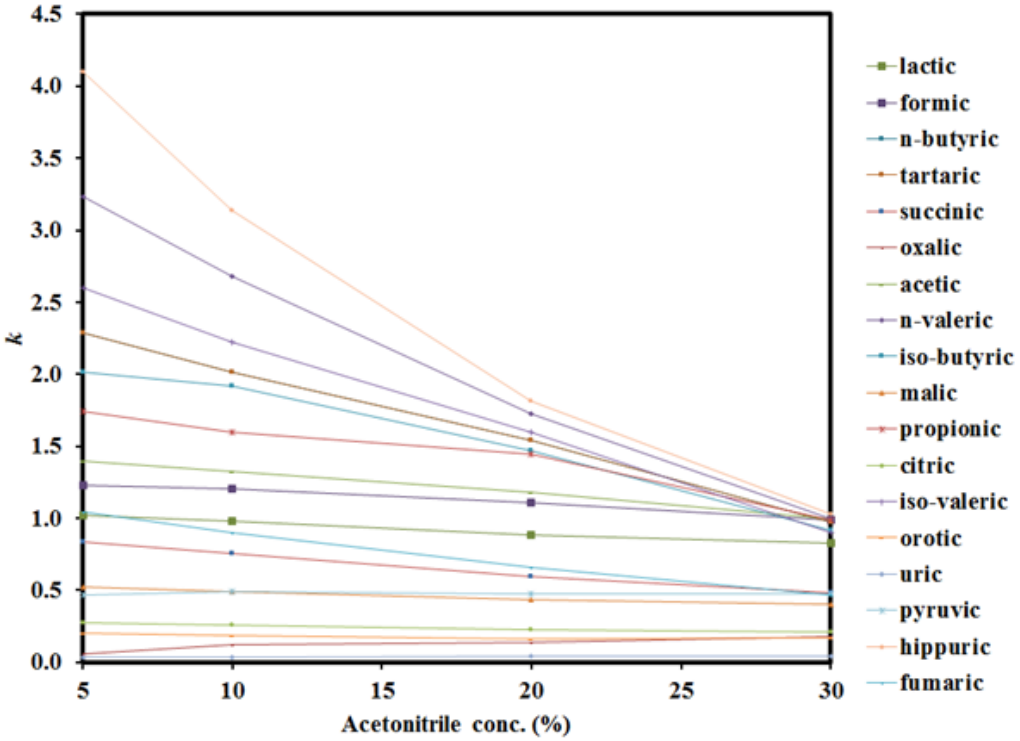

Figure 4-3. Plot of retention factor (k) versus ACN concentration (%). At concentration > 10% most analytes began to co-elute. Conditions: 2 mM H_2SO_4, mobile phase with flow rate of 0.7 mL/min at 60 ^oC.

No selectivity effects seen for the majority of the analytes, although retention times have been reduced dramatically for the more volatile organic acids such as propionic (< 43%), n and iso-butyric (< 55%), n and iso-valeric (< 64 %) and hippuric acid (<74 %). This was due to their much higher affinity for the less polar ACN which increased the elution strength of the mobile phase and therefore reduced the retention time of the analytes. These analytes have logP values ranging from (and in respective order): 0.34, 0.79, 1.02, 1.37 & 1.21. As explained in the introductory chapter, positive integers refer to hydrophobicity, the larger the number, the more hydrophobic the character, the greater the affinity for the organic

solvent. As can be seen, propionic, with its low logP of 0.34 saw a very small reduction in retention time when compared to that of the valeric and hippuric acids with their much higher logP values.

4.3.5 *Effect of column temperature*

It is evident from Figure 4-4 that there was little selectivity or retention changes for the majority of analytes due to altering the column temperature. What can be seen is a 32% decrease in the retention factor for hippuric acid which is significant due to its retention time at 20 ^{o}C. Other analytes that had a reduction in retention were the valeric and butyric species. Given this, the optimum temperature was found to be 60 ^{o}C.

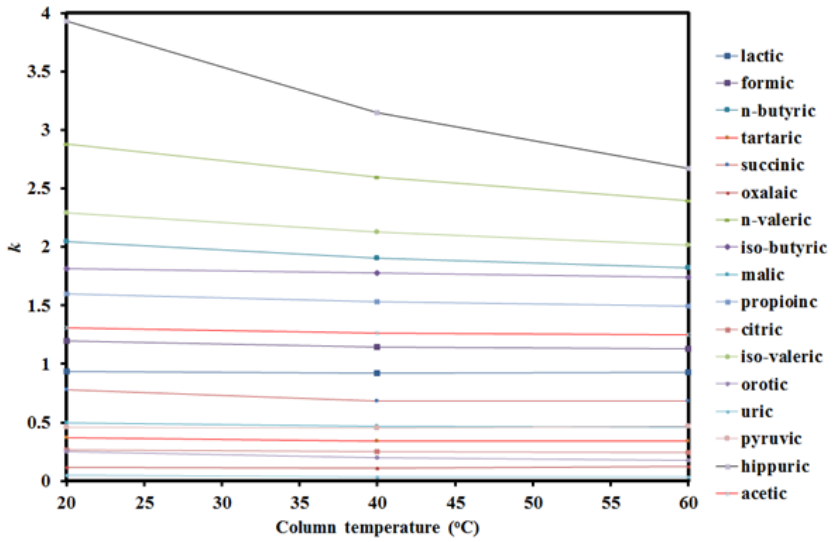

*Figure 4-4. Graph of retention factor (k) versus column temperature (^{o}C). The largest effects are seen with hippuric acid which has a substantial decrease in retention. Conditions: 2 mM H_2SO_4, 10% ACN mobile phase with flow rate of 0.7 mL/min. * Fumaric acid was removed due to contamination and was not replaced.*

4.3.6 *Effect of flow rate*

The effect of flow rate in traditional RPLC columns usually has no effect on selectivity but can affect efficiency because lower flow rates increase band

broadening due to Eddy diffusion. In comparison flow rate has a profound effect upon selectivity within IEC, though this comes at the expense of longer run times and broader peaks. Co-eluting analytes at higher flow rates can usually be separated at lower flow rates in IEC, since the analytes have more time to interact with the column packing which allows analyte bands to resolve as they interact with the polymer and sulphonic groups. The flow rate was assessed from 0.4 – 0.9 mL/min, with 0.4 mL/min producing the best results. The column manual did not advise to run separations below 0.4 mL/min [40]. The optimum flow rate was chosen by counting the number of resolved peaks. As can be seen in Figure 4-5 peaks 1 – 6 could not be baseline resolved with resolution between 0 and 1.18. Peaks 13 & 14 could not be baseline resolved with a value of 1.3. Peaks 4 & 5 and peak 9 & 10 co-eluted and could not be resolved. All other peaks gave a resolution > 1.5 as summarised in Table 4-2*2*. Un-resolved analytes were removed because they could not be quantified. This left a separation of seven organic acids (succinic, formic, acetic, propionic, iso & n-valeric and hippuric acids). Retention time, retention factor and resolution data for Figure 4-5can be found below in Table 4-2.

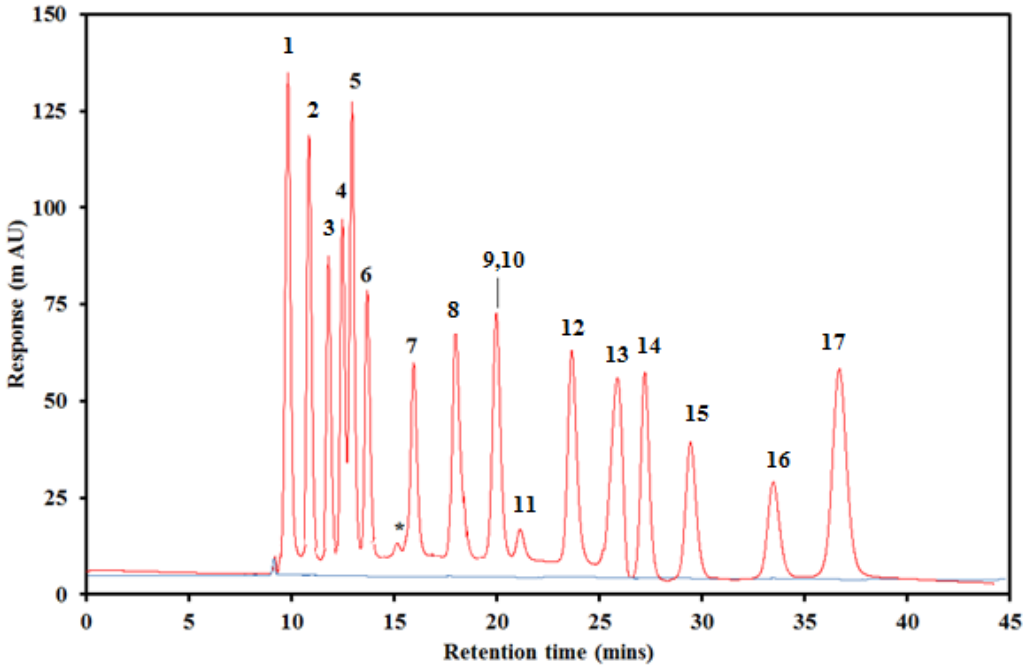

*Figure 4-5. Chromatogram obtained using a 2-mM sulphuric acid mobile phase with 10% ACN at 60 °C and a flow rate of 0.4 mL/min. Analyte I.D (mg/mL):1: oxalic (0.1); 2: orotic (0.02); 3: citric (0.4); 4: tartaric (0.3); 5: pyruvic (0.05); 6: malic (0.5); 7: succinic (0.5); 8: formic (0.5); 9,10: uric, lactic (0.01, 0.5); 11: acetic (0.8); 12: propionic (1); 13: iso-butyric (1); 14: n-butyric (1); 15: iso-valeric (1); 16: n-valeric (1); 17: hippuric acid (1); *: lactic acid isomer. Analytes 1 – 6 and 13 & 14 could not be baseline resolved and analytes 9 & 10 could not be separated, given this they were removed from development.*

Table 4-2. Retention time (mins), retention factor (k) and resolution for analytes in the above chromatogram in Figure 5 following optimisation of flow rates (t_o refers to the time of the solvent front which was 9.02 mins). N/A: not applicable; since co-eluting analytes (tartaric acid, pyruvic acid, uric acid, lactic acid and its isomer and iso/n- butyric acid) were removed the resolution between analytes to be removed and those that remain were not calculated. As an aid to understanding the resolution figures in the table, the resolution between oxalic acid and orotic acid was 1.18; resolution between orotic acid and citric acid was 1.01 and so forth.

	Oxalic acid	**Orotic acid**	**Citric acid**	**Tartaric acid**	**Pyruvic acid**	**Malic acid**	**Lactic acid isomer**	**Succinic acid**	**Formic acid**	**Uric acid and lactic acid**	**Acetic acid**	**Propionic acid**	**iso-butyric acid**	**n-butyric acid**	**iso-valeric acid**	**n-valeric acid**	**Hippuric acid**
Retention time (mins)	9.74	10.44	11.23	12.03	12.45	13.25	5.01	15.71	18.58	20.01	21.58	23.88	25.81	27.52	29.52	33.68	37.72
K	0.08	0.16	0.25	0.33	0.38	0.47	0.66	0.74	1.06	1.22	1.39	1.65	1.86	2.05	2.27	2.73	3.18
Resolution	1.18	1.01	N/A	0	N/A	N/A	N/A	1.8	N/A	N/A	1.8	N/A	0.5	N/A	2.4	1.9	N/A

4.3.7 *Optimisation of wavelength*

Many organic acids absorb UV radiation between 200 – 220 nm. The main issue here was that organic solvents such as ACN and MeOH also absorb in this region which can lead to a rising baseline. Using the HPLC wavelengths trialled to identify λ_{max} for each compound were 200 – 300 nm in 10 nm intervals. All co-eluting peaks were removed for this part of the study as resolution could not be further improved. The remaining analytes to be studied were: succinic, formic, acetic, propionic, iso-valeric, n-valeric and hippuric acids. λ_{max} was identified by using peak area as reference to measure intensity as demonstrated in Figure 4-6 & Figure 4-7 and summarised in Table 4-2. Since the absorbance of each analyte is either 200 or 210 nm, 205 nm was chosen as the optimum value

The most pronounced effects are seen on the peak areas of succinic and hippuric acid. The peak area of succinic rises significantly (850 – 1800) from 200 – 210nm, whilst the peak area of hippuric acids drops from 3600 to 1400 over the same 10 nm band width. Acetic acid is affected the least across the same 10 nm band width with a drop in peak area from 190 – 180. Formic and propionic acid have a drop in peak area from 1600 to 1570 from 200 – 120 nm. Both valeric species have a slight increase in peak area from 1360 – 1460 as wavelength increases from 200 – 210 nm. Above 210 nm all peak areas rapidly diminish, as expected, excluding hippuric acid, which increased up to 230 nm then then gradually decreased again.

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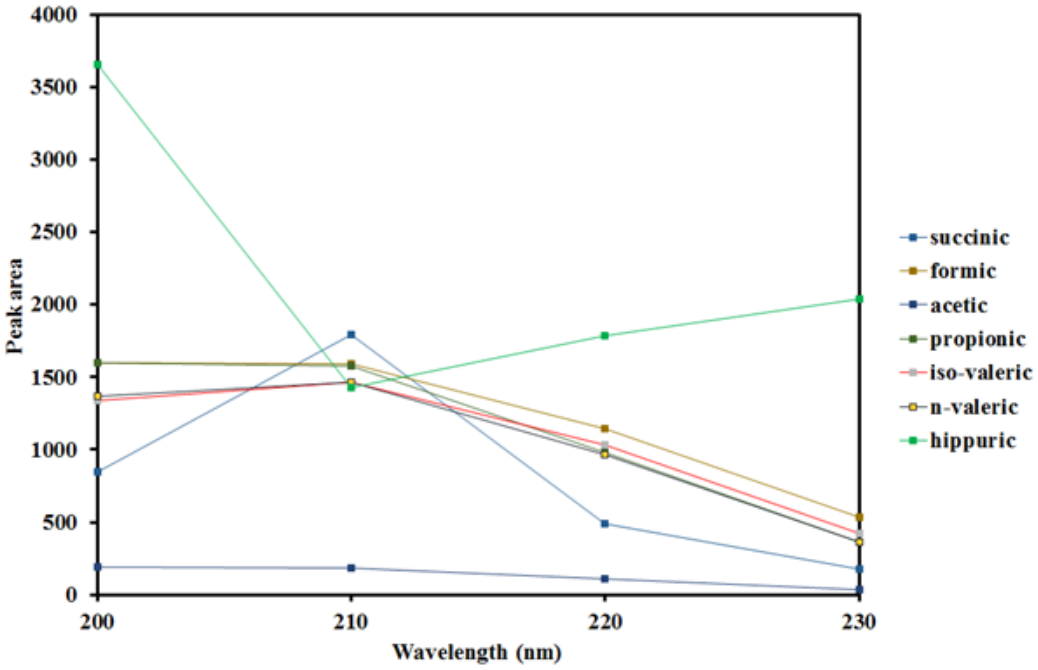

Figure 4-6. Plot of peak area versus wavelength. As wavelength increases, the general trend is a decrease in absorbance which manifests as a decrease in peak area. Analyte concentrations as per method and materials section.

Table 4-3. Summary of analyte absorbance's. Obtained using a 2 mM H_2SO_4, 10% ACN mobile phase with flow rate of 0.4 mL/min at 60 ^oC.

	Detection wavelength (nm)	**Concentration (mg/mL)**
Succinic acid	210	0.5
Formic acid	200	0.5
Acetic acid	200	0.8
Propionic acid	200	1
iso-valeric acid	210	1
n-valeric acid	210	1
Hippuric acid	200	1

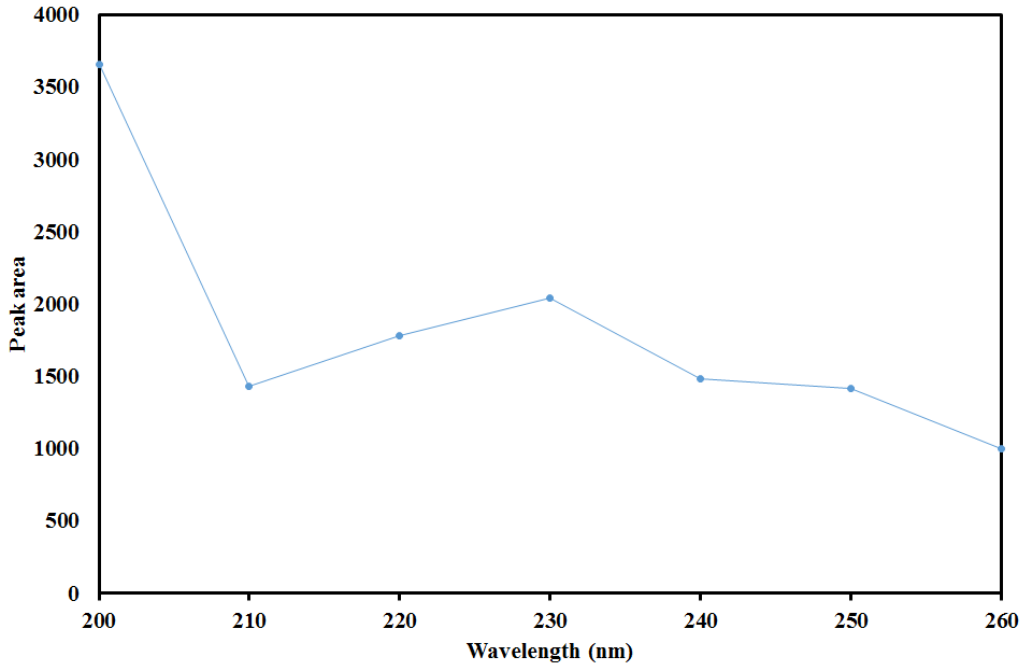

Figure 4-7. Extended absorbance plot for hippuric acid. This was extended to confirm that absorbance decreased after 230 nm.

The Agilent 1100 series HPLC available was fitted with a variable wavelength detector (VWD). Given this a variety of wavelengths can be used to give the optimum absorbance for each. It was thought that wavelength switching could initially be used, though technical issues with the VWD and time meant that only a single wavelength of, 205 nm was chosen for this analysis. This was chosen as it is the central point between the two optimum wavelengths of 200 & 210 nm.

4.3.8 *Optimization of injection volume*

A graph of theoretical plates (efficiency) on the y-axis and peak height along the secondary vertical axis against injection volume can be used to obtain the optimum injection volume. As the volume injected increases, peak height increases. This will eventually coincide with a decrease in efficiency. The area where both data

sets cross corresponds to the optimum injection volume for that specific analyte. Please see the appendix for the graphs used to evaluate this section of work.

Since the optimised injection volumes varied, it was decided to take the average and use that as the injection volume. The optimum as per the above data was 38 µL. All injection volumes are summarised in Figure 4-8.

As injection volume increases, the analyte band travelling through the column also increases as does the time required for the analyte band to pass the detector. This increases the width of each peak which has a detrimental effect upon efficiency and resolution. Resolution is defined as an instruments ability to differentiate between the apex of two eluting peaks and has a minimum value of 1.5 (unless using shorter rapid resolution columns), while the number of theoretical plates must be > 2000. Data obtained show that the values obtained were equal to or greater than outlined criteria and are summarised below in Table 4-5.

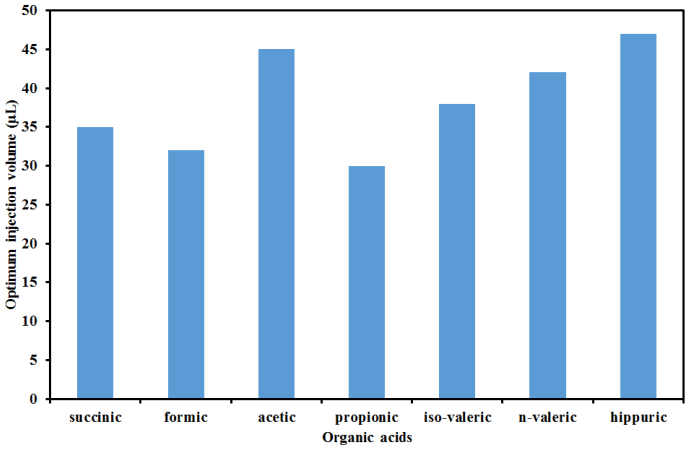

Figure 4-8. Optimum injection volumes of all organic acids, average volume: 38 µL. Conditions: 2 mM H_2SO_4, 10% ACN mobile phase with flow rate of 0.4 mL/min at 60 °C.

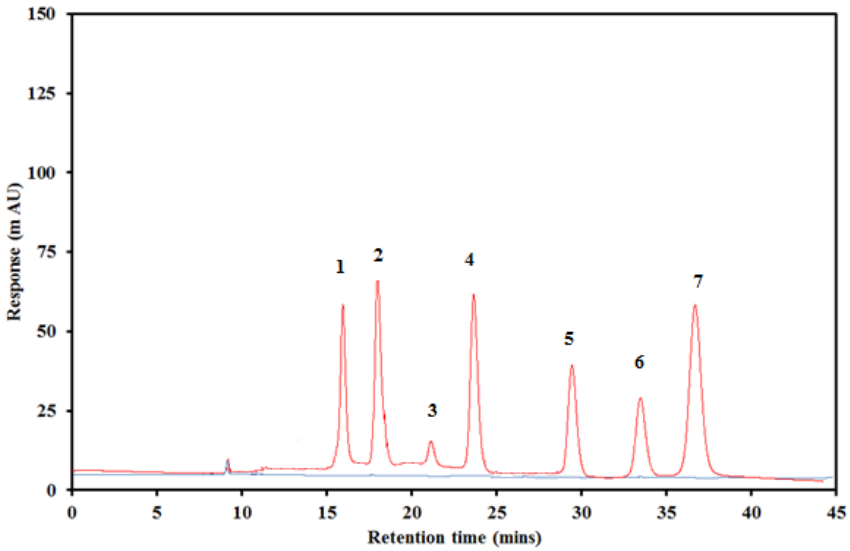

Figure 4-9. The final optimised chromatogram. Standard mix: red, blank: blue. Analyte I.D (mg/mL): 1: succinic (0.5); 2: formic (0.5); 3: acetic (0.8); 4: propionic (1); 5: iso-valeric (1); 6: n-valeric (1) and 7: hippuric acid (1). Conditions: 2 mM H_2SO_4, 10% ACN mobile phase with flow rate of 0.4 mL/min at 60 °C using an injection volume of 38 µL and detection at 205nm.

The final optimised chromatogram of organic acids in Figure 4-9 had a long run time of 45 minutes and the first elution did not occur until ~ 15 minutes. Given this, further optimisation could be possible to lower the run time by the addition of more

ACN to the mobile phase. Doing this has the potential to alter selectivity and produce more co-eluting peaks. Further to this, an increase in flow rate can also achieve shorter run times, though again, this will alter selectivity and could produce more co-eluting peaks. Given the added problems that can occur by altering these two parameters, the optimised work was validated as is. As summarised in Table 4-5, the resolution between each analyte was > 1.5 and the theoretical plate count > 2000; all criteria were met. Please see section **4.5** for validation outcomes of the IEC work.

Table 4-4 Performance characteristics of the optimised separation (optimum conditions: 2 mM H_2SO_4, 10% ACN mobile phase with flow rate of 0.4 mL/min at 60 ^oC using an injection volume of 38 µL and detection at 205nm). Resolution was > 1.5 between remaining analytes with the largest resolution between the solvent front (t_o) and the first analyte (succinic acid), the number of theoretical plates were also > 2000 which means that the development criteria as per ICH guidelines was met

	Retention time (mins)	*k*	Resolution	Theoretical plates	Peak area
t_o	9.02	N/A	4.3	N/A	N/A
Succinic acid	15.71	0.74	1.8	14071	1890
Formic acid	18.58	1.06	2.4	13234	2993
Acetic acid	21.58	1.39	1.8	16556	166
Propionic acid	23.88	1.65	3.4	84574	2767
iso-valeric acid	29.52	2.27	2.4	16413	2394
n-valeric acid	33.68	2.73	1.9	15689	1644
Hippuric acid	37.72	3.18	N/A	12981	3489

4.4 Gradient separation of organic acids using a C_{18} column

The gradient method used was published as an application note on the PerkinElmer website and was developed by Reuter [32]. The method was originally designed for the analysis of 11 organic acids: lactic, acetic, propionic, n/iso-butyric, n/iso-valeric, methylvaleric, hexanoic, heptanoic and octanoic acid. As a starting point, and to evaluate the utility of the C_{18} separation, only the key organic acids were

used at the outset for the preliminary development, those being acetic, propionic and n/iso-valeric.

Reuter used a PerkinElmer Brownlee Aqueous column (250 x 4.6 mm x 5 µm). Several column equivalence charts were consulted to find an equivalent column, though PerkinElmer/Brownlee columns were not registered on any of the sites used. Given this a HiChrom RPB C_{18} column (250 x 4.0 mm x 5 µm) was chosen from the stores.

4.4.1 *Troubleshooting*

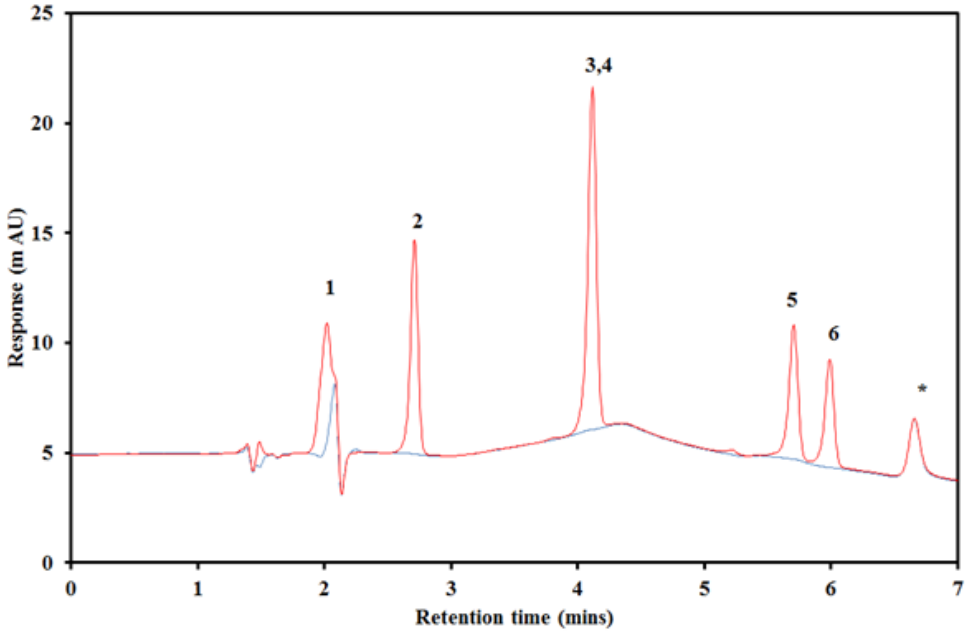

*Figure 4-10. Blue: blank (water), red: standard mix (0.25 mg/mL each in water). Peak I.D: 1: acetic acid; 2: propionic acid; 3/4: iso/n-butyric; 5: iso-valeric; 6: n-valeric acid; *: unknown. Acetic elutes on a system peak, iso/n-butyric could not be separated.*

As can be seen in *Figure 4-10* acetic acid elutes on a system peak which also distorts the tail end of the peak. To exclude contamination, fresh stocks and standards were made (and filtered) in clean volumetric flasks and vials, yet the peak persisted in each blank and standard. A ghost run was assessed and as can be seen

in *Figure 4-11* there was no peak around the two-minute mark. This suggests that the source of the problem must be with the injector or sample matrix, which in this case was water. The ghost run (no injection blank), is clean in this area. Since the peak labelled with the asterisk is present in the ghost run, this can-not come from the matrix or an analyte. One possible problem is that the peak co-eluting with acetic likely comes from the sample matrix and the peak labelled with the asterisk is due to either mobile phase impurities focusing at the head of the column or a highly retained compound from previous experiments held in the column which elutes under eluotropic changes in the gradient. To eliminate matrix issues, deionised water from a variety of sources was filtered and analysed but all results were the same and the peak remained.

The column had been used by numerous analysts over the years and many compounds passed through. To eliminate the possibility of these peaks being artefacts from previous work, the column was cleaned as per column manufacturer's web site. It states that HiChrom RPB columns should be cleaned with 10 – 20 column volumes of mobile phase, MeOH, ACN, ACN/IPA (75:25), IPA, DCM, hexane, IPA, mobile phase.

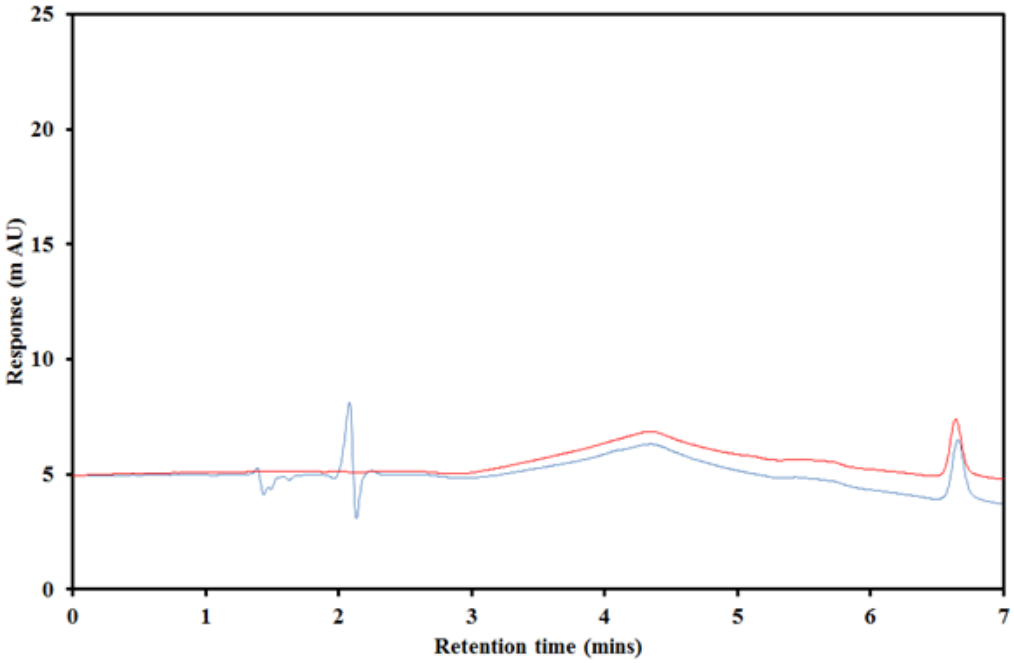

Figure 4-11. Blue: water blank, red: ghost run. Peaks could be contaminants introduced from the mobile phase, matrix (water) or a dirty column.

The cleaned column introduced more unknown peaks into the chromatogram (Figure 4-12) at around 6.2 and 6.5 minutes. These new contaminant peaks interfered with more analytes, specifically n-valeric (Figure 13) which further reduces the applicability of this separation, at least with this column, to the work and its use as an orthogonal separation.

Unfortunately, the issue could not be resolved due to time constraint, therefore, further studies into the C_{18} gradient method were terminated.

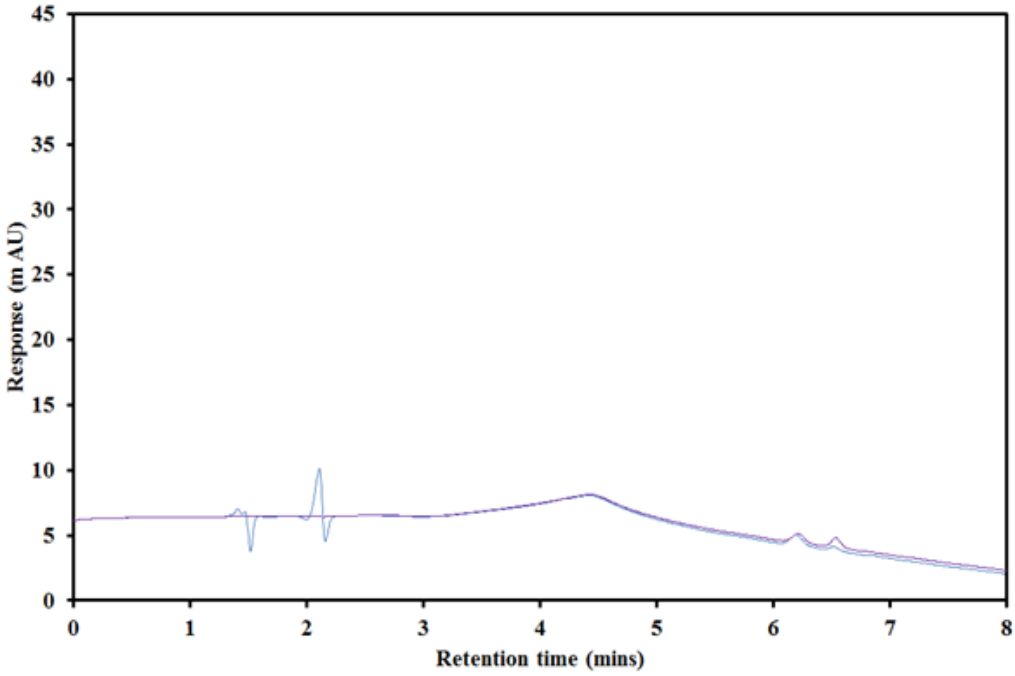

Figure 4-12. Overlay of water blank (blue) with a no injection blank/ghost run (purple) following the column cleaning schedule. As can be seen two new contaminants appear after the 6-minute mark.

