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THE APPLICATION OF DISPERSIVE LIQUID-LIQUID MICROEXTRACTION TO PRECONCENTRATION OF TRACE COMPONENTS IN MILK FOR CHROMATOGRAPHIC ANALYSIS

A dissertation submitted to WIT for the degree of Doctor of Philosophy

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Declaration

I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of PhD, is entirely my own work and has not been taken from the work of others, save and to the extent that such work has be cited and acknowledged within the text of my work.

Signed:_____

Date:_____

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%RSD	relative standard deviation
1,3-DCP	1,3-dichloro-2-proponol
2,3-DCP	2,3-dichloro-2-proponol
3-MCPD	3-chloropropane-1,2-diol
AA-DLLME	air assisted DLLME
ACN	acetonitrile
AFM_1	aflatoxin M_1
APFO	ammonium perfluorooctanoate
AQC	6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate
BBP	butyl benzyl ester
BCS	body condition score
BNZ	benznidazole
BPA	bisphenol A
BPB	bisphenol B
CI	chemical ionisation
CP-DLLME	cloud point DLLME
DAN	danofloxacin
DBP	dibutyl phthalate
DCM	dichloromethane
DEHP	di-2-ethylhexyl phthalate
DEP	diethyl phthalate
DIBP	di-isobutyl phthalate

DIOP	diisooctyl phthalate
DLLME	dispersive liquid-liquid microextraction
DMP	dimethyl phthalate
DNOP	dioctyl phthlate
DNPB	di-*n*-butyl phthalate
DoE	design of experiments
EA-DLLME	effervescent assisted DLLME
ECF	ethyl chloroformate
EF	enrichment factor
FAA	free amino acid
FAME	fatty acid methyl ester
FFA	free fatty acid
FMOC	fluorenylmethyloxycarbonyl
FSV	fat soluble vitamins
HFBI	heptafluorobutyrylimizadole
HF-LPME	hollow fibre liquid-phase microextraction
IL	ionic liquid
IL-DLLME	ionic liquid DLLME
IS	internal standard
LDS-DLLME	low density solvent-based DLLME
LLE	liquid-liquid extraction
LOD	limit of detection
LOQ	limit of quantification

LPME	liquid-phase microextraction
MEKC	micellar electrokinetic chromatography
MeOH	methanol
MNP	magnetic nanoparticles
MTBE	methyl tert-butyl ether
NFX	nifurtimox
NSAID	non-steriodial anti-inflammatory drug
PBDE	polybrominated diphenyl ether
PCB	polychlorinated biphenyls
PEG	polyethylene glycol
RR	relative recovery
SA-DLLME	surfactant assisted DLLME
SCP	sulfachloropyridazine
SD-LPME	single-drop liquid-phase microextraction
SFO-DLLME	solidified floating organic drop DLLME
SIA	sulflsoxazole
SMI	sulfamonomethoxine
SMM	sulfamonomethoxine
SMT	sulfamethizole
SMX	sulfamethoxazole
SPE	solid-phase extraction
SPME	solid-phase microextraction
ST-DLLME	solvent-terminated DLLME

TCA	Trichloroacetice acid
TFA	Trifluoroacetic acid
UA-IL/IL-DLLME	ultrasound assisted IL/IL-DLLME
UA-RM-DLLME	ultrasound assisted-reverse micelles DLLME
VA-DLLME	vortex-assisted DLLME
WSV	water soluble vitamins

Abstract

Bovine milk is an important source of energy, protein, essential vitamins, and minerals for humans. It lends itself to the manufacture of a range of dairy products. The concentration of vitamins, fatty acids, and amino acids present in milk have a marked influence on the nutritional and processability qualities of milk. Given that milk is a complex biological fluid, the analysis of these compounds presents a significant analytical challenge. The overarching aims of this study were to develop microextraction methods and chromatographic separations for the determination of selected compounds from bovine milk which would then be analysed by both High Performance Liquid Chromatography (HPLC) and Gas Chromatography (GC) coupled to various detectors (UV, PDA, MS, FID). As the selected compounds are present in trace amounts significant enrichment was required, it was for this reason that Dispersive Liquid-Liquid Microextraction (DLLME) was utilised for the analysis. DLLME involves the rapid injection of an immiscible extraction solvent into an aqueous sample in the presence of a third dispersive solvent. The third dispersive solvent must be miscible with both the extraction and aqueous phase. Typical examples of dispersive solvents include: ACN, methanol, acetone. This produces a stable emulsion of comprised of micro-droplets of extractant into which the analyte rapidly partitions. Centrifugation of the ternary mixture facilitates recovery of the sedimented extraction solvent prior to analysis. The DLLME methods were optimised using chemometric techniques. The results of the analysis were then used to investigate the changes in fatty acid content over the lactation period (palmitic acid increased from 5.73 mg/mL to 10.85 mg/mL), the effect seaweed supplementation had on the vitamin content of bovine milk (delta tocopherol increased from 3.82 to 5.96 µg/mL), and the differences in free amino acid content between different classes of commercial milk samples (alanine, glycine, and glutamic acid increased during storage).

Chapter 1

Literature review

*This review has been published in the *Journal of Chemistry* (Quigley, A; Cummins, W; Connolly, D. (2016) Dispersive liquid-liquid microextraction in the analysis of milk and dairy products: a review. *J. Chem.* **2016**: 12)

1. Literature review

1.1. Introduction

One of the most important steps in any analytical procedure is the extraction and clean-up of the sample in question. There are a variety of methods that perform these tasks, such as: liquid-liquid extraction (LLE) [1] and solid-phase extraction (SPE) [2]. While these methods perform the above tasks adequately, they also suffer a number of drawbacks. Both LLE and SPE are environmentally detrimental due to the large amounts of organic solvents used, they are slow, and labour intensive. The use of a SPE method also requires the purchase of solid phase extraction cartridges.

The development of microextraction techniques has gone some way to resolving a number of these problems. Solid-phase microextraction (SPME) was first developed in 1990 [3] and has been used extensively for a range of analytes and applications from environmental monitoring of fungicides in water [4], phthalic acid esters from vegetable oils [5], and anti-inflammatory drugs in human plasma samples [6]. SPME is an equilibrium based extraction technique. The solid extraction phase is coated onto a fibre which is then placed in contact with the sample. Several different fibres have been developed to increase the affinity for the analyte and SPME fibre. These include polydimethylsiloxne, carboxen, and polyethylene glycol. Two forms of SPME exist: headspace SPME and direct immersion SPME [7,8]. Headspace SPME has been shown to be particularly useful for the analysis of volatile compounds in complex samples when used in conjunction with GC analysis. A schematic of headspace SPME can be seen in Figure 1.1. Although SPME is more environmentally friendly than LLE and SPE as the technique does not require solvents, it still presents considerable disadvantages. The SPME fibres have a limited lifetime, are expensive, and sample carryover can be an issue.

Liquid phase microextraction (LPME) techniques offers an alternative to SPME and can be broadly divided into three classes: single-drop liquid-phase microextraction (SD-LPME) [9] hollow fibre liquid-phase microextraction (HF-LPME) [10] and dispersive liquid-liquid microextraction (DLLME) [11]. All three forms of LPME are environmentally benign since the volume of organic solvent used is typically in the

microliter range (versus millilitre volumes or greater with more conventional liquid/liquid extraction techniques)

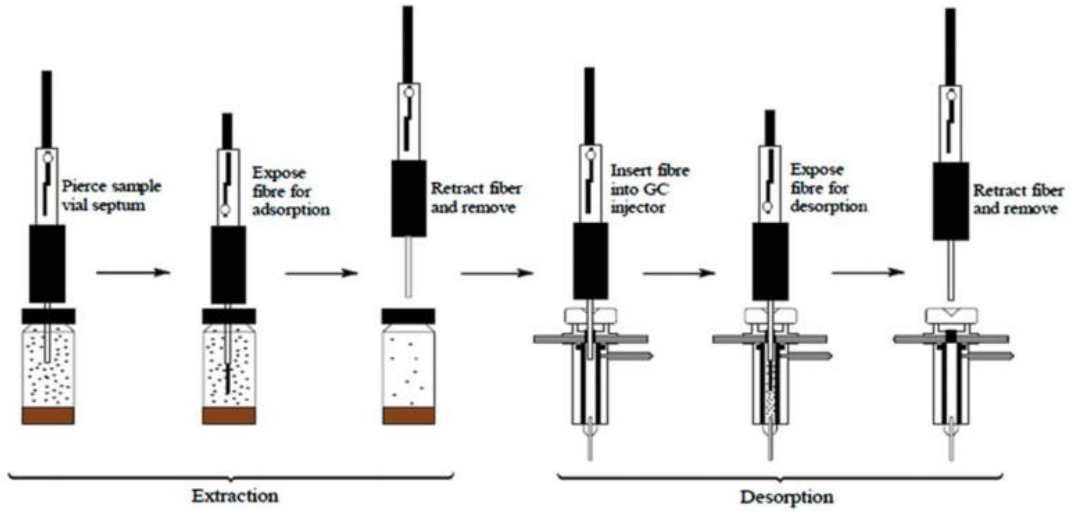

Figure 1.1: Schematic of headspace SPME [12].

These methods do not incur the high cost and sample carryover problems associated with SPME. Even though SD-LPME vastly reduces the volume of organic solvent used, there are other intrinsic problems with this method. Excessive stirring tends to break up the droplet, the extraction is time consuming, and reaching equilibrium can often prove challenging [13]. The development of HF-LPME [10] provides a way to stabilise the extraction droplet in SD-LPME by placing it in a hollow fibre. In general the method still requires long extraction times of at least 20 minutes [14] although methods have been reported using extraction times as low as eight minutes [15]. DLLME is the latest development in LPME field and is discussed in more detail below.

1.1.1. Principles of DLLME

In a typical DLLME protocol, an extraction solvent is mixed with a dispersive solvent and this solvent mixture is then rapidly injected into the aqueous sample. The rapid injection of the extraction-dispersive solvent mixture produces a cloudy solution, formed from micro-droplets of extraction solvent dispersed in the aqueous sample as shown in Figure 1.2. The formation of a cloudy solution/emulsion, allows for the instantaneous partitioning of analytes from the aqueous sample into the extraction phase (a major advantage of this technique). This is achieved by the large surface area relative to LLE created by the numerous micro-droplets. The cloudy solution is then centrifuged which breaks the emulsion into a two-phase system allowing for easy recovery of the extraction solvent for analysis.

There are several requirements that must be fulfilled in order for DLLME to be successful. The extraction solvent must be immiscible with water, miscible with the dispersive solvent, and show a high affinity for the target analytes. In what will be referred to hereafter as *"traditional DLLME"*, the extraction solvent is typically denser than water such that it will form a *"sedimented phase"* upon centrifugation for easy collection with a fine syringe needle. Conversely, the dispersive solvent has to be both miscible with the extraction solvent and the aqueous sample. Ideally, the extraction solvent will be compatible with the analytical technique being used; otherwise evaporation of the extraction solvent and reconstitution in an appropriate solvent is required. Alternatively, *in-syringe* back extraction could be used to extract the analytes into a compatible solvent [16]. Prior to analysis, the volume and type of extraction and dispersive solvent, ionic strength, pH of the aqueous phase, extraction time, and centrifugation time must be optimised to ensure quantitative extraction of analytes. A comparison of DLLME methods used for trace compound analysis in dairy compounds can be seen in Table 1.

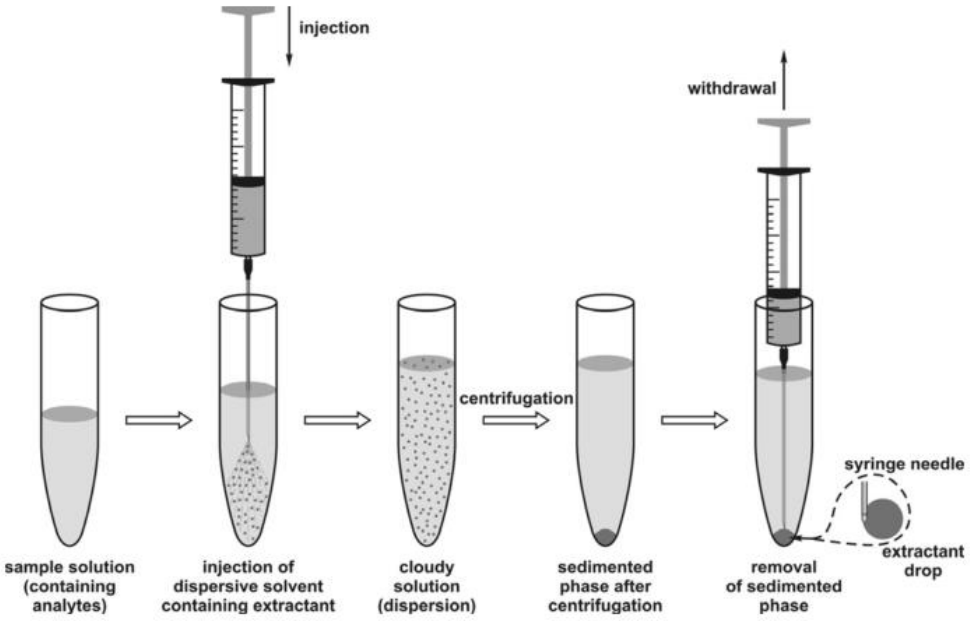

Figure 1.2: Schematic of DLLME technique [11].

An efficient DLLME method is characterised by a high enrichment factor (EF) and high relative recovery (RR). Enrichment factor is calculated as shown in Equation 1, where C_0 represents the concentration of the analyte in the original sample and C_{sed} represents the concentration of the analyte in the sedimented extraction solvent. Enrichment factors have been found that can range anywhere from single digits to several hundred. Higher enrichment factors are generally found when the analyte is in low concentration in a large sample volume, such as trace pollutants in environmental water samples.

$$Enrichment\ factor\ = \frac{C_{sed}}{C_0} \quad (1)$$

The RR is calculated according to Equation 2, where C_{found} shows total amount of analyte found after addition of standard, C_{real} is the original concentration of analyte in the sample and C_{add} is the amount of standard that was spiked into the original sample. Recoveries have varied depending on the analyte and matrix being analysed. Ideally, recoveries of 100% would be obtained but due to matrix interferences this has proven to be difficult.

$$Relative\ recovery = \frac{C_{found} - C_{real}}{C_{add}} \times 100 \quad (2)$$

1.1.2. Alternative modes of DLLME

Recently, low density solvents have been used as extraction solvents in DLLME in order to increase the range of extraction solvents compatible with the method. This mode is called low density solvent based DLLME (LDS-DLLME) [17] and the extraction solvent (including toluene, xylene, hexane, heptane etc.) floats on the surface of the aqueous phase after phase separation is induced. The solvent is recovered using a fine needle and this process is simplified when specialist glassware or other vessels are used to trap the floating solvent in a narrow restriction in the vessel [18] as shown in Figure 1.3. This technique has been used in the analysis of several different compounds, including: hydrocarbons, steroids, and dinitrobenzenes.

Solidified floating organic drop DLLME (SFO-DLLME) was developed by Yamini *et al.* [19,20] and involves the use of low-density extraction solvents having a melting point close to room temperature (typically 1-undecanol or 1-dodecanol). Yamini *et al*. used this technique to determine the concentration of aluminium in water samples. After phase separation the floating extraction solvent is frozen by placing the vessel on ice after which the frozen drop is easily collected into a separate vessel where it is usually diluted with a chromatographically suitable solvent prior to analysis.

Additional modifications to DLLME methods include the elimination of time-consuming centrifugation steps via the use of a de-emulsification solvent which causes phase separation of the emulsion upon its addition. This technique is termed solvent-terminated DLLME (ST-DLLME) [21], Chen *et al.* have determined the concentration of carbamate pesticides in water samples using this technique. Seebunrueng *et al*. have reported a similar method whereby the addition of a salt ($AlCl_3$) is used to induce phase separation due to a disruption of the interfacial tension at the droplet surface. The developers of this technique examined the concentration of pesticides in various fruit juices [22]. Alternative methods have also been developed to enhance the dispersion of the extraction solvent throughout the aqueous sample. The use of ultrasound, vortexing, or manual shaking will increase the number of micro-droplets of extraction solvent resulting in an even larger surface area [23]. Effervescence-assisted DLLME (EA-DLLME) involves the *in-situ* generation of bubbles of CO_2 to assist the dispersion of the extraction solvent, removing a need for the dispersive solvent. The CO_2 is produced by adding a mixture of

sodium carbonate and a weak acid (citric acid), usually in the form of a pressed tablet Lasarte-Aragonés *et al*. combined EA-DLLME with magnetic nanoparticles for the analysis of herbicides in water samples. Although this was carried out using river, tap, and well water; the method was not sensitive enough to detect the presence of herbicides. The samples were spiked with herbicides to show the applicability to herbicide analysis [24].

Air assisted dispersive liquid-liquid microextraction (AA-DLLME) removes the need for a dispersive solvent by repeatedly aspirating the aqueous phase and the extraction solvent into a glass syringe until a cloudy solution is formed, and has been used for the analysis of phthalate esters in aqueous samples [25]. Methods to allow easier recovery of the extraction solvent have also been developed. Shi *et al*. have derivatised magnetic nanoparticles for the easy recovery of polycyclic aromatic hydrocarbons from river water. The use of hydrophobic magnetic nanoparticles which interact with the extraction phase and can be sedimented by applying a magnet can be used to eliminate the centrifugation step [26]. Combinations of these techniques have also be used, such as the use of magnetic nanoparticles combined with effervescence assisted dispersion, mentioned above [24]. A schematic of this method can be seen in Figure 1.3 (a).

Surfactant assisted DLLME (SA-DLLME) uses surfactants as dispersive solvents. A cationic surfactant (cethyltrimethyl ammonium bromide) was used as the dispersive solvent for the analysis of chlorophenols in water samples [27]. Cloud point DLLME (CP-DLLME) uses surfactants as an extraction solvent to produce a surfactant rich sedimented phase after centrifugation. Daneshfar and Khezeli applied this technique for the analysis of organic acids in biological samples [28]. Specifically, it involves heating the sample solution containing the appropriate surfactant passed its cloud point. The cloud point is defined as the temperature at which phase separation occurs and the analytes extract into the surfactant rich phase as shown in Figure 1.3 (c). Ionic liquids have been used as an alternative to traditional organic extraction solvents in ionic liquid DLLME (IL-DLLME) because they have tuneable physicochemical properties. For example, ionic liquid miscibility in either water or organic solvents can be controlled by selecting the appropriate anion/cation combination and by incorporating the proper functional group within the IL. In addition, they often exhibit lower toxicity than organic extraction solvents [29]. Ionic liquids have also been used as both dispersive and extraction solvents

in combination with ultrasound-assisted dispersion, referred to as ultrasound-assisted ionic liquid/ionic liquid DLLME (UA-IL/IL-DLLME). This technique has been used for the determination of sulphonamides in infant formula [30]. In an effort to improve selectivity for polar or acidic/basic analytes, pH-controlled DLLME (pH-DLLME) has also been developed [31]. By performing two DLLME procedures it is possible to remove matrix interferences in the first extraction step, followed by a back-extraction after appropriate pH adjustment. This is a technique that that has been used to analyse ochratoxin A in cereals.

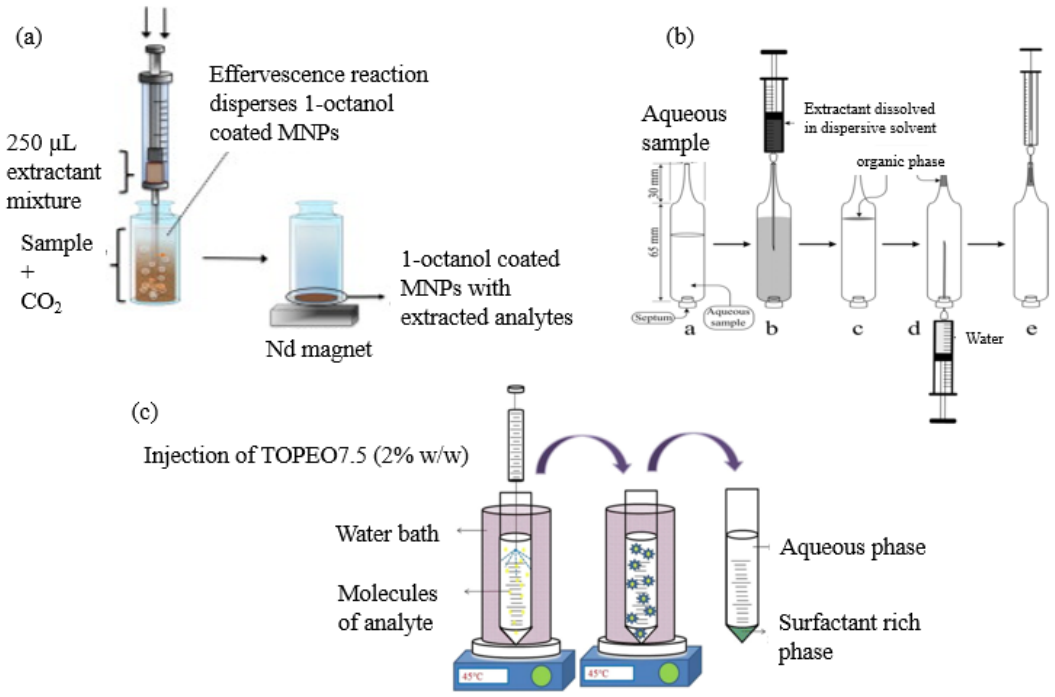

Figure 1.3: Schematic diagram of: (a) effervescence DLLME [24] (b) LDS-DLLME using specialist glassware [18] (c) CP-DLLME [28]: Schematic diagram of: (a) effervescence DLLME [24] (b) LDS-DLLME using specialist glassware [18] (c) CP-DLLME [28].