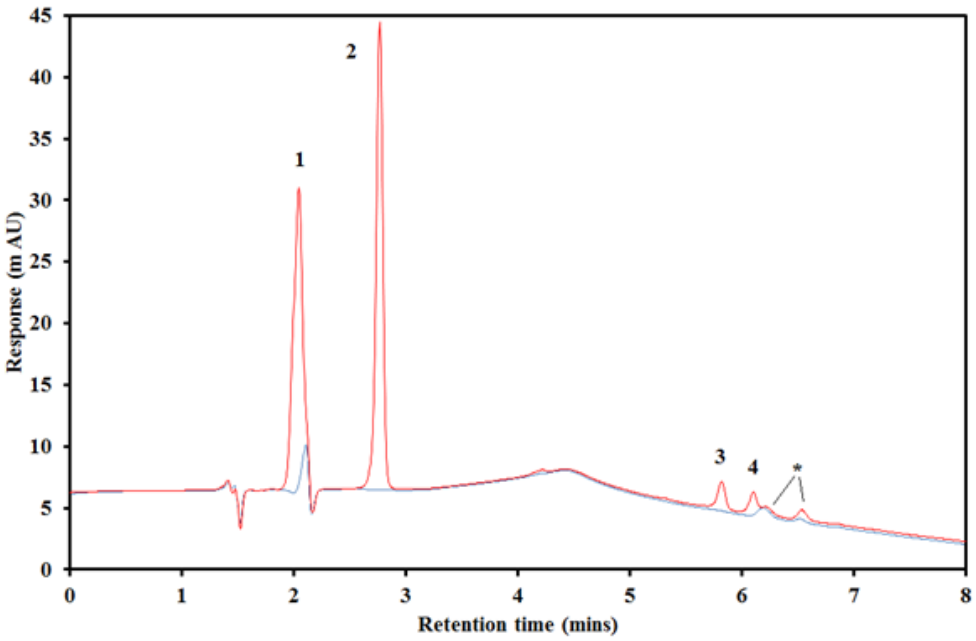

*Figure 4-13. Overlay of water blank (blue) with a standard mixture (red). Analyte I.D: 1: acetic; 2: propionic; 3: iso-valeric; 4: n-valeric; *: unknown contaminants.*

4.5 Validation

Analytical methodology should be validated using ICH Q2(R1) guidelines [41]. It is a process whereby the performance characteristics of an analytical method are established by means of laboratory studies. The output of the validation process is statistically sound methodology. This section focuses on the validation of the IEC separation of seven organic acids on the Aminex column only. The following criteria must be met: retention times must ≤ 2% RSD, have an injection precision that is ≤ 1% RSD (n=5) and have a theoretical plate count ≥ 2000.

4.5.1 *Repeatability*

Retention time repeatability is assessed using a minimum of nine determinations across the range of concentrations specified. This takes the form of triplicate injections spanning three concentration ranges; lowest (25%), middle (100%) and highest (200%) concentrations used. Alternatively, this can be done by using a

minimum of six determinations using the highest concentration of the range. The following work used triplicate injections spanning three concentration ranges to calculate retention time and retention factor (*k*) repeatability; all were < 2% RSD as per ICH guidelines. Raw retention time data (n=9) used to calculate retention factors and %RSDs for the IEC separation can be found in Table 4-5, results are summarised in Table 4-7.

Injector precision was also assessed by injecting the 100% standard five times. All were below the 1% RSD which demonstrates that the injector working correctly; results are summarised in Table 4-6.

Standard deviation (SD) was calculated in excel, the equation for SD is given by:

$$SD = \sqrt{\frac{\sum(x - \bar{x})^2}{n - 1}}$$

Equation 4-4. Equation used to calculate standard deviation of retention times and peak areas

Finally, %RSD, which was also calculated in excel, the equation for %RSD is:

$$\%\text{RSD} = \frac{SD}{average} \text{ X } 100$$

Equation 4-5. Equation used to calculate %RSD

Table 4-5 Retention time data used to calculate retention factors and %RSD from average retention times (mins), where n=9 over three levels (25, 100 & 200%). Solvent front (t_0) was 9.02 mins.

	Succinic acid	**Formic acid**	**Acetic acid**	**Propionic acid**	**iso-valeric acid**	**n-valeric acid**	**Hippuric acid**
$t_{r\,1}$ (mins)	15.76	18.61	21.63	23.96	29.5	33.63	37.99
$t_{r\,2}$ (mins)	15.77	18.61	21.65	2.97	29.51	33.64	37.00
$t_{r\,3}$ (mins)	15.77	18.62	21.65	3.98	29.52	33.65	37.02
$t_{r\,4}$ (mins)	15.75	18.61	21.63	23.95	29.96	33.57	37.89
$t_{r\,5}$ (mins)	15.78	18.63	21.65	23.97	29.5	33.61	37.96
$t_{r\,6}$ (mins)	578	18.63	21.65	23.98	29.5	33.61	37.95
$t_{r\,7}$ (mins)	5.59	18.49	2146	23.7	29.4	33.81	37.9
$t_{r\,8}$ (mins)	15.6	18.5	21.47	23.7	29.38	33.84	37.92
$t_{r\,9}$ (mins)	15.59	18.49	21.42	23.67	29.37	33.79	37.6
K	0.08	1.06	1.39	1.65	2.27	2.73	3.18
AVG (mins)	15.71	18.58	21.58	23.88	29.52	33.68	37.72
SD (mins)	0.09	0.06	0.09	0.1	0.17	0.1	0.4
%RSD	0.006	0.003	0.005	0.006	0.006	0.003	0.011

Table 4-6. Injection repeatability where n=5 at the 100% level. All results are below the 1%RSD threshold.

	Retention time (mins)	*k*	Resolution	Theoretical plates	Peak area
t_o	9.02	N/A	4.3	N/A	N/A
Succinic acid	15.71	0.74	1.8	14071	1890
Formic acid	18.58	1.06	2.4	13234	2993
Acetic acid	21.58	1.39	1.8	16556	166
Propionic acid	23.88	1.65	3.4	84574	2767
iso-valeric acid	29.52	2.27	2.4	16413	2394
n-valeric acid	33.68	2.73	1.9	15689	1644
Hippuric acid	37.72	3.18	N/A	12981	3489

4.5.2 *Linear range*

Linearity refers to the ability of a method to obtain results proportional to concentration within the specified range of concentrations. This is achieved using a minimum of five concentration ranges to obtain an output consisting of a linear regression equation in the form of y = mx + c and a correlation coefficient (R^2). The correlation coefficient must fall in the range of 0.9 – 1.1 to be considered linear [42].

Figure 7-8 Figure 7-14 in **7.2** of the **Appendix** hold all calibration curves constructed for use with the IEC method, each concentration range was injected in

triplicate. Excellent correlation coefficients of 1 were achieved following serial dilution of a stock organic acids mixture. Calibration curves were constructed by making a standard mixture at the 200% concentration range and diluting to 25, 50, 75, 100, 150%, were 100% is the expected concentration found from literature sources [1,4,43]. Results are summarised in Table 4-7.

4.5.3 *Limit of detection (LOD)*

LOD's are based on the signal to noise ratio, which is the ratio between an analytes peak height and the noise of the baseline. The LOD is reached when the signal is three times that of the noise. LOD's can be estimated using Equation 4-6 as supplied in the ICH Validation of Analytical Procedures, Q2 (R1) [41]:

$$LOD = \frac{3.3\sigma}{slope}$$

Equation 4-6. Equation used to calculate LOD

where σ is the standard deviation of the response and the slope corresponds to the m value from an analytes calibration curve. As stated in the ICH guidelines LOD's can be calculated as: '*The residual standard deviation of a regression line or the standard deviation of y-intercepts of regression*'. Given this, LOD's were calculated in excel using the regression modelling function in the Data Analysis ToolPak to calculate standard error of the y-intercepts. Standard error differs from standard deviation as it estimates the precision of a specific parameter as opposed to the scatter of the data. In this case the parameter under investigation is the mean average of peak areas (y-values). Estimated values given in Table 4-7, raw data used to calculate LODs along with a sample calculation can be found in **7.2** of the **Appendix**.

4.5.4 *Limit of quantitation (LOQ)*

LOQ's are also based on the signal to noise ratio. The LOQ is the lowest limit that an analytes concentration can be confidently and accurately calculated. The LOQ is reached when the signal is ten times that of the noise. It can be estimated via Equation 4-7 which is also defined in ICH Q2(R1) [41].

$$LOQ = \frac{10\sigma}{slope}$$

Equation 4-7. Equation used to calculate LOQ

It is acceptable for LOQ values to match the values estimated for LODs, though they may not be below the LOD [44]. Estimated values given in Table 4-7, raw data used to calculate LOQs along with a sample calculation can be found in section **7.2** of the appendix.

Table 4-7. Characteristics from method validation of IEC method where n=9. Measurements taken from low, middle and high concentration values (25, 100 & 200%) for all but %RSD of peak area where only the highest concentration value was used. All %RSD values are below the 2% required by ICH guidelines.

	%RSD of k	**%RSD of peak area**	**Regression equation**	**R^2**	**Range (µg/mL)**	**LOQ (µg/mL)**	**LOD (µg/mL)**
Succinic acid	0.006	0.74	y = 1.2702x - 0.0369	1	20-600	4.90	1.61
Formic acid	0.003	0.11	y = 3.1767x - 2.0462	1	16-500	1.74	0.57
Acetic acid	0.005	0.45	y = 0.1301x - 0.1734	1	100-3000	15.77	5.20
Propionic acid	0.006	1.49	y = 1.13188x – 2.008	1	33 - 1000	5.51	1.82
iso-valeric acid	0.006	0.83	y = 1.645x - 7.0064	1	33-1000	7.95	2.62
n-valeric acid	0.003	0.07	y = 1.2232x - 2.7701	1	33-1000	6.89	2.27
Hippuric acid	0.011	1.09	y = 89.731x - 1.8807	1	0.66-20	0.12	0.04

4.6 Discussion surrounding the preliminary results of organic acid extractions using variants of DLLME.

4.6.1 *CP-DLLME of Organic Acids*

The first extraction method researched was cloud point dispersive liquid-liquid microextraction (CP-DLLME). The CP-DLLME extraction was based on the work of Daneshfar *et al.* [45]. There are several reasons why this study was chosen as a starting point. Firstly, this was the only known amalgamation of CP with DLLME and therefore was highly novel. Secondly, surfactants are environmentally benign [46] which means this extraction will comply with the principles of sustainable development and green chemistry [47] as the majority of DLLME procedures require the use of small volumes of organic solvents. These volumes are typically $\leq$ 100 µL and whilst they are vastly more environmentally friendly, they do not fully comply with the principles of sustainable development and green chemistry. A chromatogram of the preliminary work can be found in Figure 4-14, while a summary of the results can be found in *Table 4-8*.

Table 4-8. Experimental CP-DLLME results showing that experimentation could only obtain EFs of 2.7 and 2.2 in comparison to the literature values of 41 30 respectively.

	Original concentration (µg /mL)	**Enriched concentration (µg /mL)**	**Enrichment factor**
Hippuric acid	0.5	1.34	2.7
Salicylic acid	0.5	1.1	2.2

The original concentration of hippuric and salicylic acids was 0.5 μg /mL, this value was chosen as it corresponded to the LOD of the method. The final, enriched hippuric and salicylic acid concentration found by calibration curve were 1.34 μg /mL and 1.1 μg /mL respectively which are close to the LOQ calculated from the calibration curves in Figure 4-15. This equates to an enrichment of 2.7 and 2.2 for hippuric and salicylic respectively. In comparison, literature EFs obtained by Daneshfar were higher at 41 and 30.

One amendment and one oversight was made to the extraction procedure. The amendment was the use of TX-100 and not the quoted TX-114. This will impact negatively since the carbon chain of the surfactant varies in length. TX-100 has a shorter carbon chain which equates to a lowering of hydrophobicity which leads to less attraction between polar-neutralised analyte and non-polar micelles. The oversight was the temperature used to induce CP; the temperature used was the quoted CP temperature of TX-114 (45 ^{o}C) and not that of TX-100 (65 ^{o}C). None the less the extraction was successful in that the analytes were enriched, and under sub-optimal conditions, with hippuric acid almost three times more concentrated and salicylic acid twice as concentrated. There is clear scope to improve EFs by further optimising the length of time and temperature used to heat the tube.

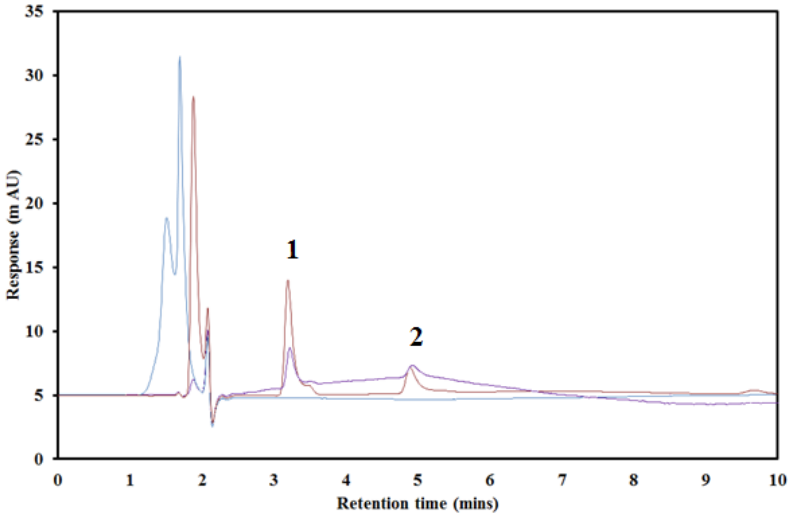

Figure 4-14. HPLC chromatogram of CP-DLLME using a C_{18} column. Hippuric acid (1) and salicylic acid (2) mixture at LOD concentrations against an enriched standard mixture. Blue: blank; purple: 0.5 µg /mL standard mixture; red: 0.5 µg/mL standard mixture following CP-DLLME.

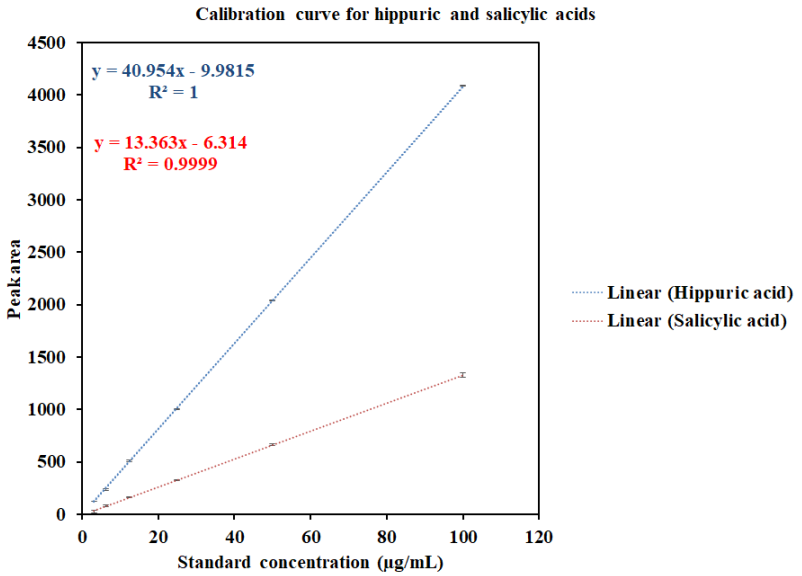

Figure 4-15. Calibration curve for hippuric acid (blue) and salicylic acid (red). A range of 3.1 – 100 µg/mL for each standard gave LOQs of 1.36 & 1.90 µg/mL and LODs of 0.47 & 0.52 µg/mL, respectively and R^2 values > 0.99.

To the authors knowledge, and time of writing, only one paper existed that combined the cloud point properties of surfactants with DLLME and this was seen as a highly novel approach [39]. However, this paper used a traditional C_{18} column that is compatible with the use of non-polar surfactants. Given the chemistry of the IEC column that the separation was developed on, it was quickly realised that passing surfactants through this column would not be an option since the surfactant will stick to the resin resulting in a fowled column. Methods such as back extraction of the organic acids from the surfactant via microwave back-extraction or ultrasonication [48] were explored in the hope of retaining the analytes in a solvent compatible with the column prior to injection, however, time, cost and feasibility became an issue and so this method of extraction could not be fully explored.

Other problematic factors to marrying DLLME with IEC were the nature of the analytes. Most of the analytes identified in the introductory chapter were small and highly polar and so will not solubilise well into many organic solvents. To compensate for this, other areas were researched such as ion-pairing with quaternary amines to increase hydrophobicity.

4.6.2 *IP-DLLME of Organic Acids*

The rationale behind the trial of an IP-DLLME method was that most of the analytes chosen are short chain organic acids. These acids are highly polar and therefore may not partition well into the surfactant or organic solvents via traditional means such as neutralisation via pH manipulation. Addition of an IP reagent can aid in lowering their solubility in the aqueous phase thus increasing their lipophilicity and the probability of greater partitioning into the hydrophobic surfactant micelles or organic solvents.

The IP-DLLME extraction was based on the work of Nojavan [38]. The method required adaptation for two reasons. The original method used a surfactant called aliquat-336 at a concentration of 0.01% in water as their IP reagent. This was not available and so CTAB was used at a concentration of 0.015%. The percentage of 0.015% was chosen and calculated from CTAB's critical micelle concentration (CMC), which is 0.9 m*M*. Any concentrations used must be below the CMC to ensure ion pairs are formed and not micelles. During Nojavan's optimisation process, several IP reagents were assessed for their effects upon EFs. Nojavan did note that CTAB gave one of the least acceptable results. Conversely, aliquat gave the most superior, which is why that reagent was used. Aliquat's superiority is most likely due to its stability in IP formation and its hydrophobicity, which gives superior partitioning of IP-analyte into octanol. Another slight adjustment was made to the volume of extraction solvent (octanol) used. The quoted value was 60 µL, however this did not produce two layers that could be easily distinguished, thus separated; therefore, the volume was increased to 100 µL. An original concentration of 2 mg/mL gave an enriched concentration of 41.59 mg/mL, this equates to an experimental EF of 21. In comparison the literature EF for folic acid is ~6 times greater at 135. The increase of extraction volume has two unfortunate disadvantages. Firstly, changing the experimentally optimised ratio of extracting solvent to dispersing solvent will have a negative effect upon enrichment into the droplets as optimum dispersion of extracting solvent throughout the aqueous sample is not achieved. Secondly, a larger volume of extracting solvent will equate to a diluting of the extract; this will lower EFs. A calibration curve was constructed (Figure 4-17) using standards in the range of 1.5 – 50 mg/mL, this gave and LOD

and LOQ of 0.16 and 0.54 mg/mL respectively and R^2 values > 0.99; the resulting chromatogram can be found below in Figure 4-16.

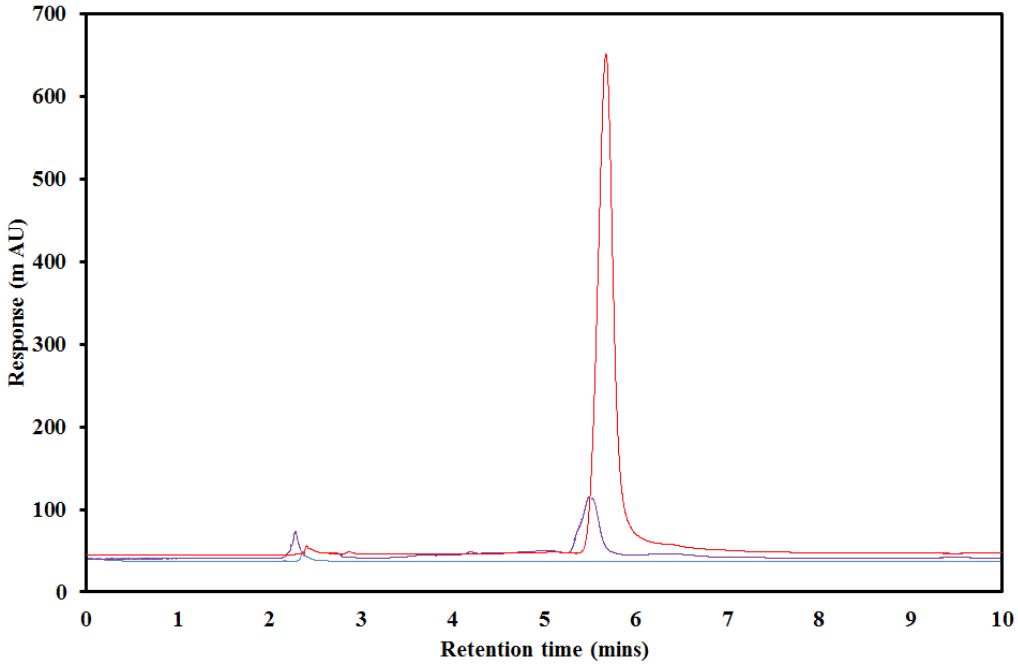

Figure 4-16. HPLC chromatogram using a traditional C_{18} column. Chromatogram shows an IP-DLLME of an enriched folic acid standard (red) against a non-enriched folic acid standard (purples) for visual comparison of peak size. Blue: blank; purple: 2 mg/mL folic acid standard; red: IP-DLLM of a 2 mg/mL folic acid standard. Peak size has significantly increased following IP-DLLME and yielded an EF of 21, in comparison the literature EF was 135.

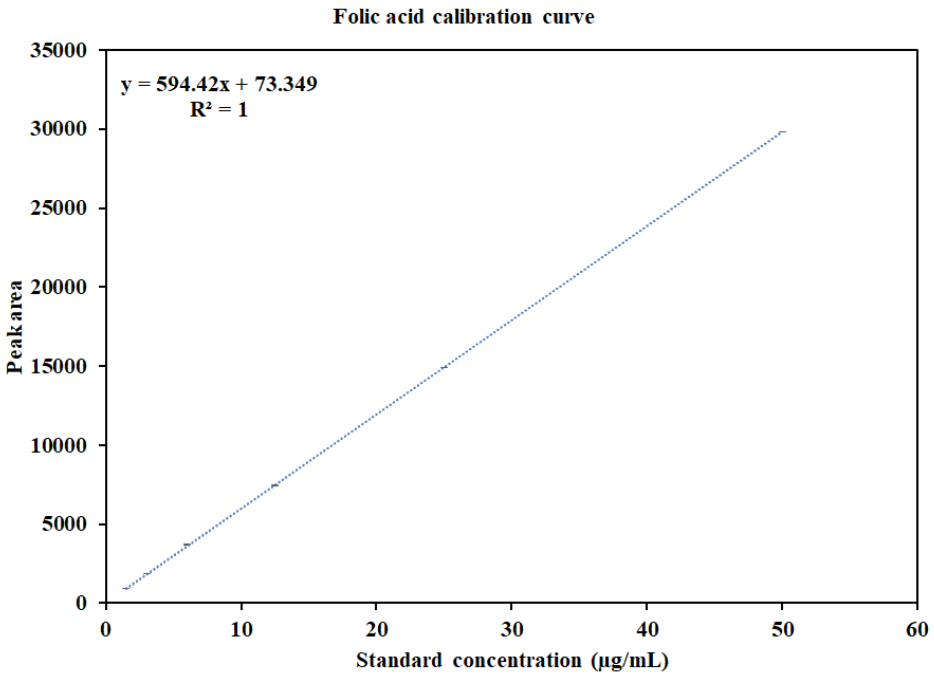

Figure 4-17. Folic acid standard curve using a linear range of 1.5 – 50 mg/mL. R^2 values >0.99 gave an LOD of 0.16 mg/mL and LOQ of 0.54 mg/mL.

Again, the issue of compatibility of solvents and surfactants with the IEC column were foreseen. To mitigate this it was thought that evaporating the solvent containing the analytes from the DLLME process and reconstituting the analytes in water would marry the extraction with the IEC separation. Unfortunately, the volatility of the analytes led them to evaporate along with the solvent. Following this, an attempt was made to remove the analytes from the organic solvent via a miniature liquid-liquid extraction with water following the IP-DLLME process. However, at this stage the IEC column had been fowled beyond repair due to small volumes of surfactant and organic solvents from previous experiments and so it is unknown whether this method would have worked. Further to this, another optimisation step would also have been required to find the optimum volume of water needed to extract in to. This extra step would also not be desirable as it increases analysis time, complexity and the introduction of errors.

The possibility of creating an IEC column by modifying a C_{18} column was explored to finish this section of work but due to the time constraints of validating another method it was decided not to go ahead.

4.6.3 *Reversal of DLLME*

To circumvent the issue of extracting into organic solvents an unorthodox approach to DLLME was experimented with which entailed reversing the DLLME process.

Reversing the DLLME process included rapidly injecting a mixture of MeOH (disperser) and water (extractor) containing the analytes into octanol. MeOH was chosen as the analytes were soluble in this solvent. MeOH is also soluble in both water and octanol, while water is soluble in MeOH but not octanol. The added benefits of using MeOH here were that it can also be used to remove proteins and lipids from dairy produce. This means that the analytes will be extracted into water. The initial extractions appeared positive, but were ultimately not repeatable. Another significant downfall was the volume of octanol used per extraction (5 mL), which meant significant waste of organic solvent which is not in keeping with the theme of producing greener extraction techniques.

4.7 Conclusion

Of the 18 organic acids commonly found in either milk or dairy produce, seven were separated via an optimised isocratic IEC method.

The IEC method successfully separated succinic, formic, acetic, propionic, iso-valeric, n-valeric and hippuric acid. LODs were: 1.74, 15.77, 5.51, 7.95, 6.89 and 0.12 µg/mL, respectively, while LOQs were: 0.57, 5.20, 1.82, 2.62, 2.27 and 0.04

µg/mL, respectively. Resolution was > 1.5 for all analytes, retention factors and peak areas all had %RSD < 2%RSD and correlation coefficients > 0.99.

Two variations of DLLME were identified and evaluated for their applicability in the extraction of organic acids from milk and dairy produce. A CP-DLLME method was trialled and gave experimental enrichment factors of 2.7 and 2.2 for hippuric acid and salicylic acid, respectively. Literature values were quoted as 41 and 29, therefore the literature values are 15 and 13 times larger than those achieved [39]. Different Triton series surfactants, with vastly different chemistries and properties (hydrophobicity and CP temperature) were used and thought to be the main factor in this. Attempts to remove the surfactant via microwave or ultrasonic back extraction did not yield satisfactory results.

An IP-DLLME method was also trialled, yielding an experimental EF of 21, despite having to alter several factors known to effect EFs such as type of surfactant, volume of extraction solvent and ratio of extractant to dispersing solvent [38]. Numerous attempts to separate organic acids from incompatible organic solvents resulted in a fouled ion exclusion column. It should be noted that these solvents are compatible with traditional C_{18} columns and could be an area for future research.

Ultimately several factors such as nature of analytes and reagents, column chemistry, time and cost of replacing the column became issues that could not be overcome. To the author's knowledge, the success of this method would have produced the first DLLME of analytes that could be separated on any ion exclusion column.

Given the nature and chemistry of the IEC column and analytes it was decided that moving forward and combining CP-DLLME with an IP-DLLME method would not be practical. Since removal of organic acids from organic solvents was not possible in this instance, gas chromatography was seen as the most complimentary technique suitable to DLLME as solvents can be directly injected into the instrument.

4.8 References

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5. Dispersive liquid-liquid microextraction and chromatographic identification of organic acids in milk and dairy produce via gas chromatography with flame ionisation detection

5.1 Introduction

It has now become necessary to provide detailed traceability of the origin of food as well as chemical composition, nutritional value and bioactivity with careful monitoring of the whole process. The findings must then be conveyed on labelling, and label claims must be monitored by both the manufacturer and independent laboratories. This ensures that regulations are upheld and that foods and beverages entering the food chain are of high quality and fit for consumption by humans and animals.

A number of instrumental methods have been employed for the analysis of organic acids in foods and beverages, such as: ion exclusion chromatography (IEC) [1–10], capillary electrophoresis (CE) [11–15] and gas chromatography (GC) [16–19]**.**

Organic acids appear in food and beverages due to hydrolysis of fats, addition of acidulants to improve taste and regulate shelf life, bovine biochemical metabolic processes, bacterial growth and adulteration [1,6,20]. Organic acids provide flavour and nutritional value to foods, although they are often the cause of spoilage as they degrade or are metabolised by bacteria. Profiling the organic acid content allows food processing laboratories to correlate individual organic acids with particular tastes and flavours. Organic acid are also added to enhance specific flavours and render the product more palatable which will then drive up consumer sales [21]. Much of this has been covered in greater depth in the introductory chapter.

5.1.1 *Gas Chromatography*

Gas chromatography is concerned with the separation of analytes in gaseous form. Since the work involves gases, analytes must be volatile or have the ability to be volatised through derivatisation. GC has been used to great effect for the separation of organic acids found in foods and beverages [17,22–25], given this, there was no requirement in the current work to develop a new method.

5.1.2 *Basic Principles of GC*

Analytes should be thermally labile and, as a rule of thumb have a molecular weight equal to or below 400 g/mol. Samples are injected into the inlet where they are rapidly heated and volatilised into their gaseous form. Due to the high efficiency of GC only a small amount of sample is required and so the function of the inlet is to allow a representative portion of the injected sample to be swept onto the column by the carrier gas. Analysis begins at temperatures far lower than that of the most volatile analyte. This focuses the analytes at the head of the column. A gradual rise in temperature then allows the more volatile analytes to vaporise first and be carried by the inert, gaseous mobile phase onto the column. Whilst on the column, analytes partition themselves and separate based on their relative vapour pressure and/or their affinity for the stationary phase and finally elute into the detector, in the case of this work a flame ionisation detector [26].

5.1.3 *Flame Ionisation Detector (FID)*

The carrier gas is made up of hydrogen and compressed air and carries the analytes directly into the detectors flame from the capillary column via the jet tip as depicted in *Figure 5-1*. The flame generates carbocations through combustion, these are then propelled toward the cathode due to a potential difference. Sensitivity is directly

proportional to the number of carbon atoms contained within the analytes, meaning, as a homologous series is traversed, sensitivity increases with chain length [27]. Ions are collected at the cathode which produces a small current which is amplified and manifests as a peak on the chromatogram [27]. This information is important since the following work is in relation to short chain organic acids. Given this, sensitivity was expected to be poorer for analytes such as acetic and propionic acid. An increase in sensitivity can be achieved by either derivatising the analyte, which will take more time and solvent as well as increase error or by enriching the analyte in a pre-concentration technique such as DLLME.

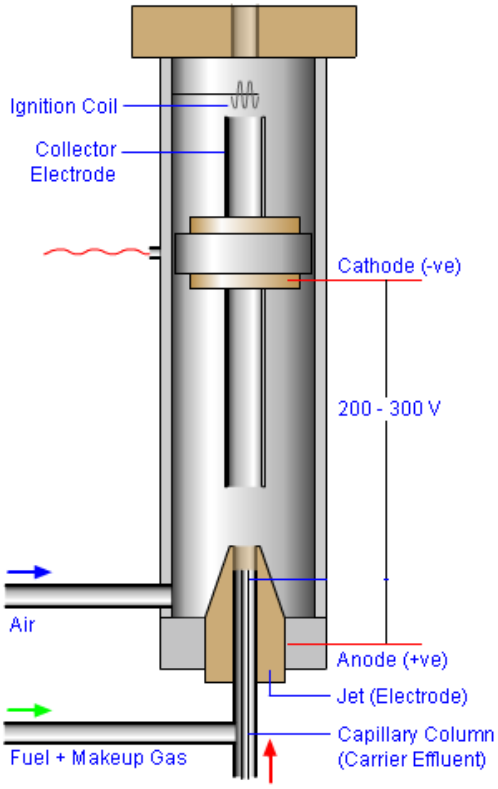


Figure 5-1. Schematic of a flame ionisation detector [27]

5.1.4 *Retention*

Retention is dependent on each analytes vapour pressure and the sum of all possible polar and non-polar interactions within the column. The strength of each varies with the type of functional group(s) present and the length of the chain. Separations occur as analytes diffuse into and out of the stationary phase, this action equates to one theoretical plate. One of the most common bonded phases is a polyethylene glycol (PEG) coating that consist of 5% diphenyl and 95% silicondimethyl. The bonded phase, also known as the stationary phase is bound to a fused silica capillary which is covered with a polyimide coating. The coating imparts strength and flexibility to the fused silica capillary, this type of column is known as a wall coated open tubular (WCOT) capillary column. Below is a cross section of a WCOT capillary (*Figure 5-2*).

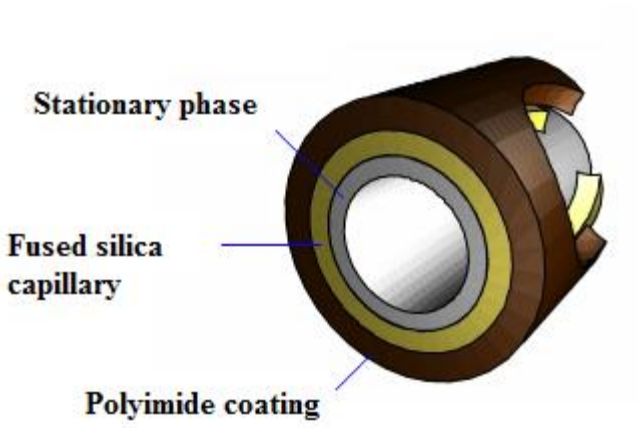


Figure 5-2. Cross section of a WCOT capillary GC column. Stationary phases are coated onto the inside of a fused silica capillary. Polyimide coatings are then applied to the outside of the fused silica capillary [28].

Molecular interaction with a PEG column are divided into three categories, hydrogen bonding, dispersive interactions and dipole interactions. A PEG column with a phenyl moiety (*Figure 5-3*) has strong dispersion forces, weak dipole interactions and no hydrogen bonding [28]. Increasing the phenyl content increases the polarity of the column (becomes less non-polar) due to π-electrons in the structure which will in turn increase the retention of polar molecules through dispersion forces. Dipole interactions are also important since the organic acids contain permanent dipoles and phenyl groups are polarisable. It is important to note that these interactions occur together and should not be thought of as singular, individual events.

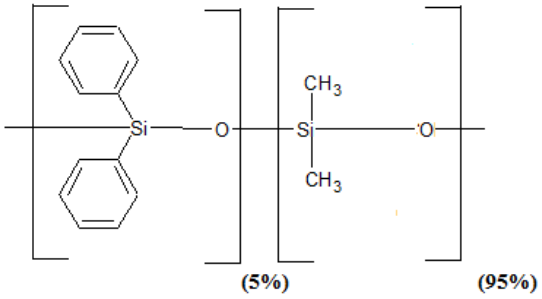


Figure 5-3. Example structure of a PEG stationary phase. The stationary phase is bound to the inside of a fused silica capillary and typically contains 5% diphenyl and 95% silicondimethyl groups. These ratios can be altered. Increasing the aromatic moiety increases the polarity of the column.

Since the volatile acids are polar they interact strongly through a mixture of dispersion and dipole interactions with the column stationary phase. This hinders the analytes ability to diffuse quickly back into the carrier gas. This is the main cause of band broadening or tailing in GC. This mismatch manifests as a slight tail on the peak and is unavoidable without specialised stationary phases [29].

5.2 Experimental

5.2.1 *GC Instrumental procedure*

Organic acids were separated on an Agilent 6890 GC system fitted with a FID and an Alltech AT-100 polyethyleneglycol (PEG) column (15 m x 530 µm i.d. x 1.2 µm) was used for the organic acid analysis; the column had a void time of 1.418 minutes. A gradient temperature program method was used for the separation of six volatile organic acids found in dairy produce. The method was based on an Agilent application note and is as follows: oven set at 100 ^{o}C and held for 5 minutes

→ 200 ºC at 10 ºC/min then held for 10 minutes with an injection volume of 1 µL and a split ration of 1:10 [30]. The injector inlet was thermostated to 250 ºC and the FID detector at 300 ºC.

Other equipment used include a Whitewater (Dublin) de-ionised water unit that was fed by an ASTD type two unit. The de-ionised water had a resistivity at 25 ºC of 18.2 MΩ/cm; conductivity of < 0.02 µS/cm and TOC: < 30 ppb.

5.2.2 *Reagents*

Stock standards of acetic, propionic, iso-butyric, n-butyric, iso-valeric & n-valeric were made at 10 000 µg /mL each in acetone (all from Sigma-Aldrich) for the separation and calibration curve, water was used for the DLLME optimisation. These were then diluted to 100 µg /mL with water to optimise the DLLME process. Other chemicals used were NaCl, potassiumhexacyanoferrate trihydrate (also known as Carrez 1, 15 g in 100 mL water), zinc sulphate heptahydrate (also known as Carrez 2, 30 g & 3 mL sulphuric acid in 100 mL water), chloroform and acetone.

5.2.3 *Sample Pre-treatment*

Proteins were removed from milk-based samples via use of two Carrez solutions, Carrez 1 and Carrez 2. Five mL milk-based sample was placed in a 15-mL centrifuge tube along with 8-mL 100 m*M* NaOH (standards used during spiking were added at this point), then shaken for one minute. 1 mL of Carrez 1 and 2 were then added to low fat samples and 1.5 mL added to high fat samples along with 1.5 mL of 500 m*M* sulphuric acid*. This was then vigorously shaken for one minute to distribute through the entire solution before centrifugation at 4500 rpm for 10 mins. Centrifugation produces a thick, white, semi-solid protein precipitate at the bottom of the tube and 10 mL supernatant. This supernatant has a pH of ~ 1.70 (n=

3), and was then filtered through a 0.45 μm PTFE syringe filter into a clean 15 mL centrifuge tube where the DLLME process took place. Blanks were made by substituting 5 mL sample for 5 mL of deionised water and subjected to the same pre-treatment and extraction protocols as the samples.

*For high fat produce it was beneficial to increase the volume of Carrez solutions 1 & 2 to 1.5 mL to remove excess lipids.

5.2.4 *Extraction protocols*

The optimised DLLME extraction protocol is based on the work of Fazeli-Bakhatari [31]. The volume of extraction solvent was altered during the investigation from 40 μL to 100 μL due to no visible organic layer. This is fully discussed in the relevant results section of this chapter.

5.2.5 *Investigational DLLME method*

A 10-mL sample containing analytes at 100 μg /mL was placed in a 15-mL centrifuge tube. 1 mL of acetone (disperser) containing 200 μL of chloroform (extractor) was then rapidly injected. The resulting emulsion was then centrifuged at 4500 rpm for 6 minutes. The organic phase was then directly injected into the GC using an injection volume of 1 μL.

5.2.6 *Optimised DLLME method*

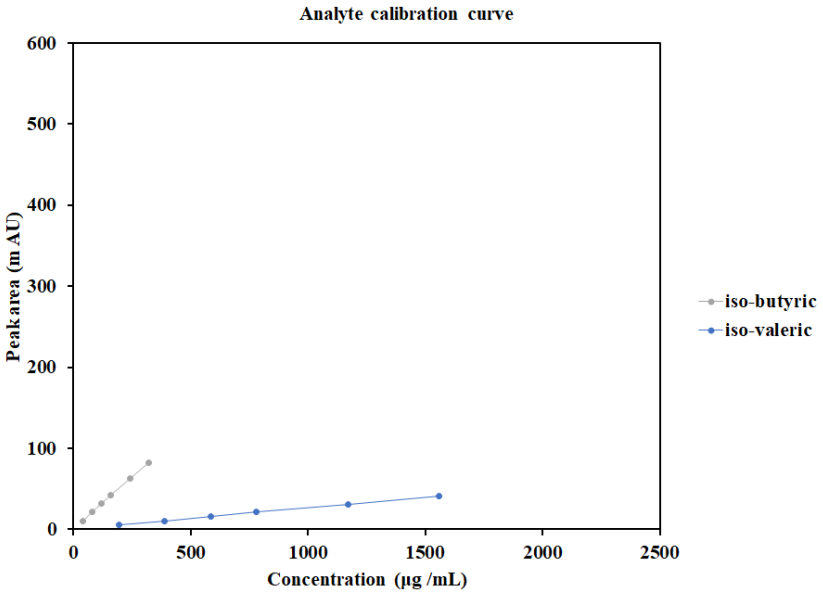
2 g NaCl was added to 10 mL of the supernatant obtained from the pre-treatment process in **5.2.3** to aid in 'salting out' the analytes. A mixture of 700 μL acetone and 100 μL chloroform was then rapidly injected through a narrow bore syringe to maximise the 'spray' of the droplets. This turbid solution was then centrifuged for

5 minutes at 4500 rpm. The supernatant was discarded and the enriched solvent injected to the GC system.

Note: The concentration of all standards used during the DLLME optimisation process had an original concentration of 100 µg/mL.

5.3 Results and discussion

An appropriate DLLME procedure for the extraction of organic acids and subsequent analysis via GC was identified from the literature [30]. Preliminary results (*Table 5-1*), chromatograms (*Figure 5-4*) and calibration curve (



) for the investigatory extraction procedure are detailed below. Following this is the discussion and results surrounding the optimisation of the parameters identified in **Chapter 2** to enable the extraction to be used for the needs of this project. Those needs were, to extract organic acids from milk and dairy produce.

5.3.1 *Preliminary DLLME investigation*

This DLLME procedure was based on the work of Fazeli-Bakhtiyari [31]. The extraction was slightly modified as the quoted volume of extraction solvent (60 µL

of chloroform) again did not produce a distinct phase separation that could be accurately removed. Increasing the extraction solvent will have a detrimental effect upon final EFs due to dilution effects. Fazeli-Bakhitiyari method was optimised for the extraction of valproic acid from human serum. Valproic acid was not available. Therefore, the extraction was trialled using six, similar organic acid standards. The chosen acids were acetic, propionic, iso-butyric, n-butyric, iso-valeric and n-valeric acid. These acids were chosen as they are volatile, thus no derivatisation was required; a readily available GC separation of these organic acids had previously been successfully trialled, as well as the reasons given in the introduction, those being their links with change in taste and quality of milk and dairy produce.

A satisfactory result was obtained in that a method optimised to extract only valproic acid (an eight-carbon molecule (logP: 3.00), had extracted two of the six organic acids chosen. Iso-valeric (logP: 1.21) and n-valeric (logP: 1.37) were extracted well and enriched to four and five times their original concentration, respectively. These were likely the best performing analytes due to their more hydrophobic nature. Propionic acid (logP: 0.33), iso and n-butyric (logP: 1.02 and 0.79 respectively) were poorly extracted and not enriched. The chromatogram in *Figure 5-4* is that of a 100 µg/mL standard mixture enriched via DLLME (red) and a non-enriched 500 µg/mL standard mixture (green) which serves as a visual comparison of peak size. As can be seen, the intensity of the propanoic acid signal is significantly reduced, as are those for both butyric species following the investigational DLLME procedure. It was expected that optimisation of this DLLME procedure could potentially extract all six acids since the current method is suboptimal for the vastly more polar acids chosen, as only valproic acid was the subject of Fazeli-Bakhitiyari work. Further to this, a number of factors known to

effect the results of DLLME were not used, such as addition of salt and alteration of pH.

Table 5-1. Preliminary DLLME enrichment factors of organic acids. Acetic and propionic were not quantifiable as they were below LOD; NQ: not quantifiable.

	Original concentration (µg /mL)	**Enriched concentration (µg /mL)**
Acetic acid	100	NQ
Propionic acid	100	NQ
iso-butyric acid	100	7.65
n-butyric acid	100	8.39
iso-valeric acid	100	407.14
n-valeric acid	100	547.48

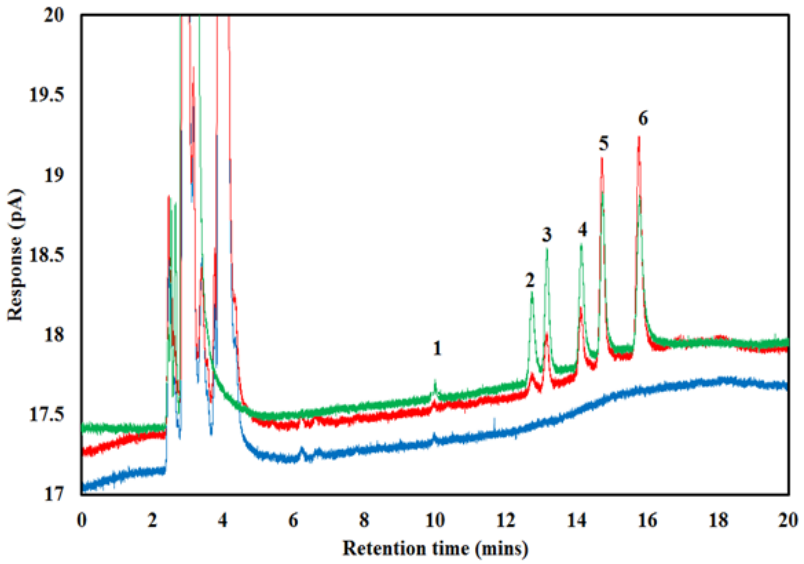


Figure 5-4: GC chromatogram of organic acids following DLLME. Blue: blank; red: DLLME of 100 µg /mL standard mixture; green: 500 µg /mL non-enriched standard mixture for comparison. Peak I.D.: 1: acetic; 2: propionic; 3: iso-butyric; 4: n-butyric; 5: iso-valeric; 6:n-valeric.

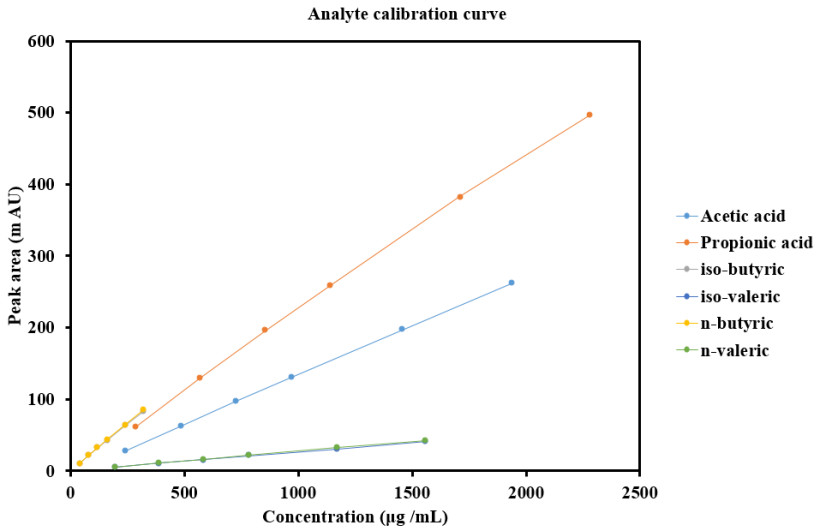


Figure 5-5. Calibration curves for all six organic acids. Linear range and R^2 values were: acetic: 242 – 1940 µg /mL, R^2: 0.9999; propionic: 285 – 2280 µg /mL, R^2: 0.9991; iso-butyric: 40 – 320 µg /mL, R^2: 0.9993; n-butyric: 40 – 320 µg /mL, R^2:0.9995; iso-valeric: 195 – 1560 µg /mL, R^2: 0.9993 and n-valeric: 195 – 1560 µg /mL, R^2: 0.9998.

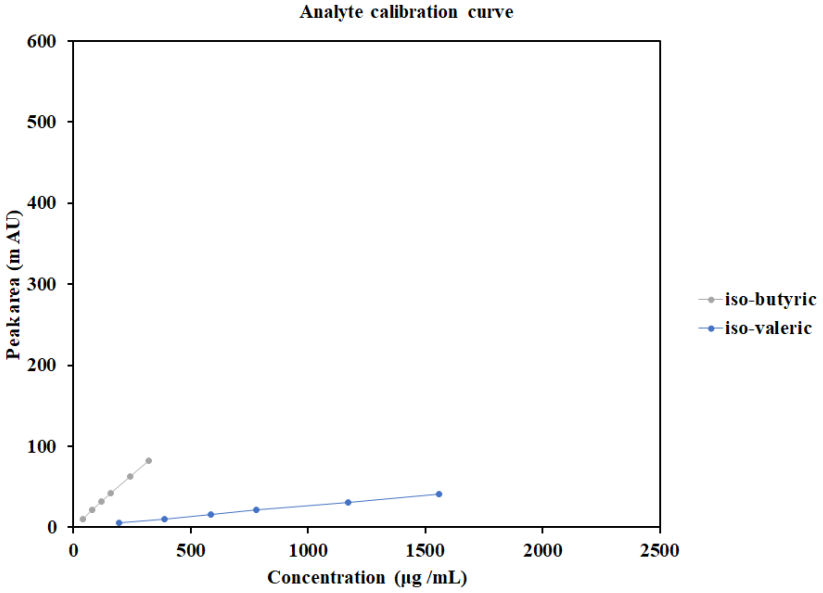


Figure 5-6 Both 'iso' species have the same linearity and range as their 'n' isomers and therefore have been masked, the bottom plot holds these two as clarification.

5.3.2 *Optimisation of the investigatory DLLME process for the extraction of organic acids*

The following DLLME factors were identified for optimisation: extraction solvent type and volume, disperser solvent type and volume, pH, salt concentration, sonication time and centrifugation time. Factor effects were assessed and recorded in terms of enrichment factors (EFs). EFs describe how much more concentrated any given analyte has become in comparison to its concentration in the original sample. Given this, the sole aim of the optimisation was to attain the largest EFs possible since larger EFs equate to a much larger signal to noise ratio which will improve the chromatography and results of trace analysis. All standard mixtures used contained organic acids at an original concentration of 100 µg/mL.

5.3.3 *Optimisation of EFs: Effect of extraction solvent type*

Two of the most important characteristics of an ideal extraction solvent are good chromatographic behaviour and its ability to extract the chosen analytes. The extraction solvent must be soluble in the dispersing solvent, but insoluble in the sample matrix. In this instance, the sample matrix is aqueous. The solvents identified and investigated were dichloromethane (DCM), logP: 1.25 [32], chloroform, logP: 1.97 [33] and octanol, logP: 3.00 [34]. Chloroform was chosen as the ideal extraction solvent as it produced the cleanest chromatogram, DCM and octanol produced peaks in the areas of interest as seen in *Figure 5-7.* All solvents were distilled prior to injection; therefore the extra peaks are likely the solvents characteristic peaks.

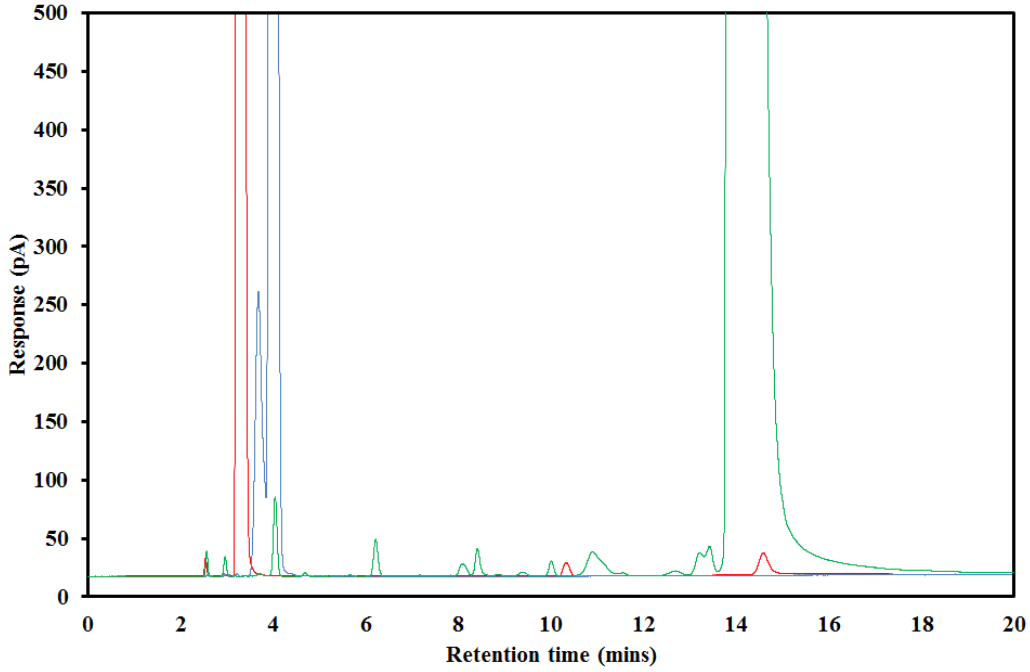


Figure 5-7. Chromatograms of distilled, extraction solvents. Blue: Chloroform; red: DCM; green: octanol.

Of the three solvents available, it was thought the highest EFs would be obtained through use of DCM since it had the lowest logP value. This suggests that the more polar analytes such as acetic and propionic would partition to a greater extent and yield the highest EFs. Ultimately, chloroform was found to have the most desirable chromatographic behaviour, i.e. no peaks in the areas of interest.

5.3.4 *Effect of extraction solvent volume*

This factor was optimised by placing varying amount of chloroform (100, 150, 200, 250 & 300 µL), which is the extraction solvent into 1 mL of dispersing solvent (acetone). Lower volumes clearly equate to larger EFs for all but propionic acid. Acetic acid was the only analyte not extracted into the extraction solvent. It is thought that this is because the DLLME conditions used were not favourable for its extraction, this was likely due to the polar nature of acetic acid and non-polar

nature of the extraction solvent droplets. Extraction solvent volume was the only parameter varied in this section and was also the first parameter to be experimented with. As can be seen in the conditions noted in the caption of *Figure 5-8* there was no pH adjustment or salt which would have had a profound effect on solubility of analytes in the extraction solvent.

5.3.5 *Example EF calculation*

Using n-valeric acid as an example: n-valeric acids regression analysis gave a standard curve of y = 0.0272x + 0.2994 (*Table 5-8*). The average peak area obtained for n-valeric acid using a standard concentration of 100 µg/mL, an extraction (chloroform) and disperser (acetone) solvent volume of 300 µL and 1000 µL, respectively, no pH adjustment (~ pH 5) and no salt at this point of the development process was 14.30.

$$X = (14.30 - 0.2994)/(0.0272) = 524\ \mu g/mL$$

The concentration found is then divided by the original concentration to obtain the EF:

$$EF = 524\ \mu g/mL\ /100\ \mu g/mL = 5.24$$

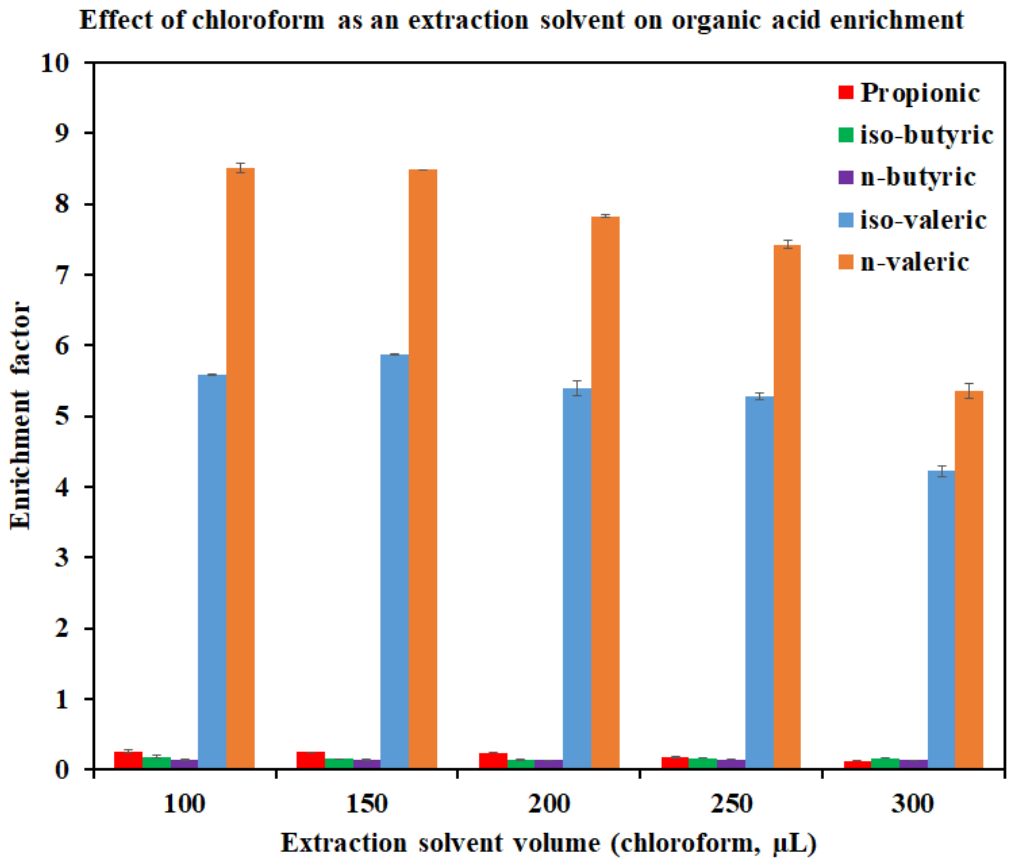


Figure 5-8. Graph depicting the increase in enrichment factors as the volume of chloroform was increased. As can be seen 100 µL produces the highest enrichment factors, although acetic has not been extracted. Conditions: 10 mL aqueous standard mixture, analytes: 100 µg /mL placed in a 15-mL centrifuge tube. 1 mL of acetone containing chloroform at 300, 250, 200, 150, 100 & 50 µL was injected directly into the sample. Centrifugation: 4500 rpm for 5 minutes. Top layer removed and 20 µL of the sediment phase placed in a vial for analysis.

It was found that volumes below 100 µL either did not produce a sedimented phase or the volume obtained was too small to work with. Therefore, the optimum volume of chloroform was found to be 100 µL. Optimised EFs were: acetic: 0, propionic: 0.25, iso-butyric: 0.15, n-butyric: 0.14, iso-valeric: 5.75 and n-valeric: 8.64. Values

lower than one indicate that mass transfer of analyte from sample to extraction is poor.

5.3.6 *Effect of salt concentration*

Addition of salts cause what is described as a salting out effect. Addition of salt causes salt molecules to become hydrated with water molecules. Since there is less water, water soluble analytes precipitate out, hence the term, salted out. This then allows salted out analytes to solubilise into the extraction solvent. NaCl was used as the salt, it was placed in varying amounts into the aqueous standard mixture while holding all other parameters constant. It was found that 22% (2.2 g) of NaCl gave the highest enrichment factors for all but acetic and propionic acid. The issue with using this amount was that NaCl had reached maximum solubility and not all salt could be consistently dissolved. Acetic acid had also been partially extracted for the first time following the addition of NaCl, although increase of NaCl did not increase acetic acids EF. The highest EFs obtained, as shown in *Figure 5-9* were: acetic: 0.33, propionic: 0.41, iso-butyric: 0.56, n-butyric: 0.76, iso-valeric: 16.53 and n-valeric: 20.69 at 20% w/v. Given the results obtained, 20% (2 g) NaCl was chosen as the optimum concentration.

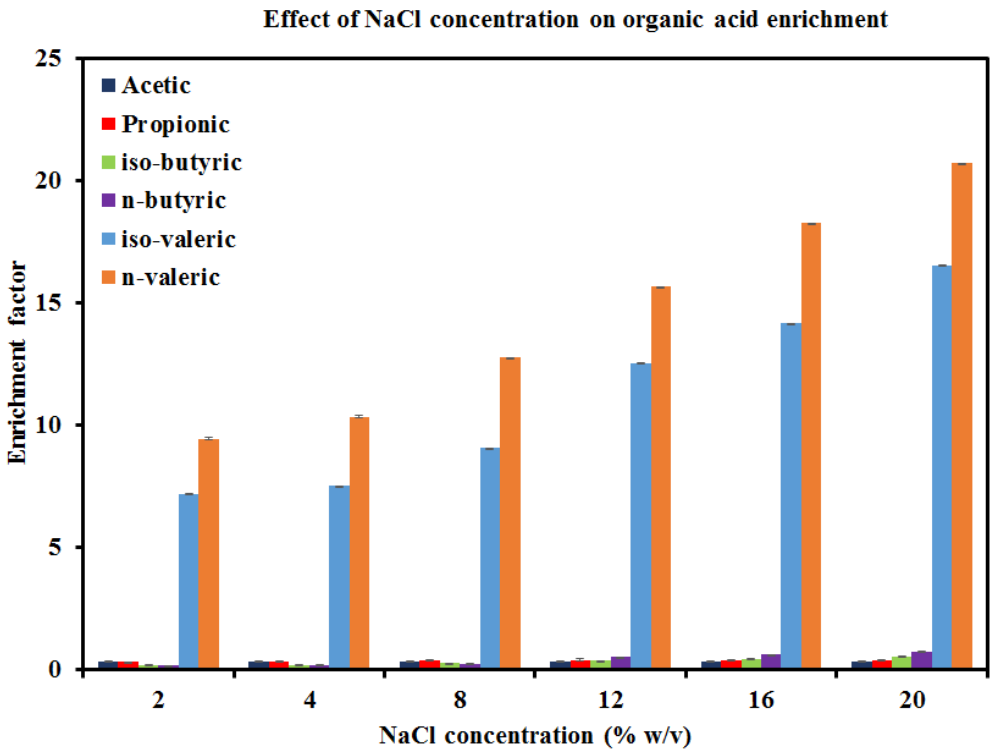


Figure 5-9. Graph of Enrichment factor versus NaCl concentration (w/v). Conditions: 10-mL aqueous standard mixture (100 µg /mL each) in water placed in 15-mL centrifuge tube. Salt was added from 2% w/v up to 30% w/v. 1-mL of acetone containing 100 µL of chloroform was injected, then centrifuged at 45000 rpm for 5 minutes. Top layer removed and 20 µL placed into a vial for analysis. Increase in NaCl increases the enrichment factor of all analytes. A positive correlation can be seen in the graph.

5.3.7 *Effect of pH*

Acidification adds an excess of hydrogen ions to the solution which neutralises acidic anions. Neutralised acids will then have a higher solubility in organic solvents. To optimise this factor the pH of the aqueous standard mixture was altered using dilute HCl or NaOH, as appropriate. The pH was trialled at pH 2.5, 7.00 and

11.50 to assess the solubility of the analytes into the extraction solvent and results graphed and laid out in *Figure 5-10*.

The optimum pH was found to be pH 2.50; as pH rises analytes become ionized due to deprotonation which has a direct effect on enrichment factors. Ionized analytes are hydrophilic and so partitioning into a hydrophobic solvent is minimised for the valeric species and fully eliminated for the more polar acetic, propionic and butyric species as the solution became more basic. The highest EFs obtained at pH 2.50 were: acetic: 0.31, propionic: 0.46, iso-butyric: 1.09, n-butyric: 1.08, iso-valeric: 34.92 and n-valeric: 42.01.

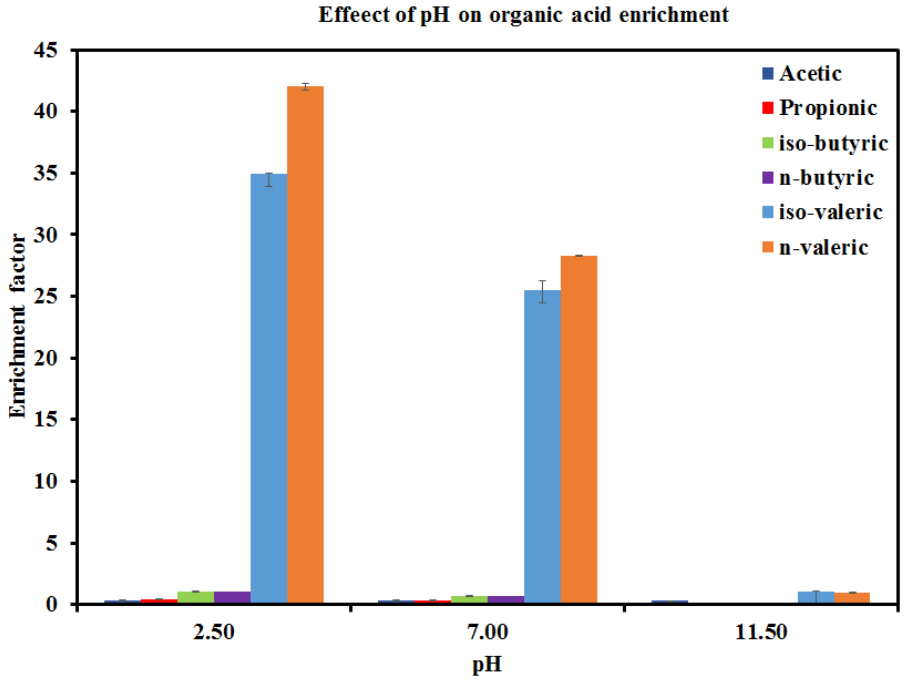


Figure 5-10. Enrichment factors of analytes increased as pH decreased. Conditions: 10-mL aqueous standard mixture (analytes 100 µg /mL each) in water was placed in a 15-mL centrifuge tube. The pH was trialled at pH 2.5, 7.00 & 11.50; 2 g of NaCl was added, 1 mL of acetone containing 100 µL of chloroform was injected followed by centrifugation at 45000 rpm for 5 minutes. Top layer removed and 20 µL taken for analysis. Ionised analytes do not solubilise well in organic solvents.