Table 1.1: Modes of DLLME used in dairy analysis

Sample	Sample preparation	Extraction/dispersive solvent	Analytes extracted	Enrichment factor	Analytical method	LOD (µg/L)	Reference
DLLME							
Infant formula, fermented milk	Acid hydrolysis, enzymatic hydrolysis and derivatisation	Tetrachloroethene (90 µL) / acetonitrile (0.5 mL)	Thiamine	Not specified	Reversed phase HPLC (RP-HPLC)	0.09	[32]
Soybean milk	Liquid-liquid extraction	Carbon tetrachloride (40 µL) / acetonitrile (1 mL)	Phthalate acid esters	200 - 260	GC-MS	0.57 - 0.79 ng/g	[33]
Full fat milk, half fat milk, skimmed milk, follow-on formula	Dilution, protein precipitation by TCA	Chloroform (200 µL) / acetonitrile (2 mL)	Macrocyclic lactones	65 - 200	HPLC-DAD coupled to APCI-IT-MS/MS	HPLC-DAD: 0.3-1.4 ng/g LC-MS/MS: 0.03-0.72 ng/g	[34]
Milk	Protein precipitation by TCA, pH adjustment, derivatisation by fluorescamine	Chloroform (1 mL) / ACN (1.9 mL)	Sulfonamides	2.2	RP-HPLC-FL	0.6-1.03	[35]

Sample	Sample preparation	Extraction/dispersive solvent	Analytes extracted	Enrichment factor	Analytical method	LOD (µg/L)	Reference
Milk, white cheese, yoghurt, unpasteurised milk	Milks: protein precipitation. Removal of fat. Yoghurt and cheese: dilution, removal of fat	Chloroform (150 µL) / ACN (2 mL)	Nonsteroidal anti inflammatory drugs	Milk: 81.1-141 Yoghurt: 45.9-81. Cheese: 145-229	Field amplified sample stacking in CE (FASS)	Milk (µg/kg): 4.8-13 Yoghurt (µg/kg): 3.0-9.7 Cheese (µg/kg): 6.1-7.7	[36]
Whole milk, skimmed milk, powdered milk	Whole milk: centrifugation Powdered milk: reconstitution with ultrapure water Protein precipitation by ACN and NaCl	Chloroform (1.5 mL) / ACN (2.4 mL)	Aflatoxin M1	33	UHPLC-MS/MS	0.6 ng/kg	[37]
Milk	Centrifugation, protein precipitation by ACN	Carbon tetrachloride (35 µL) / ethanol (800 µL)	Cholesterol	Not specified	HPLC-UV	0.01	[38]
Milk	Protein precipitation by ACN and NaCl	Chloroform (40 µL) / ACN (1 mL)	Pesticides	176-435	GC-FID, GC-MS	4-58	[39]

Sample	Sample preparation	Extraction/dispersive solvent	Analytes extracted	Enrichment factor	Analytical method	LOD (µg/L)	Reference
Milk	Protein precipitation by ACN and NaCl	1,2-dibromoethane (20 µL) / ACN (800 µL)	Phthlates	397-499	GC-FID, GC-MS	1.0-3.0	[40]
Milk	Saponification, in-tube LLE	1,1,2,2-tetrachloroethane (22 µL) / ACN (1 mL)	Polybrominated diphenyl ethers	271-307	GC-MS	0.012-0.29	[41]
Milk	QuEChERS	Chloroform (200 µL) / 10% acetic acid-ACN (1 mL)	Fluroquinolones	Not specified	RP-HPLC-UV	0.8-5.0 µg/kg	[42]
Milk	Protein precipitation and fat removal by NaOH and acetone, SPE	Chlorobenzene (19 µL) / acetone (1 mL)	Polychlorinated biphenyls, Polybrominated diphenyl ethers	Not specified	GC-MS	Polychlorinated biphenyls: 0.01-0.04 Polybrominated biphenyls: 0.2-0.4	[43]
Infant formula	Protein precipitation by TCA, pH adjustment	Tetrachloroethylene (30 µL) / ACN (440 µL)	BPA, BPB	BPA: 237, BPB: 220	GC-MS	BPA: 0.06 BPB: 0.03	[44]
Milk, yoghurt	Protein precipitation by ACN and acetic acid. Fat removal by hexane	Chloroform (110 µL)/ ACN (500 µL)	Endostrogens	50-407	MEKC-MS	1-220	[45]

Sample	Sample preparation	Extraction/dispersive solvent	Analytes extracted	Enrichment factor	Analytical method	LOD (µg/L)	Reference
UHT milk	Protein and fat removal by Carrez solution	Chloroform (250 µL)/ acetone (1.2 mL)	Benzoic acid, sorbic acid	Not specified	HPLC-UV	Benzoic acid: 0.1 µg/mL Sorbic acid: 0.08 µg/mL	[46]
Cheese	Protein precipitation by sonication	Trichloroethane (116 µL)/ ethanol (1.5 mL)	Natamycin	61.4	FAAS	1.8 ng/mL	[47]
Milk	Protein precipitation by ACN	Chloroform (400 µL)/ ACN (1.0 mL)	Chloramphenicol, florfenicol	Not specified	HPLC-UV	Chloramphenicol: 12.2 (µg/kg), florfenicol: 12.5 µg/kg)	[48]
Milk, cheese, milk powder, yoghurt	Milk: protein precipitation by ACN. Cheese/yoghurt: protein precipitation by TFA and ACN. Powdered milk: protein precipitation by acetic acid and ACN	Dichloromethane (600 µL)/ ACN(1.0 mL)	Melamine	Not specified	Positive corona discharge ion mobility spectrometry	25 µL/L	[49]

Sample	Sample preparation	Extraction/dispersive solvent	Analytes extracted	Enrichment factor	Analytical method	LOD (µg/L)	Reference
Yoghurt	Removal of fat by centrifugation. Protein precipitation by ACN and NaCl	Chloroform (1.5 mL)/ ACN (6 mL)	Aflatoxins B1, B2, G1, G2, M1	Not specified	HPLC-FLD	1.5-5.5 ng/kg	[50]
Milk	Protein precipitation by NaCl and phosphoric acid	Chloroform (250 µL)/ MeOH (1 mL)	Fatty acids	8-15	GC-FID	40-90	[51]
Ultrasound assisted DLLME							
Bottled milk	Protein precipitation by TCA and lead acetate	Carbon tetrachloride (40 µL) / methanol (0.8 µL)	Phthalate acid esters (PAEs), butyl benzyl ester (BBP), diisooctyl phthalate (DIOP),	PAEs: 226-258, BBP: 270, DIOP: 220-229	GC-FID	0.75-0.79 ng/g, 0.66 ng/g 0.64-0.76 ng/g	[52]
Skimmed milk	Protein precipitation by ACN, derivatisation by HFBI	Chloroform (100 µL) / ACN (2 mL)	Chloropropanols	Not specified	GC-MS/MS	0.9-3.6	[53]
Milk, cheese	QuEChERS	Chloroform (500 µL) / ACN (3 mL)	Alfatoxins: B1, M1	B1: 30, M1: 30	RP-HPLC-FL	B1: 0.1 µg/kg M1: 0.01 µg/kg	[54]

Sample	Sample preparation	Extraction/dispersive solvent	Analytes extracted	Enrichment factor	Analytical method	LOD (µg/L)	Reference
UA-IL/IL-DLLME							
Infant formula	Dilution	$[C_6MIM][PF_6]$ (70 µL) / $[C_4MIM][BF_4]$ (100 µL)	Sulfonamides	Not specified	RP-HPLC-PDA	2.94-16.7 µg/kg,	[30]
DLLME with back extraction							
Breast milk, ice cream	Protein precipitation by salting out with NaCl, phosphoric acid, ACN	Chloroform (200 µL) / ACN (1 mL)	Parabens	4.6-9.2	CE-DAD	300	[55]
UA-RM-DLLME							
Butter	Melting for 5 min at 40 °C	Triton X-100 (1.25% w/v) / water (400 µL)	Acetoin	245	RP-HPLC-UV	200,000	[56]
UA-IL-DLLME							
Milk	Microwave digestion with HNO_3 and H_2O_2, chelation with 1-Phenylthiosemicarbazide	$[C_6MIM][Tf_2N]$ (100 µL)	Selenium	150	Graphite furnace atomi absorption spectrometry (GFAAS)	12	[57]

Sample	Sample preparation	Extraction/dispersive solvent	Analytes extracted	Enrichment factor	Analytical method	LOD (µg/L)	Reference
LDS-DLLME							
Cheese	Protein precipitation, filtration	1-octanol (60 µL) / acetone (475 µL)	Sorbic and benzoic acids	Cheese: Sorbate (143), benzoate (170)	GC-FID	Cheese: sorbate: 150 ng/g, benzoate: 140 ng/g	[58]
Milk	Fat removal by centrifugation	1-heptanol (320 µL) / MeOH:Water (80:20) (3 mL)	Aflatoxin M1	Not specified	Fluoresence spectrophotomete	0.013	[59]
Yoghurt drinks	Protein precipitation, filtration	1-octanol (60 µL) / ethanol (450 µL)	Benzoate, sorbate	Benzoate: 162, sorbate: 181	RP-HPLC-UV	Benzoate: 0.2, sorbate: 0.5	[60]
Milk, infant formula	Protein precipitation by acetic acid and lead acetate	1-octanol (60 µL)/ ACN (1.0 mL)	Melamine	Not specified	HPLC-UV/VIS	0.1	[61]
Cheese	Microwave digestion , protein precipitation by Carrez solutions	1-octanol (60 µL)/ acetone (600 µL)	Biogenic amines	108-186	GC-MS	5.9-14.0 ng/g	[62]
Milk, yoghurt drinks	Hydrolysis, protein precipitation using Carrez solution	1-octanol (80 µL)/ ethanol (550 µL)	Cholecalciferol (D_3)	274	HPLC-UV	3	[63]

Sample	Sample preparation	Extraction/dispersive solvent	Analytes extracted	Enrichment factor	Analytical method	LOD (µg/L)	Reference
IL-DLLME							
Breast milk	Protein precipitation by $HClO_4$, H_3PO_4, MeOH	NFX: $[C_8C_1im][PF_6]$ (42 µL) / MeOH (80 µL) BNZ: $[C_8C_1im][PF_6]$ (42 µL) / MeOH (101 µL)	NFX, BNZ	NFX: 33.8, BNZ: 28.8	RP-HPLC-UV	NFX: 90 BNZ: 60	[64]

1.2. Modes of DLLME used in dairy analysis

1.2.1. Traditional DLLME

Prior to a DLLME procedure on a complex matrix such as milk, lipids and proteins must be eliminated since they can act like surfactants and disrupt the interfacial tension at the droplet surface, hindering phase separation. A list of sample pre-treatment procedures, extraction solvent type and volume, dispersive solvent type and volume, analytical method used, and analytical figures of merit can be found in Table 1.1. One of the first reports of traditional DLLME used to extract analytes from dairy products in 2009 by Daneshfar *et al.* [38] who extracted and analysed cholesterol from several food samples (egg yolk, milk, and olive oil). Previously centrifuged milk samples were subjected to acetonitrile precipitation to eliminate proteins and the aqueous supernatant (after further centrifugation) was subjected to a DLLME protocol. Acetone, ethanol and acetonitrile were trialled as dispersive solvents using carbon tetrachloride as extraction solvent. Ethanol (0.8 mL) resulted in the highest recoveries for cholesterol; lower and higher volumes resulted in either unstable emulsions or higher solubility of cholesterol respectively. Four extraction solvents (carbon disulphide, dichloromethane, chloroform, and carbon tetrachloride) were tested but only carbon tetrachloride (35 µL) yielded stable suspensions with ethanol. An extraction pH of 8.5, maximised recovery and partition of cholesterol was also deemed instantaneous upon generation of the stable emulsion (ie: extraction time was several seconds). Non-aqueous reversed phase HPLC was used to quantify the analyte; because of poor chromatographic behaviour carbon tetrachloride extracts were evaporated to dryness and reconstituted in ethanol for injection. The method proved linear in the range 0.03-10 µg/L and the LOD was 0.01 µg/L representing detection limits 100 times lower than previously reported methods for cholesterol determination in milk.

Later in 2011 Farajzadeh *et al.* used DLLME for the extraction and preconcentration of triazole pesticides from milk samples [39], using GC-FID and GC-MS to quantify the analytes. Proteins were precipitated using both acetonitrile precipitation and NaCl salting out and the pesticides pre-concentrated from 1.0 mL of the ACN supernatant by adding 40 µL of chloroform and rapidly injecting the mixture into 5 mL of deionised water. After a 5 minute centrifugation at 4,000 rpm, enrichment factors of 156 (penconazole), 166

(hexaconazole), 180 (tebuconazole), 243 (triticonazole) and 387 (difenconazole) were achieved. The linear range was as wide as 20-80,000 µg/L for penconazole and hexaconazole and the lowest recorded LOD value was 4 µg/L for hexaconazole.

That same year, Liu *et al.* combined SPE and DLLME to enable the determination of 14 different polychlorinated biphenyls (PCBs) and polybrominated diphenyl ethers (PBDEs) in milk using GC-MS [43]. To precipitate proteins, 50% NaOH and acetone were added and the samples were heated at 70 °C in a water bath. Afterwards, the analytes were extracted into 5 mL of hexane, dried over anhydrous sodium sulphate, and concentrated to 2 mL by evaporation before loading onto a SPE column. The resulting fractions from SPE were dried and reconstituted in 1 mL of acetone, which was used as the dispersive solvent in the optimised DLLME procedure. Chlorobenzene (19 µL) was mixed with the dispersive solvent and rapidly injected into 5 mL of Milli-Q water. The developed SPE-DLLME procedure proved to be effective since the sample matrix did not have a significant impact on extraction efficiencies. The method provided good recoveries and %RSD values for both polychlorinated diphenyls (recovery: 100.0-131.8%; precision: 3.20-10.20%) and polybrominated diphenyl ethers (recovery: 74.0-93.6%; 1.12-12.34%).

Cunha *et al.* expanded the range of dairy samples from milk to infant formula, while analysing bisphenol A (BPA) and bisphenol B (BPB) content using heart-cutting GC-MS [44]. The authors developed an optimised DLLME method coupled with *in-situ* derivatisation using acetic anhydride in the presence of potassium carbonate (K_2CO_3). After protein precipitation using trichloroacetic acid, K_2CO_3 was added until the pH was greater than 10; this mixture was then used as the aqueous phase in the DLLME procedure. The dispersive-extraction solvent mixture (440 µL ACN/30 µL tetrachloroethylene) was combined with 30 µL of acetic anhydride as derivatisation agent and rapidly injected into the aqueous phase and the resulting cloudy suspension allowed to react for 1 minute. Using deuterated BPA as an internal standard, recovery of BPA and BPB was found to be 114% and 68% respectively. The method was linear between 0.5-10 µg/L for both analytes and low LODs (BPA: 60.0 µg/L, BPB: 30 µg/L) were obtained corresponding to high enrichment factors (BPA: 237, BPB: 220). The method repeatability was ≤ 7 % when the analytes were at a concentration of 0.2 µg/L.

In contrast with Liu *et al.* [43], Han *et al.* combined saponification, LLE, and DLLME in the determination of polybrominated diphenyl ethers (PBDEs) in milk using GC-MS [41].

Saponification was carried out by adding a sample of milk to 50% NaOH and ethanol, this mixture was heated to 70 °C under reflux for one hour. The saponified mixture was cooled and rinsed five times with petroleum ether. The washings were collected and centrifuged. The supernatant was dried over anhydrous sodium sulfate and evaporated to dryness under nitrogen. The residue was reconstituted in 2 mL of ACN. To carry out the DLLME procedure, 1 mL of the ACN solution (dispersive solvent) and 22 µL of 1, 1, 2, 2-tetrachloroethane (extraction solvent) were combined and then rapidly injected into 5 mL of deionized water. The cloudy solution was centrifuged and the sedimented phase was removed and dried under nitrogen. The resulting residue was dissolved in 15 µL of hexane and used for GC-MS analysis. The combination of saponification, LLE, and DLLME resulted in effective matrix removal, lower LODs (0.012-0.29 µg/L), and higher recoveries (83-120%) than were reported by Liu *et al.* The above method also had high enrichment factors (270-307), and a short extraction time of 15 minutes. This method has the potential to be applied to the analysis of other organic compounds in fatty foods.

In 2012, traditional DLLME was coupled with GC-FID and GC-MS for the analysis of several phthalate esters found in milk [40]. Proteins were precipitated and phthalate esters (dimethyl phthalate (DMP), diethyl phthalate (DEP), di-isobutyl phthalate (DIBP), di-*n*-butyl phthalate (DNBP), and di-2-ethylhexyl phthalate (DEHP)) were extracted using NaCl and ACN. It was found that maximum peak area for all analytes was obtained when 0.8 mL of ACN (from the previous extraction step) was mixed with 20 µL of 1,2-dibromoethane and then rapidly injected into a 8% NaCl solution. Identification of analyte peaks found in GC-FID chromatograms were confirmed by GC-MS. Enrichment factors were very high for all analytes, 397-499. This optimised DLLME method was compared to other methods in the literature for the analysis of phthalate esters in milk. Although a reported LLE-LC-MS/MS method had a much lower LOD (LLE-LC-MS/MS: 0.01-0.5 µg/L, DLLME: 0.5-3 µg/L) the extraction time was much longer (LLE-LC-MS/MS: 100 min, DLLME: 15 min). In addition, the lower LOD may be more likely due to the use of MS/MS compared to FID as the detection method.

Viñas *et al*. determined the concentration of thiamine in infant formula, and fermented milk using traditional DLLME with HPLC fluorimetric detection [32]. All samples underwent a derivatisation reaction to differentiate between thiamine and its esters. The maximum peak area was achieved by selecting ACN (500 µL) as dispersive solvent,

tetrachloroethene (90 µL) as extraction solvent, an aqueous phase with an ionic strength (NaCl) of 24% and centrifugation for 1 minute at 4,000 rpm. The results indicated that DLLME was time-independent, as equilibrium was reached almost instantaneously. The optimised DLLME procedure resulted in lower extraction times (a few seconds) compared to a LPME method (30 min.), better extraction efficiency, an LOD of 0.09 µg/L and linearity between 0.5-10 µg/L. Recovery of thiamine in infant formula was found to be 98.7% with an RSD of 5.4%.

It was 2013 before DLLME was coupled with field-amplified sample stacking in CE; in the determination of five different non-steroidal anti-inflammatory drugs (NSAIDs) in milk, yogurt, and cheese [36]. As with other milk samples previously mentioned, proteins were precipitated using phosphoric acid, NaCl, and ACN centrifugation. Hexane was added to the supernatant to facilitate the removal of any fat present. The hexane was then discarded and the ACN layer used in the DLLME procedure. For cheese and yoghurt, the samples were homogenised with 2 mL of deionized water and the same procedure was followed as outlined above. The results from the optimised DLLME procedure were compared to other preconcentration techniques used in the extraction of NSAIDs. The extraction time was at least five times faster than other reported methods and used at least half the amount of organic solvents.

Campillo *et al.* analysed several macrocyclic lactones in milk using HPLC-DAD coupled to atmospheric pressure chemical ionization in negative ion mode ion-trap tandem mass spectrometery (APCI-IT-MS/MS) [34]. Prior to DLLME, the proteins were precipitated using TCA. The maximum peak area was achieved when ACN (2 mL) as dispersive solvent and chloroform (200 µL) as extraction solvent were used. The optimum ionic strength of the aqueous phase was obtained by adding NaCl to achieve a concentration of 24% *w/v*. Using DAD detection, the widest linearity was 5-2500 ng/g (doramectin (DOR)) while the lowest LOD was 0.3 ng/g (moxidectin (MOX) and DOR). The lowest LOD achieved by MS/MS was 0.03 ng/g. LC-MS/MS detection produced higher selectivity and improved sensitivity compared to DAD detection.

Campone *et al.* used a Box-Behnken experimental design to optimise the DLLME procedure used to determine aflatoxin M_1 (AFM_1) in whole, skimmed, and powdered milk with UHPLC-MS/MS detection [37]. The authors also compared two different methods for protein precipitation. Firstly, acetic acid was added and then the sample was heated to

100 °C for 3 minutes, centrifuged and aqueous supernatant used in the DLLME procedure. This method resulted in a recovery of only 42.7%, possibly due to proteins binding with AFM_1. The second method investigated used NaCl and ACN to simultaneously precipitate proteins and extract AFM_1 into the ACN. After centrifugation, the ACN supernatant was used as the dispersive solvent in the following DLLME procedure. The volumes of chloroform and ACN that resulted in highest recovery were: 1.5 mL and 3.8 mL, respectively. The mixture of extraction and dispersive solvent was rapidly injected into 5 mL of water. Recovery for whole, skimmed, and powdered milk was 75.3%, 74.2%, and 73.3% with precision ranging from 1.6% to 7.6%. The method was linear from 0.25-25 µg/L and had a LOD of 0.6 ng/kg, which is lower than regulations (50 ng/kg [65]).

In 2014, Arroyo-Manzanares *et al*. used traditional DLLME for the determination of several sulphonamides in milk; the analytes were detected by HPLC with fluorescence detection [35]. The authors also compared their optimised DLLME procedure to QuEChERS. Proteins were precipitated using TCA and then filtered. The DLLME extraction procedure was optimised using a central composite design. The optimum volumes for the extraction solvent (chloroform) and dispersive solvent (ACN) were 1 mL and 1.9 mL, respectively. DLLME resulted in lower LODs (0.73-1.21 µg/L) than QuEChERS (1.15-2.73 µg/L) and higher recoveries (92.9%-104.7% compared to 83.6%-97.1%, when samples were spiked with sulphonamides at 150 µg/L). QuEChERS did prove to be more reproducible than DLLME with lower %RSD values of 2.9%-7.1% and 3.0%-9.7%, respectively.

DLLME was coupled to QuEChERS in 2014 for the determination of six antibiotic fluoroquinolones with HPLC-UV detection [42]. The dried supernatant from the QuEChERS method was resuspended in 1.0 mL of a 10% acetic acid-ACN mixture, combined with 200 µL of chloroform and rapidly injected into 4 mL of deionized water. The cloudy solution was centrifuged for 5 min at 4,500 rpm. By coupling QuEChERS to DLLME, the authors have removed matrix interference, which is common problem with the detection of fluoroquinolones. The method demonstrated good recovery (74.1-101.4% for all analytes) and low LOQs (below 2.5 µg/kg for DAN and below 15 µg/kg for all other analytes).

In 2015, Alshana *et al*. determined the concentration of parabens in breast milk and ice cream using DLLME with back extraction before being analysed by CE [55]. Phosphoric acid (100 µL), ACN (1.5 mL), and saturated NaCl solution (0.5 mL) were added to samples prior to vortex mixing for 1 minute and centrifugation for 3 minutes at 4,000 rpm. The ACN supernatant (1 mL) was then used as the dispersive solvent in the DLLME step. Chloroform (200 µL) was added as the extraction solvent before the sample was made up to 8 mL with deionized water. The sample was vortexed for 1 minute which resulted in the formation of a cloudy solution. After centrifugation, the sedimented chloroform phase was transferred to into a microtube where the analytes were back extracted into 80 µL of back extraction solution (50 mM sodium hydroxide solution) for direct injection into CE. Enrichment factors for each paraben ranged from 7.0-10.7, LOD values were between 100-200 µg/L, while RSD values were from 0.6%-2.3%.

Later in 2015, DLLME was used to determine endoestrogens in whole milk, skimmed milk, semi-skimmed goat's milk, and yoghurt [45]. The separation of analytes was performed by micellar electrokinetic capillary electrophoresis. Before DLLME could take place, the samples required removal of proteins and fats. Proteins were precipitated with ACN and acetic acid while the fats were removed via extraction with hexane. Once this was completed, the samples were diluted to 7.5 mL with ultrapure water and NaCl was added (30% w/v). The extraction solvent, chloroform (110 µL), and the dispersive solvent, ACN (500 µL), were mixed together and injected into the diluted sample solution by micropipette. The solution was agitated by vortex for 2 minutes before centrifugation at 4,500 rpm for 5 minutes. The chloroform layer was evaporated to dryness and reconstituted in sample medium (75 µL) before injection onto CE. Enrichment factors ranged from 50-407 to 750-2013 depending on the analyte in question. LOD values were between 1-220 µg/L.

Javanmardi *et al.* analysed commercial milk samples for the presence of benzoic and sorbic acid by HPLC-UV [46]. Protein and fats were removed by Carrez solutions I (potassium hexaferrocyanide) and II (zinc acetate). They found that acetone (1.2 mL) and chloroform (250 µL) provided a stable cloudy solution. LOD for benzoic acid and sorbic acid were 0.1 and 0.08 µg/mL, respectively, however in this case enrichment factors were not specified.

Natamycin, a polyene macrolide antibiotic, was preconcentrated from cheese samples [47]. The analytical technique in this study was indirect flame atomic absorption spectroscopy using a metal cation which complexed with natamycin. Various metal cations were trialled and Zn (II) was found to provide the highest recovery of the analyte and so was chosen as the optimum cation. The proteins were precipitated in an ultrasonic bath in the presence of methanol. DLLME was performed using 1,1,2-tetrachloroethane (116 µL) as the extraction solvent and ethanol (1.5 mL) as the dispersive solvent. LOD for natamycin was 1.8 ng/mL, recovery was 86-96%, while a RSD of 4.7% was obtained.

Also in 2016, the concentrations of two antibiotics were determined in pasteurized milk samples [48]. Proteins were precipitated by adding ACN (10 mL) to a milk sample (5 mL) and centrifuging to pellet the precipitated proteins. Chloroform (400 µL) was mixed with the ACN/milk sample (1.0 mL) and rapidly injected into water (1.0 mL). After centrifugation, the sedimented chloroform layer was evaporated to dryness and reconstituted in mobile phase (500 µL). The sample was analysed by HPLC-UV. LOD for chloramphenicol and florfenicol was 12.2 and 12.5 µg/kg, respectively. Recoveries (inter-day and intra-day) for both analytes ranged from 69.1-79.4%.

More recently (2017), Hamed *et al.* analysed several aflatoxins in yoghurt samples by HPLC-FLD [50]. Before DLLME, samples were centrifuged to remove fats, and following this step, the proteins were precipitated by a combination of ACN (6 mL) and salting out with NaCl (1.5 g). A portion of this organic solution (5.1 mL) was taken and mixed with chloroform (1.5 mL) and rapidly injected into water (5 mL). After centrifugation to break the emulsion, the chloroform phase was dried and reconstituted in MeOH:H_2O (1:1, 500 µL). While this work seems to use high volumes of extraction and dispersive solvents than traditionally used in DLLME, the figures of merit for this work provide LOD values below the levels set by the EU commission [181]. LOD values for the aflatoxins studied ranged from 1.5-5.5 ng/kg.

In 2018, Quigley *et al.* developed a DLLME method for the analyses of fatty acids in milk [51]. Simultaneous protein precipitation and analyte extraction was achieved in one step. Proteins were precipitated by the addition of NaCl (1 mL, 2 M) and phosphoric acid (30 µL), while the analytes were extracted using folch solution (MeOH: $CHCl_3$ 2:1; 750 µL). This mixture was centrifuged and the sedimented chloroform phase was transferred to a micro reaction vial where fatty acids were derivatised to FAMEs by BF_3-MeOH (1 mL,

14% w/v). The derivatisation was carried out in a water bath at 90 °C for 30 minutes. Once the solution was cooled, the reaction mixture was rapidly injected into water (5 mL) and then centrifuged. The aqueous phase was discarded and the sedimented chloroform phase was injected onto the GC-FID. LOD for caprylic (0.08 µg/mL), capric (0.04 µg/mL), palmitic (0.04 µg/mL), stearic (0.09 mg/mL), and oleic (0.07 µg/mL) were obtained. Enrichment factors ranged from 8-15.

1.2.2. Ultrasound assisted DLLME

Ultrasound assisted DLLME was first used on a dairy product for the determination of phthalate esters (DMP, DEP, DBP, BBP, DNOP, and DIOP) in milk using GC-FID [52]. Before UA-DLLME could take place, TCA and lead acetate were added to the milk samples to precipitate the proteins. A mixture of MeOH (800 µL) and carbon tetrachloride (40 µL) were used as the dispersive and extraction solvent, respectively. Once the cloudy solution had formed, it was placed in an ultrasonic bath for two minutes. The UA-DLLME method resulted in low LODs (0.64-0.79 ng/g), high enrichment factors (220-270), and %RSD values from 2.8-4.0%.