5.3.8 *Effect of disperser solvent type and volume*

The role of the disperser solvent is to distribute the fine droplets of solubilised extraction solvent throughout the sample. Optimising the ratio of disperser to extractor solvent volume will also aid in increasing EFs. MeOH, ACN and acetone were identified as appropriate disperser solvents because they were each soluble in aqueous media and chloroform.

In regards to MeOH, the optimum volume was found to be 500 µL and produced very low EFs for all analytes as depicted in *Figure 5-11*. The highest EF was 1.36 for n-valeric acid. Neither acetic nor propionic acid produced EFs ≥1 as summarised in *Table 5-2*. Neither of the butyric species extracted, and negative concentration values were found from the calibration curve. This suggests that the concentrations fall far below the linear range. The experiment was repeated to ensure that no human error occurred. This again produced near identical results, suggesting that there was something in the sample that interfered with the extraction of these analytes. Since the only parameter that differed is MeOH as the disperser, one can only assume that these species had a far higher solubility and a greater affinity for the MeOH:water phase, with possibilities of hydrogen bonding between anions and solvent ions reducing the available acids for extraction.

ACN (*Figure 5-12*) performed slightly better than MeOH and produced slightly higher EFs. The largest seen using MeOH was 1.4, here the largest EF was more than double at 3.39 for n-valeric acid. Acetic acid was not extracted using ACN as the disperser. This is probably due to its insolubility in the extraction solvent or some unknown matrix effects, meaning acetic had a higher affinity for the ACN

droplets than the chloroform droplets. Neither propionic, iso-butyric or n-butyric produced EFs ≥1, as summarised below in *Table 5-2*.

The use of acetone (*Figure 5-13*) as the disperser solvent has clearly produced the largest EFs for all valeric species. The optimum volume was found to be 700 µL, this produced EFs that were ~13 times larger for both valeric species in comparison to using ACN and ~34 times larger than with MeOH. This is likely due to the extraction solvent, chloroform, having a higher solubility in acetone. Since solubility has increased, the disperser solvent can distribute finer extraction solvent droplets throughout the sample. This increased the surface area available for analytes to enrich into [35]. Also both butyric species now have EFs > 1 which means they can both be quantified as total mass transfer of analyte from one solvent to the other had occurred. *Table 5-2* compares the optimum volumes of each dispersing solvent and the EFs achieved.

Table 5-2. Comparison of highest EFs achieved with each dispersing solvent mixed with 100 µL of chloroform and the sample at pH 2.50 with 2% NaCl. Optimum results obtained through use of acetone as dispersing solvent.

	MeOH EF (500 µL)	**ACN EF (900 µL)**	**Acetone EF (700 µL)**
Acetic acid	0.65	0	0.34
Propionic acid	0.49	0.21	0.42
iso-butyric acid	0	0.80	1.37
n-butyric acid	0	0.78	1.26
iso-valeric acid	1.15	2.88	42.18
n-valeric acid	1.36	3.39	46.81

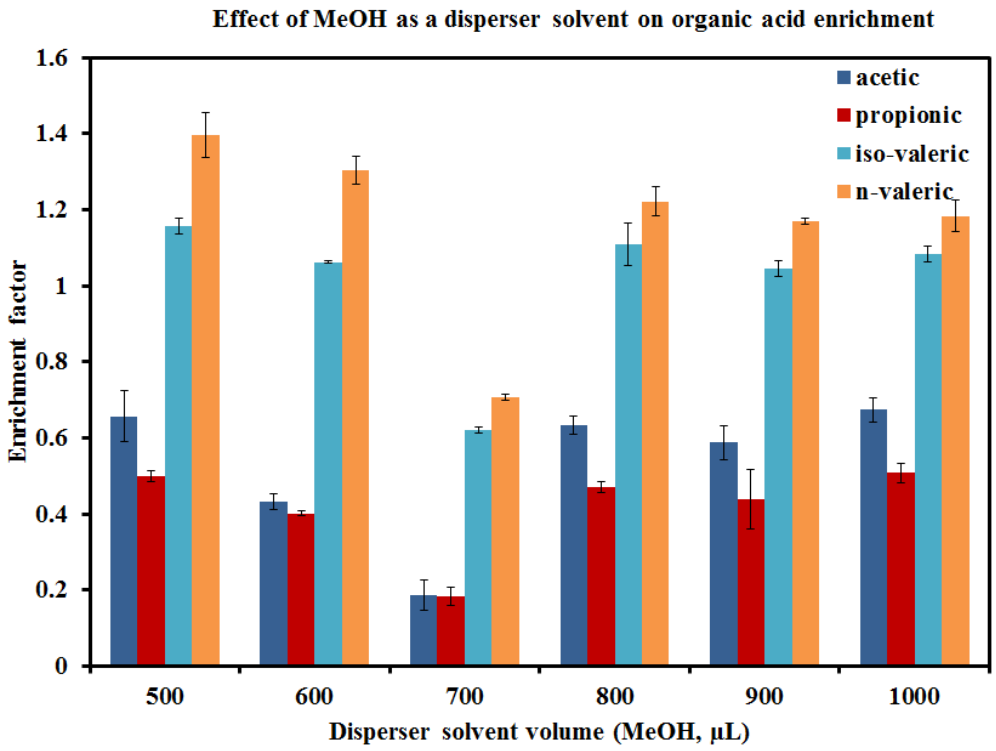


Figure 5-11. Graph of enrichment factors of organic acids using MeOH as the disperser solvent. Conditions: 10-mL aqueous standard mixture (analytes 100 µg /mL) was placed in a 15-mL centrifuge tube, 100 µL of 1 M HCl and 2 g of NaCl was added to the centrifuge. MeOH trialled at: 1000 – 500 µL in 100 µL chloroform to assess effects on EFs. Solution centrifuged at 45000 rpm for 5 minutes. Top layer discarded and 20 µL taken for analysis. The maximum enrichment factor was circa 1.4 for n-valeric acid, neither butyric species had been extracted.

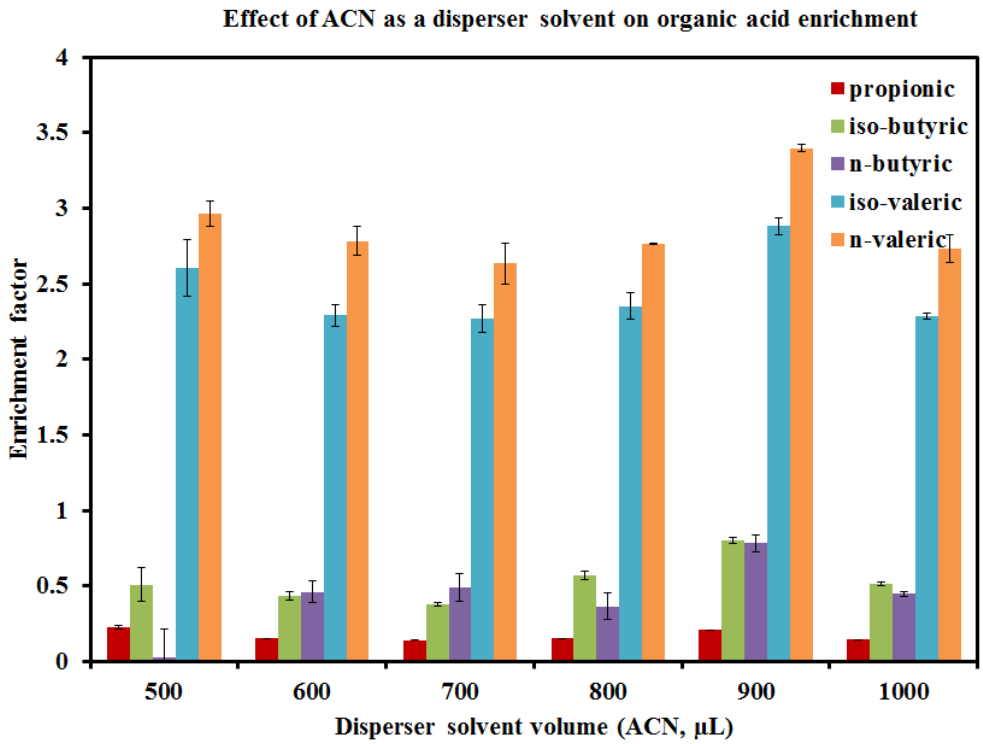


Figure 5-12. Graph of enrichment factors of organic acids using ACN as the disperser solvent. Conditions: 10-mL aqueous standard mixture (analytes at 100 µg /mL) was placed in a 15-mL centrifuge tube, 100 µL of 1 M HCl and 2 g of NaCl was added to the centrifuge tube. ACN trialled at: 1000 – 500 µL in 100 µL chloroform to assess effects on EFs. Solution centrifuged at 45000 rpm for 5 minutes. The top layer was then discarded and 20 µL taken for analysis. The largest enrichment factors were obtained for the valeric species at circa 3.5. Acetic was not extracted.

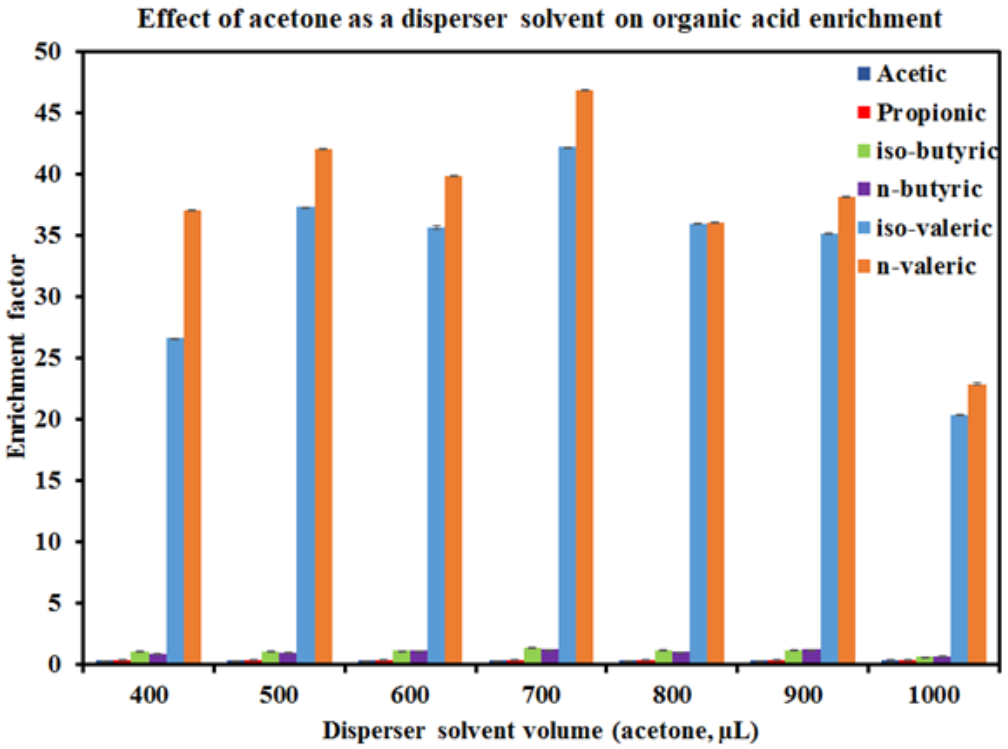


Figure 5-13. Graph of enrichment factors of organic acids using acetone as the disperser solvent. Conditions: 10-mL aqueous standard mixture (analytes 100 µg /mL) placed in a 15-mL centrifuge tube, 100 µL of 1 M HCl and 2 g of NaCl was then added. MeOH was trialled at: 1000 – 400 µL in 100 µL chloroform to assess effects on EFs. Solution centrifuged at 45000 rpm for 5 minutes. Top layer discarded and 20 µL taken for analysis. Acetic acid had been extracted with acetone, both butyric acids had EF's > 1 and large enrichment factors had been achieved for both valeric species.

5.3.9 *Effect of centrifugation time*

Centrifugal force causes a separation between insoluble solvents by pulling the densest solvent to the bottom while leaving the lighter solvent on top – the supernatant. Centrifugation time was varied to asses effects upon EFs. Low centrifugation times gave much lower enrichment factors for valeric species and

propionic, with slight differences in butyric acid, likely due to small droplets of the extraction solvent remaining in the supernatant. The optimum centrifugation time remained at five minutes as the value dropped thereafter, this can be seen in *Figure 5-14* and a chromatogram of the optimised DLLME in *Figure 5-15*. Optimum values were: acetic acid: 0.38, propionic acid: 0.97, iso-butyric acid: 1.52, n-butyric acid: 1.49, iso-valeric acid: 42.89 and n-valeric acid: 47.97.

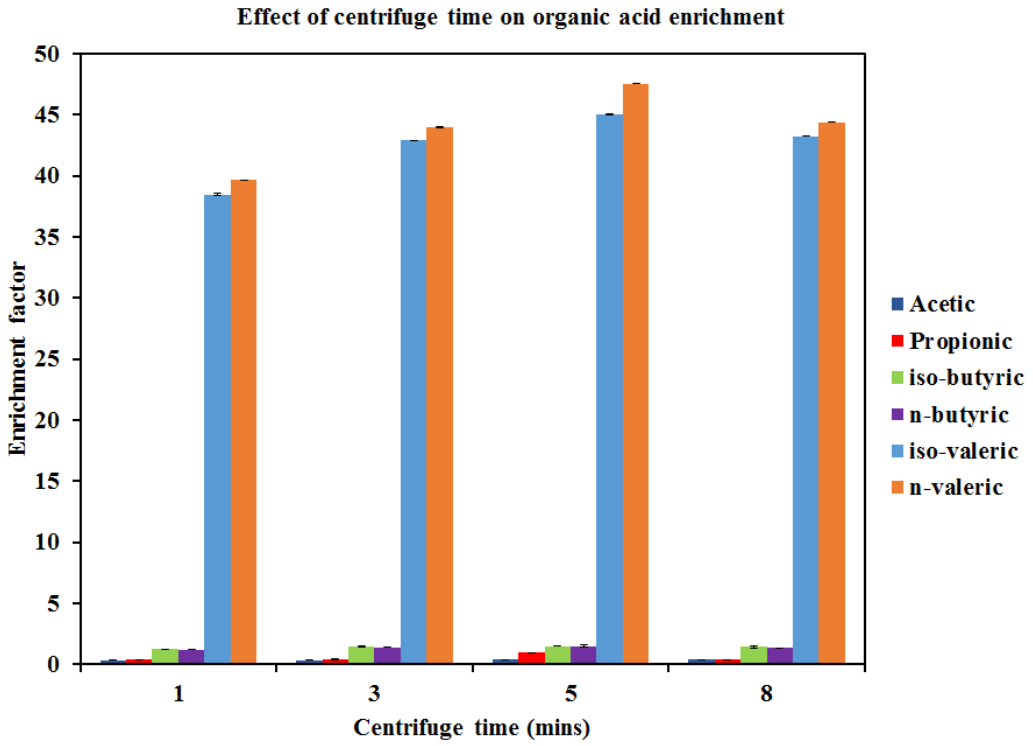


Figure 5-14. Graph of enrichment factor versus centrifugation time. Conditions: 10-mL aqueous standard mixture (STDs 100 µg /mL) placed in a 15-mL centrifuge tube, 100 µL of 1 M HCl and 2 g of NaCl was added. Mix of 100 µL chloroform & 700 µL of acetone was injected into the sample. Centrifugation: 45000 rpm for 1, 3, 5 & 8 minutes to assess effects on EF's. The top layer was discarded and 20 µL taken for analysis. Optimum centrifugation time was found to be five minutes as values dropped thereafter.

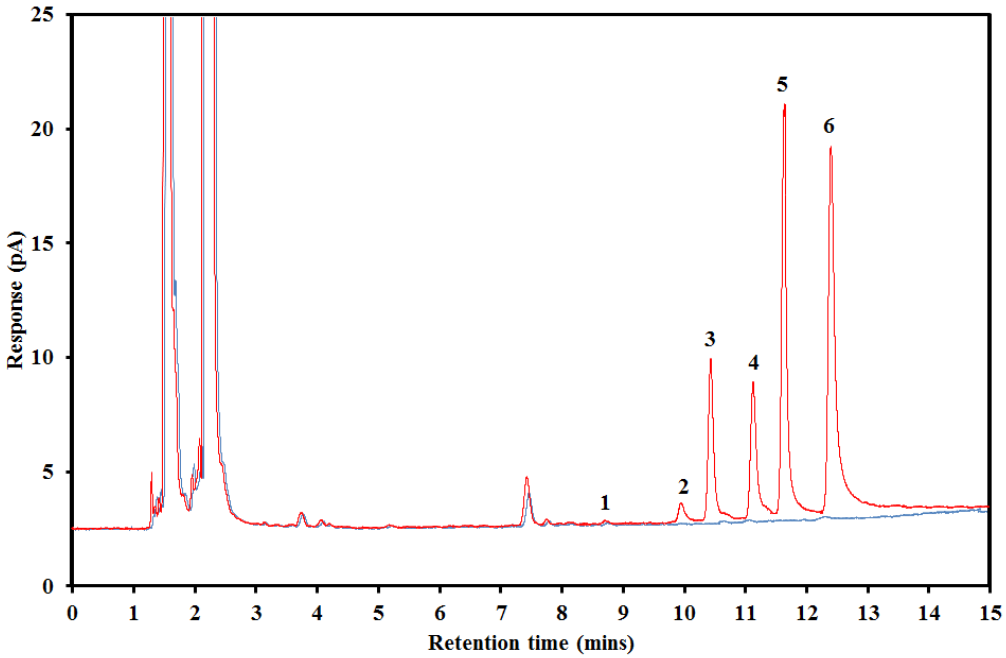


Figure 5-15. Chromatogram of optimised DLLME of chosen organic acids. Blue: reagent blank; red: standard mixture. Five-minute centrifugation has produced the largest peaks therefore five minutes was chosen as the optimum. Analyte I.D.: 1: acetic; 2: propionic; 3: iso-butyric; 4: n-butyric; 5: iso-valeric; 6: n-valeric.

5.3.10 *Effect of sonication*

Having found the optimum of the most commonly cited factors in DLLME, it was decided to assess any effects that sonication may have upon the enrichment factors, results of which are found in *Figure 5-16*. It was thought that sonication would aid in increasing EFs further as it would create finer droplets, thus further increasing surface area. Experimentation found that the EFs for both valeric species dropped by almost three quarters while EFs for both butyric species more than doubled. It is unclear why this is the case. Given such a large drop in EFs for the valeric species it was decided not to use sonication.

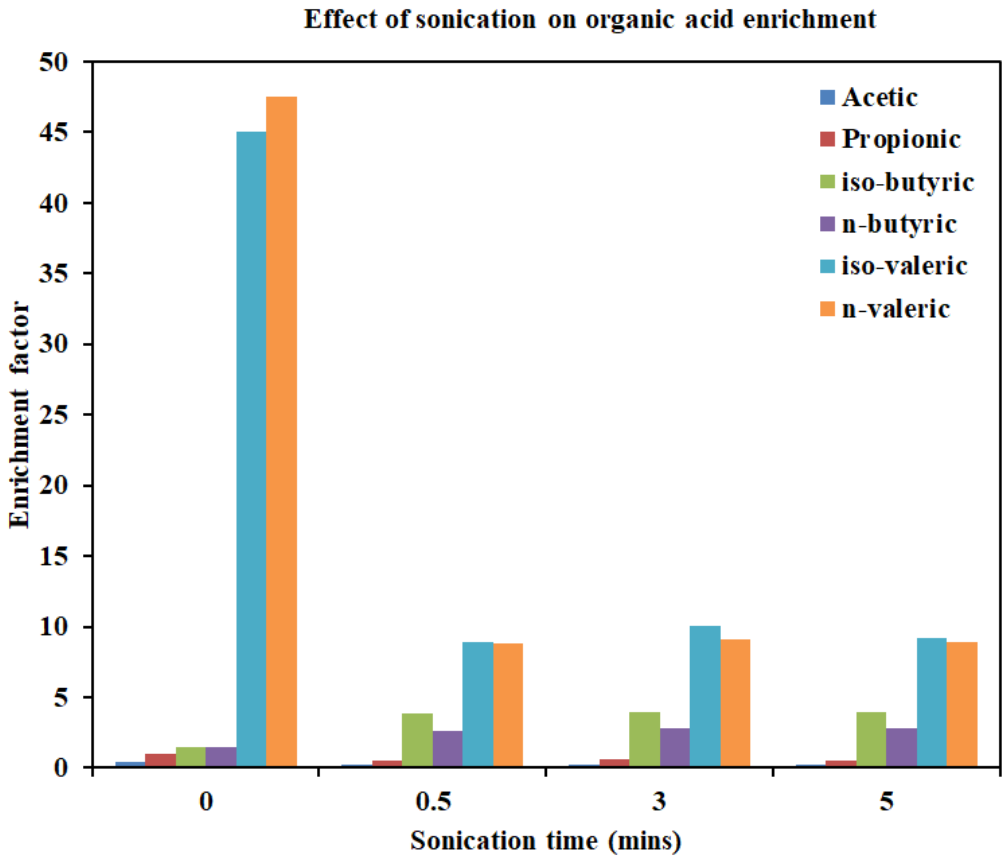


Figure 5-16. Graph of enrichment factor versus sonication time. Conditions: 10 mL aqueous standard mixture (analytes 100 µg /mL) placed in a 15-mL centrifuge tube, 100 µL of 1 M HCl and 2 g of NaCl was then added. 100 µL chloroform was added to 700 µL of acetone and injected into the sample. Sonicated for 0, 0.5, 3 & 5 minutes; centrifugation: 45000 rpm for 5 minutes. Sonication had a detrimental effect upon EFs.

The optimised DLLME gave the following enrichment factors: acetic acid: 0.34; propionic acid: 0.97; iso-butyric acid: 1.52; n-butyric acid: 1.50; iso-valeric acid: 45.04 and n-valeric acid: 47.56. Given that EFs must ≥ 1 acetic and propionic acid could not be quantified using DLLME as mass balance of both species was not obtained.

5.3.11 *Final Enrichment Factors*

The introductory chapter explained the need for rendering the sample in a form that is suitable for the extraction procedure and separation. Biological samples such as milks, and dairy produce, such as yogurts and cheeses contain interferences such as proteins and lipids that must be removed.

The mode of separation (GC) dictated that the final sample must reside in a volatile matrix. Chloroform was deemed as the ideal extraction solvent due to its chromatographic behaviour. The optimum dispersive solvent was found to be acetone and the medium that analytes are extracted from was clearly aqueous. Given this, the initial sample preparation method must render the analytes in an aqueous medium. The sample preparation method that best suited the needs of this project was the Carrez method as described in the introductory chapter (**1.6**) and by several authors [36,37]. The rationale: because the analytes will be left in an aqueous medium that was suitable for the optimised DLLME solvents (chloroform and acetone)

To take into account matrix effects and the effects of the Carrez protein precipitation method on EFs, the optimised extraction protocol was used on a spiked sample of buttermilk, an average EF was used to back calculate concentrations in the original sample; comparisons of each sample types EFs are below in *Table 5-4*.

The concentrations spiked into the buttermilk were, 200, 400 & 600 μg /mL. Since acetic and propionic acids did not produce EFs ≥ 1, they were removed from development at this stage due to the limitations of the DLLME technique at extracting highly polar compounds into chloroform. Matrix effects lowered the EFs

of n-butyric acid, iso-valeric and n-valeric acid to 1.37, 40.51 and 43.41 respectively, giving a difference of 0.13, 4.54 and 4.1 respectively with iso-butyric acid having a negligible increase of 0.07 in EF.

As can be seen in *Table 5-3*, the butyric species produced the lowest %RSD while both valeric species produced larger %RSDs. It is unclear why the %RSDs were large for the valeric species, though it could be due to several factors such as slight variation in salt, solvent volume or pH. Robustness studies should have been carried out on these factors.

Table 5-3. Average enrichment factors obtained from three concentration levels following addition of Carrez solutions and DLLME on a sample of buttermilk. The average EFs here were used to back calculate enriched concentrations in future work.

	iso-butyric acid	**n-butyric acid**	**iso-valeric acid**	**n-valeric acid**
AVG Peak area 200 µg/mL	55.93	49.09	137.63	149.27
EF for 200 µg /mL	1.58	1.35	39.04	40.57
AVG Peak area for 400 µg/mL	112.75	100.97	293.2	332.34
EF for 400 µg /mL	1.61	1.4	41.68	45.21
AVG Peak area for 600 µg/mL	164.15	147.46	430.38	490.1
EF for 600 µg /m	1.57	1.37	40.82	44.46
Average EF	1.59	1.37	40.51	43.41

Table 5-4. Comparison of EFs obtained using aqueous standards versus EFs obtained using full extraction protocol on a sample of buttermilk to screen for matrix effects on final EFs.

	iso-butyric acid	**n-butyric acid**	**iso-valeric acid**	**n-valeric acid**
EFs obtained from aqueous standards	1.52	1.5	45.04	47.56
EF obtained from buttermilk sample	1.59	1.37	40.51	43.41
Relative difference	+0.07	-0.13	-4.53	-4.15

There was clear matrix effects acting upon the analytes, this could have been due to several factors such as: analytes binding to the metal complexes formed during extraction, analytes trapped in the sedimented phase, or loss of analyte whilst transferring and filtering the supernatant from the sample preparation step. Acetic and propionic acid have consistently given EFs < 1, this suggests that they did not extract under any of the conditions assessed. It is likely that DLLME is not suited to extracting such polar compounds and is probably the reason the use of DLLME to extract such organic acids was not found in the literature, this could be improved via use of ion-pairing, time precluded this avenue of research.

In comparison, SPE with a strong anion exchange (SAX) sorbent has been used to successfully extract acetic and propionic acids from honey by Cherchi [38], as described in the introductory chapter. Given this, these analytes were not quantified in the following work, though via comparing retention times with that of standards, could still be identified.

5.3.12 *Protein precipitation*

It was found that the volume of Carrez solutions added occasionally needed to be increased for higher fat samples. Following centrifugation, occasionally a turbid solution was obtained that would often block the syringe filters and would require several new syringe filters to filter 10 mL supernatant. Obvious drawbacks to this were a decrease in sample volume obtained due to collection within the void of the syringe filter and loss of sample. Following DLLME on the sample there appeared a white floating layer of lipids. Following removal of this layer and the supernatant to access the chloroform extract a solid transparent layer would also form at the surface between the chloroform and aqueous supernatant, in extreme cases a white semi solid precipitate would form within the chloroform layer. To rectify this and ensure full removal of lipids, it was found that increasing the volume of Carrez solution by 0.5 mL to 1.5 mL, while holding all other volumes constant would remove all lipids and prevent the formation of any solids at the boundary.

5.4 Method validation

The validation parameters assessed were: repeatability of retention times (≤ 2% RSD), precision (90-110% unless sufficient justification can be made), LOD (≥ 3:1), LOQ (≥ 10:1) and linear range ($R^2 \geq 0.99$).

5.4.1 *Precision/system repeatability*

This work used triplicate injections spanning three concentration ranges to calculate retention time precision and nine determinations at the highest concentration value for peak area. Averages, standard deviation (SD) and relative standard deviations (%RSD) were calculated for peak area and retention times, results of which are found in *Table 5-5 & Table 5-6*. Supporting equations can be found in the validation section (**4.5**) of **Chapter 4**. %RSDs for retention time and peak area should be ≤ 2%. Peak area precision for acetic and iso-butyric acid were > 2% RSD at 3.09 and 2.05% RSD, respectively, which is outside of the limits set (*Table 5-5*). Possible reasons for this were the plunger on the injector needle sticking. Different wash solvents were used such as acetone, IPA and chloroform to wash the needle between injections, though this problem could not be solved due to its intermittence. Retention time precision was acceptable with < 2% RSD for all analytes (*Table 5-6*).

Table 5-5. Precision of peak area for GC separation where n=9 using the highest concentration. Acetic and iso-butyric acid were above the 2% RSD threshold.

	Acetic acid	Propionic acid	iso-butyric acid	n-butyric acid	iso-valeric acid	n-valeric acid
Average Peak Area	0.97	2.88	4.37	4.45	4.84	4.56
SD of peak area	0.03	0.05	0.09	0.05	0.09	0.07
%RSD of peak area	3.09	1.74	2.05	1.12	1.86	1.53

Table 5-6. Precision of retention time (t_r) for GC separation where n=9 using three different concentration levels (low, medium and high concentrations). All % RSDs were below the 2% threshold.

	Acetic acid	Propionic acid	n-butyric acid	iso-valeric acid	valeric acid
Average t_r (mins)	8.79	10.06	11.18	11.68	12.49
SD (mins)	0.02	0.09	0.01	0.01	0.01
%RSD	0.22	0.89	0.09	0.09	0.08

5.4.2 *Method repeatability for DLLME procedure*

Repeatability had been assessed for the DLLME procedure through GC peak area determination. This was calculated through triplicate injections of three replicate extractions using aqueous standards at an original concentration of 100 µg/mL. The results in *Table 5-7* show that all %RSDs were < 5.0 %.

Table 5-7. Precision of peak area for optimised DLLME procedure, where n=9 using an aqueous standard mixture of 100 µg/mL. Acetic and propionic acids were removed due to their inability to be extracted using this method.

	iso-butyric	**n-butyric**	**iso-valeric**	**n-valeric**
Average Peak Area	33.26	33.45	98.55	115.89
SD of peak area	1.45	1.66	4.48	4.49
%RSD of peak area	4.36	4.95	4.54	4.41

5.4.3 *Linear range*

Linear range refers to the ability of a method to obtain results which are proportional to the concentration of the analyte in the original sample. It is found by generating a calibration curve spanning the expected concentration range often quoted as 25, 50, 75, 100, 150 & 200% of the expected concentration. Linearity is a linear relationship between the chosen concentration range and the response, in this case, peak area. Plotting the data produces a regression line of the form of a straight line (*Figure 5-17* & *Figure 5-18*). The correlation coefficient must fall in

the range of 0.9 – 1.1 to be considered linear. The GC is fitted with a flame ionisation detector (FID) which has a linear range of 10^7 [39]. Concentration ranges used for all analytes are given in *Table 5-8* and were estimated from literature values obtained from Damir [40], Zeppa [3] and Ledford [41].

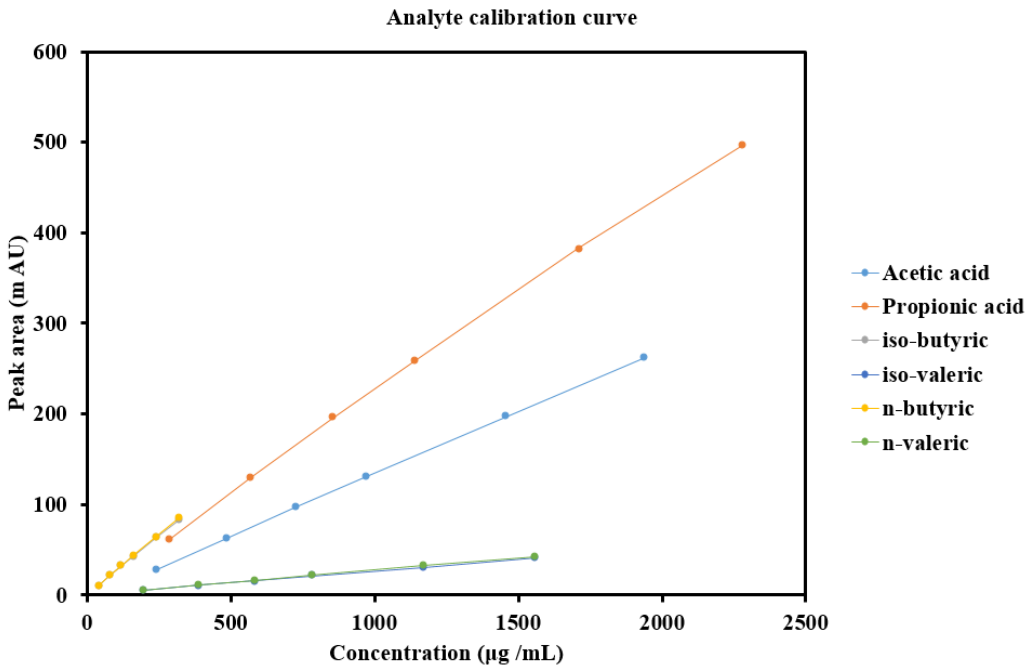


Figure 5-17. Calibration curve for all six organic acids. Linear range and R^2 values were: acetic: 242 – 1940 µg /mL, R^2: 0.9999; propionic: 285 – 2280 µg /mL, R^2: 0.9991; n-butyric: 40 – 320 µg /mL, R^2:0.9995; and n-valeric: 195 – 1560 µg /mL, R^2: 0.9998. A summary of all validation characteristics can be found in Table 5-8 and raw data in the ***Appendix****.*

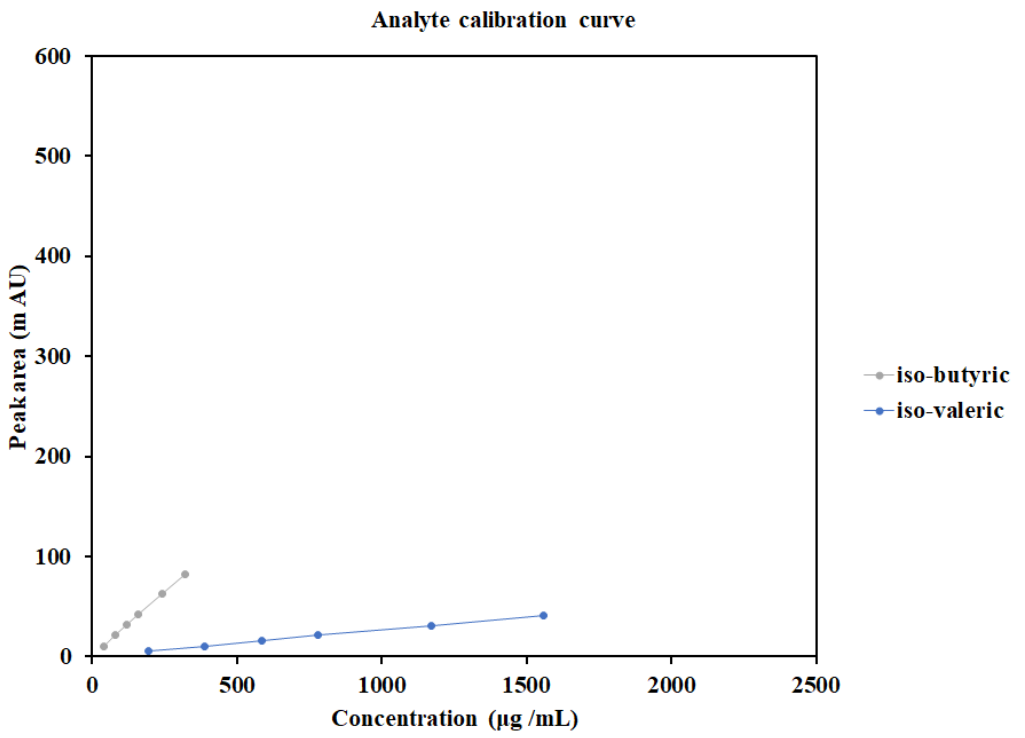


Figure 5-18. Calibration curves for iso-butyric and iso-valeric acids. Both iso-valeric and iso butyric acid calibration curves were identical to their 'n' isomers and as such, their data was hidden in Figure 5-17. Iso-butyric: 40 – 320 µg /mL, R^2: 0.9993; iso-valeric: 195 – 1560 µg /mL, R^2: 0.9993. A summary of all validation characteristics can be found in Table 5-8 and raw data in the ***Appendix****.*

5.4.4 *Limit of detection (LOD)*

LOD's are based on the signal to noise ratio, which is the ratio between an analytes peak height and the noise of the baseline. The LOD is reached when the signal is three times that of the noise. Equations and data used to calculate LODs can be found in **7.3** of the **Appendix**.

LODs were: acetic acid: 21.88 µg/mL, propionic acid: 67.25 µg/mL, iso-butyric acid: 8.04 µg/mL, n-butyric acid: 6.86 µg/mL, iso-valeric acid: 39.38 µg/mL and n-valeric acid: 21.68 µg/mL. The method produced the lowest LODs for both

species of butyric acid and the highest for propionic acid, though since propionic acid cannot be extracted it cannot be quantified in this work.

5.4.5 *Limit of quantitation (LOQ)*

LOQ's are also based on the signal to noise ratio. The LOQ is the lowest limit that an analytes concentration can be confidently and accurately calculated. The LOQ is reached when the signal is ten times that of the noise.

It is acceptable for LOQ values to match the values estimated for LODs, though they may not be below the LOD [42]. LOQs achieved were: acetic acid: 66.32 µg/mL, propionic acid: 203.79 µg/mL, iso-butyric acid: 24.38 µg/mL, n-butyric acid: 20.82 µg/mL, iso-valeric acid: 119.36 µg/mL and n-valeric acid: 65.71 µg/mL. The method again produced the lowest LOQs for both species of butyric acid and the highest for propionic acid. Any value, lower than the quoted LOQ, but above the LOD can-not be seen as an absolute concentration but can confidently be used qualitatively as identification of an analyte band. Equations and data used to calculate LOQs can be found in the appendix in **7.3** of the **Appendix**.

Table 5-8. Calculated validation parameters; linear range, regression equation, R^2 values, LODs, LOQs, recovery and EFs. All concentrations in (µg /mL). No recovery data available for acetic and propionic acid as their EFs were below 1. NQ: not quantifiable since EFs < 1.

	Acetic acid	**Propionic acid**	**iso-butyric acid**	**n-butyric acid**	**iso-valeric acid**	**n-valeric acid**
Linear range (µg /mL)	242 – 1940	285 – 2280	40 – 320	40 – 320	195 – 1560	195 - 1560
Regression equation	y = 0.1381x -4.2657	y = 0.2178x +5.8819	y = 0.257x +1.062	y = 0.2643x +0.9403	y = 0.026x +0.5842	y = 0.0272x +0.2994
R^2	0.9999	0.9991	0.9993	0.9995	0.9993	0.9998
LOD (µg /mL)	21.88	67.25	8.04	6.86	39.38	21.68
LOQ (µg /mL)	66.32	203.79	24.38	20.82	119.36	65.71
%Recovery	NQ	NQ	98.25	83.67	87.09	87.41
EF	NQ	NQ	1.59	1.37	40.51	43.41

5.4.6 *Accuracy*

Recovery of the DLLME procedure coupled with the Carrez protein and lipid precipitation was carried out on a sample of buttermilk. Three replicate extractions were undertaken, with each injected in triplicate. Please see *Table 5-9* for a summary of results.

Table 5-9. Recovery data for organic acids spiked into buttermilk.

	Original concentration (µg /mL)	**Concentration of spike (µg /mL)**	**Final concentration (µg /mL)**	**% Recovery**
iso-butyric acid	6.01	10	15.83	98.25
n-butyric acid	6.05	10	14.41	83.67
iso-valeric acid	3.93	10	12.64	87.09
n-valeric acid	4.13	10	12.87	87.41

An acceptable range of recovery lay between 90 – 110 % with those figures closer to 100 % demonstrating minimal loss and almost complete mass balance of the standard. However, a low recovery that yields a consistent result is also acceptable.

5.4.7 *Intermediate precision*

Intermediate precision was carried out to ascertain if there was any difference between results obtained in the morning and in the night. Three replicate standard

mixtures were subjected to the full DLLME process then injected in triplicate on three consecutive mornings and three consecutive evenings. A one way analysis of variance (ANOVA) was then used to screen for variances within and between data sets. This identified whether there were factors that caused variation beyond experimental variation. The null hypothesis was that the means were equal ($H_o = \mu_1 = \mu_2$). The proposed hypothesis was that the means were not equal ($H_1 = \mu_1 \neq \mu_2$). The output of the one way ANOVA shows that all calculated *F* values < *F crit* which means that the null hypothesis, H_o is accepted – all means were equal – no difference between data sets. A summary of results can be found in *Table 5-10*, all raw data and a sample calculation can be found in the appendix in **section 7.4.**

Table 5-10. Summary of ANOVA. $H_o = \mu_1 = \mu_2$ is accepted because all F values were below F crit.

	Iso-butyric acid	**n-butyric acid**	**Iso-valeric acid**	**n-valeric acid**
Count	3	3	3	3
F	0.01112	1.05445	0.01563	0.65424
P-value	0.9211	0.3625	0.9653	0.46454
F crit	1.78064	7.70865	7.70865	7.70865

5.5 Application to Real Samples

Samples tested were pasteurised cow's milk, probiotic yogurt, buttermilk, Greek style yogurt, brie cheese, cottage cheese and goat's cheese. The concentrations quoted below are the concentrations in the original sample. These were back-calculated using the EFs quoted above. A summary of all results obtained in this section of work can be found in *Table 5-19.*

All samples (sourced from Tesco) and blanks were subjected to the optimised extraction procedure outlined in **5.2.3** and **5.2.4** and detected via the GC-FID procedure outlined in **5.2.1** to determine their organic acid content. All samples tested were fresh and within their respected 'use by' dates. Unidentified peaks were present in several chromatograms, those peaks that do not appear in the blank are likely to be other organic compounds such as aldehydes, ketones, alcohols and diols [44,45]. Due to time constraints, it was not possible to test a number of standards to assess what compounds they were. Acetic acid was present in several samples, though due to poor extraction performance (EF < 1) was not quantifiable (NQ) using the current DLLME method. Propionic acid was not detected in any sample using the current DLLME method.

Due to a shift in retention times a chromatogram of a standard mixture has been given (*Figure 5-19*) and overlain with each sample in this section to give added confidence that the peaks present in the samples are the analytes under investigation.

Retention time shifts in GC can occur due column issues (stationary phase degradation, length etc.), carrier flow and temperature control. All carrier flow rates were left as per instrument default; unlike Empower3, ChemStation software does

not offer the peace of mind and functionality of locking instrument methods via passwords or restricted access to instrument functions, meaning that they are all open to change – this could have occurred quite easily given the volume of staff, postgraduate and undergraduate students that used that particular instrument.

The research environment at third level educational facilities do not have stringent GxP policies in place such as usage logbooks to track analyst usage, changes to the column (new column fitted or portions periodically cut from the end) or septum replacement. Given this, it is not possible to know when these things have taken place.

Due to the public sector not having the disposable income a private sector company would have, instruments may not be functioning at their optimum performance which can mean temperature controls can vary which can have a direct impact on retention times. Without a proper instrument qualification system in place or preventative maintenance such as weekly/monthly cleaning or 6 and 12 calibrations by the manufacturer it is not possible to know the degree to which the instruments performance may be affected.

Given the variety of factors speculated above, it was not possible to ascertain the root cause of the shift in retention time, though via the use of standards as a comparison under the same conditions the analytes can still be identified.

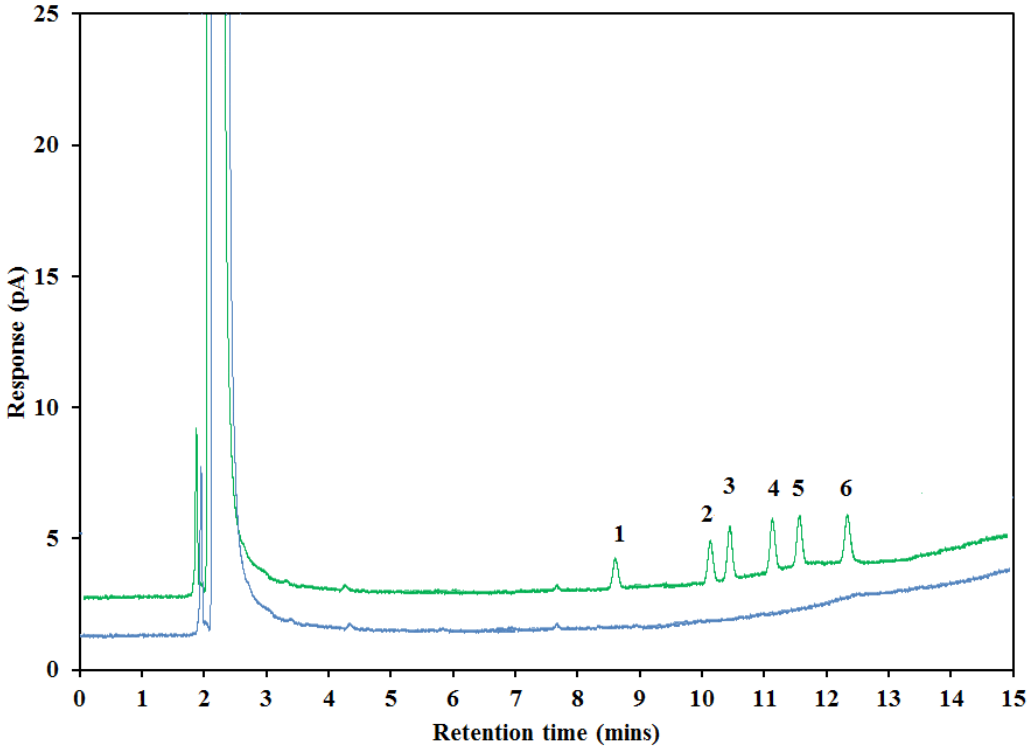


Figure 5-19. Chromatogram of standard mixture of organic acids made in acetone (green) at 500 µg/mL, and an acetone blank (blue). Peak ID (t_r in mins): 1: acetic acid (8.49); 2: propionic acid (10.099); 3: iso-butyric acid (10.21); 4: n-butyric acid (11.09); 5: iso-valeric acid (11.54); 6: n-valeric acid (12.25).

5.5.1 *Milks*

5.5.1.1 Goat's milk

The analysis determined that the original concentration of organic acids present in goat milk were: iso-butyric acid at 13.23 µg /mL, n-butyric acid at 14.64 µg /mL, iso-valeric acid at 13.12 µg /mL and n-valeric acid at 12.70 µg /mL. Acetic acid was present, but not quantifiable. Propionic acid was not found using this method. A number of the studies on the nutritional content and composition of goat's milk gave no reference to several of the organic acids studied herein. The majority, such as those by Jenness [46] and Posati [47] only analysed even numbered fatty acids

starting at C4:0 (n-butyric acid). Given this, the only comparison found at the time of writing was that of butyric acid. The figures produced for butyric acid concentration in goat's milk were: 1300 μg/mL (Posati) and 4500 μg/mL (Jenness). In comparison, this work found n-butyric acid at 5.24 μg/mL. These studies made no reference to how the acids were extracted so it is not possible to comment on that aspect, though the figures produced are extremely low in comparison. It is unclear why these figures are so low, though diet, breed and season do contribute enormously to milk composition and fluctuations can be large. These factors have been discussed in the introductory chapter. The resulting chromatogram can be found in *Figure 5-20* and a comparison of concentrations obtained with literature values in *Table 5-11*.

Sample calculation

The linearity study for n-valeric acid gave a regression equation of: $Y = 0.0272x + 0.2994$, n-valeric acid had an average peak area of 15.32 following the extraction process. Plugging that value into the above equation and rearranging to find x gives an enriched concentration of 552 μg/mL.

To find the original concentration, pre-enrichment, divide the enriched concentration (552 μg/mL) by the EF for n-valeric acid (43.41). Doing so gives a pre-enrichment concentration of 12.72 μg/mL.

Table 5-11. Comparison of organic acid concentrations in goat's milk with literature values. ND: not detected.

	Acetic acid	Propionic acid	iso-butyric acid	n-butyric acid	iso-valeric acid	n-valeric acid
AVG peak area			6.42	6.28	14.42	15.32
AVG retention time (mins)	8.51		10.12	11.05	11.56	12.21
EF			1.59	1.37	40.51	43.41
Enriched concentration (µg/mL)			20.99	20.1	531.57	551.49
Original concentration (µg/mL)			13.23	14.64	13.12	12.72
Posati [47] (µg/L)				1300		
Jenness [46] (µg/mL)				4500		

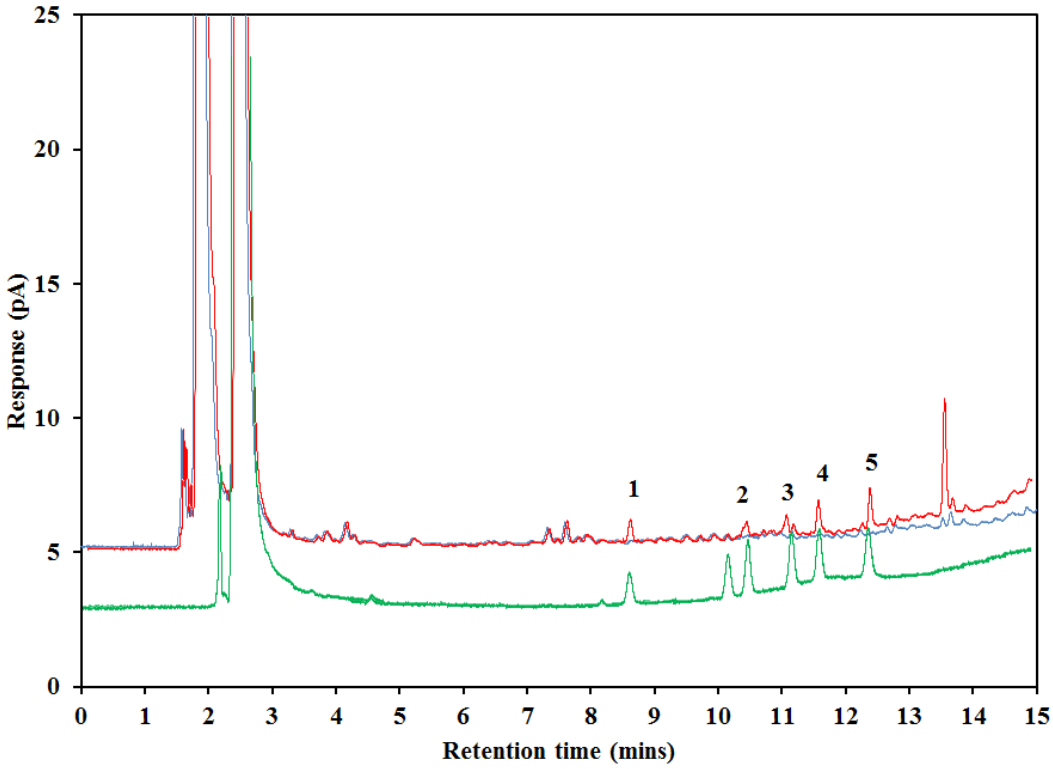


Figure 5-20. Chromatogram of DLLME on goat's milk (red) overlain with a blank (blue) and a standard mixture in acetone (green). Analyte I.D. (µg /mL): 1: acetic acid; 2: iso-butyric acid (13.23); 3: n-butyric acid (14.64); 4: iso-valeric acid (13.12); 5: n-valeric acid (12.70). Propionic acid was not detected

5.5.1.2 Buttermilk

The analysis determined that the original concentration of organic acids in buttermilk were: acetic acid (NQ), n-butyric acid at 14.83 µg /mL, iso-valeric acid at 12.22 µg /mL and n-valeric acid at 12.78 µg /mL. Propionic acid was not detected with this method. Marsili found a number of organic acids in buttermilk, those relevant to this work were acetic and propionic acid at 850 µg/mL and 60 µg/mL, respectively [1]. Kristensen also looked at the composition of buttermilk, though Kristensen only looked at acids with even carbon numbers starting from C4 (butyric acid) to C18; they noted butyric acid at 2970 µg/mL [48]. There is a large

difference between the two literature values as well as the value obtained for this work. Since butyric acid is a fermentation product, it is not an unreasonable assumption that this could be due to different cultures used in the manufacturing process [49,50] as well as any dietary influences [51]. Since most authors look at even numbered acids and both valeric species (pentanoic acid) were found, this method could be useful for any future studies into valeric acid and buttermilk. The resulting chromatogram can be found in *Figure 5-21* and a comparison of concentrations obtained with literature values in *Table 5-12.*

Table 5-12. Comparison of organic acid concentrations in buttermilk with literature values. ND: not detected, NQ: not quantifiable

	Acetic acid	**Propionic acid**	**iso-butyric acid**	**n-butyric acid**	**iso-valeric acid**	**n-valeric acid**
AVG peak area				6.32	13.46	15.4
AVG retention time (mins)	8.59			11.04	11.59	12.2
EF			1.59	1.37	40.51	43.41
Enriched concentration (µg/mL)			24.64	20.37	495.09	555.01
Original concentration (µg/mL)	NQ	ND	15.54	14.38	12.22	12.78
Kristensen [48] (µg/mL)				2970		
Marsili [1] (µg/mL)	850	60				

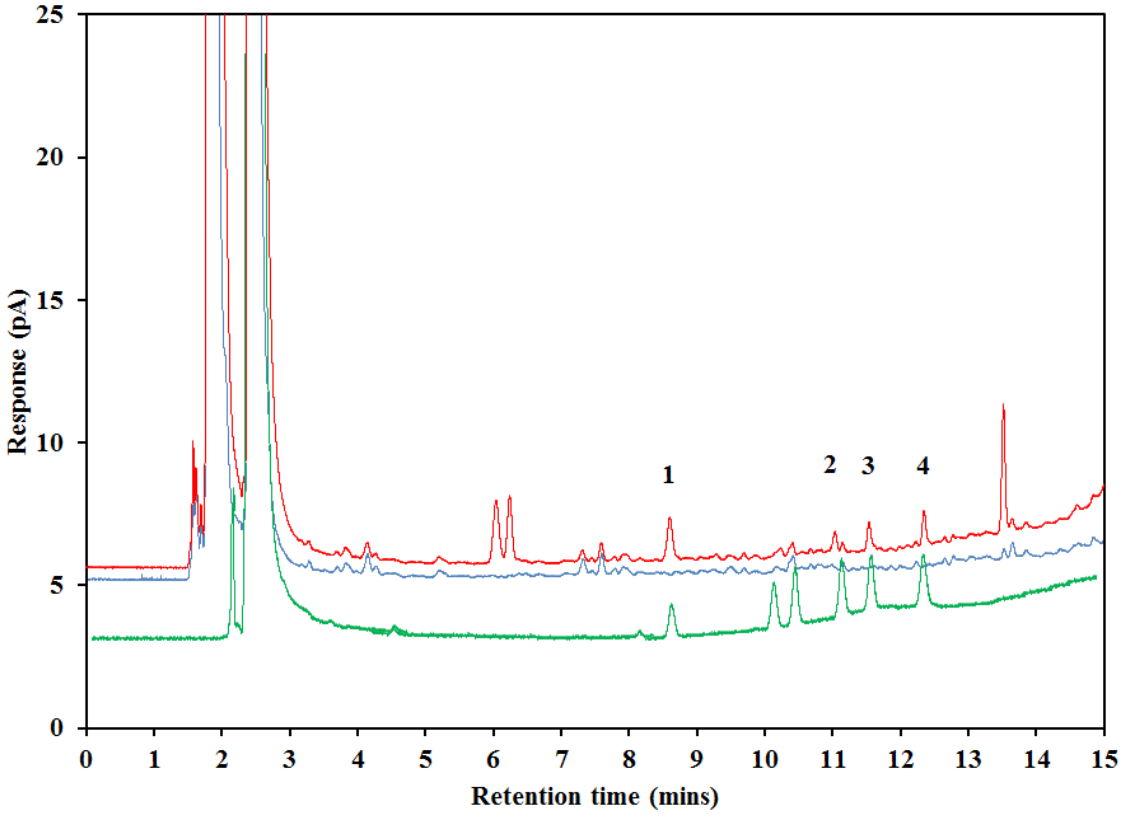


Figure 5-21. Chromatogram of DLLME on buttermilk (red) overlain with a reagent blank (blue) and a standard mixture in acetone (green). Analyte I.D. (µg /mL): 1: acetic acid; 2: n-butyric acid (20.37); 4: iso-valeric acid (12.22); 5: n-valeric acid (12.78). Propionic acid was not detected.

5.5.1.3 Cow's milk

The analysis determined that the original concentration of organic acids present in cow's milk was: acetic acid (NQ) and n-butyric acid at 10.41 µg /mL, iso-butyric, iso-valeric, n-valeric acid and propionic acid were not detected with this method. Ledford found that milk contained acetic acid at 8.16 µg/mL, propionic acid at 7.77 µg/mL and n-butyric acid at 8.98 µg/mL [41]. Marsili found acetic and propionic at 100 µg/mL and 120 µg/mL, respectively [1]. None of the other organic acids were present using their extraction techniques, which were not preconcentration techniques, which indicates that they may not be present in cow's milk. The higher

value of n-butyric found in this work (14.04 μg/mL) in comparison to the value obtained by Ledford (8.98 μg/mL) could be due to the DLLME preconcentrating the analyte. This could equally be due other factors such as geographical distribution, breed, nutrition and microbial activity [52–54]. The resulting chromatogram can be found in *Figure 5-22* and a comparison of concentrations obtained with literature values in *Table 5-13.*

Table 5-13. Comparison of organic acid concentrations in cow's milk with literature values. ND: not detected, NQ: not quantifiable

	Acetic acid	Propionic acid	iso-butyric acid	n-butyric acid	Iso-valeric acid	n-valeric acid
AVG peak area				4.72		
AVG retention time (mins)	8.49			11.03		
EF			1.59	1.37	40.51	43.41
Enriched concentration (µg/mL)	ND	ND	ND	14.3	ND	ND
Original concentration (µg/mL)	NQ	ND	ND	10.41	ND	
Ledford (µg/mL)	8.16	7.77		8.98		
Marsili (µg/mL)	100	120				

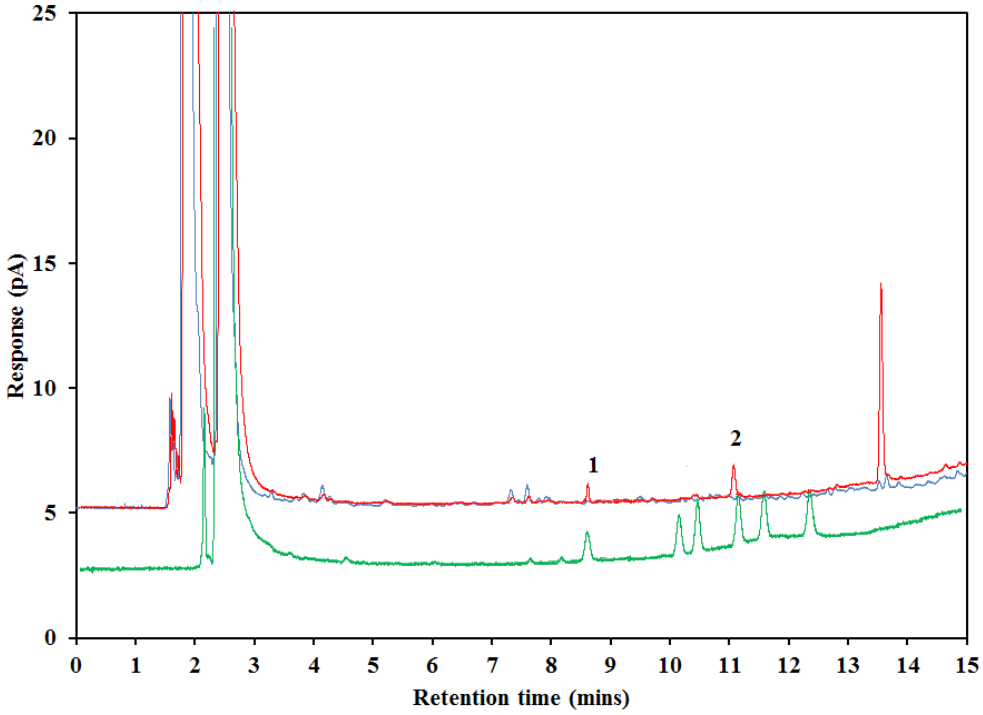


Figure 5-22. Chromatogram of DLLME on cow's milk (red) overlain with a reagent blank (blue) and a standard mixture in acetone (green). Analyte I.D. (µg /mL): 1: acetic acid and 2: n-butyric acid (10.41). Iso-butyric acid, iso-valeric acid, n-valeric acid and propionic acid were not detected.

5.5.2 *Cheese's*

5.5.2.1 Goat's Cheese

The analysis determined that the original concentration of organic acids present in goat's cheese was: acetic acid (NQ), iso-butyric acid at 5.68 µg /g, n-butyric acid at 7.51 µg /g, iso-valeric acid at 5.06 µg /g and n-valeric acid at 5.32 µg /g. Propionic acid was not detected using this method.

Numerous literature sources such as Fontecha [55] and Park [56] have again only looked at even numbered saturated fatty acids, starting at butyric acid. Fontecha reported a concentration of 744 µg/g for butyric acid and Park an average of 21800

μg/g. These figures are much larger than the 11.13 μg/g found using this method, though Park noted a minimum and maximum of 19700 – 24400 μg/g, so there is quite a large spread between those obtained in a single study. Conversely, Fontecha's figure of 744 μg/g is also much lower than Park's. The spread in data can be due to a number of factors discussed in the introductory chapter, such as breed, diet, season, health and management of the animal(s) [46,47]. A further study by Attaie was assessed as the author specifically looked at the volatile acid fraction of goat's cheese using HS-GC [57]. Attaie found only acetic acid and n-valeric acid at 1.16 μg/g and 1.68 μg/g. The concentration of n-valeric found in this work (5.29 μg/g) is much closer than the other values. One possible reason that the figure is higher in this work is due to the preconcentration technique used. Another reason could again be due to diet, breed, season etc. The resulting chromatogram can be found in *Figure 5-23* and a comparison of concentrations obtained with literature values in *Table 5-14.*

Table 5-14. Comparison of organic acid concentrations in goat cheese with literature values. ND: not detected, NQ: not quantifiable

	Acetic acid	Propionic acid	iso-butyric acid	n-butyric acid	iso-valeric acid	n-valeric acid
AVG peak area			3.38	3.67	5.91	6.58
AVG retention time (mins)			10.12	11.05	11.54	12.19
EF			1.59	1.37	40.51	43.41
Enriched concentration (µg/g)			9.01	10.32	204.93	231.06
Original concentration (µg/g)	NQ	ND	5.68	7.51	5.06	5.32
Park [56] (µg/g)				21800		
Attaie [57] (µg/g)	1.16					1.68
Fontecha [55] (µg/g)				744		

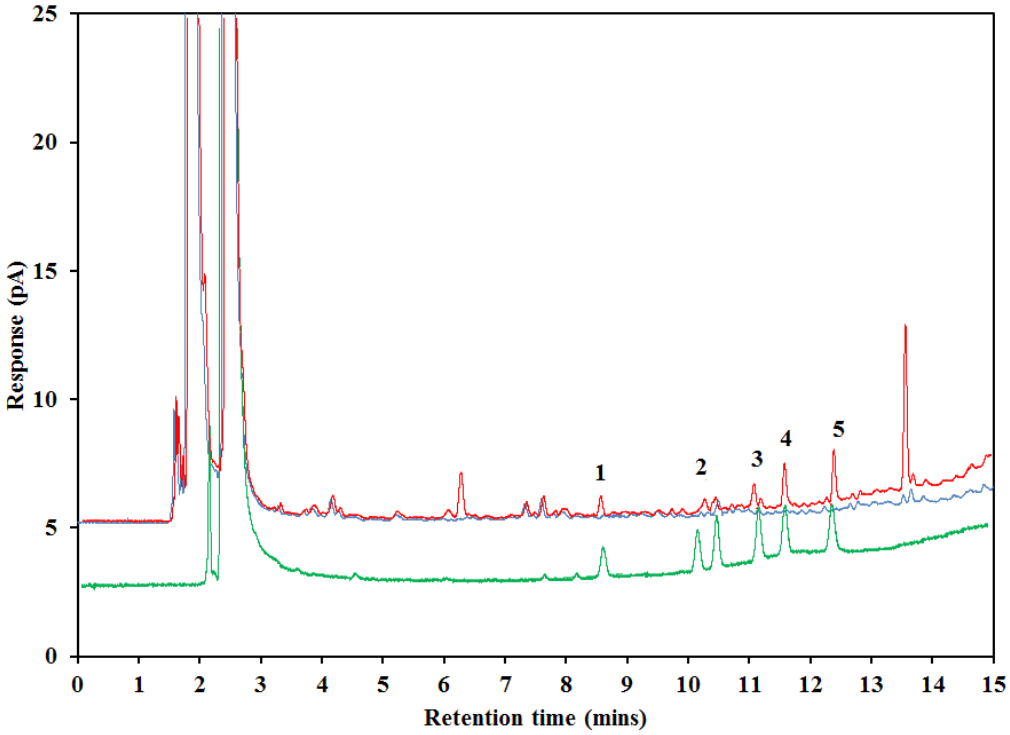


Figure 5-23. Chromatogram of DLLME on goat's cheese (red) overlain with a reagent blank (blue) and a standard mixture in acetone (green). Analyte I.D. (µg /g): 1: acetic acid; 2: iso-butyric acid (5.60); 3: n-butyric acid (7.51); 4: iso-valeric acid (5.06); 5: n-valeric acid (5.32). Propionic acid was not detected.