In 2013, simultaneous derivatisation and UA-DLLME was developed for the determination of chlorophenols (1,3-DCP, 2,3-DCP, 3-MCPD) in milk using GC-MS [53]. Proteins were precipitated by ACN (2 mL), which was also used as the dispersive solvent. Both the extraction solvent, chloroform (100 µL), and the derivatisation reagent, N-heptafluorobutyrylimizadole (HFBI) (50 µL), were mixed with ACN. After the formation of the cloudy solution, the sample was placed in an ultrasonic bath heated to 30 °C for five minutes. This was to aid emulsion formation and to ensure derivatisation was complete. The extraction parameters were optimised by central composite design. LODs as low as 0.9-3.6 µg/L were achieved along with recoveries ranging from 99%-102%.

Karaseva *et al.* coupled QuEChERS to UA-DLLME for the determination of aflatoxins B1 and M1 in milk and cheese samples using HPLC with fluorescence detection [54]. QuEChERS was used as a sample pretreatment protocol and to initially extract the

aflatoxins from the milk samples. ACN (3 mL) and chloroform (500 µL) were used as dispersive and extraction solvents, respectively. Once a cloudy solution had formed, it was placed in an ultrasonic bath for two minutes. The sedimented phase that was produced after centrifugation was dried under nitrogen. The residue was then reconstituted in ACN and subjected to HPLC analysis. The limits of detection for both B1 and M1 were 0.1 µg/kg and 0.01 µg/kg, respectively. Recoveries for B1 for all samples were between 51.2%-75.4%, while M1 had recoveries between 52.5%-72.2%. Total sample preparation time was approximately 1.5 hours.

1.2.3. Low-density solvent DLLME

Solvents that have a density lower than water were used as extraction solvents in the determination of benzoate and sorbate in yoghurt drinks [60]. Sample preparation involved protein precipitation by NaOH, H_2SO_4, potassium hexaferrocyanide (Carrez solution I), and zinc acetate (Carrez solution II). The supernatant from the previous step was used as the aqueous phase for LDS-DLLME. Ethanol (450 µL) and 1-octanol (60 µL) were used as the dispersive and extraction solvents, respectively. After centrifugation of the cloudy solution, the 1-octanol was removed and injected into HPLC-UV system for analysis. The LDS-DLLME parameters were optimised by a central composite experimental design. This method was compared to several other procedures reported in the literature for the analysis of benzoate and sorbate. LODs for this method (benzoate: 0.06 µg/L, sorbate: 0.15 µg/L) were much lower than those found in other methods (benzoate: 1.22-900 µg/L, sorbate: 2-500 µg/L). The method also provided good recovery of both benzoate (91.25%) and sorbate (106%).

Abedi *et al.* also determined benzoate and sorbate concentration in milk, cheese, and yogurt drinks by LDS-DLLME, this time using GC-FID as the detection method [58]. Many aspects of the papers are the same: both methods are optimised by central composite design, both use similar sample pretreatment procedures, and both have found that the optimum extraction solvent was 60 µL of 1-octanol. Abdol-Samad *et al.* have found that 475 µL of acetone was the optimum dispersive solvent. The newly developed LDS-DLLME-GC-FID method showed recoveries of benzoate (103.7%) and sorbate

(88%) that differ from the previous paper. LODs were 140 ng/g and 150 ng/g for benzoate and sorbate, respectively.

In 2015, Amoli-Diva *et al.* coupled LDS-DLLME with vortex-assisted dispersive solid phase extraction (VA-D-SPE) for the analysis of AFM_1 in milk samples [59]. Once the optimised LDS-DLLME emulsion had been formed (extraction solvent: 1-heptanol; 320 µL, dispersive solvent: MeOH/water (80:20); 3 mL), 500 µL of adsorbant (containing acid modified magnetic nanoparticles (MNPs)) were added and the sample was agitated on a vortex. An external magnet was applied which allowed the safe removal of supernatant. The analyte was desorbed from the adsorbant by the addition of 2 mL of ACN. Finally, the analyte was separated from the MNPs by magnetic decantation. The ACN eluent was evaporated to dryness and the residue reconstituted in Triton X-100 before analysis by fluorescence spectrophotometer. The method had an LOD for aflatoxin M1 of 0.013 µg/L, a linear range between 0.02-200 µg L^{-1}, and an extraction time of 20 minutes.

The next application of LDS-DLLME for dairy products was by Faraji *et al* in 2017 [61]. This group analysed melamine in milk and powdered infant formula. Proteins were precipitated by the addition of TCA (8.0 mL, 5% w/v) and lead acetate solution (1.0 mL, 2.2% w/v). The sample and protein precipitation solutions were placed in an ultrasonic bath for 10 minutes prior to centrifugation. To increase the detectability of melamine, it was derivatised using dabsyl chloride (100 µL, 4 mg/mL), in the presence of a sodium carbonate buffer (pH 9.0), by heating the deproteinsed sample for 10 minutes at 70 °C. The reaction was quenched by the addition of cold ACN to a volume of 1 mL. This ACN solution was then used as the dispersive solvent for the DLLME procedure. The extraction solvent used was 1-octanol (60 µL). This was mixed with the ACN solution and water (5 mL) was rapidly injected into the dispersive/extraction solvent mixture. Centrifugation broke the resulting emulsion and allowed for the recovery of the extraction solvent which was floating at the top of the tube. LOD for melamine was reported as 0.1 µg/L.Unfortunately the enrichment factor was not reported.

This method achieves a much lower LOD (0.1 µg/L) than Mirzajani and Tavaf (25 µg/L) [49]. The precipitation procedure used by Mirzajani may not have been sufficient as a small volume of ACN (0.4 mL) was used to precipitate proteins from a sample of milk (1.0 mL). Typically for ACN to function as an effective protein precipitation method, it

needs to be present in a 2:1 or 3:1 ratio to the sample. The ineffective precipitation of proteins may have led to problems with recovery of the DCM extraction solvent, or melamine may have not partitioned into the extraction solvent due to a greater affinity for the proteins. Alternatively, melamine may be more soluble in 1-octanol than DCM, and so this would lead to the lower LOD that was found by Faraji *et al.*

LDS-DLLME was again used in 2017 for the analysis of several biogenic amines in cheese samples. The biogenic amines included: cadaverine (CAD), histamine (HIA), putrescine (PUT), and tyramine (TYA), the structures of these compounds can be seen in Figure 1.4. The sample was spiked with analytes prior to microwave acidic digestion. Once digestion was complete, proteins were precipitated with Carrez solutions and then centrifuged. The supernatant was transferred to another container and the pH was increased to 11. To increase volatility of the analytes, they were derivatised using isobutyl chloroformate. Once this reaction was completed, a mixture of acetone (600 µL) and 1-octanol (60 µL) was rapidly injected into the sample; 1-octanol was recovered after centrifugation and analysed by GC-MS. LOD for CAD (8.8 ng/g), HIA (10.0 ng/g), PUT (14.0 ng/g), and TYA (5.9 ng/g) was achieved. The use of the microwave resulted in a more rapid digestion (3.0 minutes) than would be obtained using conventional methods.

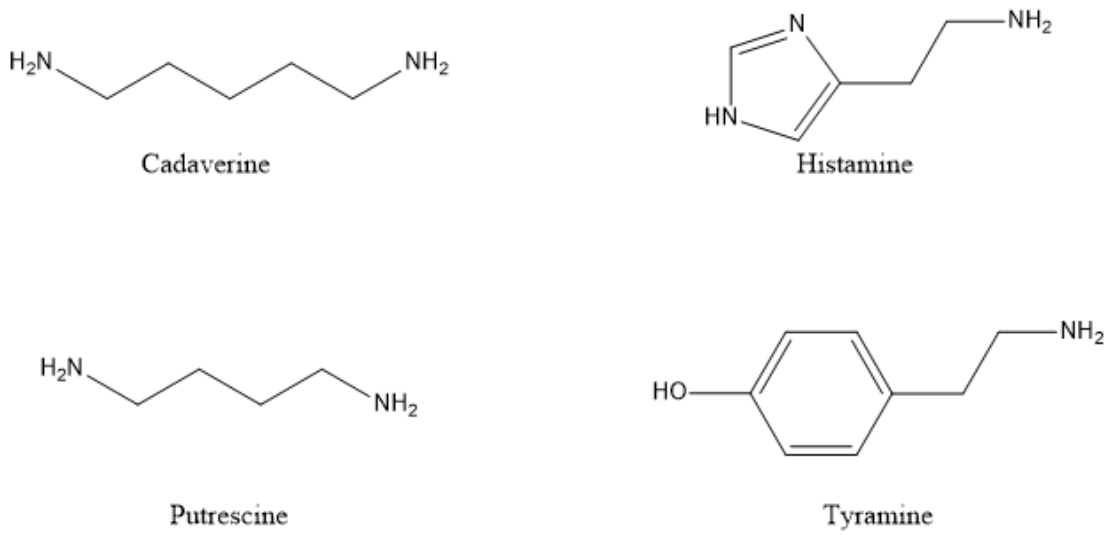

Figure 1.4: Structures of cadaverine, histamine, putrescine, and tyramine.

In 2018, Kamankesh *et al.* analysed cholecalciferol (vitamin D_3) in milk and yoghurt drinks. To remove any lipids present in the samples, base hydrolysis was performed on both the yoghurt and milk samples by the addition of KOH and ethanol (80:20, 8 mL) containing sodium ascorbate (2% w/v). Once hydrolysis was completed, the samples were centrifuged. The supernatant pH was adjusted to 4.5 and Carrez solutions were used to precipitate the proteins. The supernatant (10 mL) obtained after removal of proteins was used as the aqueous phase for the DLLME procedure. In this method, 1-octanol (80 µL) was used as the extraction solvent while ethanol (550 µL) was used as the dispersive solvent. A mixture of these two solvents was rapidly injected into the aqueous sample solution obtained after protein removal. An LOD of 3 µg/L was obtained for cholecalciferol and a recovery of 97% was achieved.

1.2.4. UA-RM-DLLME

Previously, all analytes mentioned have largely been non-polar, hydrophobic compounds. Roosta *et al.* have developed a method using a surfactant that forms reverse micelles (Triton X-100) for the determination of acetoin (structure shown in Figure 1.5), a polar compound, in butter using an ultrasound assisted-reverse micelle-DLLME procedure coupled with HPLC-UV detection [56].

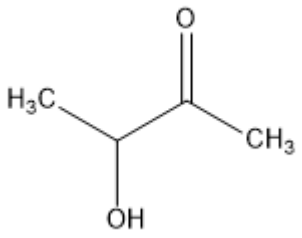

Figure 1.5: Structure of acetoin.

The butter samples (2 g) were melted by heating at 40 °C for 5 minutes before dilution with 2 mL of hexane and adding Triton X-100 (1.25% *w/v*). The sample was mixed by vortex for 1 minute. Distilled water (400 µL) was added as a modifier and the formation of a cloudy solution was produced by placing the sample in an ultrasonic bath for 4 minutes followed by centrifugation. The extraction process was optimised by a Box-Behnken experimental design. The LOD for the developed method was found to be 200 µg L^{-1}, while extraction recovery and repeatability were 96.40% and 2.86%, respectively.

1.2.5. IL-DLLME

Room temperature ionic liquids are another alternative green extraction solvent. In 2015 an IL-DLLME procedure was developed for the determination of nifurtimox (NFX), and benznidazole (BNZ) in breast milk coupled to HPLC-UV [64]. Proteins and lipids were removed by the addition of a precipitation mixture ($HClO_4$, H_3PO_4, and methanol) followed by incubation at 80 °C for 60 minutes. After centrifugation, the supernatant was separated from the solid material (proteins and lipids). This process was repeated and the supernatents combined. Analysis of NFX and BNZ were carried out separately using two different IL-DLLME procedures. For NFX: a mixture of NaOH (50 µL; 2 M) and KCl (150 µL; 30% *w/v*) were added to the supernatant. Then 42 µL of $[C_6C_1im][PF_6]$, as extraction solvent, and 80 µL of MeOH, as dispersive solvent, were rapidly injected into the above supernatant. For BNZ: a mixture of NaOH (45 µL; 2 M) and KCl (100 µL; 30% *w/v*) were added to the supernatant obtained from the pretreatment step. Both $[C_6C_1im][PF_6]$ (42 µL) and MeOH (101 µL) were mixed and rapidly injected into the above supernatant. The NFX and BNZ samples were shaken on a vortex for 6 minutes and centrifuged at 10,000 rpm for 20 min. The extraction solvent was sedimented at the bottom of the centrifuge tube. The supernatant was removed and the extraction solvent was injected for analysis. The NFX procedure had an LOD of 290 µg/L, a linear range from 300-34,400 µg/L and an enrichment factor of 33.8. The BNZ procedure had a LOD of 180 µg/L, a linear range from 200-29,160 µg/L, and an enrichment factor of 28.8.

1.2.6. UA-IL-DLLME

The use of ionic liquids as extraction solvents has been combined with UA-DLLME in technique termed: ultrasound assisted-ionic liquid- DLLME (UA-IL-DLLME). Tuzen *et al.* developed an US-IL-DLLME method for the determination of selenium in milk using graphite furnace atomic absorption spectrometric detection [57]. Prior to microextraction, the pH of the sample was lowered to pH 2 with dilute HCl. Chelation of selenium was achieved through adding 0.1% 1-phenylthiosemicarbazide (1 mL). The extraction solvent, $[C_6MIM][Tf_2N]$ (100 µL), was added and the sample placed in an ultrasonic bath for 10 minutes. The resulting cloudy solution was centrifuged and, afterwards, placed on ice to increase the viscosity of the now sedimented extraction solvent. The aqueous phase was removed by simple decantation. A mixture of HNO_3 and ethanol (1:1 *v/v*) was added to the extraction solvent to decrease viscosity and allow for easier retrieval. The authors found that without the use of ultrasound, recovery of selenium was below 25% while quantitative recovery was achieved when the sample was sonicated for 10 minutes. The UA-IL-DLLME method had an LOD of 0.012 µg/L, a linear range between 0.04-3.0 µg/L, a %RSD value of 4.2%, and an enrichment factor of 150.

1.2.7. UA-IL/IL-DLLME

Ionic liquids have also been used as both dispersive (hydrophilic IL) and extraction (hydrophobic IL) solvents in the same method. Gao *et al.* have developed a UA-IL/IL-DLLME method to determine the concentration of sulphonamides in infant formula using HPLC-PDA detection [30]. A sample of milk powder was weighed and dissolved in distilled water (50 °C); the ratio of infant formula to water was 1:8. Orthophosphoric acid (20 µL) and $[C_6MIM][BF_4]$ (70 µL), as extraction solvent, were added to the sample and intensely shaken for 5 min. When complete, $[C_4MIM][BF_4]$ (100 µL), as dispersive solvent, was added and the sample was transferred to an ultrasonic bath for 2 minutes. The resulting cloudy solution was then centrifuged and the sedimented extraction phase was collected. The IL was diluted with ACN and 0.1% formic acid to 200 µL before being filtered and injected into HPLC for analysis. The optimised method was used to determine

the concentration of six different sulphonamides: sulfamerazine (SMI), sulfamethizole (SMT), sulfachlopryridazine (SCP), sulfamonomethoxine (SMM), sulfmethoxazole (SMX), and sulflsoxazole (SIA). The LODs for each sulphonamide ranged from 2.94-16.7 µg kg^{-1}. Recovery for all the sulphonamides were all above 95% with RSD values less than 6.5%.

1.2.8. VA-DLLME

D'Orazio *et al*. developed a vortex assisted-DLLME (VA-DLLME) method to determine estrogenic compounds in milk and yogurt coupled to micellar electrokinetic chromatography with mass spectrometry [45]. The removal of proteins was achieved by adding ACN (4 mL) and acetic acid (100 µL). The sample was vortexed for 2 minutes and left in the dark for 15 minutes before centrifugation for 10 minutes at 4400 rpm. The supernatant was treated with 2 mL of hexane and the above vortex and centrifugation process was repeated, to remove fats. The aqueous layer was evaporated to 1.5 mL using a rotavapor (40 °C; 180 mbar). The extract was diluted to 7.5 mL with Milli-Q water and NaCl was added (30% *w/v*). After filtration, a mixture of dispersive solvent (ACN; 500 µL) and extraction solvent (chloroform; 110 µL) were added and the sample was vortexed for 2 minutes. After centrifugation, the sedimented chloroform phase was collected and evaporated to dryness, before being reconstituted in 75 µL of the sample medium (11.25 mM ammonium pentaflurooactanoic acid (APFO), pH 9 containing 10% *v/v* MeOH) and injected into the MEKC-MS system. The LOD for the 11 estrogenic compounds ranged from 1-220 µg/L. The method showed good recoveries of between 84-112%.

1.3. Conclusion

This is the first review of the use of DLLME in dairy samples. It can be seen that the various modes of DLLME can be applied to a range of analytes in different samples, while being coupled to various analytical techniques. The technique is compatible with a

range of solvents. The most commonly used solvent for DLLME analysis in dairy products is chloroform (Figure 1.6). Although there are health risks associated with using chloroform, the small volumes utilised in DLLME reduce the chance of harmful side effects from the solvent. To further reduce the risk of exposure to harmful solvents, 1-octanol is the most common solvent in LDS-DLLME. Ionic liquids are also an emerging trend in the quest for safe, green extraction solvents. In the coming years it will be interesting to observe any change in the frequency that each extraction solvent is used.

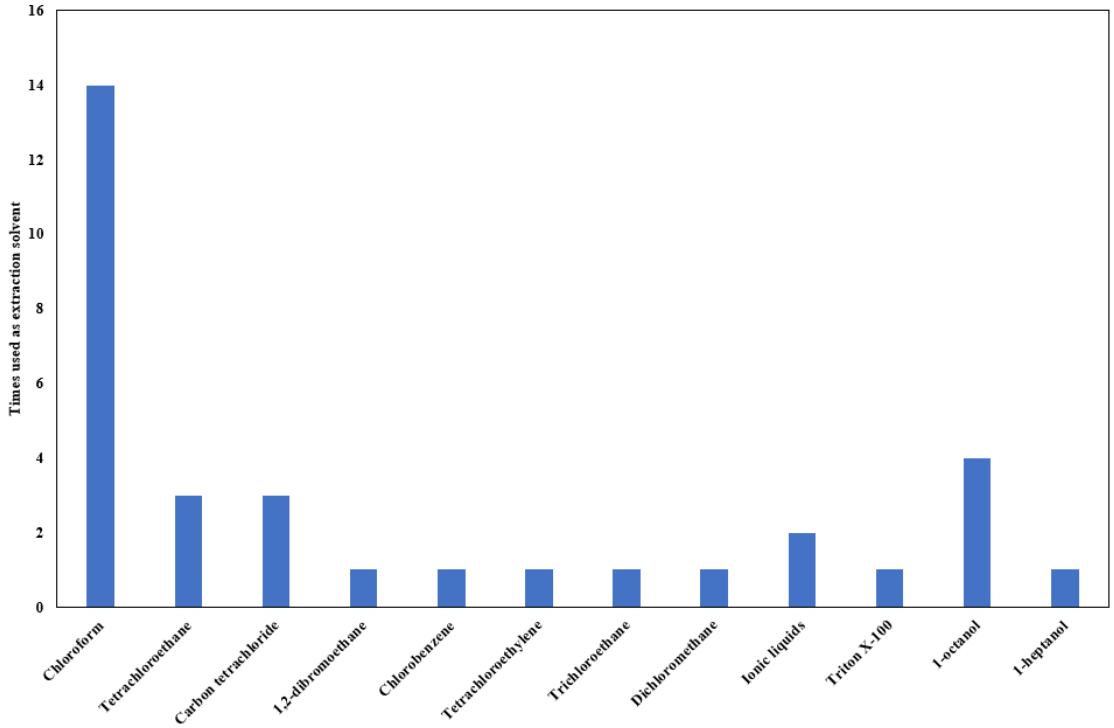

Figure 1.6: Extraction solvents for DLLME used in the analysis of dairy products (data from Table 1.1).

Several authors have evaporated the extraction solvent and reconstituted in a more suitable solvent before analysis by the respective method. This practice seems to negate the use of DLLME in the first place as it would be simpler to extract multiple times in a larger volume of extraction solvent and evaporate this to dryness before reconstituting in a small volume of suitable solvent. It would be interesting to see comparisons between

LLE and DLLME that both use the same volume of extraction solvent to test for extraction efficiency.

The review also highlights the importance of the sample pretreatment step in carrying out a successful DLLME method. With the correct sample pretreatment, DLLME can be a powerful tool in the analysis of analytes in dairy products; affording high enrichment factors while using minimal organic solvents. From Table 1.1, protein precipitation is an essential step in pretreatment. The removal of proteins from the sample allows for optimum phase separation between the extraction and dispersive solvents after centrifugation.

The extraction technique can be coupled to several different analytical techniques which increases the number of potential analytes that can be tested. The choice of analytical technique depends on the analyte being quantified.

In general, the above modes of DLLME are both quick and easy to use, but they do have some drawbacks. Each sample can require: pH adjustment, filtration, or centrifugation; depending on the sample pretreatment required. This can increase total sample preparation time.

Chapter 2

Determination of selected fat soluble vitamins using DLLME in bovine milk after seaweed supplementation

*Some of this data has been published in the *Journal of Chromatography B* (Quigley, A; Walsh S.W; Hayes E; Connolly, D; Cummins, W. (2018) Effect of seaweed supplementation on tocopherol concentrations in bovine milk using dispersive liquid-liquid microextraction. *J. Chrom. B.* **1092**: 152-157)

2. Effect of seaweed supplementation on vitamin content

2.1. Introduction

Vitamins are a group of compounds that are essential for human health, they catalyse various biochemical reactions and have a role in metabolic processes. They are typically divided into two classes: fat soluble vitamins (FSV) and water soluble vitamins (WSV). Fat soluble vitamins include retinol (vitamin A), tocopherol (vitamin E), radiostol (vitamin D), and antihemorrhagic vitamins (vitamin K) [66]. While an excess of both classes of vitamins poses health risks to humans, an over consumption of FSVs can also have an impact; they are not easily excreted from the body as they are stored in the liver and other fatty tissues [67]. Low consumption of FSVs, particularly vitamins A and D, have been linked to deficiency syndromes [68]. This is in contrast to WSVs which can be easily excreted through urine. Conditions that may arise from an excess of FSV include: depression, cardiovascular disease, kidney stones, and anaemia [69]. These vitamins are also added to various foodstuffs, including milk, to provide a greater nutritional benefit for the consumer. For example, both vitamins A and E are added as antioxidants and pigments [70].

To protect from vitamin degradation, fat soluble vitamins are added to commercial milk in derivatised forms. Vitamin A can be added in its palmitate or acetate form, while vitamin E is added as its acetate form [71]. Due to the instability of these vitamins, they are rarely added in their native form. Vitamin D can be added to products as either D_2 (ergocalciferol) or D_3 (cholecalciferol). The structures of these vitamins can be seen in Figure 2.1. Modifying the chemical structure of the vitamins in this way helps to ensure that the concentration of vitamins does not change during manufacturing or storage.

There are many commercial supplements available on the market to increase the quality of milk produced from a dairy cow e.g. FlowMag Liquid Mineral, NutriBuff Dairy, etc. which aim to increase the mineral content in the cows diet. Although *Ascophyllum nodosum* is a seaweed that is widely available both in fresh and processed forms, it's use as a supplement is relatively underutilised. Several groups have explored the effect of *Ascophyllum nodosum* supplementation on the inorganic mineral content of milk [72] and fatty acid content [73] but the use of *A. nodosum* as a vitamin supplement is relatively unexplored. Kidane *et al.* showed that supplementation with seaweed resulted in an

increased immuno response and α tocopherol levels in Norwegian Red Cows [74]. It would be important to determine if a similar increase in FSV levels would be observed using Irish seaweed supplementation for Freisan cows. Increased tocopherol levels can result in numerous health benefits for the cow. These include: reduction in testicular degradation, prevention of muscle dystrophy [74], and a reduction in oxidative blood damage [75].

Dispersive liquid-liquid microextraction (DLLME) was originally developed in 2006 for the analysis of organic compounds in water [76]. DLLME is a three phase extraction system comprising of an extraction solvent, a dispersive solvent, and an aqueous phase. To be considered for use in DLLME, both the extraction and dispersive solvents must fulfil certain criteria: the extraction solvent must be immiscible with the aqueous phase and miscible with the dispersive solvent, the analytes must show affinity for the extraction solvent, and the dispersive solvent must be miscible with both the aqueous phase and the extraction solvent. Originally, the extraction solvent used was always denser than water but solvents with a density lower than water have also been used [17]. A breakdown of extraction solvents for DLLME in dairy analysis can be seen in Section 1.3. Recently, ionic liquids [29], deep eutectic solvents [77], and supramolecular solvents [78] have also been utilised as extraction solvents in DLLME. A more detailed overview of DLLME can be seen in Section 1.1.1.

Traditional methods to pre-concentrate and extract FSVs in milk include solid phase extraction (SPE) [79], super critical fluid extraction [80], and liquid-liquid extraction [81]. These methods require expensive instrumentation or the use of relatively large volumes of solvent. As a result there is a need for the development of a rapid, sensitive and environmentally friendly method to analyse trace levels of vitamins in complex sample matrices, such as milk. Recently, DLLME has been used in the analysis of vitamins in foodstuffs such as fruit juices [82], plant based foods [83], and urine [84]. To the best of the author's knowledge, this is the first time DLLME has been applied to FSV in bovine milk.

The presented work in this chapter developed a novel DLLME method which required less organic solvents, and decreased analysis time. This method was then used to determine if supplementing cow feed with *A. nodosum* resulted in an increase in FSV in British Friesian cows. As *A. nodosum* is a widely available, renewable resource in Ireland;

its potential use as a supplement could reduce costs for dairy farmers. Friesian cows are among most popular milking breeds in Ireland so any improvement in milk quality would be beneficial to both the agricultural and food sectors in Ireland.

2.2. Overarching aims of this chapter

The aim of this chapter is to develop and optimise both a non-aqueous reversed phase HPLC separation and DLLME procedure for the separation and extraction of retinol acetate, K_2, Δ-tocopherol, D_3, and tocopherol acetate. The newly developed microextraction method was used to determine if increased levels of Δtocopherol would be detected in British Freisan cows that had been fed *A. nodosum* harvested from the west coast of Ireland.

2.3. Materials and methods

2.3.1. Chemicals and materials

HPLC grade methanol, acetonitrile, and ethanol were purchased from Lennox (Dublin, Ireland). BD Precisionglide syringe needles gauge 30 L 1.0 inch, menaquinone (K_2) (98%), Δ tocopherol (96%), ascorbic acid (99%), and tocopherol acetate (96%) were purchased from Sigma Aldrich (Dublin, Ireland). Ergocalciferol (D_3) (98%) and calciferol (D_2) (98%), was purchased from Tokyo Chemical Industry (Oxford, United Kingdom). Ultrapure water was provided by a Whitewater purification system (Dublin, Ireland).

2.3.2. HPLC method

Chromatography was performed on an Agilent 1100 HPLC system equipped with an Agilent Zorbax Eclipse Plus C_{18} column (50 x 4.6 mm; 1.8 µm). ACN was used as an isocratic mobile phase at a flow rate of 1.0 mL/min. The injection volume was 1 µL. The separation was carried out at 30 °C. Detection was carried out using a wavelength

switching timetable at wavelengths of 216 nm (0-2 min) and 327 nm (2- 6 min). Statistical analysis was carried out using Minitab (v18.0). A separation of the selected FSV can be seen in Figure 2.3.

2.3.3. Stock standard preparation

Stock standard solutions of each vitamin were prepared as follows: retinol acetate (1.46 mg/mL), tocopherol (1.32 mg/mL), D_3 (1.56 mg/mL), and tocopherol acetate (1.51 mg/mL) were dissolved in MeOH. K_2 (0.97 mg/mL) was dissolved in acetone. Structures of selected vitamins can be seen in Figure 2.1.

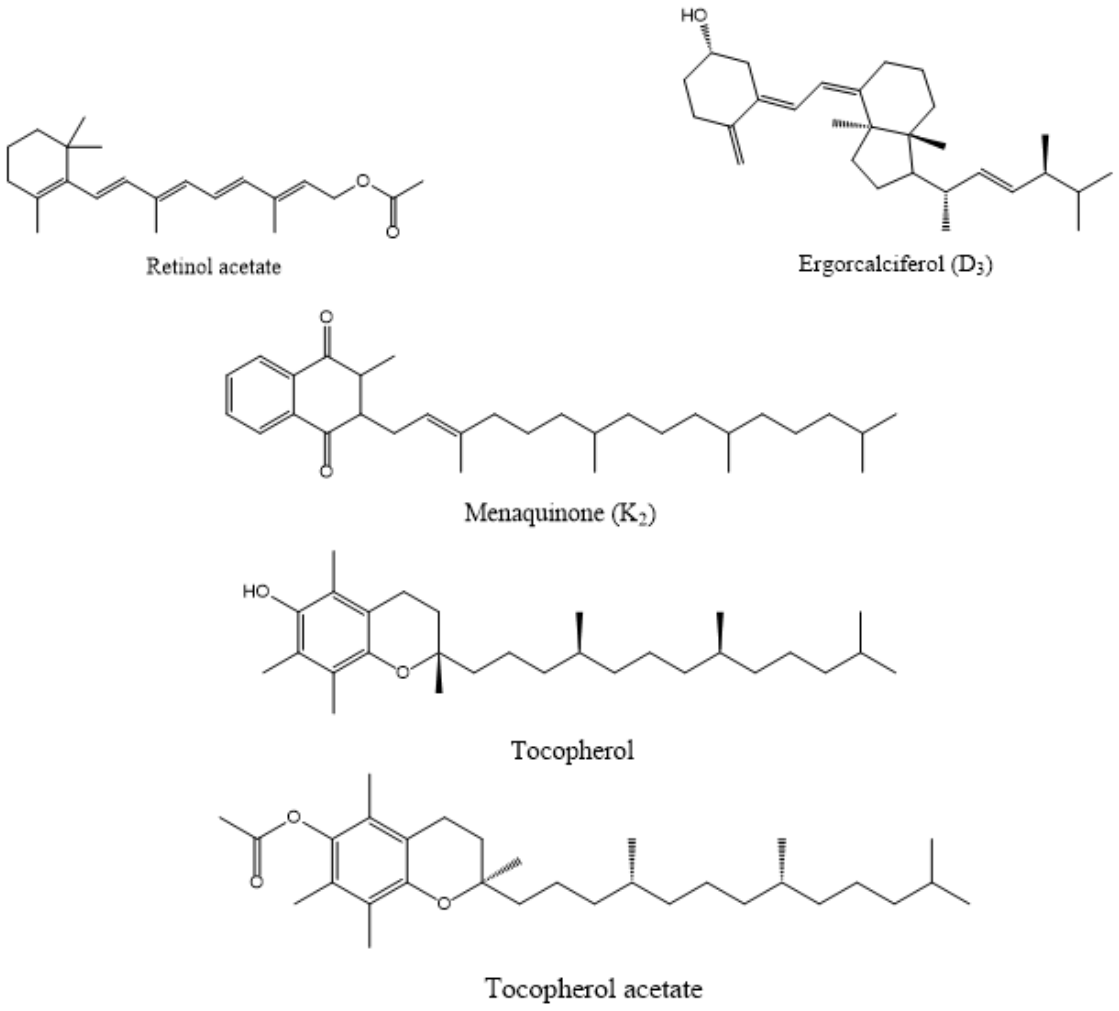

Figure 2.1: Structures of selected vitamins.

2.3.4. Seaweed supplementation

British Friesian cows (n=12) were divided into two groups. One group (n=6) had feed supplemented with 20% *A. nodosum*, while the other group (n=6) had no supplementation. All cows were dosed for gastrointestinal roundworms, lungworms, tapeworms, and adult liver fluke before the trial began. The groups of cows were of the same age, body condition score, and lactation cycle. Cows in the supplementation group were tested for acceptance prior to the trial and no cow refused the supplement. The two groups of cows were housed in the typical loose housing system with a slatted floor and cubicles that were lined with rubber matting and limed regularly, they had access to *ad lib* silage and fresh water and were supplemented with 1-2 kg of super beef nuts and 100 g of pre-calver minerals during the dry off period. The duration of the trial was 98 days.

2.3.5. DLLME procedure

The milk samples were obtained from cows and frozen at -20 °C until analysis. The samples were thawed and shaken prior to extraction. The samples were prepared as follows. 1.0 mL of milk, 9.0 mL of ethanol (containing ascorbic acid; 5 g/L) were added together. The samples were heated at 78 °C for 30 minutes and shaken at 10 minute intervals. Post heating, samples were cooled on ice and centrifuged for 5 minutes at 4,500 rpm. 1.0 mL of supernatant was mixed with 200 µL of extraction solvent and rapidly injected into 5 mL of ultrapure water. The resulting cloudy solution was centrifuged for 5 minutes at 4,500 rpm. The organic phase was again centrifuged for 10 minutes at 13,500 rpm to before injection onto the HPLC system.

2.4. Results

2.4.1. Development of fat soluble vitamin separation

2.4.1.1. Stationary phase selection

Given the lipophilic properties of FSVs, it was decided to use reversed phase HPLC to separate analytes of interest. Different combinations of C_{18} stationary phase, particle size, and particle type were screened to achieve resolution between analytes in the shortest time possible.

Column manufacturers have been designing stationary phases that increase the efficiency of HPLC separations. Efficiency can be maximized when band broadening is minimized. The band broadening process is due to a combination of three factors: Eddy diffusion, Longitudinal diffusion, and Mass transfer [85,86]. A decrease in any of these factors through improved design of columns and stationary phases will result in improved efficiency and ultimately better separations.

One of the most common ways to reduce band broadening is to reduce Eddy diffusion. Eddy diffusion refers to the broadening of bands due to the many different paths that an analyte molecule can take as it interacts with the stationary phase. As Eddy diffusion has a more pronounced negative effect on efficiency as particle size increases, this has led to manufacturers reducing the size of silica particles. The reduction from 5 μm to sub 2 μm particle size has led to higher efficiencies but also a substantial increase in column backpressure. The increase in column backpressure is inversely proportional to the square of the particle diameter meaning a system and pump capable of maintaining such pressure is required. Smaller particle sizes also provide a reduction in Mass transfer, as the analyte molecules have a smaller diffusion path into and out of the particle.

To reduce the column backpressure while still maintaining the increased efficiency gained by sub 2 μm particles, manufacturers developed superficially porous particles. These particles have a solid core onto which the stationary phase is bonded. This results in a reduced Eddy diffusion as there are fewer paths that the analyte molecule can take through the stationary phase. A depiction of a superficially porous particle can be seen in Figure 2.2. Like with sub 2 μm particles, Mass transfer is also reduced due to the shorter diffusion paths provided by the solid core.

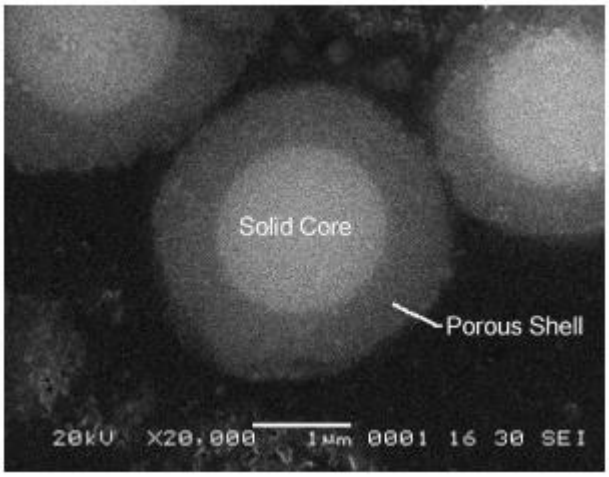

Figure 2.2: Superficially porous particle showing the solid core with stationary phase bonded to the surface [87].

Columns used in the screening experiment of this study included: Agilent Zorbax (250 x 4.6 mm; 5 μm), Agilent RapidRes (50 x 4.6 mm;1.8 μm), and Coretecs (150 x 2.1 mm; 2.7 μm). The Agilent columns were both fully porous silica particles while the Coretecs column had superficially porous particles. A comparison of the column parameters can be seen below in Table 2.1.

Table 2.1: Comparison of column dimensions.

Name	Stationary phase	Dimensions (mm)	Particle size (μm)	Particle type
Agilent Zorbax	C_{18}	250 x 4.6	5	Fully porous
Agilent RapidRes	C_{18}	50 x 4.6	1.8	Fully porous
Coretecs	C_{18}	150 x 2.1	2.7	Superficially porous

Chromatographic conditions for each column were optimised for the analytes chosen. Columns were then evaluated based on the run time needed to complete the analysis. A

shorter run time would increase the throughput of samples and also reduce mobile phase consumption; leading to more economically and environmentally friendly separations. The Zorbax column required the longest run time (23 min) using a gradient mobile phase system (MeOH, ACN), and needed a longer run time to elute analytes. Although both the Coretecs and RapidRes columns had similar run times, the Coretecs column required a lower flow rate than the RapidRes column. Unfortunately, backpressure on the Coretecs reached the upper limit of the HPLC system. Neither back flushing or washing with alternative solvents removed the blockage and so the RapidRes column was used for further experiments. A comparison of the separations can be seen in Table 2.2.

Table 2.2: Initial separation conditions.

Column	Mobile phase	Flow rate (mL/min)	Run time (min)
Zorbax	Mobile phase A: ACN Mobile phase B: MeOH	2.0	23
RapidRes	ACN (isocratic)	1.0	6
Coretecs	ACN (isocratic)	1.0	6

2.4.1.2. Mobile phase selection

Initially a scouting gradient was developed to optimise the separation of the vitamin standard mix on the RapidRes column. Mobile phase A was H_2O and mobile phase B was MeOH or ACN. The gradient time was calculated according to the equation below [88]:

$$t_g = \frac{k^* 1.15 \text{ S } \Delta\Phi \text{ } V_m}{F}$$

$$t_g = \frac{5 \; x \; 1.15 \; x \; 4 \; x \; 1 \; x \; 0.564}{1}$$

$$t_g = 12.972 \approx 13 \text{ min}$$

All peaks eluted early in the gradient, with the first peak eluting at 0.660 min and the last at 1.059 min. If the difference in retention time between the first and last peak is less than 25% of the gradient time, then an isocratic separation should be possible [89].

$$\Delta t_r = 1.059 - 0.660 = 0.399$$

As Δt_r is less than 25% of the gradient time (3.25 min) than an isocratic gradient should theoretically be possible. Both MeOH and ACN were evaluated as potential isocratic mobile phases. Although resolution improved in the MeOH isocratic separation compared to gradient separation, peaks were still poorly resolved ($R_s < 2$, resolution was too poor to accurately measure). Resolution was greatly improved using an ACN isocratic mobile phase, with all peaks baseline resolved apart from vitamins D_2 and D_3.

2.4.1.3. Separation temperature

A change in the temperature at which a separation is carried out can change selectivity, which in turn leads to changes in resolution. To increase resolution between vitamins D_2 and D_3, the separation temperature was varied from the initial 20 °C to 30 °C, 40 °C, 50 °C, and 60 °C. Maximum resolution was obtained at 30 °C. At temperatures higher than this, peaks co-eluted.

2.4.1.4. Reduction in extra column band broadening

As mentioned previously, the use of sub 2 µm particles results in smaller analyte bands. These smaller bands can result in increased resolution compared to a separation carried out on traditional 5 µm particle stationary phase. Without the proper system modifications, extra column band broadening can occur. System modifications include: narrow bore tubing, low volume needle seat, bypassing the heat exchanger, and a low volume detector cell. These modifications prevent the narrow analyte band becoming

diluted in a relatively large volume of mobile phase [90]. Modifying the HPLC system with a low volume needle seat was not possible due to budgetary constraints.

The system was already equipped with narrow bore tubing (0.005 inch) and so the effect of bypassing the heat exchanger was examined. An Agilent 1200 HPLC system was equipped with a semi-micro flow cell. This has a flow cell volume of 5 µL, compared to the standard Agilent flow cell volume of 13 µL. The vitamin standard mix was ran on the 1200 system modified with narrow bore tubing and bypassing the heat exchanger. As the separation was carried out in isocratic mode, no adjustment to the mobile phase was needed. Separations could be compared between the two systems by using relative retention times of each vitamin. The resolution of the D vitamins using the standard flow cell volume (13 µL) and the semi-micro flow cell volume (6 µL), with both systems equipped with 0.005 inch polyether ether ketone (PEEK) tubing and bypassed heat exchanger, is given below in Table 2.3.

Table 2.3: Comparison of resolution on Agilent 1100 and 1200 systems.

Analytes	Resolution on 1100	Resolution on 1200
D_2 and D_3	1.05	1.37

2.4.1.5. Wavelength switching

To maximise detectability of each vitamin, a wavelength switching experiment was carried out. The λ_{max} for each vitamin was obtained from a Dionex application note [91] and used to construct a wavelength switching timetable. The peak areas obtained using the wavelength switching timetable were compared to running the separation at the original detection wavelength (210 nm). The wavelength switching timetable can be seen in Table 2.4.

Table 2.4: Wavelength switching timetable.

Time (min)	Wavelength (nm)
0	327
2	210

Wavelength switching between tocopherol, D_2, D_3, and tocopherol acetate was not possible due to the similar retention times of these compounds. They were detected at 210 nm which did not result in a significant difference in peak area.

Resolution between vitamins D_2 and D_3 could not be achieved with the available equipment. To enable accurate quantification of D_3, vitamin D_2 was removed from the standard mix. D_3 is more commonly found in bovine milk [92,93] The optimised separation can be seen in Figure 2.3.

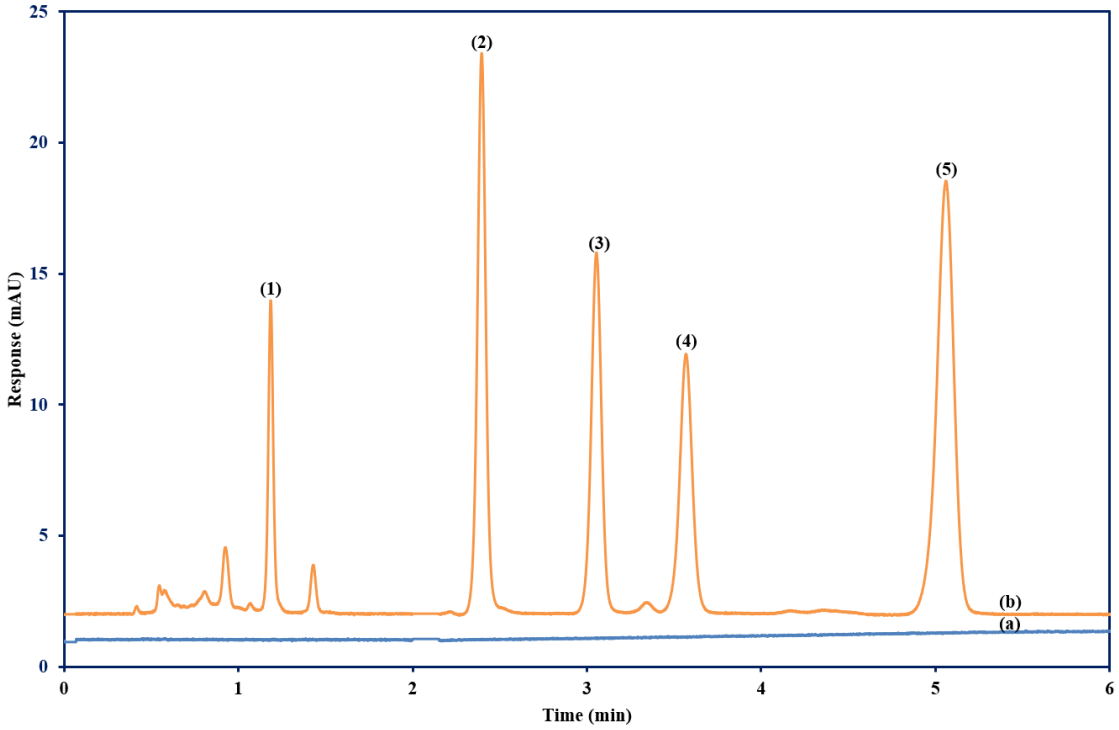

Figure 2.3: Chromatogram of selected vitamins. Chromatogram (a) blank, (b) selected vitamins. Peak identification: (1) retinol acetate (20 µg/mL), (2) K_2 (20 µg/mL), (3)

tocopherol (20 µg/mL), (4) D_3 (20 µg/mL), (5) Tocopherol acetate (20 µg/mL). Chromatographic conditions: stationary phase: C_{18} (50 x 4.6 mm; 1.8 µm), mobile phase: ACN, flow rate: 1.0 mL/min, temperature: 30 °C, detection wavelength: 327 nm and 216 nm.

2.4.1.6. Peak purity

Tocopherol peak purity was determined using a PDA detector and Chemstation software. The spectra can be seen below in Figure 2.4. The peak shape is uniform across all wavelengths which indicates that there are no impurities co-eluting with tocopherol.

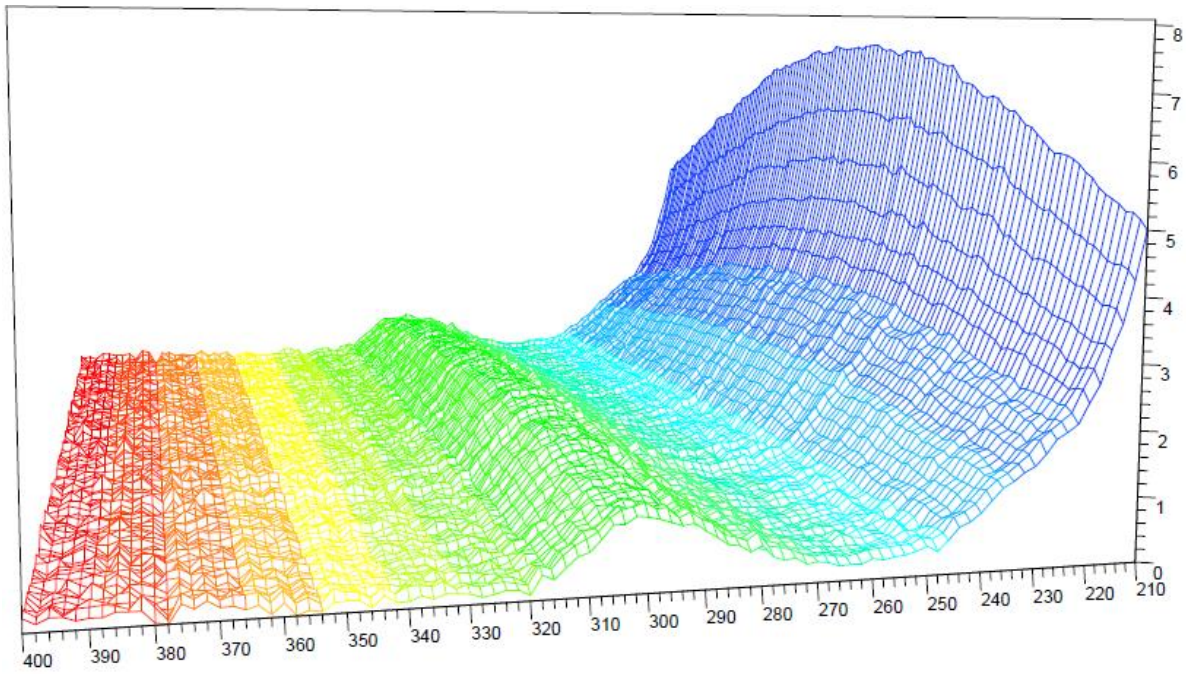

Figure 2.4: Tocopherol peak purity analysis by PDA. X-axis: wavelength (nm), Y-axis: response (mAU), Z-axis: time (min).

2.4.2. Optimisation of DLLME procedure

In the present work, parameters such as the protein precipitation solvent, extraction solvent, volume of extraction solvent, volume of dispersive solvent, and volume of aqueous phase were optimised. The protein precipitation and extraction solvent were optimised first. The volumes of the solvents used were then optimised by design of experiments (DoE) using a factorial screening and a central composite design approach. The optimisation process was carried out using milk purchased from a local shop before applying the optimised process to untreated milk samples. Parameters such as ascorbic acid concentration, heating time and temperature were previously optimised [94] and adapted for this work.

2.4.3. Selection of organic extraction solvent

Any extraction solvent for DLLME must fulfil the criteria outlined in the introduction. Solvents that had both higher and lower densities than water were examined as potential extraction solvents. Solvents were evaluated by extracting analytes using 200 µL of solvent from milk samples that had proteins precipitated by 9 mL of EtOH. The screened solvents were chloroform ($CHCl_3$), dichloromethane (DCM) (CH_2Cl_2), and 1-octanol. These solvents were chosen as they cover a broad range of polarity which would influence analyte solubility. As Figure 2.5 shows, chloroform displayed the highest extraction efficiency and thus was selected as the optimum extraction solvent.