5.5.2.2 Cottage cheese

The analysis determined that the following organic acids were present in cottage cheese: acetic acid (NQ) and n-butyric acid at 4.04 µg /g. Propionic, iso-butyric, iso-valeric and n-valeric acid were not detected with this method. The resulting chromatogram can be found in *Figure 5-24.*

Mullin *et al.* published a paper that determined the levels of citric, formic, lactic and acetic acid in a variety of cheese's, including cottage cheese [58]. Mullin noted acetic acid at a concentration of 9760 µg/g. Marsili also determined the

concentration of a number of organic acids, both volatile and non-volatile in cottage cheese [1]. Marsili noted that acetic and propionic acid were present at ~ 100 μg/g and 120 μg/g, respectively. While acetic was found using this method, it could not be quantified and no propionic acid was detected. As can be seen by the literature values, there is again a big difference between both studies. Neither used a preconcentration technique to isolate their acids though two different chromatographic techniques were used. Mullin used a Dionex ion chromatography system fitted with a conductivity detector, while Marsili used a HPLC fitted with an ion exclusion column and UV detector. Organic acids do not have a chromophore and therefore absorb weakly in the UV meaning that the ion chromatography system is far superior. This could be one possibility of such large differences in the values. Other possible reasons could be different cultures used, breed, diet etc. as well as starter distillates used to improve taste and aroma of produce such as cheese's [59]. All of which have been discussed and cited numerous times through this entire body of work. The resulting chromatogram can be found in *Figure 5-24* and a comparison of concentrations obtained with literature values in *Table 5-15.*

Table 5-15. Comparison of organic acid concentrations in cottage cheese with literature values. ND: not detected, NQ: not quantifiable

	Acetic acid	**Propionic acid**	**iso-butyric acid**	**n-butyric acid**	**iso-valeric acid**	**n-valeric acid**
AVG peak area				2.4		
AVG retention time (mins)				11.10		
EF			1.59	1.37	40.51	43.41
Enriched concentration (µg/g)	NQ	ND	ND	5.55	ND	ND
Original concentration (µg/g)	NQ	ND	ND	4.04	ND	ND
Marsili [1] (µg/g)	100	120				
Mullin [58] (µg/g)	9760			744		

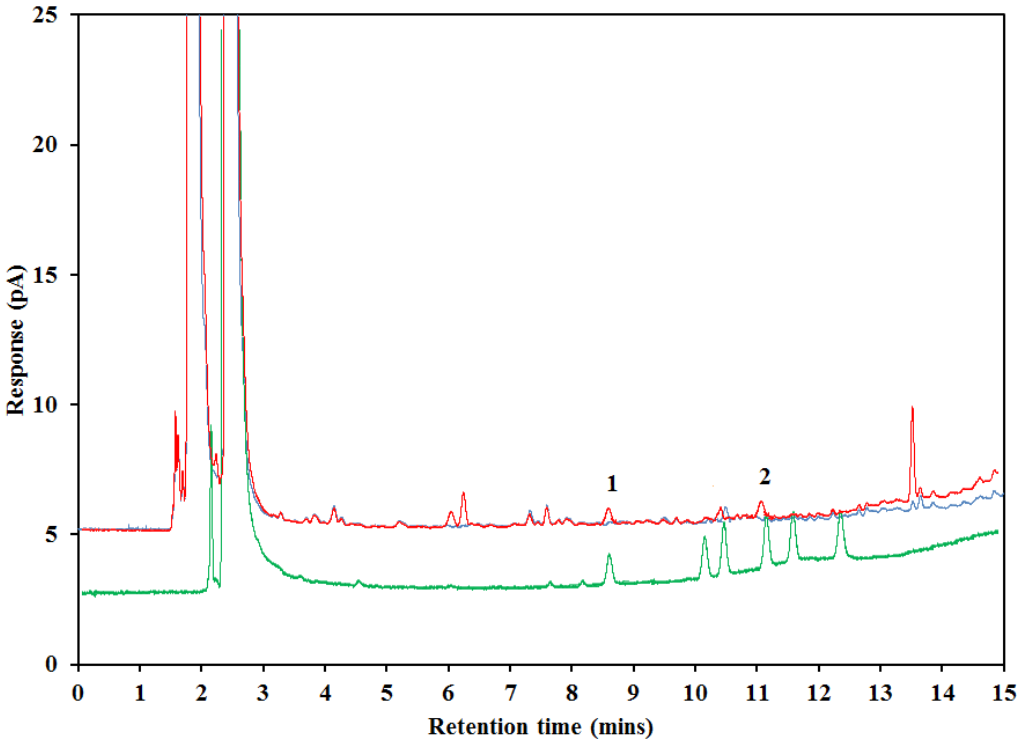


Figure 5-24. DLLME of cottage cheese (red) overlain with a reagent blank (blue) and a standard mixture in acetone (green). Analyte I.D. (µg /mL): 1: acetic acid, 2: n-butyric acid (4.04). Propionic, n-butyric, iso-valeric and n-valeric were not detected.

5.5.2.3 Brie cheese

The analysis determined that the following organic acids were present in brie cheese: acetic acid (NQ) and n-butyric acid at 42.30 µg /g; iso-valeric: 0.61 µg/g and n-valeric: 0.91 µg/g. Propionic, iso-butyric were not detected using this method.

Only one paper could be found that cited any experimentation on the organic acid content of brie cheese [58]. Mullin noted that acetic acid was present at 1206 µg/g. Acetic was identified using this method qualitatively and so no comparison can be

made in this instance. The resulting chromatogram can be found in *Figure 5-25* and a comparison of concentrations obtained with literature values in *Table 5-16.*

Table 5-16. Comparison of organic acid concentrations in brie cheese with literature values. ND: not detected, NQ: not quantifiable

	Acetic acid	**Propionic acid**	**iso-butyric acid**	**n-butyric acid**	**iso-valeric acid**	**n-valeric acid**
AVG peak area				16.3		
AVG retention time (mins)	8.49			11.08		
EF			1.59	1.37	40.51	43.41
Enriched concentration (µg/g)				58.1	ND	ND
Original concentration (µg/g)	NQ	ND	ND	42.31	ND	ND
Mullin [58] (µg/g)	1260					

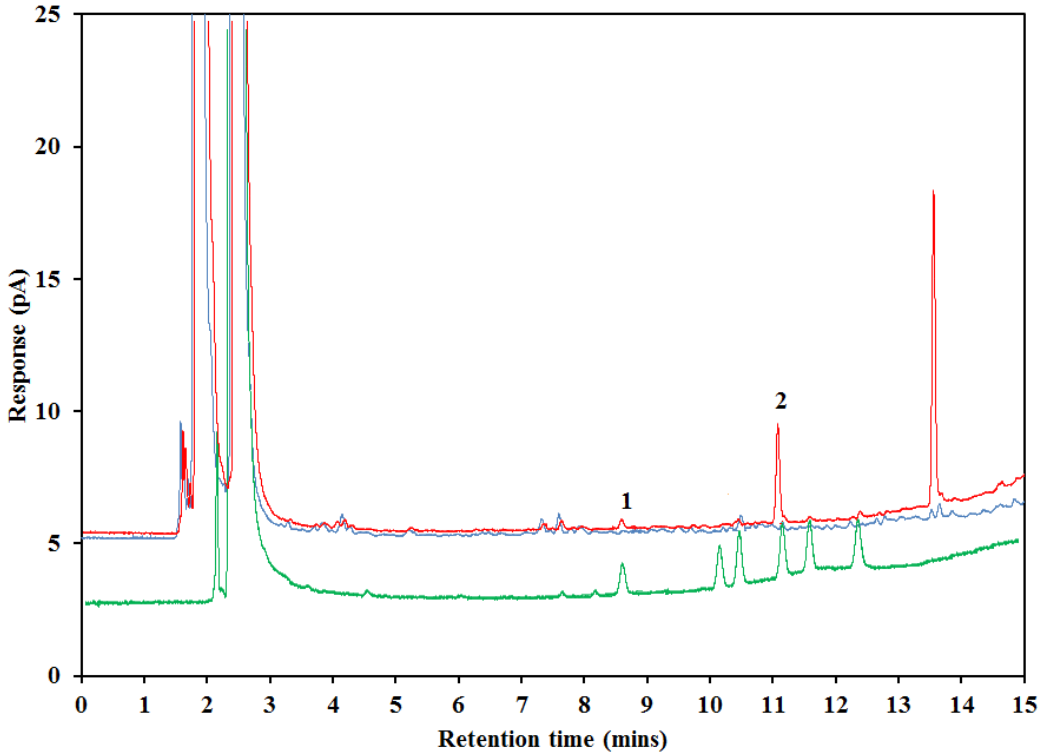


Figure 5-25. Chromatogram of DLLME on brie cheese (red) overlain with a reagent blank (blue) and a standard mixture in acetone (green). Analyte I.D. (µg /mL): 1: acetic acid; 2: n-butyric acid (42.31). Propionic, iso-butyric, iso-valeric and n-valeric acids were not detected.

Since these are different types of cheese, one would expect different organic acids present at different levels as they contribute to different flavours and appear in higher or lower concentrations depending on cultures used, stage of ripening, breed, animal nutrition, seasonal changes etc. All the cheeses contain acetic acid though it is not quantifiable with the current method and so no direct comparison can be made with this work for this analyte since it cannot be extracted.

Concentrations of iso-butyric acid were not cited in any of the literature sources. The only cheese sample tested that contained iso-butyric was goat's cheese (and

goat milk) at a concentration of: 5.6 µg/g. It is possible that it has not been detected in the works cited above as it is present at trace amount that could not be detected without a preconcentration technique such as DLLME. A DLLME technique such as the method proposed could therefore be useful.

5.5.3 *Yogurt's*

5.5.3.1 Probiotic yogurt

The analysis determined that the original concentration of organic acids present in probiotic yogurt were: acetic acid (NQ), iso-butyric acid at 6.12 µg /g, n-butyric acid at 6.90 µg /g, iso-valeric acid at 5.91 µg /g and n-valeric acid at 6.06 µg /g, propionic acid was not detected.

Literature sources cite the general health benefits of probiotic yogurts [60,61]. Though only one literature source could be found that studied organic acids similar to those in this body of work, and that source looked only at butyric acid [18]. Mojgani found butyric acid at 2 µg/g in comparison to this works 6.90 µg/g. Again, differences can be due to factors such as cultures used. Mojgani also noted that butyric acid levels were consistently higher in probiotic yogurts in comparison with standard yogurts. The resulting chromatogram can be found in *Figure 5-26* and a comparison of concentrations obtained with literature values in *Table 5-17.*

Table 5-17. Comparison of organic acid concentrations in probiotic yogurt with literature values. ND: not detected, NQ: not quantifiable

	acetic acid	propionic acid	iso-butyric acid	n-butyric acid	iso-valeric acid	n-valeric acid
Average peak area			3.56	3.45	6.81	7.45
AVG retention time (mins)	8.5		10.12	11.02	11.51	12.23
EF			1.59	1.37	40.51	43.41
Enriched concentration (µg/g)			9.72	9.48	239.62	263.04
original concentration (µg/g)			6.12	6.9	5.91	6.06
Mojgani [18], probiotic (µg/g)				45.2		

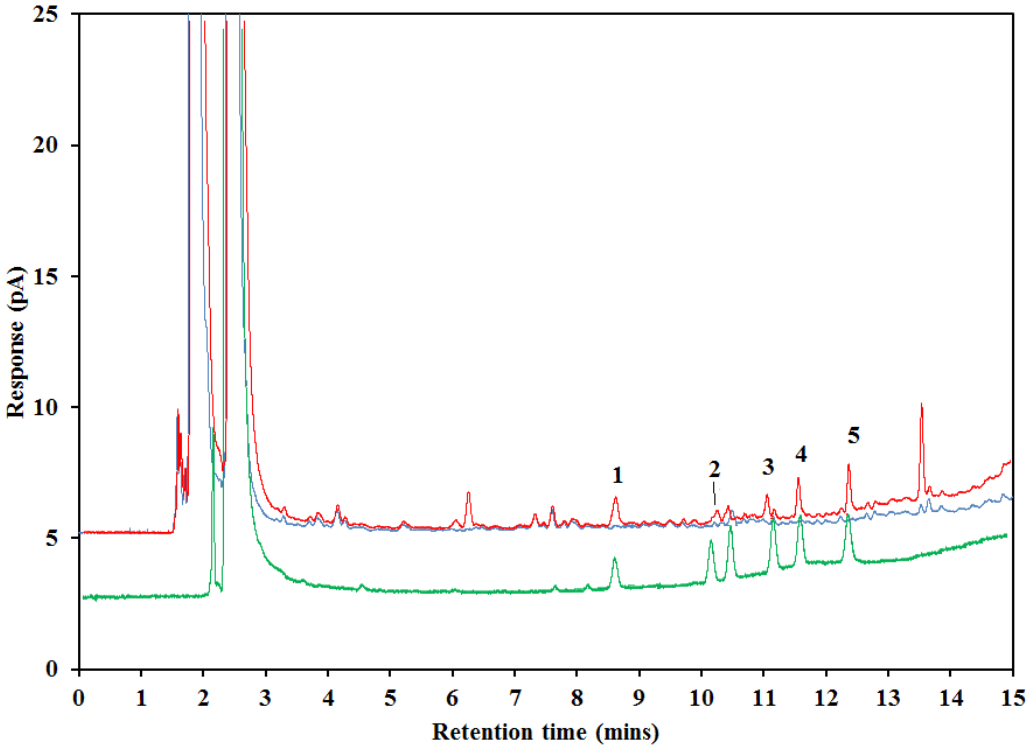


Figure 5-26. Chromatogram of DLLME on a well-known probiotic yogurt (red) overlain with a blank (blue) and standard mixture in acetone (green). Analyte I.D. (µg /g): 1: acetic acid (not quantifiable); 2: iso-butyric acid (6.12); 3: n-butyric acid (6.90); 4: iso-valeric acid (5.91); 5: n-valeric acid (6.06). Propionic acid was not detected.

5.5.3.2 Greek yogurt

The analysis determined that the original concentration of organic acids present in Greek yogurt were: acetic acid (NQ) and n-butyric acid at 11.00 µg /g. Propionic, n-butyric, iso-valeric and n-valeric acid were not detected.

Serafeimidou *et al.* quantified the concentration of fatty acids in Greek yogurt, though like many others, cited above, the research group only looked at saturated

fatty acids with even number carbon atoms; starting from butyric [62]. The group noted that n-butyric was found at ~ 45.2 μg/g.

Marsili looked at natural yogurt and found several organic acids, those relevant to this work were acetic and propionic acid [1]. These two acids were both found at ~ 120 μg/g. Given this, it is difficult to give any comparisons.

Several sources cited above claim that probiotic yogurts contain much higher concentrations of n-butyric acid. This does not appear to be the case in this instance since the literature values here for Greek yogurt have vastly higher amounts. In comparison, this work found no butyric acid in Greek yogurt. Again, this can be due to different strains of starter cultures used in its manufacture. It should also be noted that this work tested only one sample of Probiotic, given such a small sample size, it is not possible to state. The resulting chromatogram can be found in *Figure 5-27* and a comparison of concentrations obtained with literature values in *Table 5-18.*

Table 5-18. Comparison of organic acid concentrations in Greek yogurt with literature values. ND: not detected, NQ: not quantifiable

	acetic acid	propionic acid	iso-butyric acid	n-butyric acid	iso-valeric acid	n-valeric acid
Average peak area				5.55		
AVG retention time (mins)	8.52			11.03		
EF			1.58	1.37	40.51	43.418
Enriched concentration (µg/g)				17.45		
original concentration (µg/g)				11		
Serafeimidou [62] (µg/g)			45.2			
Marsili [1], (µg/g)	120	120				

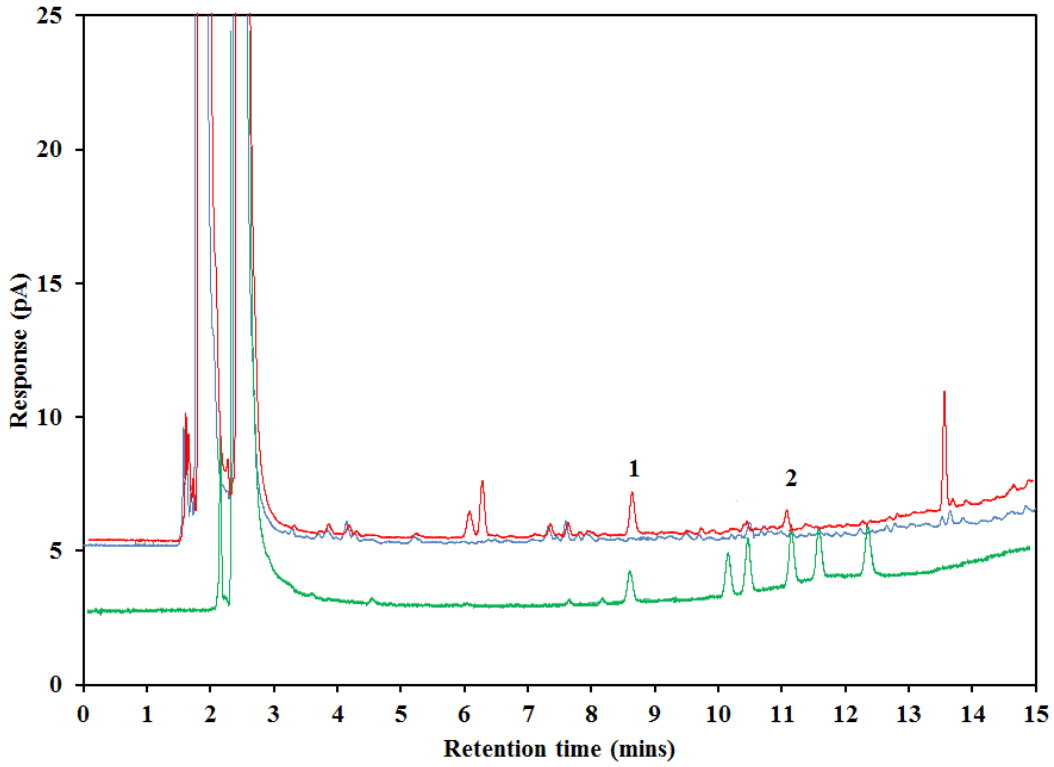


Figure 5-27. Chromatogram of DLLME on Greek yogurt (red) overlain with a blank (blue) and standard mixture in acetone (green). Analyte I.D. (µg /g): 1: acetic acid (not quantifiable); 2: n-butyric acid (11.00). Propionic, n-butyric, iso-valeric and n-valeric were not detected.

Table 5-19. Summary of results for this work. Organic acids (except for propionic acid) were detected via comparison with retention times of a standard mixture, n-butyric acid was quantified in all samples except Greek yogurt. Acetic acid was detected in all samples, though not quantifiable (NQ). Propionic acid was not detected (ND) in any sample using this method. This could be due to the extraction procedure rather than not being present.

	Acetic acid	**Propionic acid**	**iso-butyric acid**	**n-butyric acid**	**iso-valeric acid**	**n-valeric acid**
Goat milk (µg/mL)	NQ	ND	13.23	14.64	13.12	12.72
Buttermilk (µg/mL)	NQ	ND		14.38	12.22	12.78
Cow milk (µg/mL)	NQ	ND	ND	10.41	ND	ND
Goat cheese (µg/g)	NQ	ND	5.68	7.51	5.06	5.32
Cottage cheese (µg/g)	NQ	ND	ND	4.04	ND	ND
Brie (µg/g)	NQ	ND	ND	42.31	0.39	0.72
Probiotic (µg/g)	NQ	ND	6.13	6.9	5.91	6.06
Greek (µg/g)	NQ	ND	ND	11	ND	ND

5.6 Conclusion

An existing GC method was validated for the analysis of six volatile organic acids found in milk and dairy produce [30]. Those acids were acetic, propionic, iso-butyric, n-butyric, iso-valeric and n-valeric acid. These analytes were chosen due to their volatility and appearance in milk and dairy produce [3,41,63]. The method gave retention times of 8.79, 10.06, 10.67, 11.18, 11.68 and 12.49 minutes,

respectively and %RSD of: < 0.06%. Peak area was also assessed and gave %RSD of ≤ 2.0 % for analytes, excluding acetic which was 3.0%. Coefficients of determination (R^2) were all ≥ 0.99. LODs for acetic, propionic, iso-butyric, n-butyric, iso-valeric and n-valeric acid were estimated to be: 21.88, 67.25, 8.04, 6.86, 39.38 and 21.68 μg/mL, respectively and LOQs were: 66.32, 203.79, 24.38, 20.82, 119.36 and 65.71 μg/mL, respectively.

A novel preconcentration technique, DLLME, based on the work of Fazeli-Bakhitiary was optimised and validated for the analysis of the organic acids noted above [31]. The optimum extraction and dispersing solvents were found to be chloroform (100 μL) and acetone (700 μL); optimum pH was found to be pH 2.50, optimum NaCl concentration was found to be 20% w/v and the optimum centrifugation time was found to be 5 minutes; sonication was found to be detrimental to the analysis as it lowered EFs. Because EFs describe how much more concentrated the analyte is in comparison with its original concentration, the results of each optimised factor were reported in terms of EFs with preference given to obtaining the highest EFs possible. EFs for acetic, propionic, iso-butyric, n-butyric, iso-valeric and n-valeric acid were: 0.17, 0.19, 1.59, 1.37, 40.51 and 43.41, respectively. EFs for both acetic and propionic acid were < 1. This indicates that mass transfer of analyte was not achieved. Given this acetic acid and propionic acid could only be determined qualitatively. Recoveries for iso-butyric, n-butyric, iso-valeric and n-valeric acid were: 98.25, 83.67, 87.09 and 87.41%, respectively. Intermediate precision was carried out to ascertain if there was any difference between results obtained in the morning and in the night and found no significant difference in results.

The optimised and validated method was then applied to real samples of milk and dairy produce, the following results were obtained: cow's milk: acetic: (NQ); n-butyric: 10.41 μg/mL. Buttermilk: acetic: (NQ); n-butyric: 14.38 μg/mL; iso-valeric: 12.22 μg/mL; n-valeric: 12.78 μg/mL. Goat's milk: acetic acid (NQ); iso-butyric: 13.23 μg/mL; n-butyric: 16.46 μg/mL; iso-valeric: 13.12 μg/mL; n-valeric: 12.72 μg/mL. Cottage cheese: acetic acid (NQ); n-butyric: 4.04 μg/g. Brie cheese: acetic acid (NQ); n-butyric: 42.31 μg/g; iso-valeric: 0.39 μg/g; n-valeric: 0.72 μg/g. Probiotic yogurt: acetic acid (NQ); iso-butyric: 6.13 μg/g; n-butyric: 6.90 μg/g; iso-valeric: 5.91 μg/g; n-valeric: 6.06 μg/g. Greek yogurt: acetic acid (NQ); n-butyric: 11.00 μg/g.

This work found that while there were large variations between the concentrations found through use of this GC-DLLME method and those cited in the literature, there were also large variations found between literature sources and with the results quoted within single studies. With regard to this study, only one sample type was used from one shop, not a variety of the same brand or different brands of, for example, buttermilk (though this is true for all samples) from a variety of sources. The studies cited in the relevant sections have all used vast numbers of different sample types from different sources.

There are several hugely important factors and even more combinations of these factors that can explain the variations in the organic acid profiles. Those factors are physiological (breed of animal, age, stage of lactation and health), nutrition and supplements (grass, silage, grain, soy fortified with vitamins and minerals), seasonal changes, geographical changes and the types of cultures used in the manufacturing process [49–52,54,64–69].

This work was designed as a proof of concept, to show that this type of extraction can be used to extract the quoted acids from milk and dairy produce, rather than to compare the levels of these organic acids in milk and dairy produce; though this work has clearly shown that the optimised DLLME can be used for this purpose, especially for the analysis of valeric acid since the clear majority of authors looking at fatty acid levels show preference to even carbon acids starting at C4 – the reason for this was unclear.

Most of the extraction procedures available have used large quantities of solvents as demonstrated in the literature review. This method has addressed solvent consumption and uses only 800 µL in total, significantly cutting down cost and waste. As described in the DLLME literature review, extractions using DLLME are almost instantaneous [70–72]. This means that even though miniaturised extraction techniques such as SPE have also addressed the issue of solvent consumption, DLLME excels due to the length of time it takes to undertake the extraction; this will increase sample throughput in busy laboratories. DLLME is also far simpler since the extraction only entails injecting a binary mixture into a pH adjusted sample containing a salt, rather than a laborious preconditioning, loading, washing and elution step as seen in SPE. DLLME also produces very high EFs as does SPE, though DLLME was found to be unsuitable for highly polar analytes such as acetic and propionic acid using the chosen solvents. Others have researched the use of ionic liquids in DLLME; this could be an avenue of future work.

Several of the organic acids extracted also have significance in a clinical setting as acetic, propionic and valeric acid are often used as bio-markers for several diseases. Acetic acid can be used to identify urinary tract diseases in pregnant women [73],

propionic acid can be used to identify patients with vitamin B_{12} deficiency [74] and valeric acid can be used to identify a genetic disease called valeric acidosis [75]. This is significant as it shows the versatility of the method and that that this work has potential outside of food analysis.

5.7 References

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6. Conclusion

The previous chapters contain an in-depth investigation into the appearance of organic acids in milk and dairy produce, their extraction using a novel preconcentration technique called dispersive liquid-liquid microextraction (DLLME) and identification using several chromatographic techniques, such as: high performance liquid chromatography with ultra violet detections (HPLC-UV), gas chromatography with flame ionisation detection (GC-FID) and capillary electrophoresis with diode array UV detection (CE-DAD).

This work began by discussing the most common organic acids in milk and dairy produce as established in a variety of literature sources [1–5]. Further to this, their physiochemical properties, occurrence and role of organic acids were also established. It was found that organic acids occur in milk and dairy produce due to factors such as: natural biochemical processes within the animal, additives to improve quality and shelf life, adulteration, bacterial growth and types of starter cultures used [5,6]. It has also been well established in the literature that the levels of these organic acids vary widely due to nutrition, season, breed, age, stage of lactation and mastitis [7–11].

Milk and dairy produce contain several interferences such as proteins, peptides, amino acids, vitamins, minerals, carbohydrates and lipids. All of which require removal prior to analysis. The Carrez method, as described by Kamankesh [12] and Ghasemian [13] proved to be the most useful of all protein removal techniques as the analytes then reside in an aqueous matrix which is ideal for extraction using DLLME. During experimentation, it was found that higher fat produce required larger volumes of the Carrez solutions to fully remove proteins and lipids.

The literature reviews, found in **Chapter 1** and **2** discussed the theory and use of the more traditional extraction techniques: liquid-liquid extraction (LLE), solid-liquid extraction (SLE) and distillation, as well as the newer, miniaturised extraction techniques such as: solid phase extraction (SPE), solid phase microextraction (SPME), cloud point extraction (CP) and DLLME. It was established that SPE, SPME and DLLME in particular were developed to address the principles of sustainable development and green chemistry [14] and that this body of work should follow that principle. The literature reviews noted that there was a gap in the literature regarding the use of DLLME for the extraction of small, highly polar organic acids.

The technique was easily optimised by experimenting with the following variables: extraction solvent type and volume, dispersing solvent type and volume, chromatographic behaviour of the chosen extraction solvent, concentration of salt, pH of aqueous sample, sonication time and centrifugation time.

The optimum extraction and dispersing solvents were found to be chloroform (100 µL) and acetone (700 µL); optimum pH was found to be pH 2.50, optimum NaCl concentration was found to be 20% w/v and the optimum centrifugation time was found to be 5 minutes; sonication was found to be detrimental to the analysis as it lowered EFs. EFs for acetic, propionic, iso-butyric, n-butyric, iso-valeric and n-valeric acid were: 0.17, 0.19, 1.59, 1.37, 40.51 and 43.41, respectively. EFs for both acetic and propionic acid were < 1. This indicates that complete mass transfer of analyte was not achieved, they were therefore only qualitatively assessed. Recoveries were performed by spiking known amounts of standards into buttermilk. Recoveries for iso-butyric, n-butyric, iso-valeric and n-valeric acid were: 98.25, 83.67, 87.09 and 87.41%, respectively. Intermediate precision was

carried out to ascertain if there was any difference between results obtained in the morning and in the night and found no significant difference in results. The DLLME process was optimised using GC-FID, detail of which are below.

CE was the first separation technique used due to the variety of anions that could potentially be separated in one analysis. Anions such as ionised organic acids, amino acids, minerals such as phosphates, sulphates, nitrites and nitrates, chloride and fluoride. This wide variety of analytes could potentially have given a wider scope for the use of the extraction. Unfortunately, this was not to be the case as a base line anomaly appeared that could not be resolved. Given this the work then focused on producing orthogonal separations using HPLC and GC.

Initially, 18 organic acids were identified as common constituents of milk and dairy produce, those being: lactic, tartaric, malic, n/iso-butyric, n/iso-valeric, propionic, oxalic, citric, pyruvic, formic, acetic, succinic, fumaric, orotic, uric and hippuric acid. Two different reversed phase (RP) columns were used for this work. A traditional C_{18} column and a more specialised ion exclusion column (IEC). The most successful in terms of the achieved separation was found to be IEC. The IEC method separated seven of the 18 analytes, those being: succinic, formic, acetic, propionic, iso-valeric, n-valeric and hippuric acid in < 40 minutes. LODs were, 1.61, 0.57, 5.20, 1.82, 2.62, 2.27 and 0.04 μg/mL respectively, while LOQs were, 4.90, 1.74, 15.77, 5.51, 7.95, 6.89 and 0.12 μg/mL respectively. R^2 values were all equal to 1 showing excellent linearity, resolution was > 1.5 for all analytes and peak area and retention time %RSD were all below 2 %. Problems arose with marrying the extraction to the column. This was due to the nature of the solvents used and the chemistry of the column. The solvents resided in a surfactant or chloroform matrix and such solvents are not compatible with the polymer

divinylbenzene resin that the sulphonic groups were bound to as they adsorb strongly and swell the resin, resulting in a fouled column. It is possible to back-extract the analytes from chloroform into an aqueous matrix thus allowing compatibility with such a column. Adding an extra step would obviously require optimisation and possibly lower recoveries. This could be experimented with in any future work.

The C_{18} gradient method was found toward the end of the project and was based on a PerkinElmer application note [15]. This method had the ability to separate 11 organic acids: lactic, acetic, propionic, n/iso-butyric, n/iso-valeric, methylvaleric, hexanoic, heptanoic and octanoic acid. Unfortunately, several issues arose with this separation mainly due to the use of a very old column found in the stores. A lack of time meant that troubleshooting this issue was not possible. This method had the potential to accommodate the use of DLLME since chloroform can be injected into this type of column. Hexanoic, heptanoic and octanoic acids are also found in milk and dairy produce and due to their hydrophobicity, will extract well into chloroform [16]. Given this, future work could entail purchasing a new column and expanding the range of compounds extracted and analysed via this gradient HPLC method and further optimising this DLLME procedure.

Finally, the most successful work can be found in the GC-FID chapter. A separation of six organic acids, chosen due to their volatility was achieved using a gradient method on a polyethylene glycol column (PEG) with a 5% phenyl moiety. This method was validated for the analysis of six volatile organic acids found in milk and dairy produce. Those acids were acetic, propionic, iso-butyric, n-butyric, iso-valeric and n-valeric acid. The method gave a retention time %RSD of: < 0.06%. Peak area was also assessed and gave %RSD of < 0.0%. Coefficients of

determination (R^2) were all > 0.999. LODs for acetic, propionic, iso-butyric, n-butyric, iso-valeric and n-valeric acid were estimated to be: 21.88, 67.25, 8.04, 6.86, 39.38 and 21.68 μg/mL, respectively and LOQs were: 66.32, 203.79, 24.38, 20.82, 119.36 and 65.71 μg/mL, respectively.