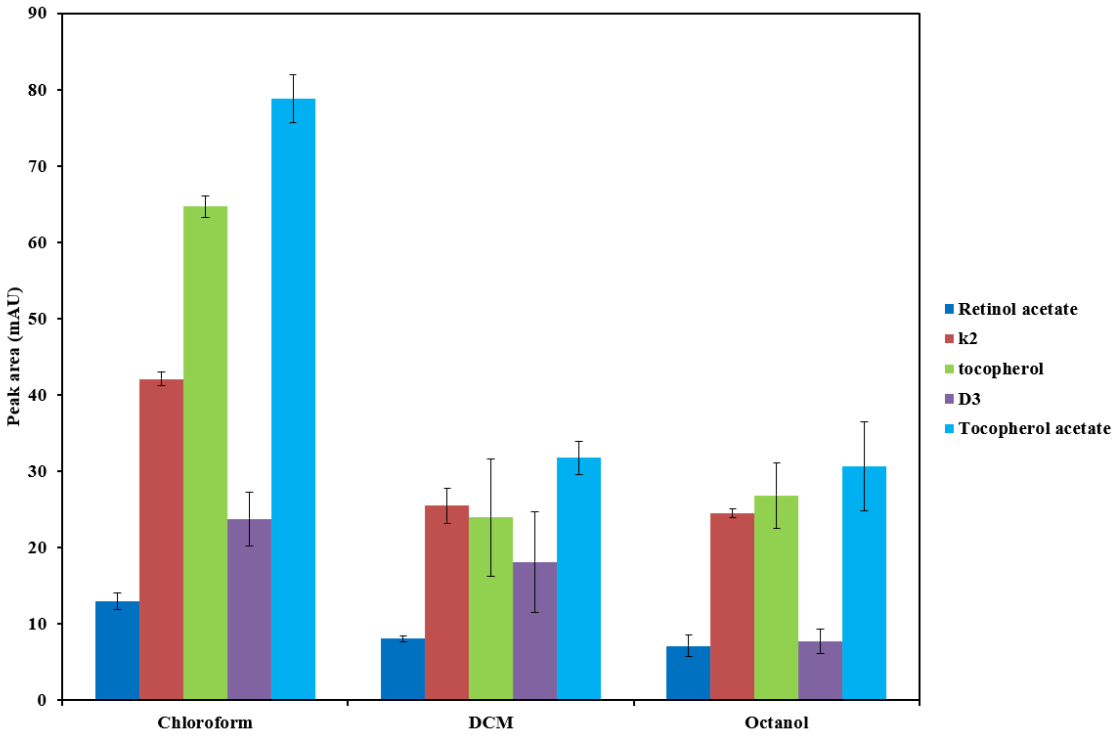

Figure 2.5: Evaluation of extraction solvents (n=3).

2.4.4. Selection of protein precipitation solvent

The method of protein precipitation must be compatible with DLLME. Simultaneous protein precipitation and analyte extraction using organic solvents was trialled, as protein precipitation without efficient extraction would result in poor recovery and higher LOD. ACN and ethanol were screened as potential protein precipitation and extraction solvents. The extraction efficiency for both protein precipitation solvents with chloroform was tested as this would ultimately affect the LOD for the subsequent DLLME procedure. These solvents are common dispersive solvents in DLLME methods and also commonly used in protein precipitation applications [40,95]. Figure 2.6 shows that ethanol had greater extraction efficiency than ACN and so ethanol was used as the dispersive solvent in the DoE. The possible explanation for the better extraction efficiency when ethanol is used compared to ACN is two-fold. Firstly, ethanol produced a more stable cloudy solution upon rapid injection into the aqueous phase. This facilitates a more rapid transfer of the analytes into the extraction phase, resulting in a more efficient extraction. Secondly, the solubility of the analytes could be greater in ethanol than ACN; also resulting in a

more efficient extraction [96]. Direct measurement of protein precipitation levels was not explored as analyte recovery was the most important factor in determining the optimum protein precipitation solvent.

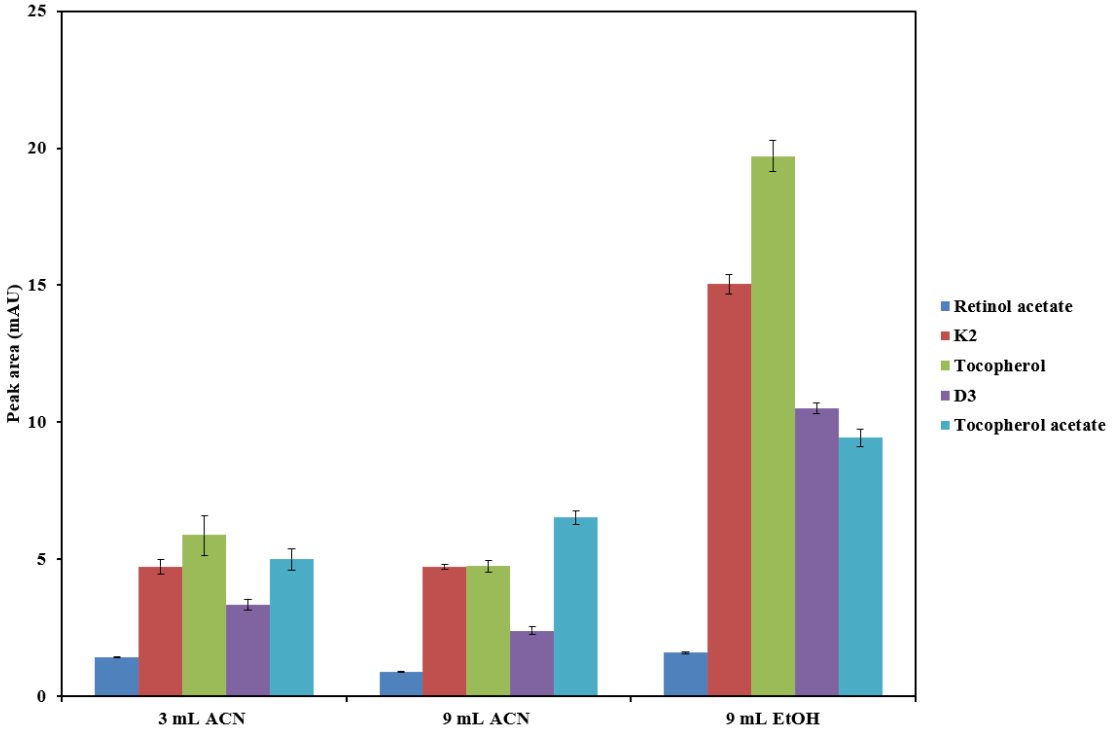

Figure 2.6: Screening of potential protein precipitation solvents (n=3).

2.4.5. Factorial screening

A 3 factor, 2 level factorial design (2^3) was used to screen for significant factors. A list of the factors and levels can be found in Table 2.5. Preliminary experiments determined the minimum and maximum levels for each factor. Values outside these ranges resulted in poor extraction performance due to an unstable cloudy solution. In the case of the minimum extraction solvent volume, when less than 200 µL was used the resulting organic phase volume was too loo low to be practically analysed.

Table 2.5: Screened factors and levels.

Factor	-1	+1
Dispersive solvent volume (EtOH) (mL)	0.5	1.0
Extraction solvent ($CHCl_3$) (µL)	200	400
Aqueous phase volume (H_2O) (mL)	5	10

The resulting Pareto chart (Figure 2.7) showed that only the volume of extraction solvent was significant. This is evident as the volume of extraction solvent is the only factor that crosses the significance line. As the extraction efficiency of DLLME is dependent on the stability of the cloudy solution formed from the interaction of extraction and dispersive solvents, it was decided to further optimise the DLLME procedure.

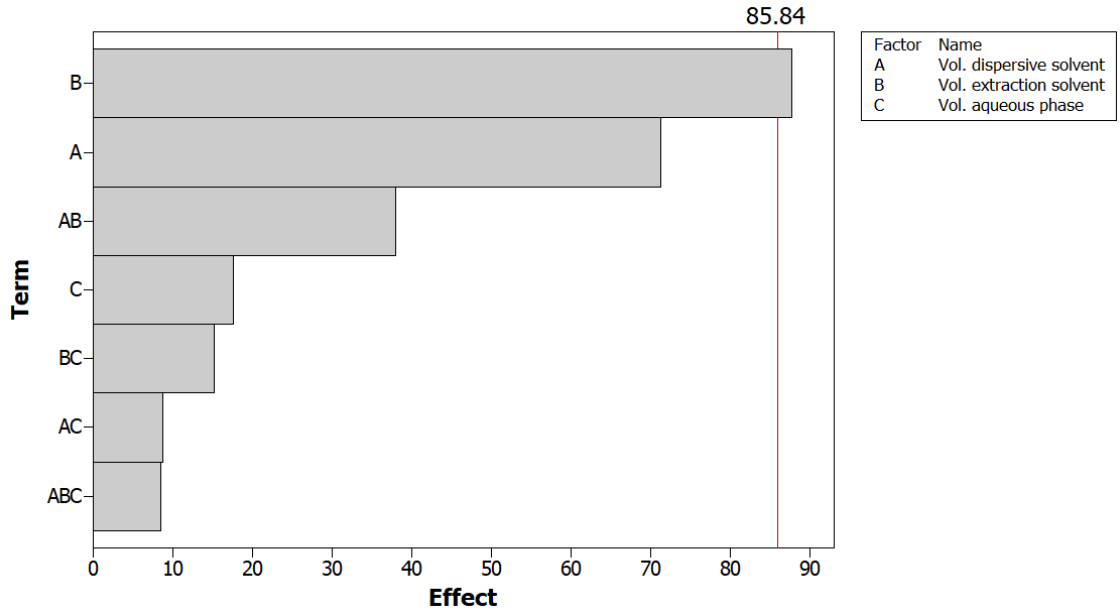

Figure 2.7: Pareto chart of factorial screening experiment ($p<0.05$).

2.4.6. Central composite design

A central composite design was chosen to further optimise the DLLME process by varying the volumes of dispersive and extraction solvents. The goal was to maximise the response, in this case response was analyte peak area. The central composite design

consisted of a 2^2 full factorial design which was augmented with both star (± α) and centre points (0). The levels for each factor are given in Table 2.6 and the resulting response surface can be seen in Figure 2.9. The response was maximised when 200 µL of extraction volume and 1.0 mL of dispersive solvent was used.

Table 2.6: Factors and levels in central composite design.

Factor	-α	-1	0	+1	+α
Dispersive solvent (mL)	0.293	0.5	0.75	1.0	1.41
Extraction solvent (µL)	117.16	200	300	400	565.68

The resulting ANOVA table and response surface can be seen in Figure 2.8 and Figure 2.9, respectively. The ANOVA table shows that the volumes of dispersive and extraction solvents are significant. A significant quadratic interaction was also detected: extraction*extraction solvent volume had a p value = 0.09. This indicated that the relationship between response and extraction volume was not linear.

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	5	27695.4	5539.1	30.10	0.000
Linear	2	24123.4	12061.7	65.54	0.000
Dispersive	1	9075.2	9075.2	49.32	0.000
Extraction	1	15048.3	15048.3	81.77	0.000
Square	2	2783.2	1391.6	7.56	0.018
Dispersive*Dispersive	1	655.4	655.4	3.56	0.101
Extraction*Extraction	1	2408.1	2408.1	13.09	0.009
2-Way Interaction	1	788.8	788.8	4.29	0.077
Dispersive*Extraction	1	788.8	788.8	4.29	0.077
Error	7	1288.2	184.0		
Lack-of-Fit	3	347.5	115.8	0.49	0.706
Pure Error	4	940.7	235.2		
Total	12	28983.6			

Figure 2.8: ANOVA table from central composite design.

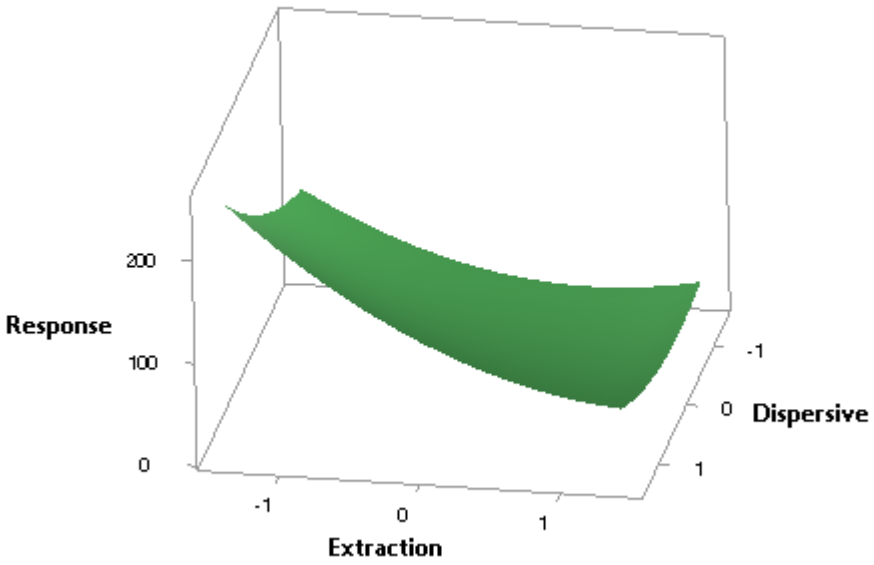

Figure 2.9: Response surface generated from the central composite design.

The regression equation generated from the central composite design can be seen in below in Figure 2.10. This equation can be used to predict responses inside the experimental space.

```
Response = 56.92 + 33.68 Dispersive - 43.37 Extraction
           + 9.71 Dispersive*Dispersive + 18.61 Extraction*Extraction
           - 14.04 Dispersive*Extraction
```

Figure 2.10: Regression equation.

2.4.7. Validation of DLLME procedure

To evaluate the applicability of the developed DLLME method to fat soluble vitamin analysis in bovine milk, linearity, LOD, reproducibility, recovery, and enrichment factor were determined; these were evaluated with spiked samples. The figures of merit are shown in Table 2.7. Linearity for retinol acetate, K_2, delta tocopherol, D_3, and tocopherol acetate was obtained in the range 0.1 to 8 µg/mL. Analytes were spiked in at the following

concentrations: 0.1, 2, 4, 6, 8 µg/mL. The linearity was determined by plotting calibration curves of peak area versus the concentration of each analyte. The coefficients of each analyte ranged from 0.989- 0.998. The LOD was obtained from the slopes of the linearity curves, according to Equation 2 where σ is standard deviation of the calibration curve and s is the slope of the calibration curve. The inter-day reproducibility relative standard deviation (%RSD) for each analyte was below 7% (n=6). Recovery was calculated by spiking sample with analyte and checking for a proportionate increase in peak area. Recovery for each analyte was between 78% and 92%, while the enrichment factors were 64 to 89. An example of the chromatography obtained for the LOD can be seen below in Figure 2.11.

$$LOD = 3.3 \times (\sigma/s) \quad (2)$$

Table 2.7: Figures of merit for fat soluble vitamin analysis.

Analyte	**Linearity (R^2)**	**LOD (µg/mL)**	**Reproducibility (%RSD) (n=6)**	**Recovery (%) (n=6)**	**EF**
Retinol acetate	0.992	0.01	6.8	94	72
K_2	0.989	0.03	4.9	78	83
Delta tocopherol	0.998	0.10	2.9	81	89
D_3	0.991	0.05	4.8	82	64
Tocopherol acetate	0.996	0.02	6.2	89	71

A recently published method by Kamankesh *et al.* [63] also analysed D_3 in milk samples using LDS-DLLME. This paper has been reviewed in Section 1.2.3. Kamankesh *et al.* have achieved an LOD of 0.0009 µg/mL which is lower than what was achieved in the method presented in this chapter. Ethanol was used as the dispersive solvent for both methods but Kamankesh *et al*. used 1-octanol as the extraction solvent. When 1-octanol

was evaluated as a potential extraction solvent in this work it was found to be less efficient than chloroform (Figure 2.5).

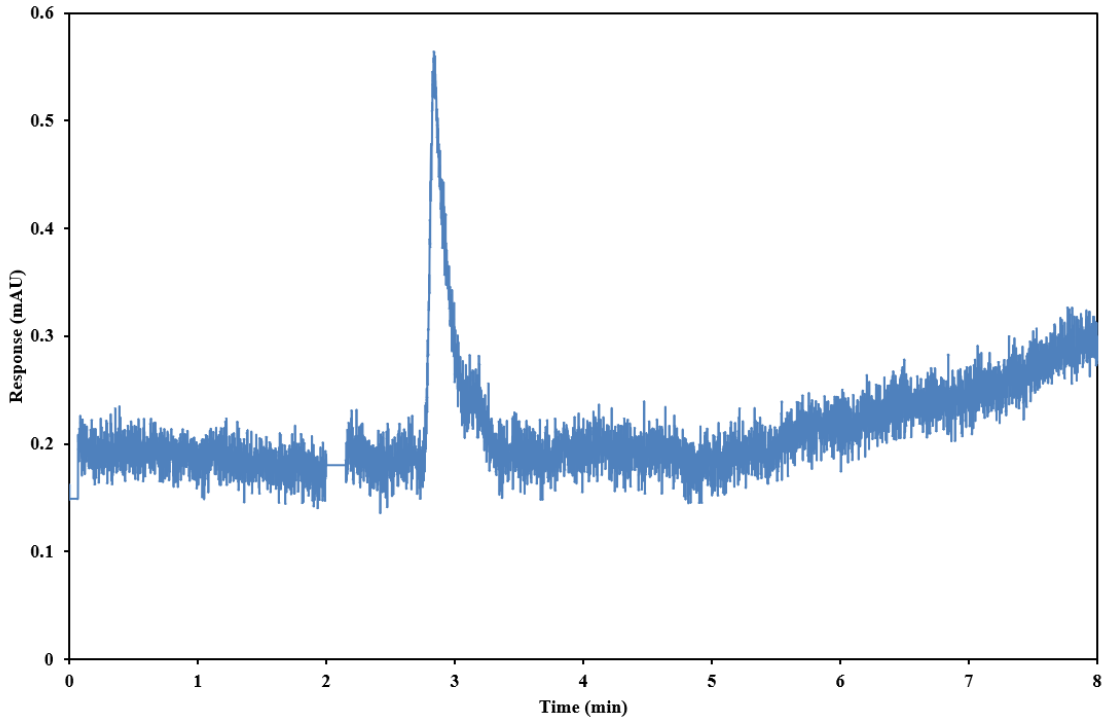

Figure 2.11: LOD chromatogram for tocopherol.

There are more significant differences in the sample treatment prior to DLLME between the two methods. Kamankesh *et al*. have used Carrez solutions to precipitate proteins. This is a commercially available kit that can be used to precipitate proteins, among other applications. The kit contains two solutions: Carrez solution I and Carrez solutions II. Carrez solution I is an aqueous solution of potassium hexacyanoferrate (II) trihydrate ($K_4[Fe(CN)_6]$ x $3H_2O$) while Carrez solution II is an aqueous solution of zinc sulfate heptahydrate ($ZnSO_4$ x $7H_2O$). Upon addition of both solutions to an aqueous sample, the salts react to form $Zn_2[Fe(CN)_6]$. This is a precipitate that has limited solubility and high molecular weight compounds, such as proteins, will be adsorbed to the newly formed complex allowing for precipitation by centrifugation. The use of the Carrez kits may denature the proteins more effectively than ethanol. This would release any D_3 that may have held by the hydrophobic regions of the proteins, allowing for a more quantitative extraction. Carrez solutions were not evaluated for this work due to budgetary constraints.

While both Kamankesh *et al.* and this work both analysed milk samples, Kamankesh *et al.* determind D_3 in pasteurised and homogenised commercial milk samples. In contrast, this work developed a method and applied it to untreated milk samples obtained directly from the cow. Untreated milk samples present an even more complex sample matrix than commercial milk and the added matrix interference may be responsible for the higher LOD values obtained in this work.

2.4.8. Comparison with recently published methods

To ensure that this method represented a step forward in scientific knowledge, it was compared to recently published methods for tocopherol analysis (Table 2.8). The presented work provided lower LOD and reproducibility than published methods.

Table 2.8: Comparison with recently published methods for tocopherol analysis. N.R: not reported.

Analyte	LOD (µg/mL)	Reproducibility (%RSD)	Reference
Tocopherol acetate	N.R	3.95	[97](2016)
Tocopherol	0.5	1.2	[71] (2014)
Tocopherol	0.13	5	[98] (2012)
Tocopherol	0.10	2.9	Presented work

2.5. Sample analysis

2.5.1. Standard addition

As mentioned previously, bovine milk is a complex biological fluid and as such, quantification of trace compounds is a challenge. Traditionally, calibration and quantification has been carried out using one of three methods: external calibration, internal calibration, or standard addition.

External calibration does not account for matrix effects, and an accurate blank matrix could not be developed [99]. Simulated milk ultrafiltrate is often used as an analytical substitute for milk, but this lacks the complexity of natural milk [100]. Several internal standards were explored but none of the screened compounds were sufficiently retained using the above chromatographic separation, or co-eluted with an analyte of interest, see Table 2.9. Isotope-labelled internal standards, for LC-MS detection, were not explored due to their cost. Also, distinguishing between analyte and isotope-labelled internal standard is not possible using UV detection and requires instruments coupled to mass spectrometers.

Table 2.9: Retention factors of compounds screened for internal standard.

Compound name	Retention factor (k)
Anthacene	0.3
Benzophenone	0.3
Cinnamaldehyde	0.3
Cumene	0.3
Dodecanophenone	0.8 (co-eluted with retinol acetate)
Hyrdorxyphenylacetate	0.3
Hydroxypropiophenone	0.3
Phenantherene	0.3
Phenylethylacetate	0.3

Standard addition was chosen as the calibration method for this work as it can account for matrix effects in complex samples [101]. Standard addition is performed first by dividing the sample into four or more aliquots. The first aliquot is analysed directly, while the other three aliquots have the analyte added to the sample in increasing concentrations.

The original concentration of the analyte can be then be determined by extrapolation from the resulting calibration curve [101].

The first point in the standard addition curve was obtained from analysing sample without added standards, while the remaining four points were obtained from adding increasing amounts of standards. The concentration of analytes added at each stage can be seen in Table 2.10. Standard addition was not evaluated for other compounds as they were not detected without the added standards. Standard addition for both delta tocopherol and D_3 resulted in linear curves with R^2 values greater than 0.98

Table 2.10: Analyte concentration at each standard addition level.

Analyte	**Level 1 (µg/mL)**	**Level 2 (µg/mL)**	**Level 3 (µg/mL)**	**Level 4 (µg/mL)**
Delta tocopherol	2.0	4.0	6.0	8.0
D_3	0.20	0.30	0.50	0.70

2.5.2. Effect of seaweed supplementation

The effect of seaweed supplementation on the FSV content of bovine milk was investigated with the newly developed DLLME method. The analysis was carried out as detailed in Section 2.3.5. The results were analysed by a t-test and the null hypothesis stated that the group means were not significantly different. It was found that seaweed supplementation had a statistically significant ($p > 0.05$) effect on the concentrations of Δ tocopherol, increasing from 3.82 µg/mL to 5.96 µg/mL. An example of the chromatograms obtained can be seen in Figure 2.12 and a bar chart showing error bars in Figure 2.13. As outlined in the introduction, an increase in the levels of tocopherol have numerous benefits. The use of a locally grown, renewable resource grown to increase the nutritional benefits of bovine milk could have a wide impact on the agricultural and food industries in Ireland.

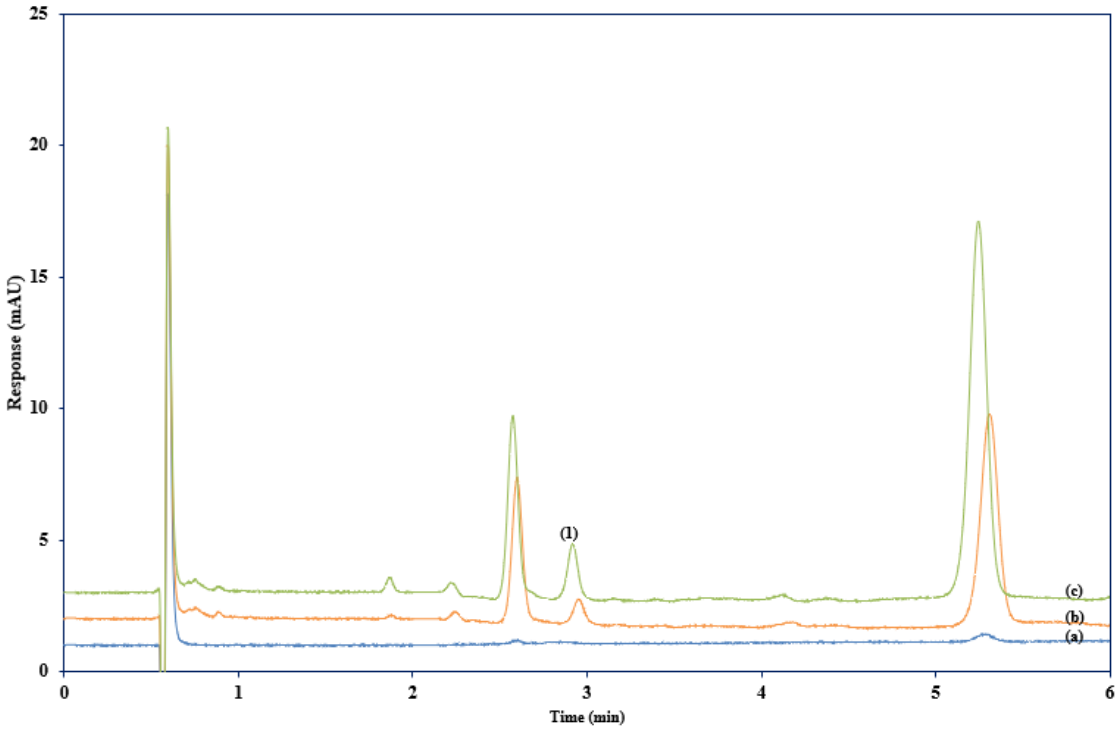

Figure 2.12: Comparison of chromatograms showing the effect of seaweed supplementation on tocopherol levels in milk. Chromatogram identification: (a) blank, (b) no seaweed supplementation, (c) seaweed supplementation. Peak identification: (1) Δ tocopherol. Chromatographic conditions as outlined in Figure 2.3.

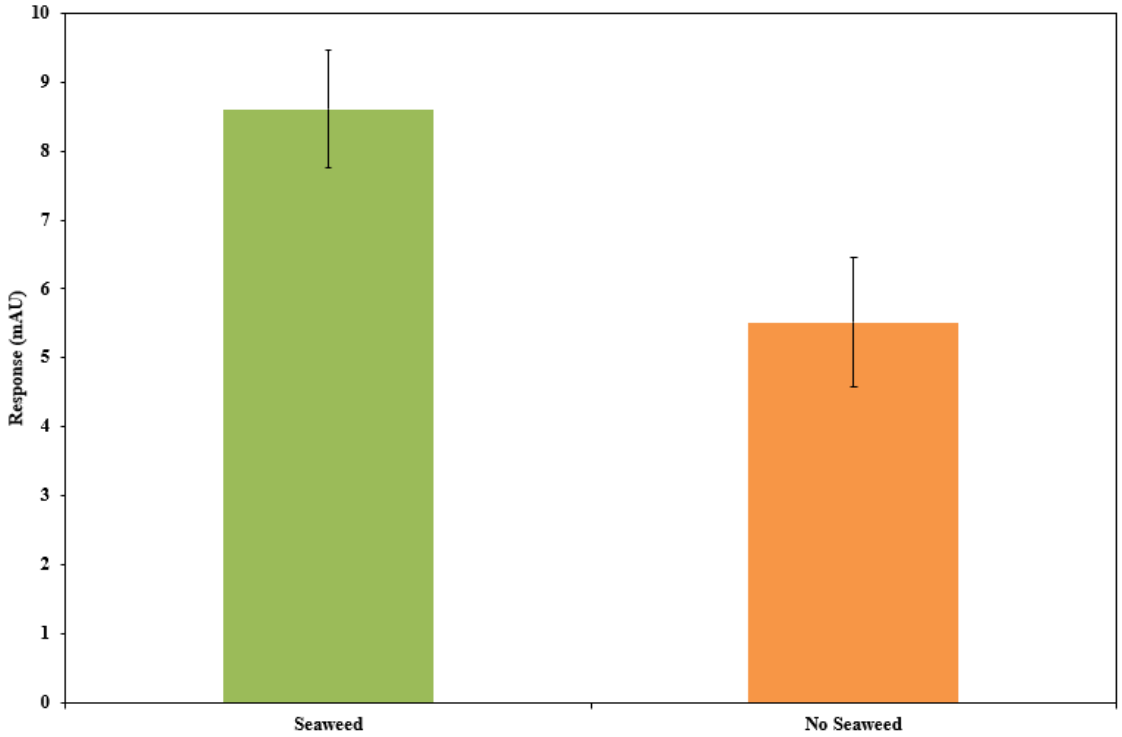

Figure 2.13: Effect of seaweed supplementation on tocopherol response. P-value < 0.05

2.6. Conclusion

The novel DLLME sample preparation method combined with HPLC-UV was developed and used to investigate the effect of *A. nodosum* on the vitamin content of British Friesian milk. The DLLME method was optimised by DoE which resulted in an environmentally friendly method which used minimal organic solvents. It afforded high enrichment factors, low detection limits, and good repeatability. The supplementation resulted in an increase in delta tocopherol content from 3.82 µg/mL to 5.96 µg/mL.

Chapter 3

Development of DLLME procedures for the extraction and preconcentration of fatty acids from bovine milk

*Some of this data has been published in the *Journal of Chromatography B* (Quigley, A; Connolly, D; Cummins, W. (2018) The application of dispersive liquid-liquid microextraction in the analyses of the fatty acid profile in bovine milk in response to changes in body condition score. *J. Chrom. B.* **1073**: 130-135)

3. Development of DLLME procedure for the extraction and derivatisation of fatty acids from milk

3.1. Introduction

Fatty acids can be broadly classified into two classes: short chain and long chain fatty acids. Short chain fatty acids have a chain length of between 2 and 8 carbons, while long chain fatty acids can have up to a 9 to 30 carbon length chain. Fatty acids can be further characterised on the basis of saturation. The absence of double bonds in the carbon chain results in a saturated fatty acid, these fatty acids are not affected by hydrogenation or halogenation. When a single or multiple double bonds are present in the carbon chain, the fatty acid is referred to as mono-unsaturated or poly-unsaturated, respectively. Structures of saturated and unsaturated fatty acids can be seen in the below Figure 3.1.

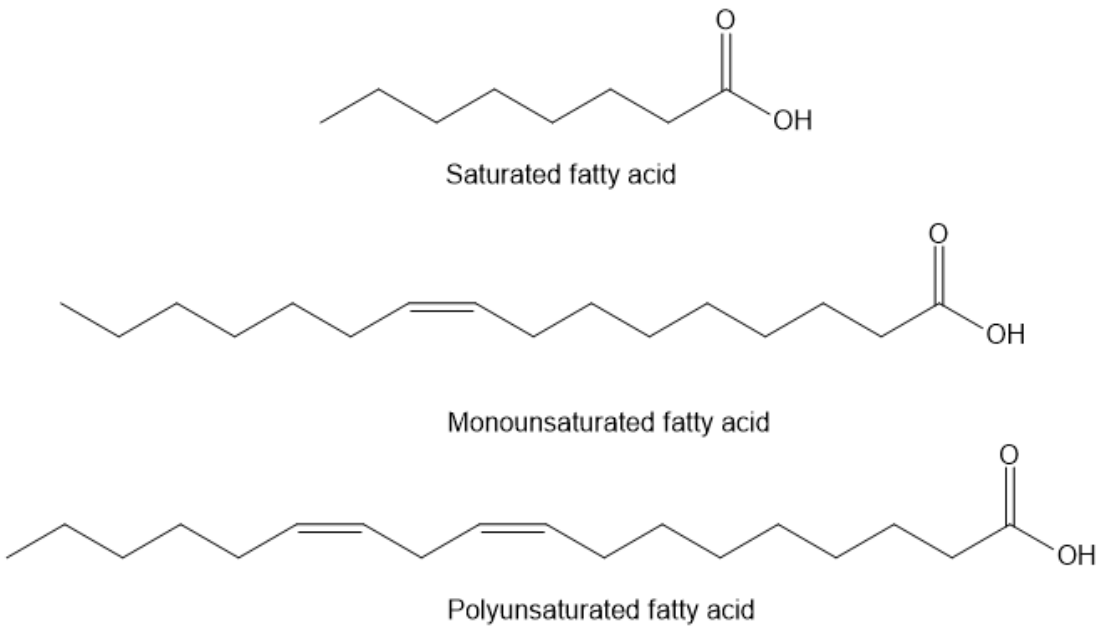

Figure 3.1: Structures of saturated and unsaturated fatty acids.

Fatty acids can be identified by shorthand notation, based on the length of the carbon chain and degree of unsaturation. For example, palmitic acid, has a saturated 16 carbon

chain and can be described as C16:0. Where C16 is the length of the carbon chain and 0 represents the degree of unsaturation. Similarly, palmitoleic acid has a 16 carbon chain with one double present and so is denoted as C16:1. Structures of these fatty acids can be seen in Figure 3.2.

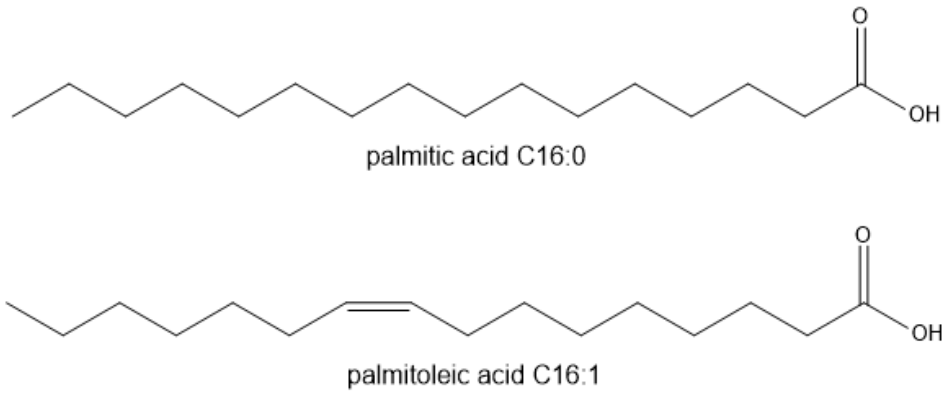

Figure 3.2: Structures of palmitic acid (C16:0) and palmitoleic acid (C16:1).

3.1.1. Fatty acids in bovine milk

Bovine milk is composed of a suspension of fat (4.2%), protein (3.4%), minerals (0.8%), vitamins (0.1%), and carbohydrates (4.6%) in water (87%). Of the 4.2% fat, 99% is present in triglyceride form, with the remaining lipids present as free fatty acids. A triglyceride consists of three fatty acids bound to a glycerol back bone, a typical example can be seen in Figure 3.3 below.

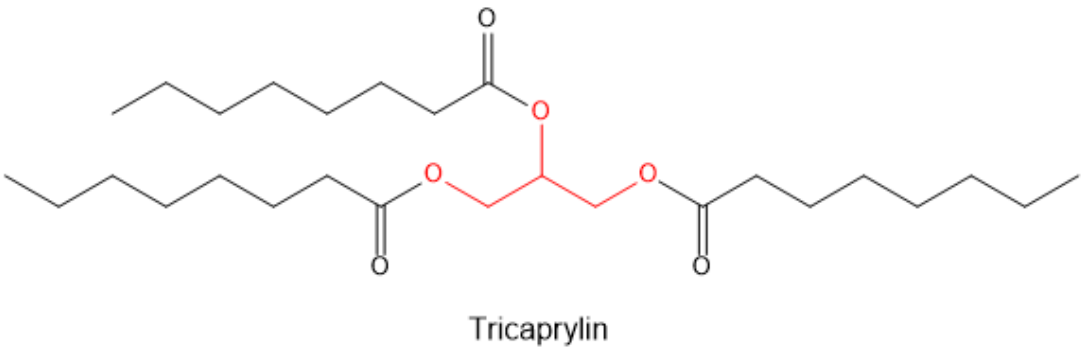

Figure 3.3: Structure of tricaprylin. Gylcerol backbone shown in red.

Milk fat is composed of approximately 400 different fatty acids. They are obtained from two main sources: *de novo* synthesis in the mammary gland, and uptake from feed. Fatty acids from C4:0 to C14:0 are synthesised in the mammary gland. The carbon chain length is extended in the mammary gland by the addition of acetate and β-hydroxybutyrate, which are produced from fermentation of feed components. *De novo* synthesis accounts for approximately half the C16:0 produced, the other half, and longer chain fatty acids are obtained from lipids in the diet and lipolysis of adipose tissue [102].

3.1.2. Current methods to analyse fatty acids

For analysis by gas chromatography (GC), non-volatile fatty acids require derivatisation. Typically, fatty acids are derivatised to fatty acid methyl esters (FAMEs); which are volatile and compatible with GC analysis. This derivatisation is typically carried out by either acid catalysed derivatisation or base catalysed derivatisation.

3.1.2.1. Acid catalysed derivatisation

Acid catalysed derivatisation is capable of derivatising both fatty acids as free fatty acids (esterification), and triglyceride molecules (transesterification). A reaction mechanism

can be seen below for free fatty acids in Figure 3.4, and for triglyceride molecules in Figure 3.5. The lone pair of the carbonyl oxygen acts as a nucleophile and attacks the electrophilic boron. The loss of an electron results in a formal positive charge on the carbonyl oxygen. This further polarises the carbonyl bond, making the carbonyl carbon more electrophilic. The increased electrophilic character of the carbonyl carbon allows nucleophilic attack by methanol to take place. Specifically, it is the lone pair on hydroxyl group that acts as the nucleophile. Several proton transfers take place which ultimately means water is expelled, due to its strong leaving group ability. The last step in the mechanism is the regeneration of the catalyst.

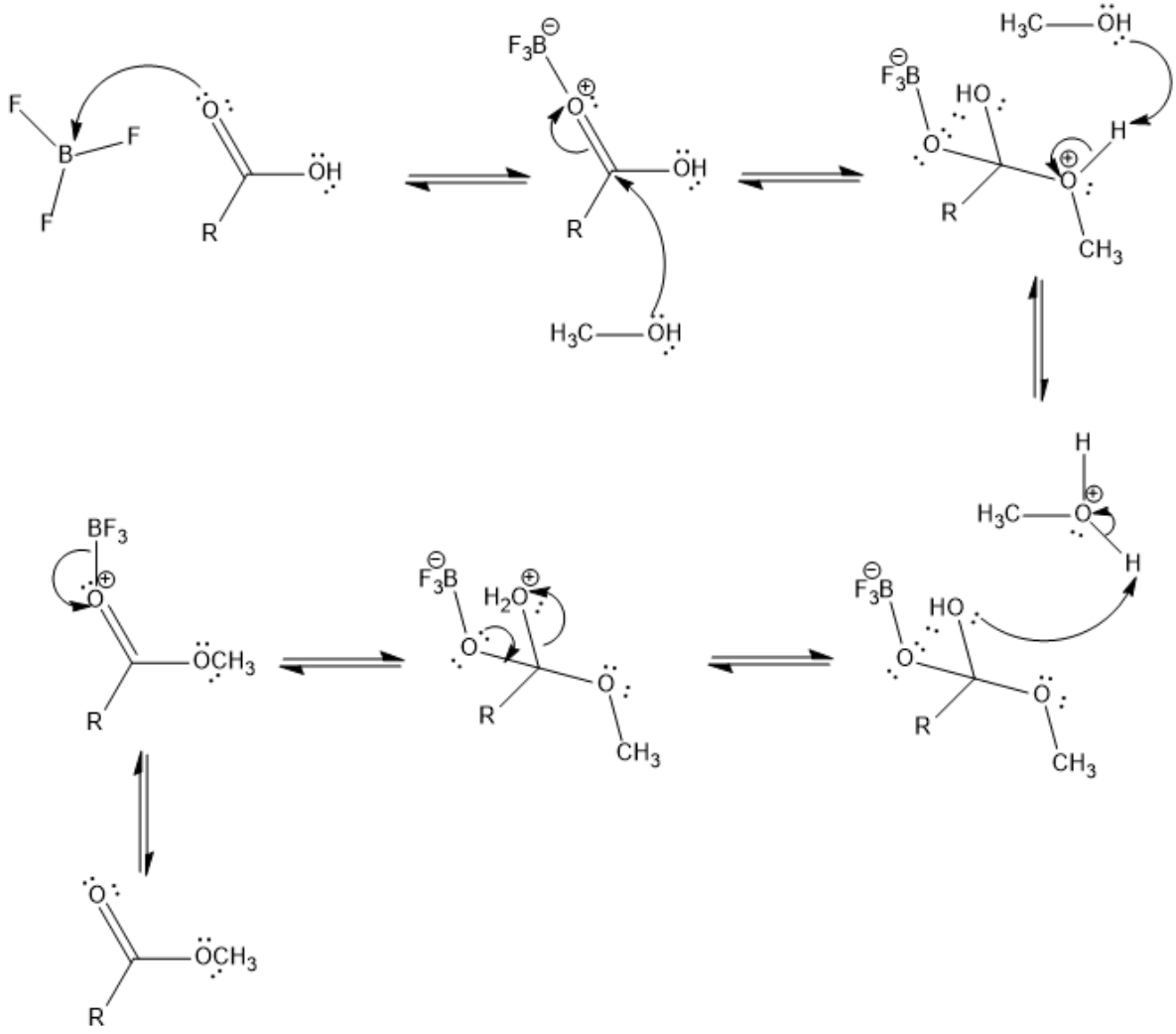

Figure 3.4: Reaction mechanism for the derivatisation of free fatty acids to methyl esters by lewis acid catalysis.

As in the reaction mechanism for the derivatisation for free fatty acids to methyl esters, BF_3 coordinates to the carboxyl oxygen. This increases the electrophilic character of the carboxyl carbon allowing for nucleophilic attack by methanol. A carbocation is formed and collapses when the fatty acid leaves the glycerol backbone of the triglyceride. A proton transfer results in the FAME being formed

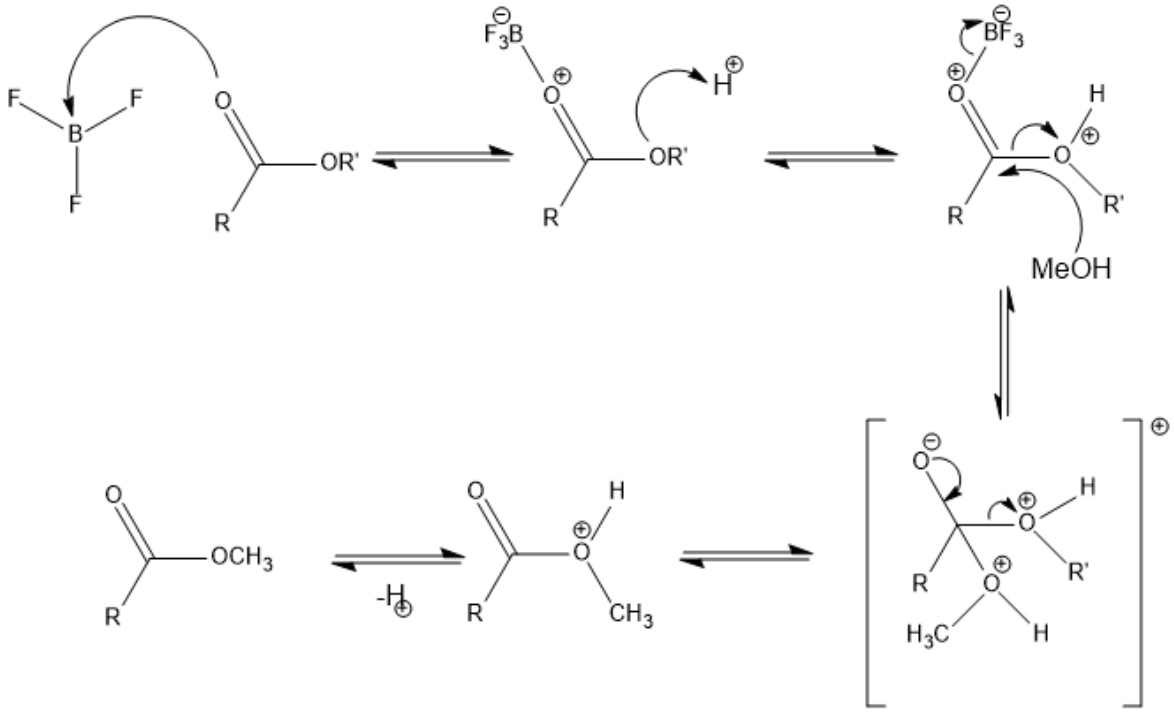

Figure 3.5: Reaction mechanism for the derivatisation of fatty acids in a triglyceride molecule to methyl esters by transesterification using lewis acid catalysis.

It is important to note that these reactions are equilibrium based and so steps must be taken in experimental design to ensure the equilibrium is pushed to form the desired methyl ester product. Such steps include carrying out the reaction in an excess of methanol and/or removing water as it is formed.

Several acid catalysts can be used in the production of FAMEs. These include BF_3-MeOH, BCl_3-MeOH, H_2SO_4-MeOH, and HCl-MeOH. While all the acid catalysts

mentioned above derivatise lipids according to the same reaction mechanism the reaction time and temperature needed for complete derivatisation varies between them.

3.1.2.2. Base derivatisation

In contrast to acid catalysed derivatisation, base derivatisation is unable to produce FAMEs from free fatty acids. The reaction mechanism for ester derivatisation can be seen below in Figure 3.6.

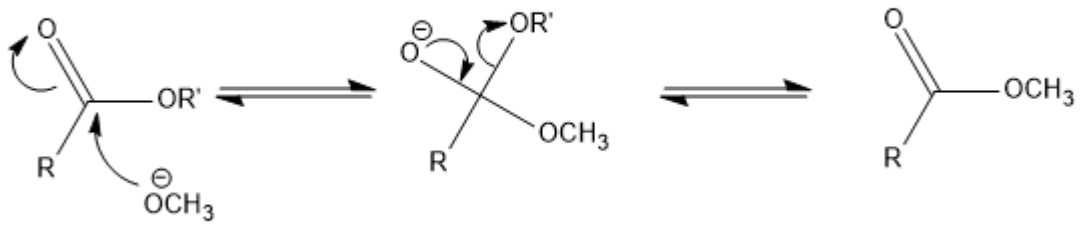

Figure 3.6: Base transesterification of esterified fatty acids.

The carbon atom has a partial positive charge due to the electron withdrawing effects of both oxygens. The methoxide group acts as a strong nucleophile and attacks the electrophilic carbonyl carbon. Reformation of the carbonyl carbon results in the expulsion of the alkoxide and formation of the methyl ester.

As with acid catalysed derivatisation, this mechanism is equilibrium driven. Reaction conditions must ensure that the equilibrium is pushed to the formation of the desired methyl ester. This is typically achieved by ensuring a significant excess of the methoxide nucleophile relative to the target ester.

Several bases can be used in the production of FAMEs. These are prepared by dissolving sodium hydroxide or potassium hydroxide in methanol, producing sodium or potassium methoxide, respectively.

3.1.2.3. Pyrolysis

Fatty acids can also be derivatised using pyrolysis. This is a reaction that takes place at elevated temperatures (>250 °C) between methanolic tetramethylammonium compounds and the carboxylic group of a fatty acid. The resulting salt decomposes at high temperature to form a FAME [103]. This process takes place at high temperatures in the GC inlet in the presence of a salt. Tetramethylammonium ammonium salts were first used to produce pyrolysed methyl esters (330 – 365 °C), but this method did not yield quantitative recovery without careful sample drying [104]. The replacement of tetramethylammonium salts with tetramethylsulfonium and trialkylselenonium salts allowed for pyrolysis to occur at lower temperatures (approx. 200 °C). The by-products produced in this derivatisation are volatile but typically do not interfere with GC analysis [105].

Pyrolysis has not been used extensively for the analysis of fatty acids from bovine milk. Recently, pyrolysis derivatisation of fatty acids has been compared to a direct on-column approach. The results indicate that the pyrolysis method is more robust although direct injection onto the column resulted in lower LOD values (0.7 mg/L compared to 5 mg/L). Direct injection onto the column is an injection technique in which all of the sample is transferred to the column. This is in contrast to splitless injection where the remaining sample is flushed from the inlet after a predetermined time.

3.1.3. Influence of body condition score on fatty acid profile

Body condition score (BCS) is a visual method for the assessment of the fatness of a dairy cow. It is a 5-point scale with 0.25 increments. A maximum score of 5 indicates that the

cow is obese, while a minimum score of 1 indicates that the cow is emaciated. The optimum body condition score for a cow at calving is between 3.0 and 3.5. Cows that have a body condition score outside of this range produce a lower quantity of milk, possibly because of negative energy balance. Negative energy balance in dairy cows occurs when the cow is expending more energy than it consumes from feed. This condition has been seen approximately one week pre-calving when dry matter intake reduces. As a result of this condition, cows will begin to deplete stores of body fat in an attempt to reduce the calorie deficit [106].

Ensuring the correct conditioning of cows is vital not just for milk production, but for the overall health of the cow. A cow that is excessively fat will be immunocompromised and thus more likely to suffer from infections and may encounter problems during calving. A cow that has a poor body condition score, outside of the optimum 3.0 – 3.5, range may not cycle in time for the next calving [107].

A reduction in milk fat in is experienced in the beginning of the lactation cycle due to negative energy balance. This occurs because energy demand for lactation exceeds the energy obtained from the diet. To counter this deficit, mobilisation of fat reserves occur which can result in a lower body condition score if the energy requirements of the cows are not controlled. This effect can be more pronounced on cows that have a body condition score greater than 3.5 at prepartum. [107].

The optimum body condition score of a cow needs to be accurately assessed to ensure a negative energy balance does not impact on milk yield and cow health.

3.1.4. Influence of lactation cycle on dairy cows

The lactation cycle is defined as the period between one calving and the next. The lactation cycle can be divided into four phases: early, mid, late lactation, and dry period. The dry period is used to recondition the cows to ensure that they have adequate reserves for the next lactation cycle. A diagram of a typical lactation cycle can be seen below in Figure 3.7.