The optimised DLLME and GC-FID analysis was then applied to the determination of organic acids in real samples. Samples analysed were: goat's milk, cow's milk, buttermilk, goat's cheese, cottage cheese, brie cheese, a well-known probiotic yogurt and Greek yogurt; results of which can be found in **5.5**. This work found large variations between the concentrations found and those cited in the literature, though there were also large variations between literature sources. Several reasons exist as to why these variations occur such as breed, nutrition and season, to name but a few. All of which are discussed above and in more depth in the relevant chapters.

This work was designed as a proof of concept, to show that this type of extraction can be used to extract the quoted acids from milk and dairy produce, rather than to compare the levels of these organic acids in milk and dairy produce; though this work has clearly shown that the optimised DLLME can be used for this purpose, especially for the analysis of valeric acid since the clear majority of authors quoted throughout, looking at fatty acid levels show preference to even numbered carbon acids starting at C4. Again, this is an area that can be explored in any future work.

This method is also highly versatile and can be used outside of the agri-food industry, as several of the analytes (acetic, propionic and valeric acid) are used as bio-markers for diseases of the urinary tract [17], vitamin B_{12} deficiency [18] and valeric acidosis [19].

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7. Appendix

7.1 Injection volume graphs for ion exclusion chromatographic (IEC) work

As the volume injected increases, peak height (orange data lines) increases. This coincides with a decrease in efficiency (blue data lines). The area where both data sets cross corresponds to the optimum injection volume for that specific analyte. Below are the graphs (Figure 7-1 - Figure 7-7) used to determine injection volume in the IEC work.

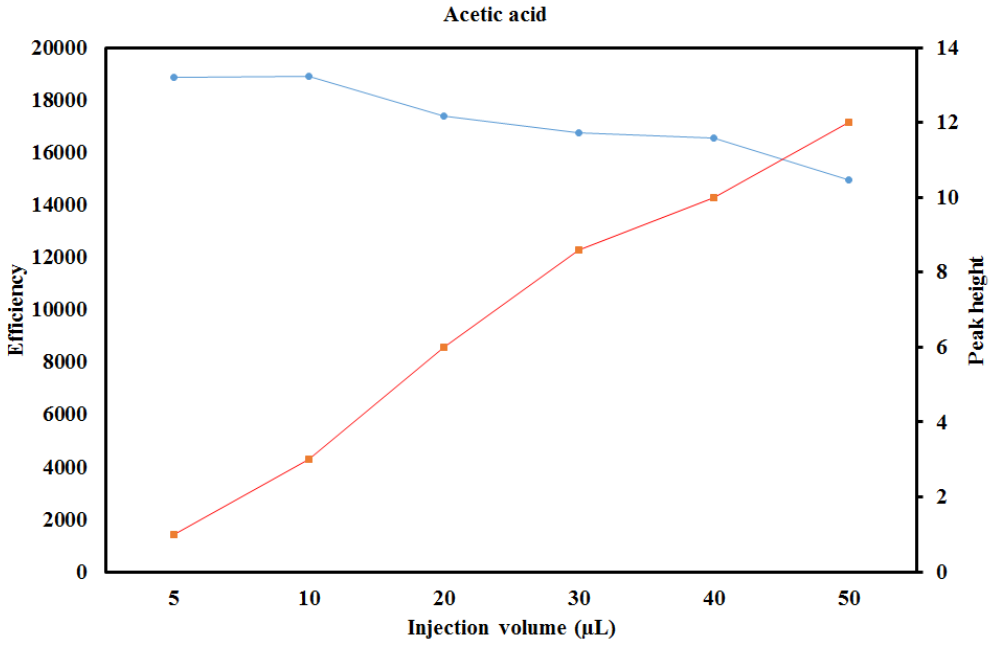

Figure 7-1Graph of efficiency (blue) versus peak height (orange) versus injection volume (µL) for acetic acid; optimum injection volume: 45 µL.

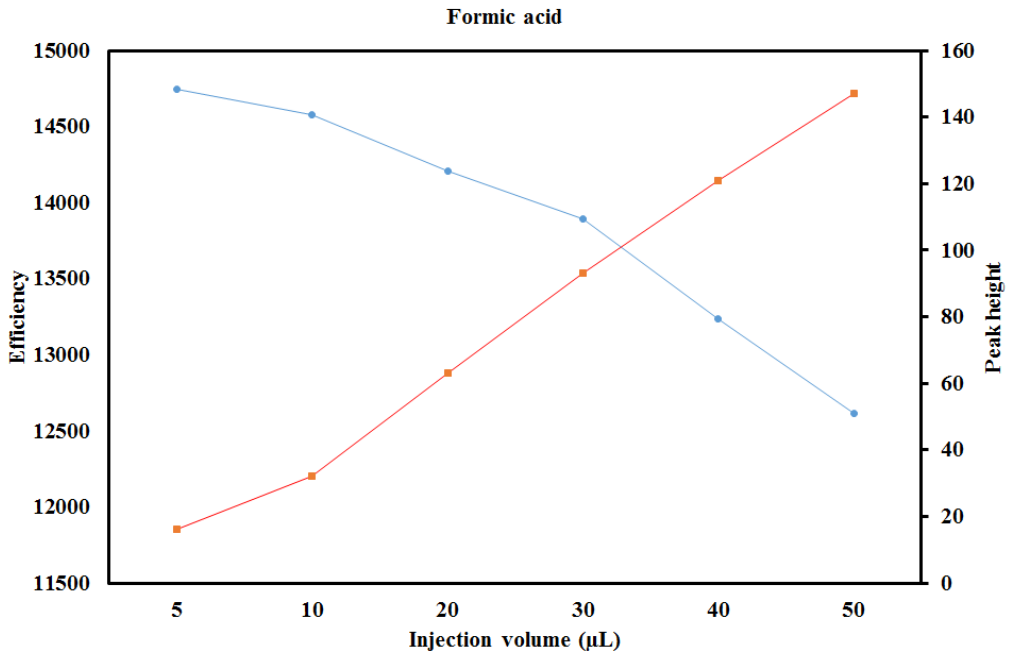

Figure 7-2. Graph of efficiency (blue) versus peak height (orange) versus injection volume (µL) for formic acid; optimum injection volume: 32 µL.

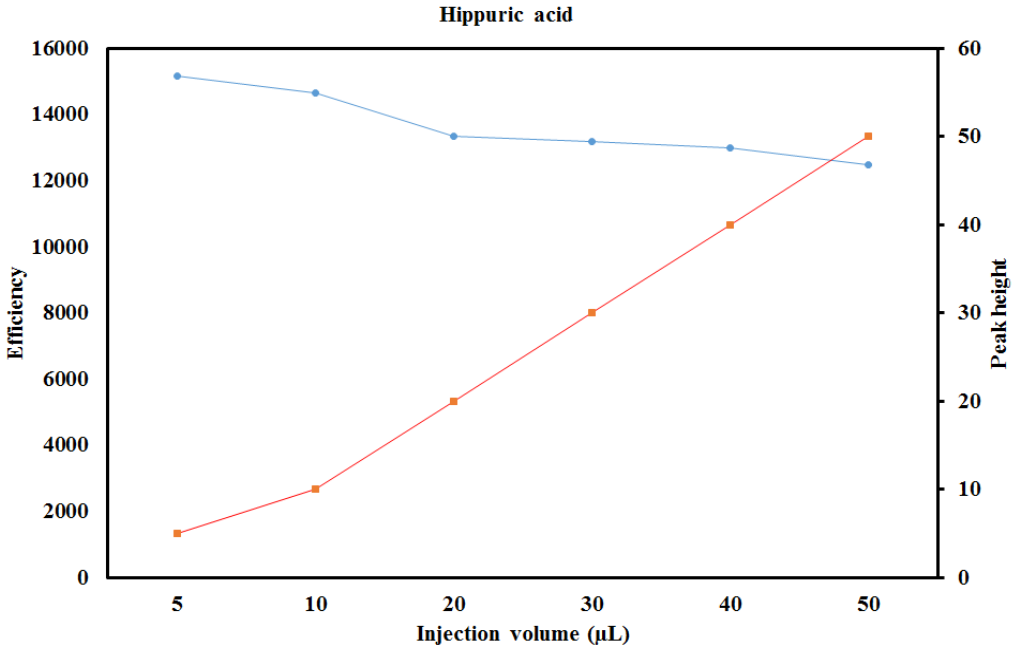

Figure 7-3. Graph of efficiency (blue) versus peak height (orange) versus injection volume (µL) for hippuric acid; optimum injection volume: 47 µL.

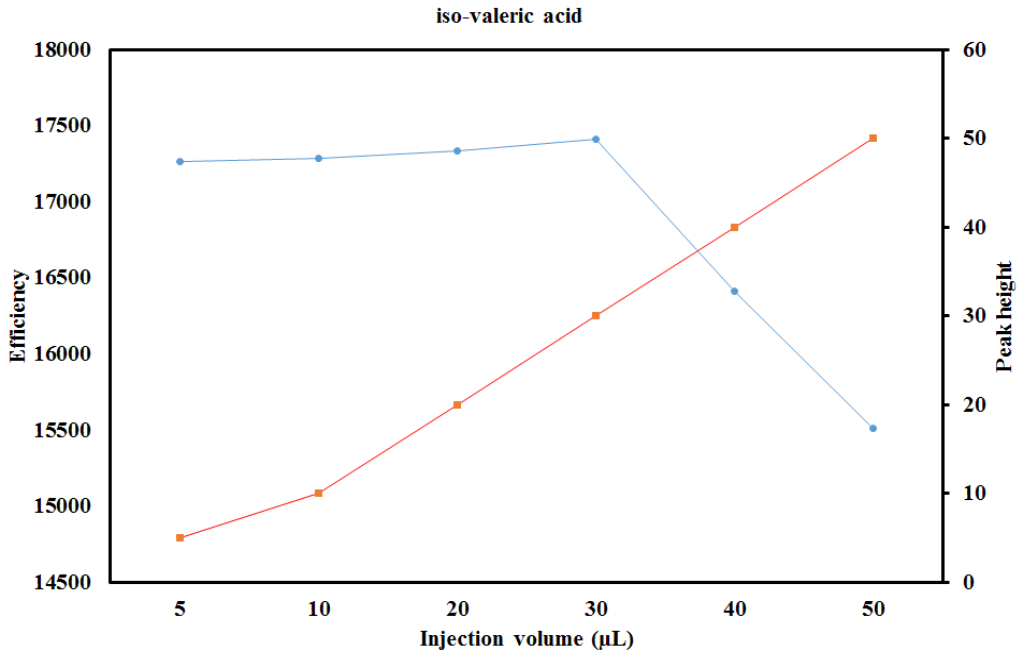

Figure 7-4. Graph of efficiency (blue) versus peak height (orange) versus injection volume (µL) for iso-valeric acid; optimum injection volume: 38 µL.

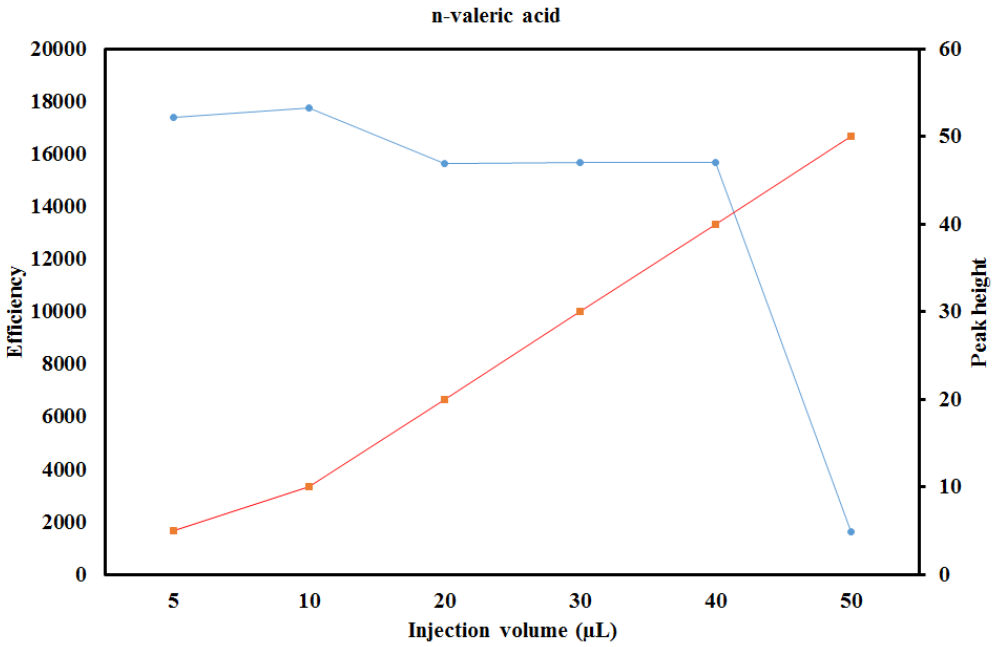

Figure 7-5. Graph of efficiency (blue) versus peak height (orange) versus injection volume (µL) for n-valeric acid; optimum injection volume: 42 µL.

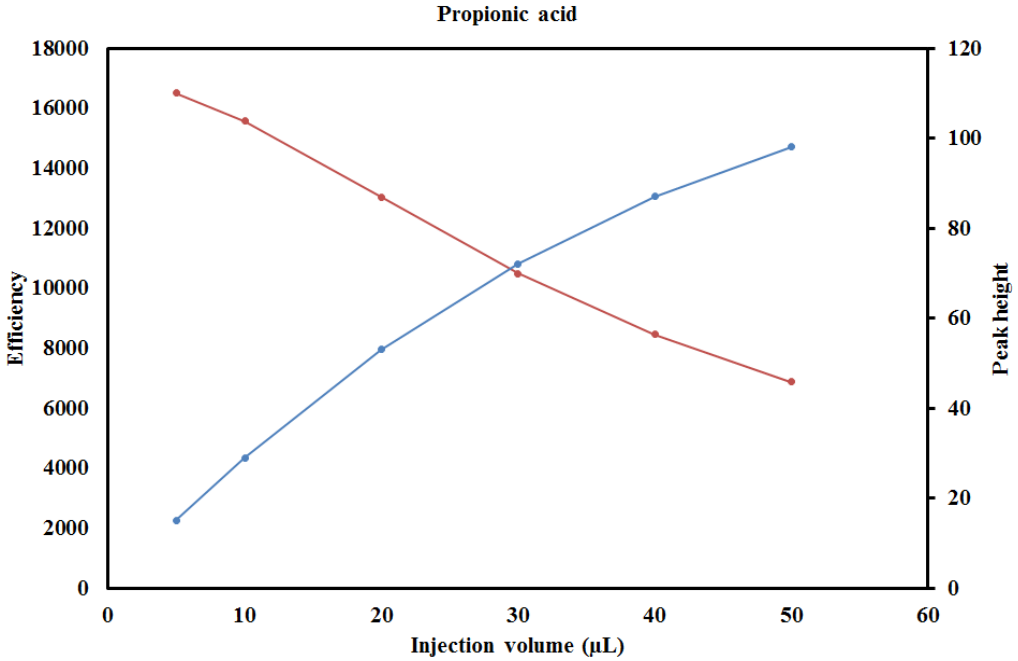

Figure 7-6. Graph of efficiency (blue) versus peak height (orange) versus injection volume (µL) for propionic acid; optimum injection volume: 30 µL.

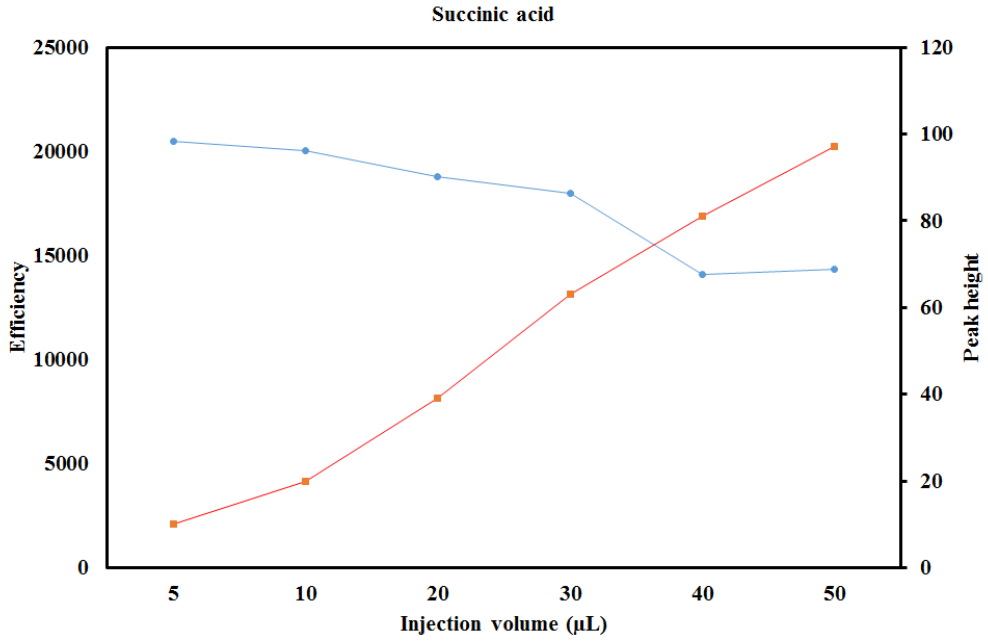

Figure 7-7. Graph of efficiency (blue) versus peak height (orange) versus injection volume (µL) for succinic acid; optimum injection volume: 35 µL.

7.2 Ion exclusion chromatography linearity graphs and data used to estimate LODs & LOQs

7.2.1 *Succinic acid*

LOQ: 4.91 µg/mL, LOD 1.62 µg/mL.

	Concentration (µg/mL)	**AVG peak area**
Succinic acid	600.00	763.00
	300.00	379.67
	120.00	151.00
	60.00	75.67
	40.00	50.67
	30.00	38.33
	24.00	33.00
	20.00	25.00

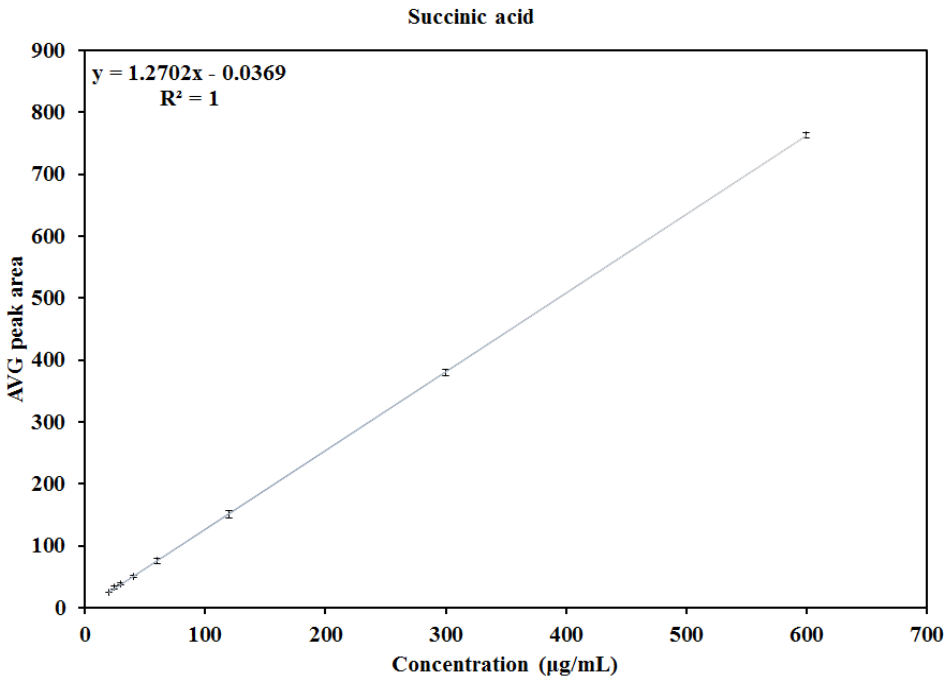

Figure 7-8. IEC calibration curve for succinic acid. Range: 20 – 600 µg/mL, R^2: 1.

SUMMARY OUTPUT

Regression Statistics	
Multiple R	1
R Square	1
Adjusted R Square	1
Standard Error	1.39
Observations	8

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	5E+05	473172.8	244917	4.59E-15
Residual	6	11.59	1.931973		
Total	7	5E+05			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	-0.04	0.623	-0.05928	0.95466	-1.561579	1.487711	-1.56	1.488
X Variable 1	1.27	0.003	494.8908	4.6E-15	1.263928	1.276489	1.264	1.276

Sample calculation for LOQ

$$LOQ = \frac{10\sigma}{slope}$$

$$LOQ = (10)\ (standard\ error\ of\ intercept))/\ (coefficient\ of\ variable)$$

$LOQ = 10\ x\ 0.623/1.270$ = 4.905 µg/mL.

Sample calculation for LOD

$$LOD = \frac{3\sigma}{slope}$$

$$LOQ = (3)\ (standard\ error\ of\ intercept))/\ (coefficient\ of\ variable)$$

$LOQ = 3\ x\ 0.623/1.270$ = 1.618 µg/mL.

7.2.2 *Formic acid*

LOQ: 1.74 µg/mL; LOD: 0.58 µg/mL.

	Concentration (µg/mL)	**AVG peak area**
Formic acid	500.00	1586.67
	250.00	791.67
	100.00	314.33
	50.00	157.00
	33.33	105.33
	25.00	78.67
	20.00	61.67
	16.00	47.00

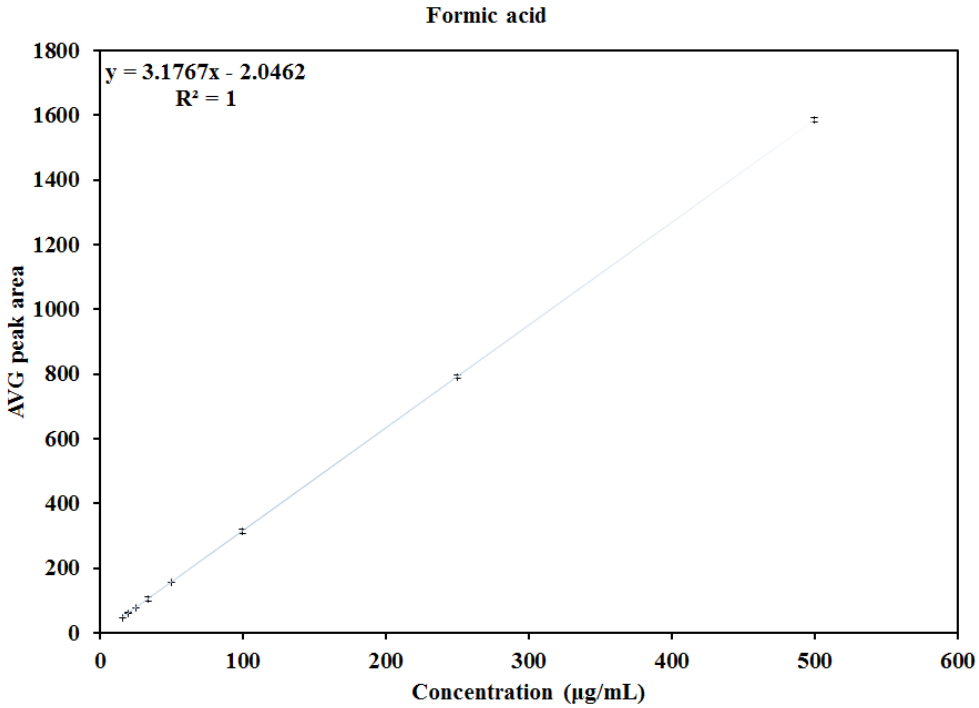

Figure 7-9. IEC calibration curve for formic acid. Range: 16 – 500 µg/mL, R^2: 1

Regression Statistics	
Multiple R	0.99999777
R Square	0.99999553
Adjusted R Square	0.99999479
Standard Error	1.23742828
Observations	8

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	2056705.465	2056705	1343173	2.7855E-17
Residual	6	9.187372516	1.531229		
Total	7	2056714.653			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	-2.0461985	0.55449954	-3.69017	0.010207	-3.403009988	-0.689387	-3.40300999	-0.689387
X Variable 1	3.17671489	0.00274102	1158.954	2.79E-17	3.17000786	3.1834219	3.17000786	3.183421929

7.2.3 *Acetic acid*

LOQ: 15.77 µg/mL, LOD: 5.21 µg/mL.

	Concentration (µg/mL)	AVG peak area
Acetic acid	3000	390.33
	1500	194.67
	600	77.67
	300	39.00
	200	25.67
	150	18.67
	120	16.00
	100	13.33

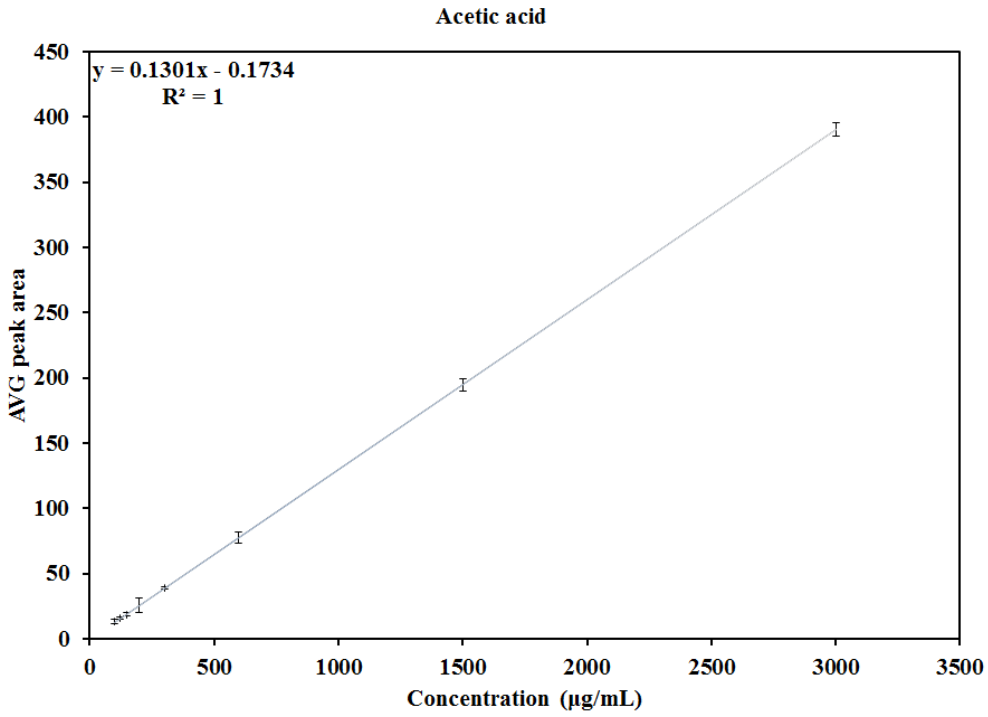

Figure 7-10. IEC calibration curve for acetic acid. Range: 100 – 3000 µg/mL, R^2:

1.

Regression Statistics	
Multiple R	0.999994934
R Square	0.999989869
Adjusted R Square	0.99998818
Standard Error	0.457780088
Observations	8

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	124105.3537	124105.4	592211.3	3.24984E-16
Residual	6	1.257375655	0.209563		
Total	7	124106.6111			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	-0.17337064	0.205213898	-0.84483	0.430599	-0.675510956	0.32876968	-0.67551096	0.32876968
X Variable 1	0.130103903	0.000169064	769.5527	3.25E-16	0.129690217	0.13051759	0.129690217	0.13051759

7.2.4 *Iso-valeric acid*

LOQ: 7.95 µg/mL, LOD: 2.62 µg/mL.

	Concentration (µg/mL)	**AVG peak area**
Iso-valeric acid	1000	1639.67
	500	813.67
	200	318.33
	100	155.33
	66.67	102.33
	50	75.00
	40	63.67
	33	49.00

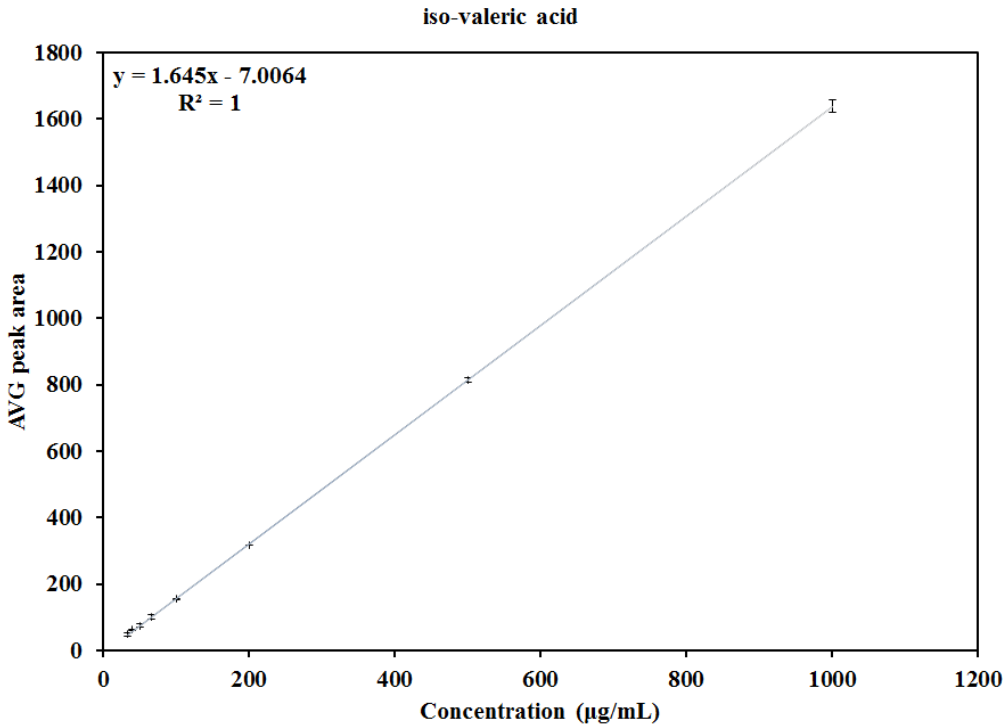

Figure 7-11. IEC calibration for iso-valeric acid. Range: 33 – 1000 µg/mL, R^2: 1.

Regression Statistics	
Multiple R	0.999988417
R Square	0.999976834
Adjusted R Square	0.999972973
Standard Error	2.917771469
Observations	8

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	2204889.128	2204889	258990.7	3.8853E-15
Residual	6	51.08034208	8.51339		
Total	7	2204940.208			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	-7.006373201	1.307854927	-5.35715	0.001733	-10.206579	-3.8061675	-10.2065789	-3.80616748
X Variable 1	1.645022032	0.003232434	508.9113	3.89E-15	1.63711255	1.6529315	1.63711255	1.652931512

7.2.5 *n-valeric acid*

LOQ: 6.90 µg/mL, LOD: 2.28 µg/mL.

	Concentration (µg/mL)	**AVG peak area**
n-valeric acid	1000.00	1221.67
	500.00	606.33
	200.00	241.00
	100.00	120.67
	66.67	80.33
	50.00	59.67
	40.00	47.00
	33.00	35.00

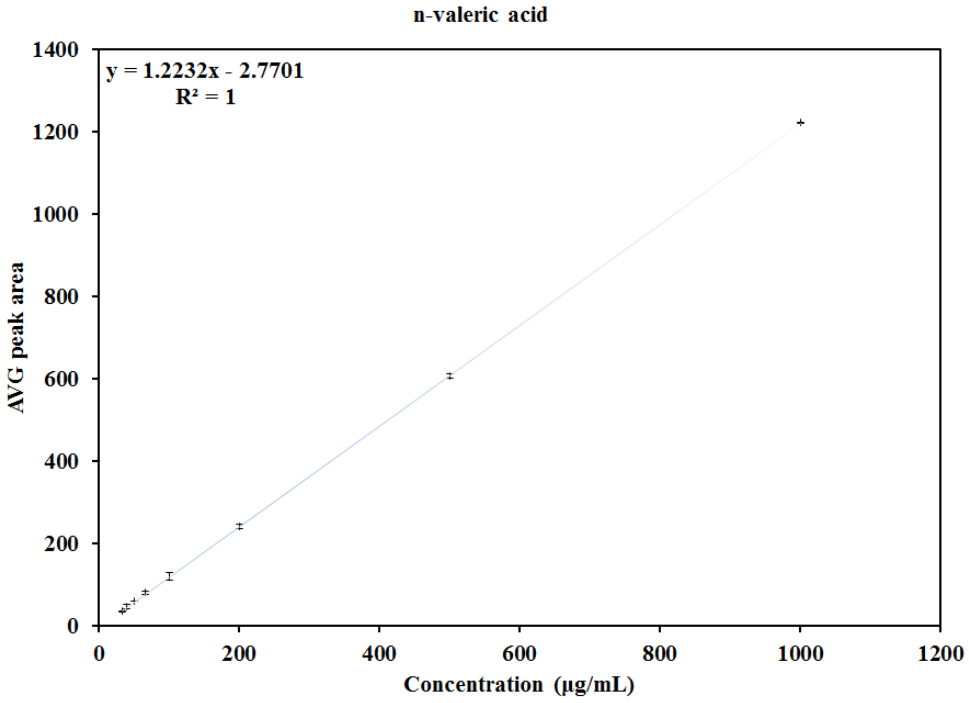

Figure 7-12. IEC calibration curve for n-valeric acid. Range: 33 – 1000 µg/mL, R^2: 1.