Milk fat will be at highest in early lactation due to the nutrient rich colostrum produced. The levels of fatty acids in milk decline for the following two months and before slowly increasing as the lactation cycle progresses.

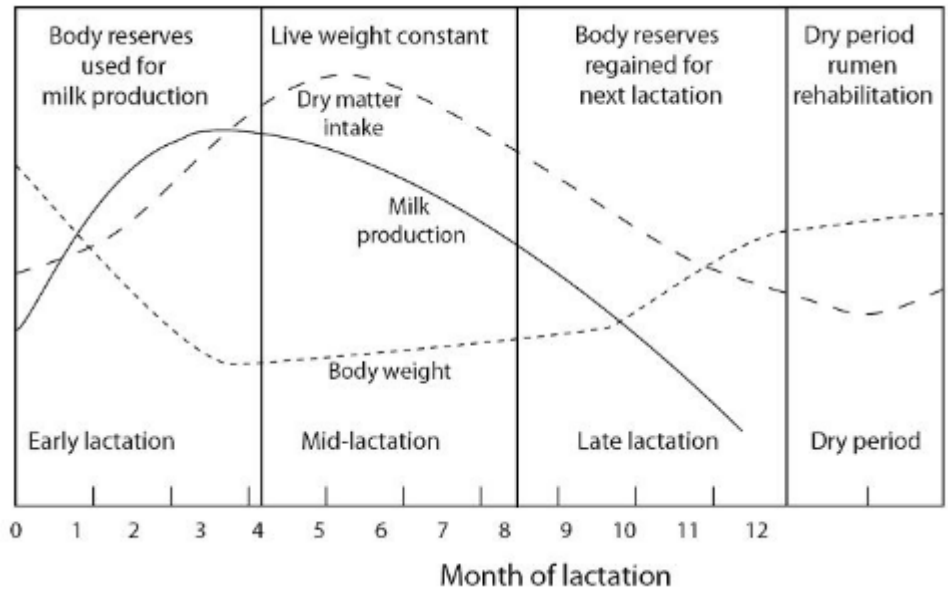

Figure 3.7: Stages of lactation cycle in dairy cows.

3.2. Overarching aims of this chapter

The aim of this chapter is to develop a temperature programmed GC separation and DLLME procedure for the quantification of fatty acids found in bovine milk. The optimised method will be used to analyse milk from cows that have varying body condition scores and are at different stages in their lactation cycle.

3.3. Materials and methods

3.3.1. Reagents and materials

BF_3-MeOH (14% w/v) purchased from Sigma-Aldrich. Phosphoric acid, sodium chloride, ultra-pure water, chloroform, methanol, fatty acid standard mix, nonanoic acid

methyl ester, and glyceryl triheptadecanoate were purchased from Sigma Aldrich (Ireland). Ultrapure water was provided by a Whitewater purification system

Chromatography was preformed using an Agilent 6890 GC system equipped with an Alltech AT-1000 capillary column (polyethylene glycol (PEG), 15 m x 0.52 mm; 1.2 µm). Helium was used as the carrier gas at a flow rate of 4 mL/min, inlet temperature: 300 °C, detector temperature: 280 °C, oven temperature: initial: 40 °C, final: 220 °C split: 10:1.

3.3.2. Standard preparation

Standard solutions of each fatty acid were prepared as follows: octanoic acid: 21.83 mg/mL, capric acid: 20.54 mg/mL, palmitic acid: 20.05 mg/mL, stearic acid: 20.03 mg/mL, oleic acid 21.83 mg/mL, C9: 3.03 mg/mL, and C17: 3.17 mg/mL. All standards were dissolved in hexane. Supelco FAME standard mix, originally dissolved in dichloromethane (DCM) was diluted 1:10 with hexane. The individual FAMEs were chosen as they cover short chain, long chain, and unsaturated fatty acids, which show the applicability of the DLLME method to a broad range of FAMEs.

3.4. Results

3.4.1. Development of FAME separation

3.4.1.1. Selecting a stationary phase

Although there were many published methods available on the separation of FAMEs, a method was developed using the column available in the lab to ensure maximum resolution was achieved for all FAMEs. An initial screening of potential stationary phases to carry out the separation of 37 FAMEs was performed. A polar, PEG (polyethylene glycol) column and a medium polarity 6% cyanopropyl column were selected due to their difference in polarity. The dimensions of each column can be seen in Table 3.1. Separations on both columns were carried out at 5 °C/min ramp rate. A scouting gradient

of 5 °C/min for both columns was used as it provided a shallow gradient. A steeper gradient would reduce resolution between peaks, while a shallower gradient would result in excessively long run times. The separation was evaluated based on the number of peaks fully resolved and the resolution between critical peak pairs. A comparison of both separations can be seen in Figure 3.8. Two different final temperatures for each separation were used as columns had different maximum operating temperatures. The PEG column had a maximum operating temperature of 220 °C while the cyanopropyl column had a maximum operating temperature of 260 °C.

Table 3.1: Comparison of GC columns.

Stationary phase	**Length (m)**	**Internal diameter (mm)**	**Film thickness (µm)**	**Maximum temperature (°C)**	**Polarity**
PEG	15	0.53	1.2	220	High
6% cyanopropyl	30	0.32	1.8	260	Mid

The separation was more efficient using the PEG stationary phase (even though it was the shorter column) and so comparisons with stationary phases less polar than 6% cyanopropyl were not explored. The increase in the number of peaks in the early part of the chromatogram (0-5 min) can be attributed to the mixture of solvents in the FAME mixture. As stated previously, the FAME mix was originally dissolved in DCM before being diluted with hexane (1:10). As the cyanopropyl column is less polar than the PEG column, it can partially separate these solvents. In general, longer columns (e.g 100 m) would be used for the above separation, however, these were the only columns available for use.

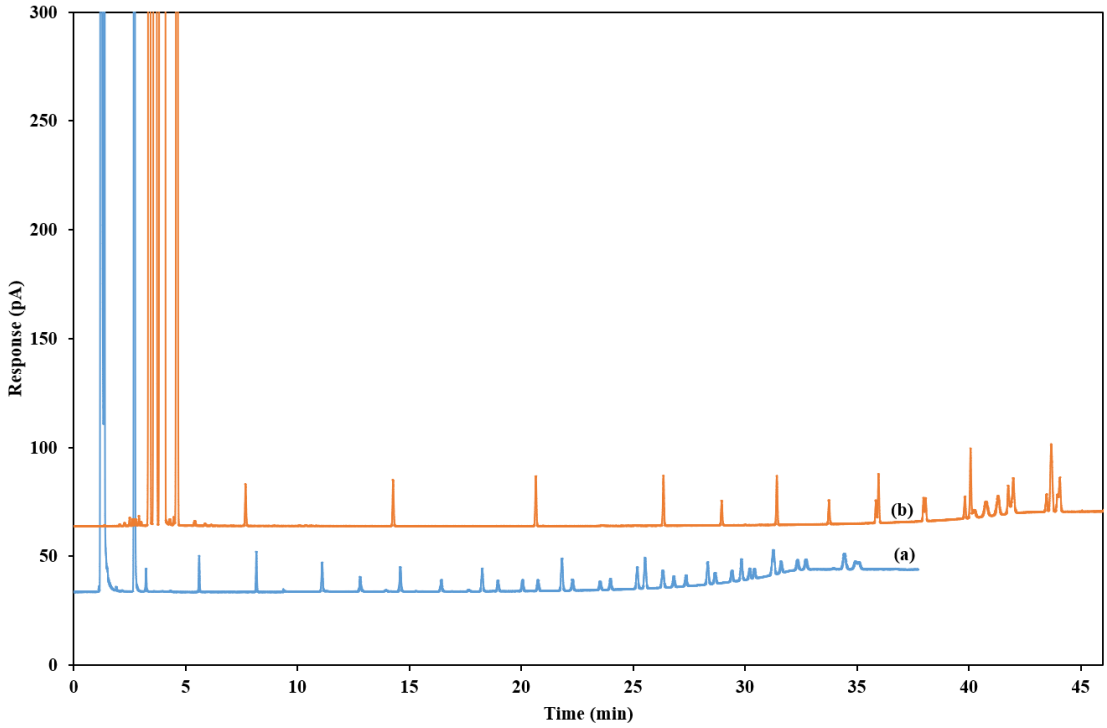

Figure 3.8: Comparison of stationary phases separating FAME mix. Chromatogram (a) PEG column, chromatogram (b): 6% cyanopropyl column. Chromatographic conditions: carrier gas: helium, flow rate: 4 mL/min, ramp rate: 5 °C/min, initial: 40 °C, final: 220 °C, 240 °C for (b), inlet temperature: 300 °C, detector temperature: 280 °C.

Although the Supelco 37 FAME mix standard was used, it was not possible to formally identify the peaks. In general, members of a homologous family of compounds (like fatty acids) will elute based on increasing boiling point as they all possess the same functional interactions with the stationary phase. However the Supelco mix also contains unsaturated FAMEs. The presence of double bonds can alter the elution order and so it is not possible to identify peaks by comparing relative retention times with the test chromatogram. A list of the compounds found in the 37 FAME mix can be seen in Figure 3.9.

Description
cis-13,16-Docosadienoic acid methyl ester 2 wt. %
cis-4,7,10,13,16,19-Docosahexaenoic acid methyl ester 2 wt. %
cis-11,14-Eicosadienoic acid methyl ester 2 wt. %
cis-5,8,11,14,17-Eicosapentaenoic acid methyl ester 2 wt. %
cis-8,11,14-Eicosatrienoic acid methyl ester 2 wt. %
cis-11,14,17-Eicosatrienoic acid methyl ester 2 wt. %
cis-11-Eicosenoic acid methyl ester 2 wt. %
Methyl *cis*-10-heptadecenoate 2 wt. %
Methyl hexanoate 4 wt. %
Methyl γ-linolenate 2 wt. %
Methyl arachidate 4 wt. %
Methyl arachidonate 2 wt. %
Methyl behenate 4 wt. %
Methyl butyrate 4 wt. %
Methyl decanoate 4 wt. %
Methyl dodecanoate 4 wt. %
Methyl elaidate 2 wt. %
Methyl erucate 2 wt. %
Methyl heneicosanoate 2 wt. %
Methyl heptadecanoate 2 wt. %
Methyl linoleate 2 wt. %
Methyl linolelaidate 2 wt. %
Methyl linolenate 2 wt. %
Methyl myristate 4 wt. %
Methyl myristoleate 2 wt. %
Methyl oleate 4 wt. %
Methyl octanoate 4 wt. %
Methyl palmitate 6 wt. %
Methyl palmitoleate 2 wt. %
Methyl pentadecanoate 2 wt. %
Methyl *cis*-10-pentadecenoate 2 wt. %
Methyl stearate 4 wt. %
Methyl tricosanoate 2 wt. %
Methyl tetracosanoate 4 wt. %
Methyl tridecanoate 2 wt. %
Methyl undecanoate 2 wt. %
Methyl *cis*-15-tetracosenoate 2 wt. %

Figure 3.9: FAMEs present in 37 mix.

3.4.1.2. Separation optimisation

As can be seen from Figure 3.10, the separation on the PEG stationary phase still has peaks exhibiting poor resolution and peaks that are co-eluting. The early eluting peaks, peaks eluting before 30 min, are all well resolved with a resolution greater than 2 in all cases. This facilitated an increase in the ramp rate in order to reduce the overall run time for this segment of the chromatography. Resolution was assessed using chromatography software (Chemstation).

The elution temperature at 30 min was 150 °C (30 min x 5 °C/min). The ramp rate from 40 °C to 150 °C was increased from 5 °C/min to 10 °C/min, followed by 5 °C/min until the final temperature of 220 °C. This change in the method has decreased the run time while still providing adequate resolution between early eluting peaks. In addition, the improved temperature programming has also resulted in an increase in the number of peaks resolved. This can be clearly seen from the below chromatograms.

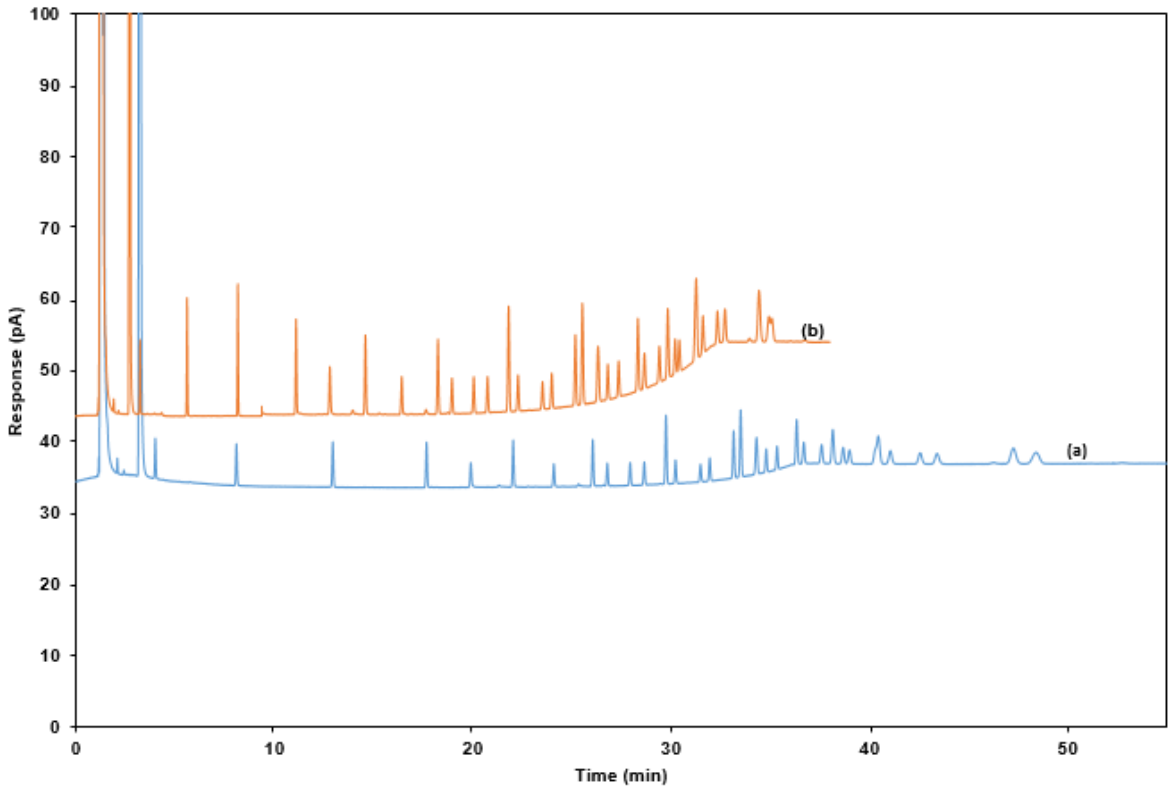

Figure 3.10: The effect of ramp rate on early eluting peaks. Chromatogram (a): 5 °C/ min ramp rate, chromatogram (b): 10 °C/min ramp rate to 30 min continuing at 5 °C/min until 220 °C. Chromatographic conditions: stationary phase: AT-1000 (PEG 15 m x 0.53 mm; 1.2 µm), carrier gas: helium, flow rate: 4 mL/min, initial: 40 °C, final: 220 °C, inlet temperature: 300 °C, detector temperature: 280 °C.

The elution of the first critical peak pair occured at 27 minutes, Giddings approximation was employed to try to separate these peaks. Giddings approximation is a GC tool used tentatively identify an isothermal temperature hold to separate two or more closely eluting peaks in a temperature programme. Its calculation is shown below:

$$T' = T_f - 45\ °C$$

Where *T'* is the isothermal hold temperature and T_f is the analyte elution temperature. Using this formula, an isothermal hold for 1 minute should be inserted at 114 °C to increase resolution between the critical peak pair eluting at 27 minutes. The optimised chromatogram can be seen below in Figure 3.11. This method was used in all subsequent experiments.

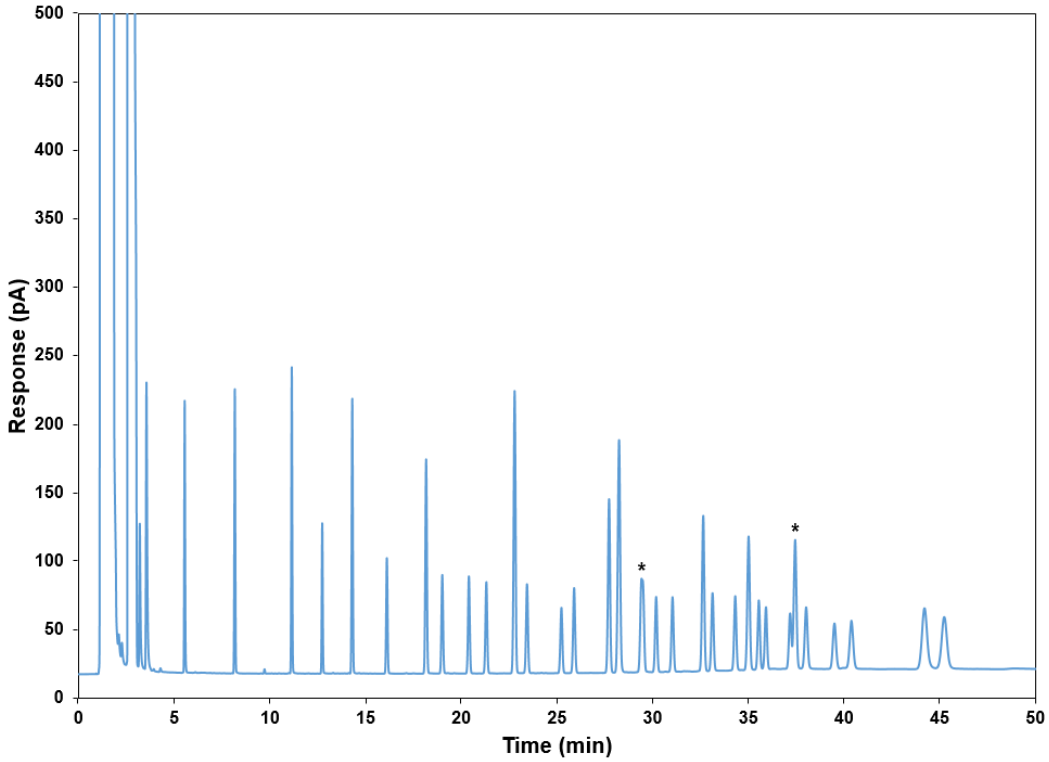

Figure 3.11: Optimised separation of FAME mix. Peak identification: asterisk indicates critical peak pairs whose resolution could not be improved. Chromatographic conditions: stationary phase: AT-1000 (PEG 15 m x 0.53 mm; 1.2 µm), carrier gas: helium, flow rate: 4 mL/min, initial: 40 °C, final: 220 °C, inlet temperature: 300 °C, detector temperature: 280 °C.

3.4.1.3. Individual FAME retention time markers

As mentioned above, the presence of unsaturated FAMEs can alter the predicted elution order of FAMEs. Individual standards of octanoic, capric, palmitic, stearic, and oleic were derivatised to form FAMEs using the following Supelco method [108]: Fatty acid standards (1 mL) and BCl_3 – MeOH (2 mL) were placed in micro-reaction vial which was then heated at 60 °C for 15 min. After being cooled to room temperature, hexane (1 mL) and water (1 mL) were added and the vials were shaken for 1 minute. The upper hexane layer was taken for analysis by GC.

The structures of each fatty acid and FAME can be seen below in Figure 3.12. The selected fatty acids have been shown to have some beneficial effects for human health.

Medium chain fatty acids (caprylic and capric) have been shown to increase the amount of good cholesterol. While long chain fatty acids (oleic and stearic) can reduce blood pressure and help burn excess fat. Not all long chain fatty acids are beneficial to human health. Palmitic acid intake has been linked to weight gain and obesity.

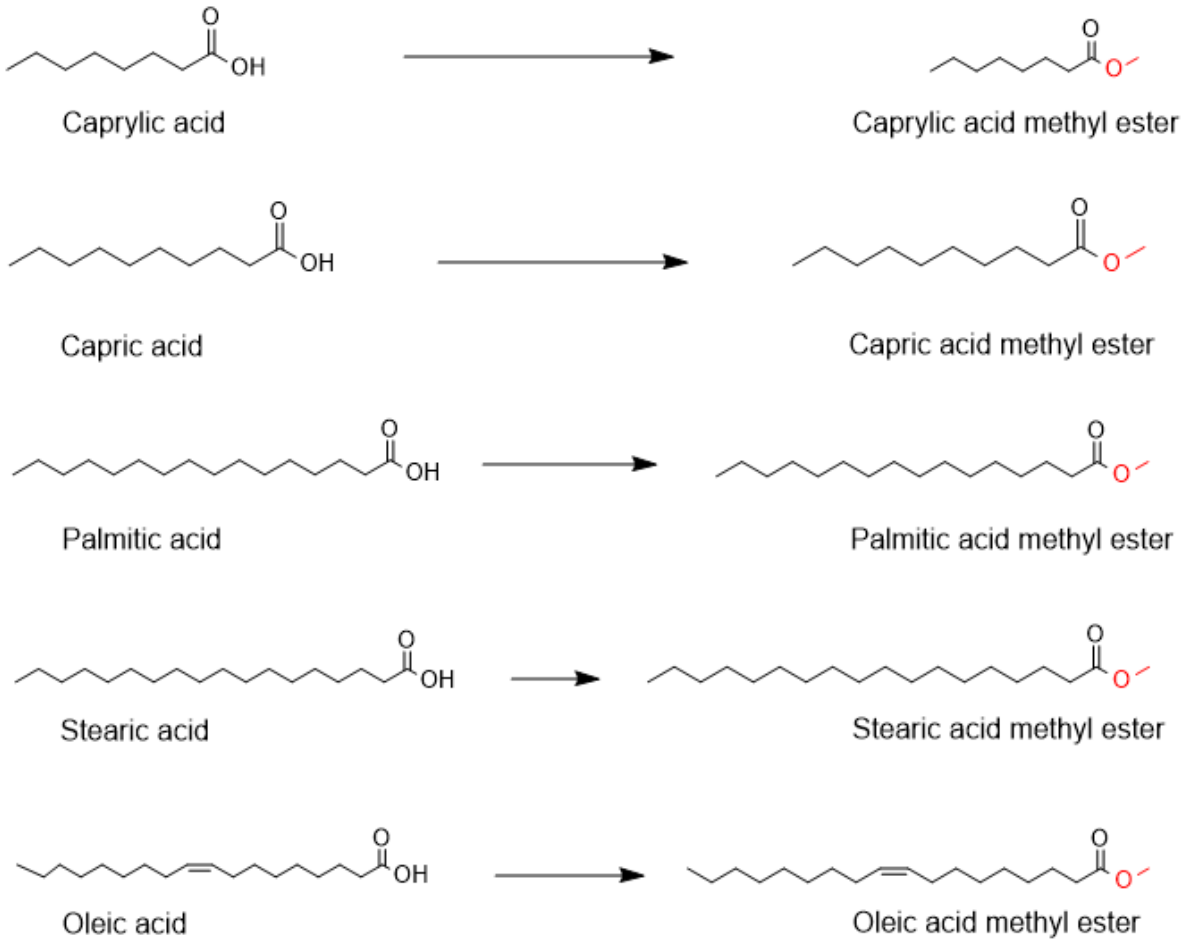

Figure 3.12: Structures of fatty acids and corresponding FAMEs.