Regression Statistics	
Multiple R	0.99999128
R Square	0.99998257
Adjusted R Square	0.99997966
Standard Error	1.88200873
Observations	8

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	1219160.29	1219160	344205.3	1.65513E-15
Residual	6	21.25174124	3.541957		
Total	7	1219181.542			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	-2.7701062	0.843587108	-3.28372	0.016745	-4.834289471	-0.705923	-4.834289471	-0.70592289
X Variable 1	1.22323175	0.002084971	586.6901	1.66E-15	1.21813001	1.2283335	1.21813001	1.22833349

7.2.6 *Hippuric acid*

LOQ: 0.13 µg/mL, LOD: 0.04 µg/mL.

	Concentration (µg/mL)	**AVG peak area**
Hippuric acid	20.00	1792.67
	10.00	895.67
	4.00	355.33
	2.00	179.33
	1.33	120.00
	1.00	89.67
	0.80	70.00
	0.66	52.67

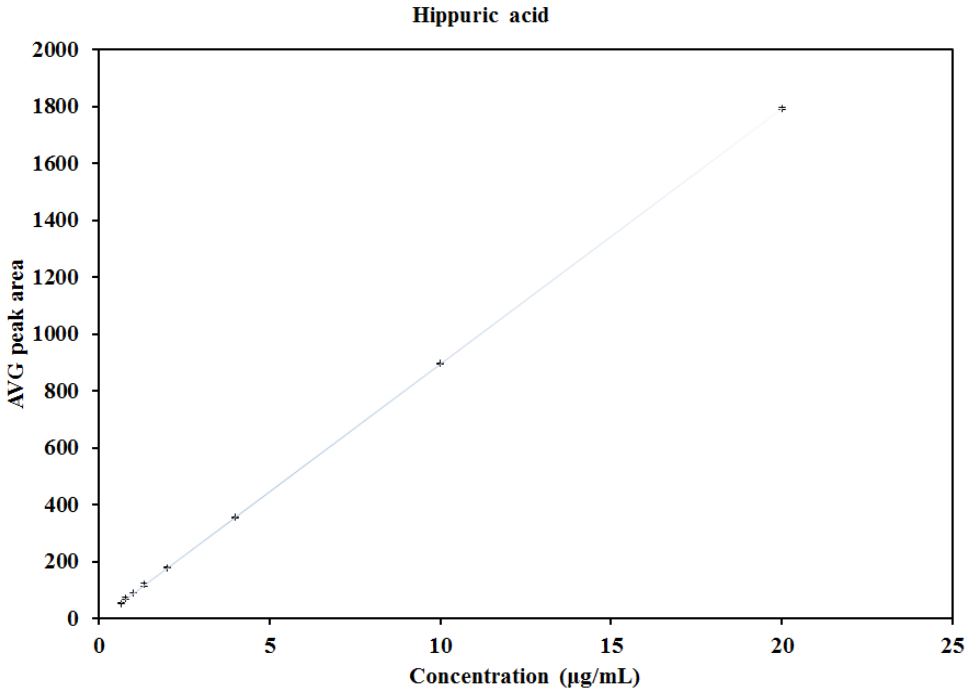

Figure 7-13. IEC calibration curve for hippuric acid. Range: 0.66 – 20 µg/mL, R^2: 1.

Regression Statistics	
Multiple R	0.999992824
R Square	0.999985648
Adjusted R Square	0.999983256
Standard Error	2.505455237
Observations	8

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	2624321.614	2624321.614	418064.9528	9.23754E-16
Residual	6	37.66383567	6.277305945		
Total	7	2624359.278			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	-1.880700423	1.122986907	-1.6747305	0.145008418	-4.628550393	0.867149547	-4.62855039	0.867149547
X Variable 1	89.73055885	0.138777319	646.579425	9.23754E-16	89.39098299	90.07013472	89.390983	90.07013472

7.2.7 *Propionic acid*

LOQ: 5.51 µg/mL, LOD: 1.82 µg/mL.

Analyte	Concentration (µg/mL)	AVG peak area
Propionic acid	1000	1317.00
	500	657.33
	200	260.33
	100	130.33
	66.67	87.67
	50	65.33
	40	51.33
	33	38.67

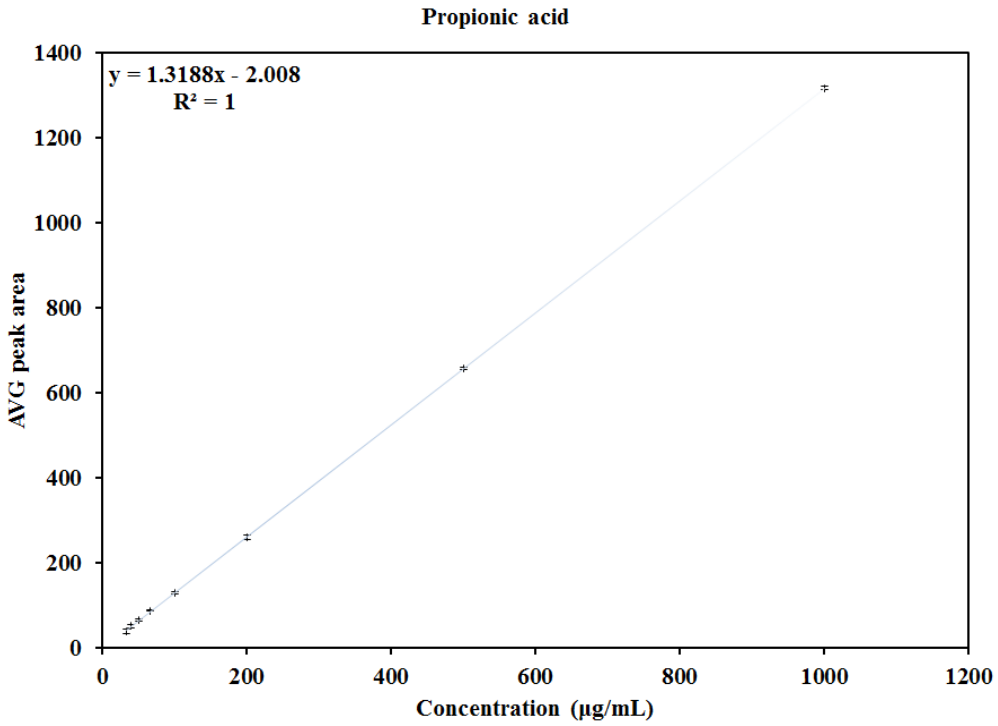

Figure 7-14. IEC calibration curve for propionic acid. Range: 33 – 1000 µg/mL, R^2: 1.

Regression Statistics	
Multiple R	0.99999445
R Square	0.99998889
Adjusted R Square	0.99998704
Standard Error	1.61979013
Observations	8

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	1417196.702	1417197	540147.8	4.28305E-16
Residual	6	15.74232032	2.62372		
Total	7	1417212.444			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	-2.0080271	0.726050865	-2.76568	0.032608	-3.7846096	-0.2314447	-3.7846096	-0.23144467
X Variable 1	1.31884394	0.001794474	734.9475	4.28E-16	1.314453019	1.32323486	1.31445302	1.323234856

7.3 Gas chromatography linearity graphs and data used to estimate LODs & LOQs

7.3.1 *Acetic acid*

LOD: 21.89 µg/mL, LOQ: 66.32 µg/mL.

Acetic acid	**Concentration (µg/mL)**	**AVG Peak area**
	242.50	28.16
	485.00	62.21
	727.50	97.00
	970.00	131.01
	1455.00	197.15
	1940.00	262.52

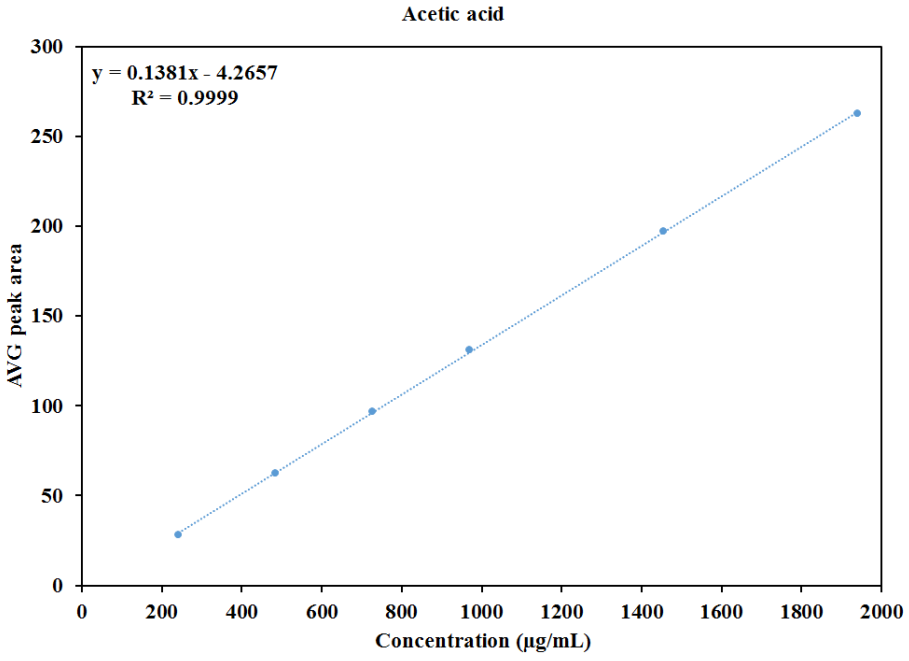

Figure 7-15. GC calibration curve for acetic acid, range: 242 – 1940 µg/mL.

Regression Statistics	
Multiple R	0.999930965
R Square	0.999861934
Adjusted R Square	0.999827418
Standard Error	1.147194675
Observations	6

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	38123.1212	38123.12	28967.71	7.14863E-09
Residual	4	5.264222487	1.316056		
Total	5	38128.38543			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	-4.265709804	0.915785399	-4.65798	0.009604	-6.808337691	-1.723082	-6.8083377	-1.72308192
X Variable 1	0.138083893	0.000811308	170.199	7.15E-09	0.13583134	0.1403364	0.13583134	0.140336446

7.3.2 *Propionic acid*

LOD: 67.25 µg/mL, LOQ: 203.79 µg/mL.

Propionic acid	Concentration (µg/mL)	AVG peak area
	285	61.9013
	570	129.59
	855	195.901
	1140	258.376
	1710	382.595
	2280	496.431

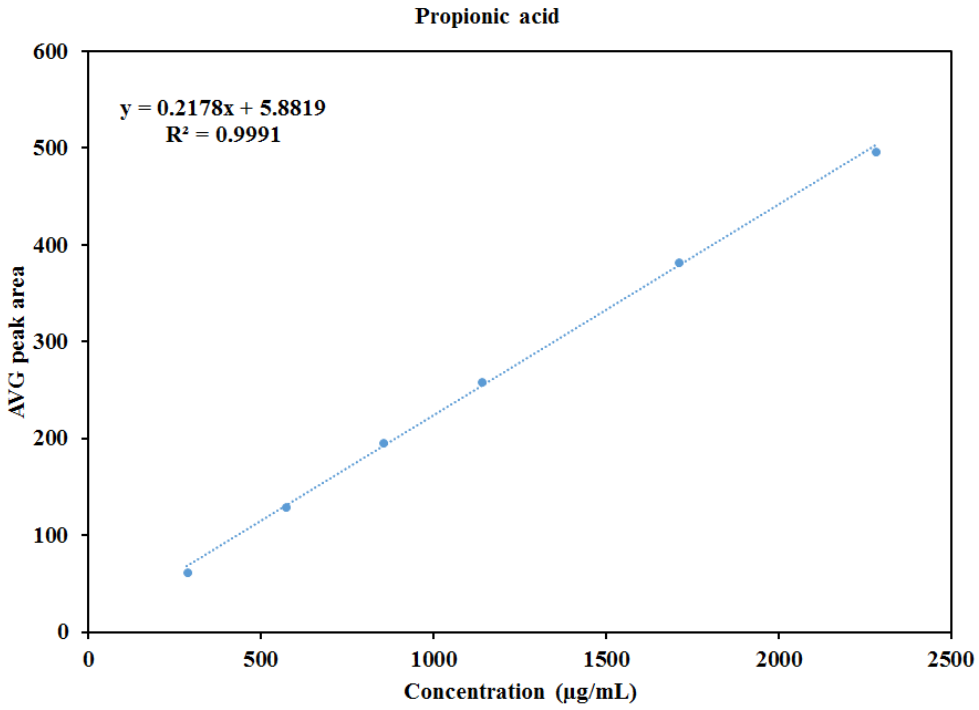

Figure 7-16. GC calibration curve for propionic acid, range: 285 – 2280 µg/mL.

Regression Statistics	
Multiple R	0.999528363
R Square	0.999056949
Adjusted R Square	0.998821186
Standard Error	5.559197944
Observations	6

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	130960.1417	130960.1	4237.55	3.336E-07
Residual	4	123.6187271	30.90468		
Total	5	131083.7605			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	5.88190098	4.437810266	1.325406	0.25567	-6.439436	18.203238	-6.4394356	18.20323757
X Variable 1	0.217763581	0.003345244	65.09647	3.34E-07	0.2084757	0.2270515	0.20847569	0.227051468

7.3.3 *Iso-butyric acid*

LOD: 8.05 µg/mL, LOQ: 24.39 µg/mL.

Iso-butyric acid	**Concentration (µg.ml)**	**AVG peak area**
	40	10.4487
	80	21.626
	120	32.408
	160	42.801
	240	63.3133
	320	82.464

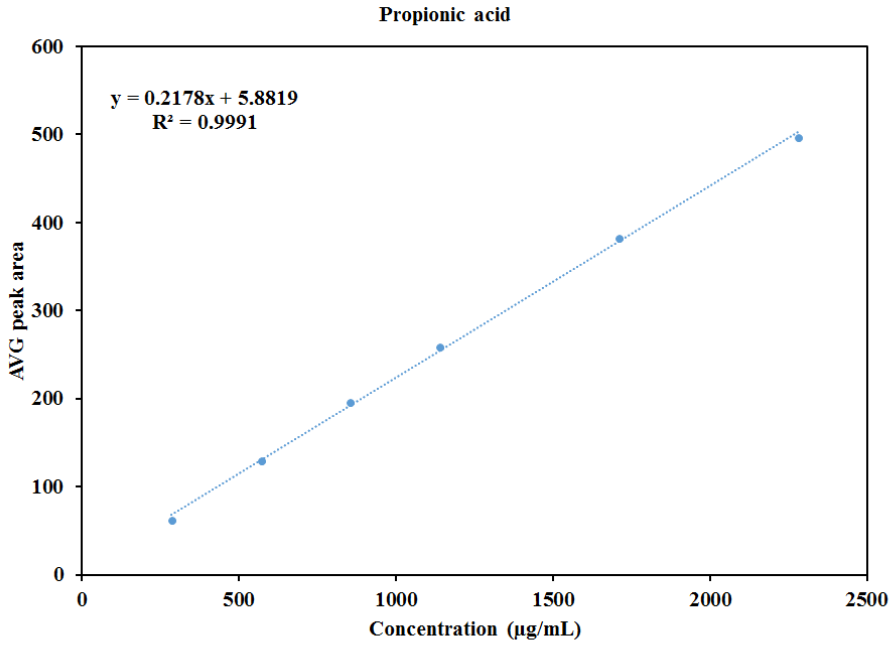

Figure 7-17. GC calibration curve for iso-butyric acid, range: 40 – 320 µg/mL.

Regression Statistics	
Multiple R	0.999656984
R Square	0.999314087
Adjusted R Square	0.999142608
Standard Error	0.785113011
Observations	6

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	3592.171296	3592.171	5827.64	1.76E-07
Residual	4	2.465609759	0.616402		
Total	5	3594.636905			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	1.06195098	0.626741954	1.694399	0.165437	-0.67816	2.802066	-0.67816	2.802066
X Variable 1	0.256968015	0.003366144	76.33898	1.76E-07	0.247622	0.266314	0.247622	0.266314

7.3.4 *n-butyric acid*

LOD: 6.87 µg/mL, LOQ: 20.82 µg/mL.

n-butyric acid	**Concentration (µg/mL)**	**AVG peak area**
	40	10.654
	80	22.205
	120	33.017
	160	44.001
	240	64.617
	320	84.9143

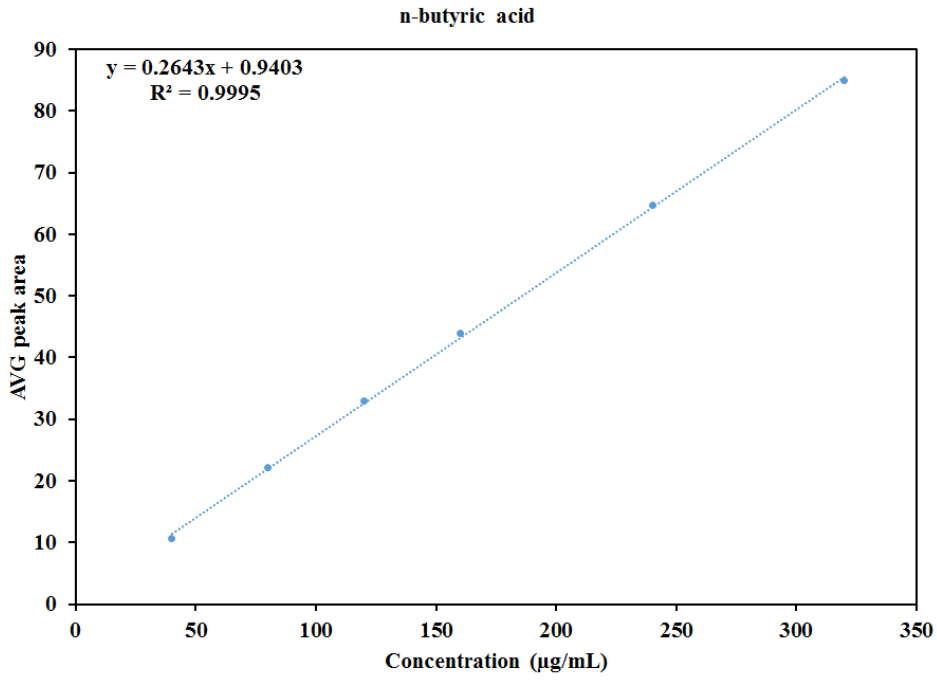

Figure 7-18. GC calibration curve for n-butyric acid, range: 40 – 320 µg/mL.

Regression Statistics	
Multiple R	0.999750126
R Square	0.999500315
Adjusted R Square	0.999375394
Standard Error	0.689268781
Observations	6

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	3801.230347	3801.23	8001.05	9.36E-08
Residual	4	1.900365807	0.475091		
Total	5	3803.130713			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	0.940340196	0.550231185	1.708991	0.162633	-0.58735	2.468027	-0.58735	2.468027
X Variable 1	0.264339853	0.002955216	89.44859	9.36E-08	0.256135	0.272545	0.256135	0.272545

7.3.5 *Iso-valeric acid*

LOD: 39.39 µg/mL, LOQ: 119.36 µg/mL.

Iso-valeric acid	**Concentration (µg/mL)**	**AVG peak area**
	195	5.2333
	390	10.7473
	585	15.8163
	780	21.4063
	1170	31.0133
	1560	40.771

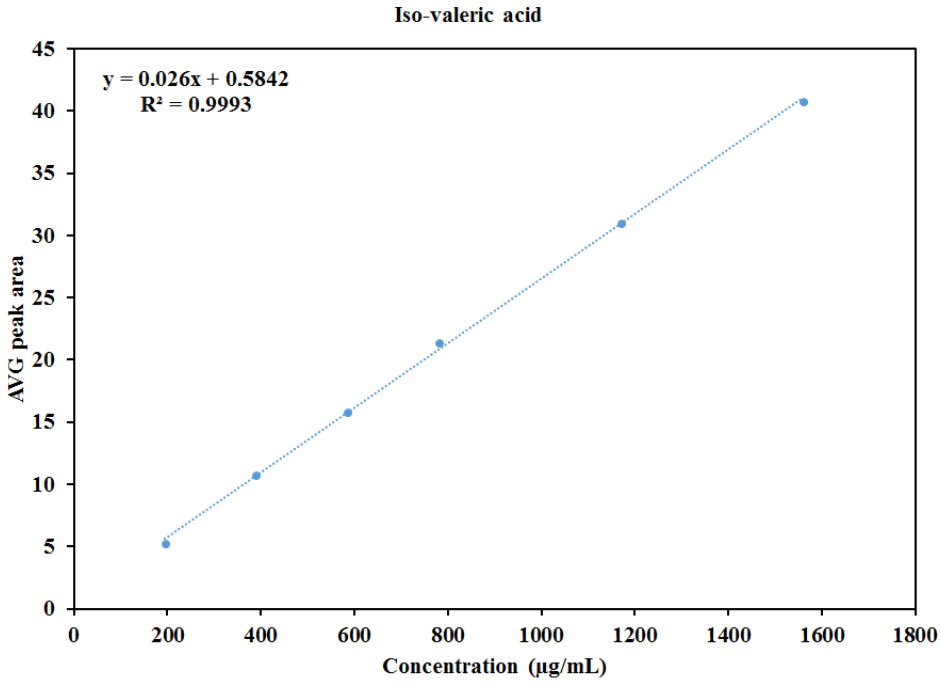

Figure 7-19. GC calibration curve for iso-valeric acid, range: 195 – 1560 µg/mL.

Regression Statistics	
Multiple R	0.999654312
R Square	0.999308744
Adjusted R Square	0.99913593
Standard Error	0.388132998
Observations	6

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	871.1276812	871.1277	5782.567	1.79E-07
Residual	4	0.602588897	0.150647		
Total	5	871.7302701			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	0.584214706	0.309839769	1.885538	0.132431	-0.27604	1.444468	-0.27604	1.444468
X Variable 1	0.025957738	0.000341355	76.04319	1.79E-07	0.02501	0.026905	0.02501	0.026905

7.3.6 *n-valeric acid*

LOD: 21.68 µg/mL, LOQ: 65.71 µg/mL.

n-valeric acid	**Concentration (µg/mL)**	**AVG peak area**
	195	5.34
	390	10.9857
	585	16.4003
	780	21.5143
	1170	32.3287
	1560	42.5143

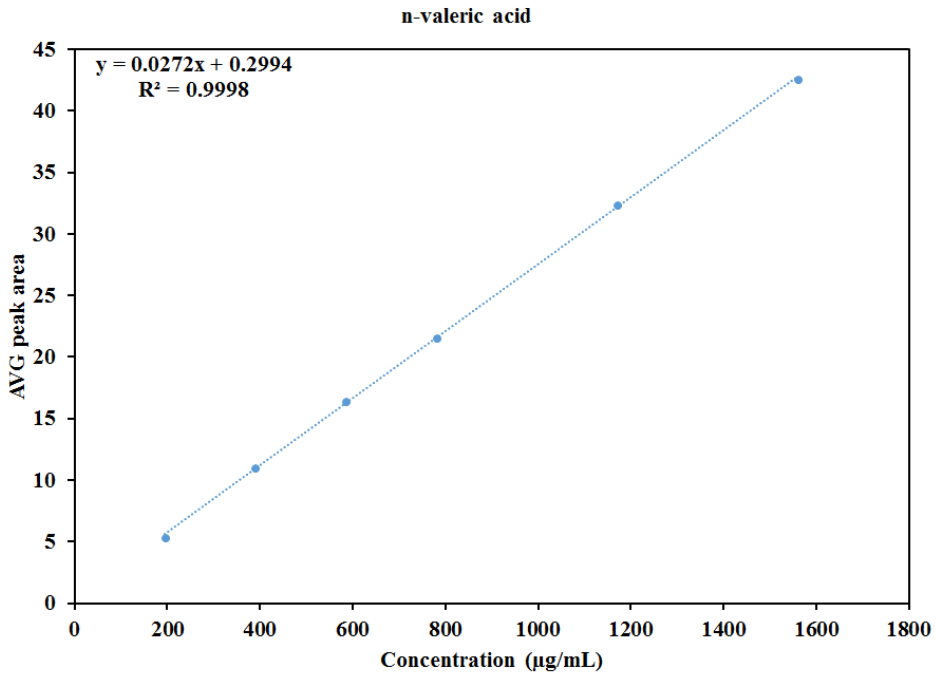

Figure 7-20. GC calibration curve for n-valeric acid, range: 195 – 1560 µg/mL.

Regression Statistics	
Multiple R	0.999895215
R Square	0.999790441
Adjusted R Square	0.999738051
Standard Error	0.223861855
Observations	6

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	956.3631842	956.3632	19083.7	1.65E-08
Residual	4	0.200456521	0.050114		
Total	5	956.5636408			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	0.29942451	0.178704995	1.675524	0.169141	-0.19674	0.795589	-0.19674	0.795589
X Variable 1	0.027198024	0.000196882	138.1438	1.65E-08	0.026651	0.027745	0.026651	0.027745

7.4 Analysis of variance for intermediate precision between peak areas obtained in the morning and night following DLLME.

The model data used in the below calculation is that of the peak areas obtained in the morning and night for iso-butyric acid. Null hypothesis (H_0) was that both group means were equal.

$H_o = \mu_1 = \mu_2$, $H_1 = \mu_1 \neq \mu_2$, $\alpha = 0.05$

$$MSS_w = \frac{\Sigma_{g,G}\,(x - \bar{x})^2}{n - k}$$

Equation 7-1. Equation used to calculate mean sum of squares, where Σ_{gG} refers to the sum of within group means ($\bar{x}_g$) and sum of all means between groups of data ($\bar{x}_G$), n refers to the total number of data sets between all groups (6) and k refers to the number of groups of data (2).

$$MSS_B = \frac{\Sigma_{g,G}\, n_g\left(\bar{x}_g - \bar{x}_G\right)^2}{k - 1}$$

Equation 7-2. Equation used to calculate the mean sum of squares between groups of data.

$$d_{fB} = k - 1$$

Equation 7-3. Equation used to calculate degrees of freedom between groups. The value obtained here is used to find the correct column in the table of critical F values at 0.05. ***k-1 = 2-1 = 1****.*

$$d_{fW} = n\text{-}k$$

Equation 7-4. Equation used to calculate degrees of freedom within groups. The value obtained here is used to find the correct row in the table of critical F values at 0.05. ***n-k = 6 – 2 = 4.***

$$F = \frac{MSS_B}{MSS_W}$$

Equation 7-5. Equation used to obtain the value for F. If F < F crit (found using Equation 3 and 4 from table of critical F values) the null hypothesis must be accepted.

$$(x_1 - \bar{x})^2$$

Equation 7-6. Equation used to calculate sum of squares within groups (SS_W). Where x_1 refers to the average peak areas found in the morning column in Table 7-1 below and $\bar{x}$ refers to the within group mean ($\bar{x}_g$) of the morning peak area averages.

$$(x_2 - \bar{x})^2$$

Equation 7-7. Equation used to calculate sum of squares within groups (SS_W). Where x_2 refers to the average peak areas found in the night column in Table 7-1 below and $\bar{x}$ refers to the within group mean ($\bar{x}_g$) of the night peak area averages.

To calculate the MSS_W the sum of square for the morning and night were added together. This was then divided by n-k (6-2 = 4) as show in Equation 7-8 *8.*

$$MSS_w = \frac{0.5012 + 2.3785}{6 - 2}$$

$$\mathbf{MSS_W = 0.719925}$$

Equation 7-8. Calculation for MSS_W

SS_B was then calculated by subtracting the group mean ($\overline{x}_g$) and mean of all data sets ($\overline{x}_G$) ($\overline{x}_g$ - $\overline{x}_G$) and squaring the answer. The answer obtained was then multiplied by the number of data sets in the group (n_x), in this case three. These figures were then added together and divided by k-1 (2-1 = 1). An example is shown below in Equation 7-9.

$$SS_B = n_1(\overline{x}_1 - \overline{x}_G) = 3(28.45\text{-}28.49)^2 = 0.0048$$

$$SS_B = n_2(\overline{x}_2 - \overline{x}_G) = 3(28.52\text{-}28.49)^2 = 0.0027$$

$$MSS_B = \frac{0.0048 + 0.0027}{2 - 1}$$

$$\mathbf{MSS_B = 0.0075}$$

Equation 7-9. Example calculations to obtain sum of squares between groups (SS_B). The answers to SS_B were then added together and divided by 1 to obtain MSS_B.

The value for *F* was then found by using Equation 7-5*:*

$$\boldsymbol{F = 0.01}$$

*Table 7-1. A summary of all data required to calculate MSS_w and MSS_B. Average peak areas were obtained via triplicate injections of three replicate extractions using the optimised DLLME procedure in **Chapter 5**. There are three within group data sets (peak area averages) and two groups of data (morning & night). Within group mean for morning and night was: 28.45 and 28.52, respectively. The mean of all 6 data sets in both groups was 28.49. The total sum of square for peak areas found in the morning and night was 2.375 and 0.5012, respectively. Peak areas obtained from iso-butyric acid.*

Iso-butyric acid	**Peak area averages (morning)**	**Peak area averages (night)**	$(x_1 - \bar{x})^2$	$(x_2 - \bar{x})^2$
	28.85	29.04	0.16	0.2704
	27.22	28.04	1.5129	0.2304
	29.22	28.5	0.756	0.0004
(# in group) n_g	3	3	*Σ 2.375*	*Σ 0.5012*
(overall number) n_G	6			
(within group mean) $\bar{x}_g$	28.45	28.52		
(mean of all data sets) $\bar{x}_G$	28.49			
(number of groups) k	2			

The answers from d_{fB} and d_{fW} which were 1 and 4, respectively were then used to find the value of *F* crit in a table of critical *F* values [1] by going to column 1, row 4. H_0 accepted because $F < F$ crit.

F crit = 7.71

7.4.1 *Data for iso-butyric acid peak areas (morning and night)*

iso-butyric acid

Peak are averages (morning)	Peak area averages (night)
28.85	29.04
27.22	28.04
29.29	28.50

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	3	85.36164	28.45388	1.189229
Column 2	3	85.58067	28.52689	0.248991

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.007995203	1	0.007995	0.011118	0.921101	7.708647422
Within Groups	2.876439528	4	0.71911			
Total	2.884434731	5				

7.4.2 *Data for n-butyric acid peak areas (morning and night)*

n-butyric acid

Peak are averages (morning)	Peak area averages (night)
22.88	22.30
22.35	21.91
22.64	22.77

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	3	67.87373	22.62458	0.071009
Column 2	3	66.97473	22.32491	0.18448

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.1347	1	0.1347	1.05445	0.362522	7.708647
Within Groups	0.510978	4	0.127745			
Total	0.645678	5				

7.4.3 *Data for iso-valeric acid peak areas (morning and night)*

iso-valeric acid

Peak are averages (morning)	Peak area averages (night)
86.64	85.73
83.84	85.97
88.94	87.14

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	3	259.413	86.47101	6.51382
Column 2	3	258.8367	86.27889	0.570137

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.05536	1	0.055366	0.015631	0.906535	7.708647
Within Groups	14.16791	4	3.541978			
Total	14.22328	5				

7.4.4 *Data for n-valeric acid peak areas (morning and night)*

n-valeric acid

Peak are averages (morning)	Peak area averages (night)
106.98	105.28
104.25	104.07
104.53	104.08

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*

Column 1	3	315.754	105.2513	2.261629
Column 2	3	313.437	104.479	0.481229

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.894748	1	0.894748	0.65242	0.464543	7.708647
Within Groups	5.485717	4	1.371429			
Total	6.380466	5				

7.4.5 *ANOVA summary*

Table 7-2. Summary of ANOVA outputs. F < F crit for all analytes, there for the null hypothesis that both means are the same was accepted.

Analyte	***Count***	***F***	***P-value***	***F crit***
Iso-butyric acid	3	0.01112	0.9211	1.78064
n-butyric acid	3	1.05445	0.3625	7.70865
Iso-valeric acid	3	0.01563	0.9653	7.70865
n-valeric acid	3	0.65424	0.46454	7.70865

7.5 References

[1] G. Currell, in:, Essent. Math. Stat. Sci., John Wiley & Sons Ltd, West Sussex, 2009, p. Appendix IV.