The derivatised fatty acids were then compared to the 37 FAME standard mix. This comparison is shown below in Figure 3.13. The retention time markers shown in Figure 3.12 correspond with analytes in the Supelco FAME mix (injected individually and as a mixture). The developed gradient separation provided baseline resolution between the target analytes and other FAMEs that may be present in milk samples. Linear Retention Indices were not used in this optimisation

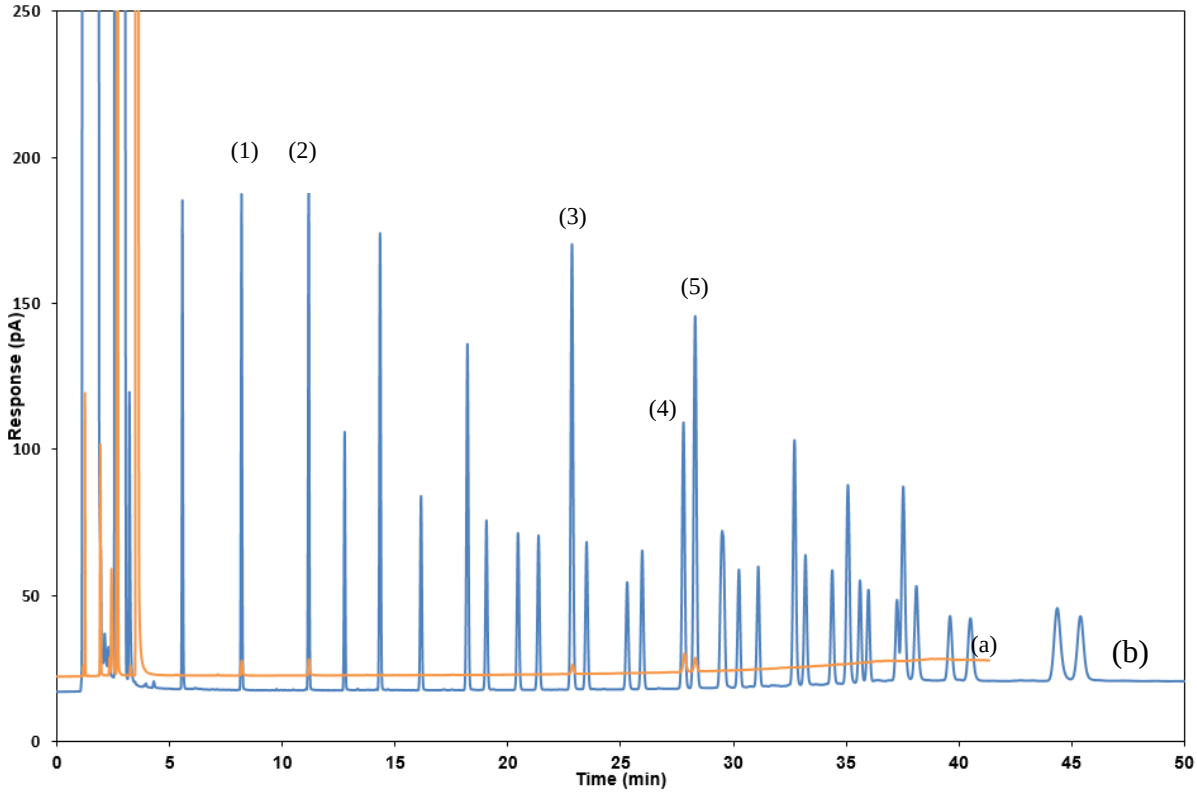

Figure 3.13: Comparison of FAME mix and selected fatty acid standards. Chromatogram (a) selected fatty acid standards, chromatogram (b): FAME mix. Peak identification: (1) octanoic, (2) capric, (3) palmitic, (4) stearic, (5) oleic. Chromatographic conditions: stationary phase: AT-1000 (PEG 15 m x 0.53 mm; 1.2 µm), carrier gas: helium, flow rate: 4 mL/min, initial: 40 °C, final: 220 °C, inlet temperature: 300 °C, detector temperature: 280 °C.

3.4.2. Development of derivatisation and liquid-liquid extraction procedure

3.4.2.1. Comparison of acid and base catalysed transesterification

Fatty acids can be derivatised into FAMEs by both acid and base catalysis, a general reaction mechanism for both reactions have been discussed previously and can be seen in the introduction (Section 3.1.2). Although both catalysts produced FAMEs as the end product, there are differences in each pathway. As can be seen from the reaction mechanism, base catalysed derivatisation will only produce FAMEs from tri or diglycerides. Any free fatty acids (FFA) that were present in the sample will not be converted to FAME form. This can be seen in the reaction pathways detailed in Section

3.1.2.2. Acid catalysed derivatisation will convert both tri and di glycerides as well as FFA to their respective FAME forms.

Acid and base catalysis was compared by derivatising tripalmitate and dipalmitin, these are triglycerides and diglycerides, respectively. Their structures can be seen in Figure 3.12 below. The constituent fatty acid that makes up both tripalmitate and dipalmitin is palmitic acid (C15:0).

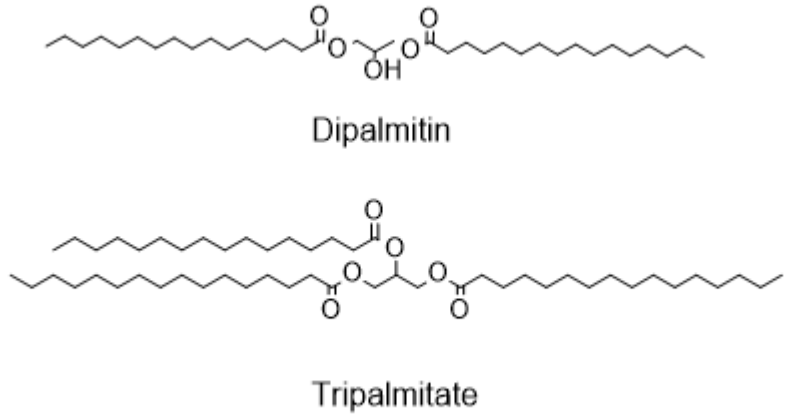

Figure 3.14: Structures of dipalmitin and tripalmitate.

When both dipalmitin and tripalmitate are derivatised they produce palmitic acid methyl esters. When complete derivatisation of both compounds is achieved, the ratio of palmitic acid methyl ester from both reactions should be 3:2, as tripalmitate contains three molecules of palmitic acid whereas dipalmitin contains only two. Dipalmitin and tripalmitate were derivatised according to the methods outlined below. A previously prepared stearic acid methyl ester (Section 3.1.3) was used as an internal standard (IS). Derivatisation temperatures and times were taken from literature.

Acid catalysed transesterification: dipalmitin (0.2 mg/mL), tripalmitate (0.2 mg/mL), and stearic acid methyl ester (0.045 mg/mL), diluted to 1 mL with hexane, were added to a micro-reaction vial. BCl_3-MeOH (2 mL) was added to each micro-reaction vial and heated for 15 min at 50 °C. The vials were cooled on ice for 5 min. Water (5 mL) and

hexane (1 mL) were added and the vials were shaken by hand for 2 min. After phase separation, the supernatant was analysed by GC.

Base catalysed transesterification: dipalmitin (0.2 mg/mL), tripalmitate (0.2 mg/mL), and stearic acid methyl ester (0.045 mg/mL), diluted to 1 mL with hexane, were added to a micro-reaction vial. Sodium methoxide (NaOMe) (2 mL; 1 M) was added to each vial. The vials were heated for 15 min at 50 °C and then placed on ice for 5 min. Water (5 mL) and hexane (1 mL) were added and the vials were shaken by hand for 2 min. After phase separation, the supernatant was analysed by GC.

Analysis of the acid catalysed derivatisations showed no peak at the expected retention time of palmitic acid methyl ester. It appears the derivatisation temperature of 50 °C was insufficient for the derivatisation to occur.

This was not the case for the base catalysed derivatisation of dipalmitin and tripalmitate. When the peak ratio of IS and palmitic acid methyl ester was compared from dipalmitin and tripalmitate, it was found that the ratio increased by a factor of 0.33 in the tripalmitate chromatogram. The peak areas and ratios of the derivatisation of dipalmitin and tripalmitate are shown below in Table 3.2.

Table 3.2: Base derivatisation of dipalmitin and tripalmitate.

	Stearic (n=3) peak area	**Palmitic (n=3) peak area**	**Palmitic/Stearic**	**Increase (%)**
Dipalmitin	392.50	235.65	0.6	-
Tripalmitate	441.63	380.53	0.8	33

As the above base catalysed reaction resulted in complete derivatisation of both dipalmitin and tripalmitate, this procedure was carried forward to the analysis of real milk samples.

3.4.3. Comparison of extraction methods

Traditionally, lipids have been extracted before derivatisation using Folch reagent (chloroform: methanol (2:1)) [109] or hexane:MTBE (1:1) [110]. There have been reports of lipid derivatisation *in-situ* without the need for prior extraction or protein precipitation [111]. Extraction of lipids prior to derivatisation has been considered to be advantageous as it has the potential to remove matrix interferences while also potentially providing some preconcentration of analytes. Although extraction of lipids prior to derivatisation can provide some benefits, the addition of an extra step in the protocol can introduce errors in the procedure. *In-situ* derivatisation has attempted to overcome that uncertainty by derivatising lipids in the sample solution, without extracting them first. The following sections compare published methods for that have analysed lipids from complex samples that have used liquid-liquid extraction or *in-situ* derivatisation. Published methods were used as they have been previously optimised for the extraction of FAMEs. This approach has saved considerable time in the method development process as the alternative would be to optimise several methods and then compare the extraction efficiency.

3.4.3.1. Liquid-liquid extraction

Hexane:MTBE (1:1) has been shown to be as efficient as Folch reagent [110], A liquid-liquid extraction using hexane:MTBE (1:1) and subsequent derivatisation was compared to *in-situ* derivatisation using the methods outlined below:

Liquid-liquid extraction: milk samples were homogenised for 5 minutes at 38 °C, to homogenize milk fat throughout the sample. Milk (1 mL), sodium chloride (50 mg), H_2SO_4 (80 µL; 50% *v/v*), and hexane:MTBE (1:1) (2 mL) were added to a 15 mL

centrifuge tube. Sodium chloride was added to decrease analyte solubility in the aqueous phase by changing the ionic strength. H_2SO_4 precipitated the proteins found in milk. The tubes were shaken by hand for 2 minutes before centrifugation for 5 minutes at 4,500 rpm. Supernatant (1 mL) transferred to micro reaction vial. Sodium methoxide in MeOH (2 mL; 1 M) was added and micro reaction vials were heated for 15 min at 80 °C. After cooling on ice, hexane (1 mL) and water (1 mL) were added. Micro reaction vials were agitated for 2 minutes, and after phase separation, the supernatant was transferred to a GC vial for analyses.

3.4.3.2. In-situ derivatisation and extraction

In-situ derivatisation: milk samples were homogenised for 5 minutes at 38 °C. Milk (100 µL), and sodium methoxide in MeOH (2.5 mL) were placed in a micro reaction vial and heated for 10 minutes at 80 °C. After cooling on ice, BCl_3 – MeOH (2.5 mL) was added and samples were heated for 3 minutes at 80 °C. Samples were cooled on ice before the addition of the hexane (1 mL) and water (1 mL). Micro reaction vials were shaken by hand for 2 minutes and then centrifuged at 4,500 rpm for 5 minutes. The supernatant was transferred to a GC vial for analyses.

An over lay of the resulting chromatograms from each extraction and derivatisation method can be seen below (Figure 3.15). There are several FAMEs which have been extracted into hexane:MTBE which were not extracted by the *in-situ* derivatisation method. As *in-situ* derivatisation has been shown to have a lower extraction efficiency than liquid-liquid extraction, lipids will be extracted in a simultaneous protein precipitation – lipid extraction step, and not an *in-situ* derivatisation method.

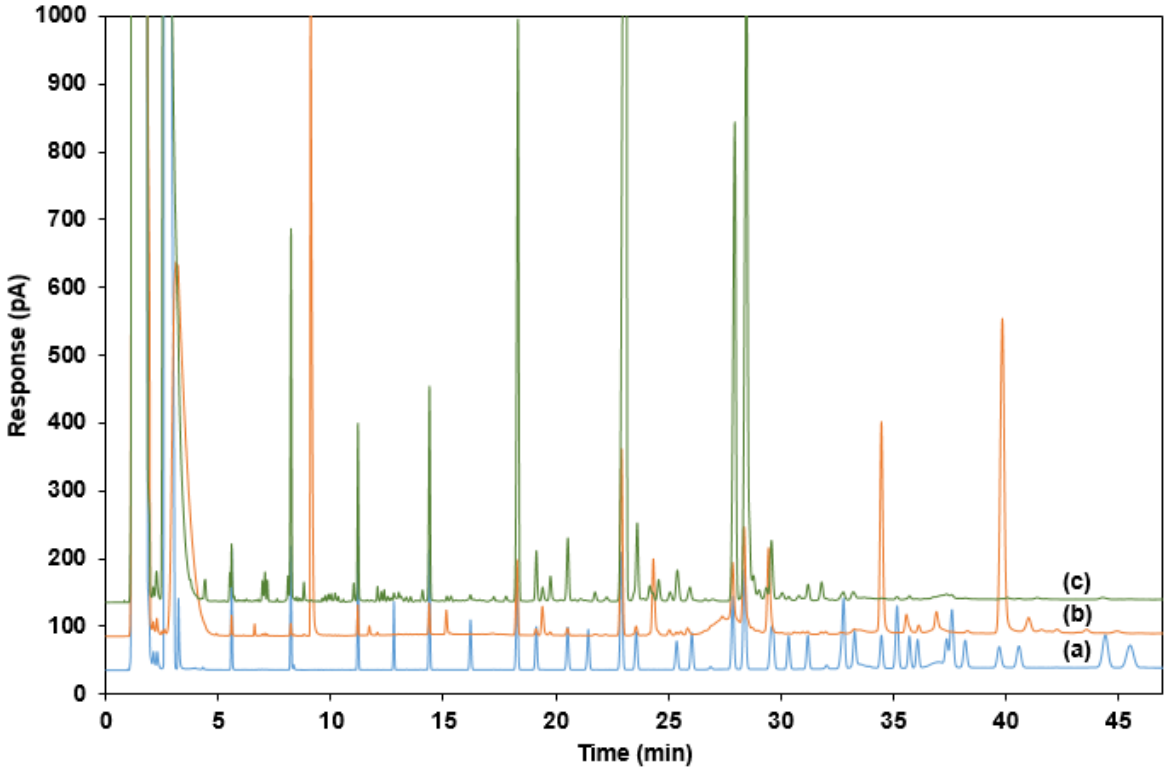

Figure 3.15: Comparison of liquid-liquid extraction and *in-situ* derivatisation. Chromatogram (a): FAME mix, chromatogram (b): liquid-liquid extraction, chromatogram (c): *in-situ* derivatisation. Chromatographic conditions: Chromatographic conditions: column: Alltech AT-1000 (PEG, 15 m x 0.53 mm; 1.8 µm), carrier gas: helium, temperature programming: initial temperature: 40 °C, final temperature: 220 °C, injection volume: 1 µL, inlet temperature: 300 °C, detector temperature: 280 °C.

3.4.4. Development and optimisation of derivatisation protocol

A preliminary DLLME protocol was developed based on the hexane:MTBE (1:1) liquid-liquid extraction method mentioned above. The hexane from the simultaneous protein precipitation and extraction step was used as the extraction solvent in the final DLLME step. Methanol (used as the derivatisation reagent) was used as the dispersive solvent. The proposed protocol for the DLLME procedure is shown below.

DLLME: milk (1 mL), NaCl (50 mg), H_2SO_4 (80 µL; 50% v/v), and hexane:MTBE (1:1) (500 µL) added to a centrifuge tube. Samples were centrifuged for 5 minutes at 4,500 rpm and 250 µL of the supernatant was transferred to a micro reaction vial with sodium

methoxide (1 mL). The micro reaction vials were heated in a water bath for 15 min at 80 °C. Samples were cooled on ice and 1 mL of the reaction mixture was injected rapidly into 5 mL of water. A cloudy solution was formed and then centrifuged for 5 min at 4,500 rpm. The supernatant was then taken for analyses by GC.

Although centrifugation achieved the separation of the extraction solvent from the aqueous phase, recovery of the extraction solvent was not possible. At the organic – aqueous interface, there was a thin emulsion which prevented recovery of the extraction solvent without contamination. The addition of salt did not sufficiently change the ionic strength to break the emulsion. It was suspected that this emulsion was the result of underivatised free fatty acids. Only fatty acids that are part of a triglyceride or diglyceride structure will be derivatised by sodium methoxide to their respective FAMEs. It was assumed that any free fatty acids still present in the reaction mixture would be ionised through deprotonation of the carboxyl group as previously described. It is postulated that at sufficient levels these ionised fatty acids would have the potential to be partially soluble in both the aqueous and organic phases, thus preventing recovery of the extraction solvent without contamination.

Due to the above issues an alternative derivatisation method using acid catalysis was explored. Both free fatty acids and fatty acids that are part of a triglyceride or diglyceride structure will be methylated using acid catalysis. The method developed and utilised is outlined below:

Acid catalysed derivatisation with DLLME: milk (1 mL), conc. phosphoric acid (100 µL), NaCl solution (500 µL; 2 M), and hexane: MTBE (1:1) (500 µL) were placed in a centrifuge tube. Samples were agitated for 2 minutes before centrifugation at 4,500 rpm for 5 minutes. Supernatant (250 µL) was transferred to a micro reaction vial along with BF_3 - MeOH (1 mL). The micro reaction vials were heated for 15 minutes at 80 °C. After being cooled on ice, 1 mL of the reaction mixture was rapidly injected into 5 mL of water, where it was allowed to equilibrate for 1 minute. The resulting cloudy solution was centrifuged for 5 minutes at 4,500rpm. The supernatant was recovered and transferred to a GC vial for analyses. The resulting chromatogram can be seen below.

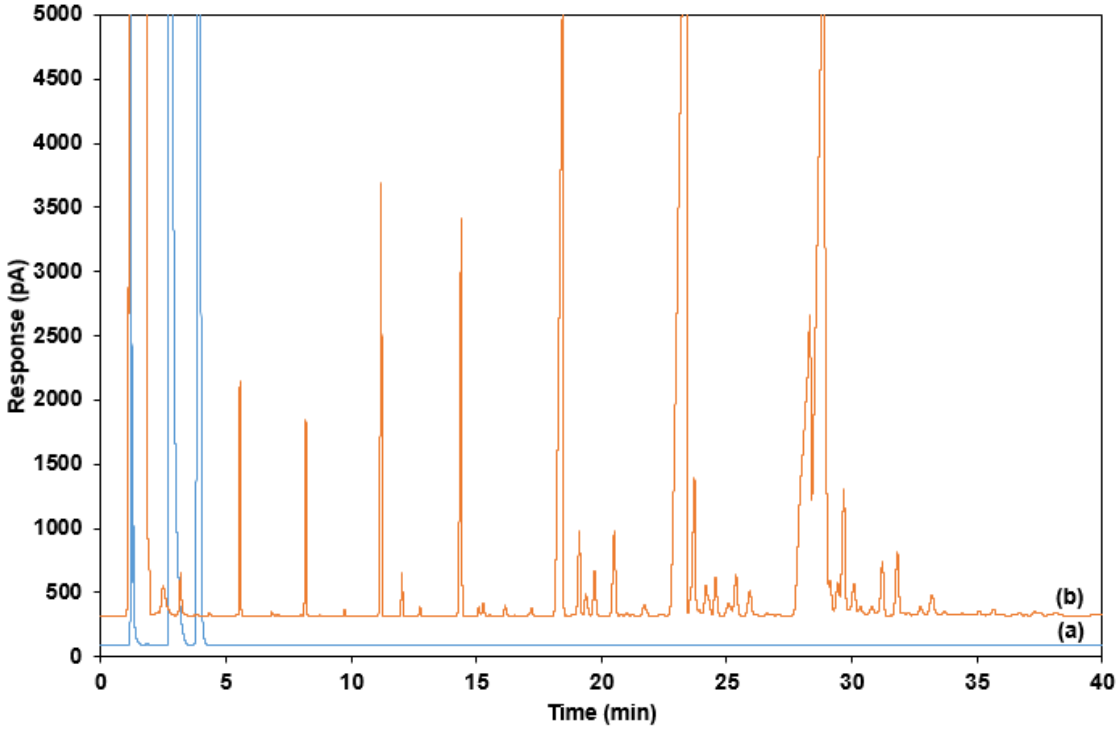

Figure 3.16: Preliminary DLLME using BF_3 as a derivatisation catalyst on a milk sample. Chromatogram (a): extraction blank, chromatogram (b): DLLME. Chromatographic conditions: column: Alltech AT-1000 (PEG, 15 m x 0.53 mm; 1.2 µm), carrier gas: helium, temperature programming: initial temperature: 40 °C, final temperature: 220 °C, injection volume: 1 µL, inlet temperature: 300 °C, detector temperature: 280 °C.

Acid catalysed methylation allowed for easy recovery of the reaction solvent. This supports the prior suggestion that phase separation is hindered by the presence of free fatty acids when base catalysed derivatisation was used.

3.4.4.1. Split ratio optimisation

It can be seen from the above chromatogram, DLLME has pre-concentrated the FAMEs to such an extent that several FAME peaks are fronting; a common symptom of column overloading. Peak fronting occurs when the active sites of stationary phase become saturated with a particular analyte, this inhibits stationary phase interaction with other analyte molecules. The analyte is free to travel down the column until it encounters

stationary phase that is not saturated, which produces a smearing effect down the column, this manifests itself as peak fronting in the chromatography produced.

To reduce the amount of sample on the column, samples were injected in split mode. Due to limits on the capability of the GC instrument, the maximum split ratio that could be investigated was 15:1. Changing the injection mode to split eliminated peak fronting, allowing for more accurate quantification. The chromatography from both injections can be seen in Figure 3.17. A split of 10:1 was used to avoid using conditions at the maximum operating capacity of the instrument.

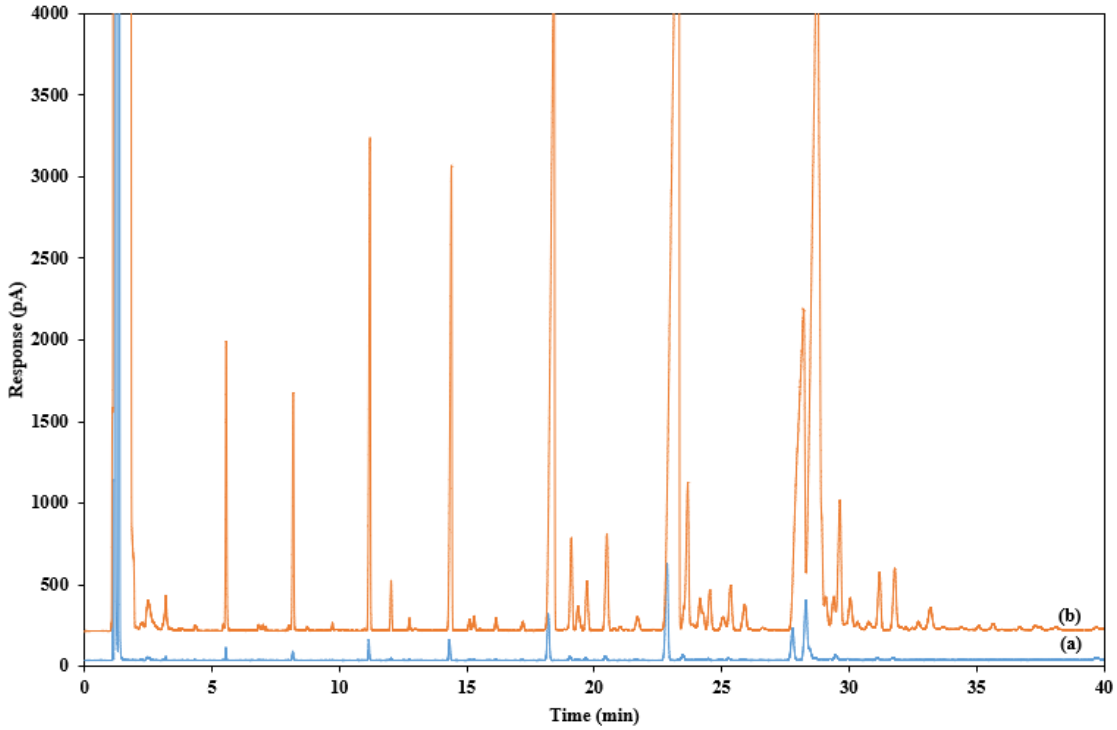

Figure 3.17: Comparison of split and splitless injections. Chromatogram identification: (a) 10:1 split injection (b) splitless injection. Chromatographic conditions as detailed in Figure 3.11.

3.4.4.2. Nonanoic acid methyl ester as internal standard

Internal standards are used to compensate for both errors in sample preparation, when analytes require derivatisation, and sample introduction with respect to the chromatographic system. Sample introduction in GC can be a source of high variability. Sample discrimination in the inlet, analyte evaporation, and injection volume variations are all noted sources of variability. The introduction of an internal standard will partially account for these errors. An internal should mimic the chemical and physical properties of the analytes of interest but not be naturally present in the sample itself.

Nonanoic acid methyl ester (C9:0), structure shown below in Figure 7, was chosen as an internal standard as fatty acids with an odd numbered carbon chain are not present in bovine milk, with the exception of (C17:0).

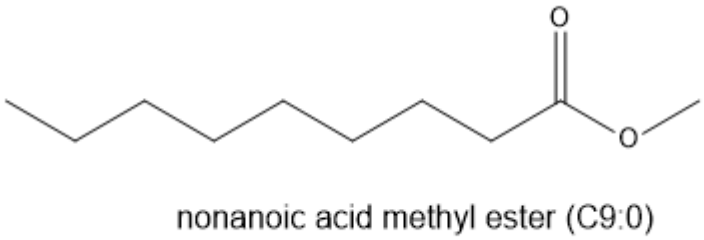

Figure 3.18: Structure of nonanoic acid methyl ester.

It was predicted that nonanoic acid methyl ester would elute between caprylic acid methyl ester (C8:0) and capric acid methyl ester (C10:0) as it is part of the same homologous family of compounds. When the chromatograms for nonanoic acid methyl ester and the FAME mix are compared, nonanoic acid methyl ester has eluted between octanoic acid methyl ester and capric acid methyl ester as expected.

3.4.5. Optimisation of derivatisation procedure

Derivatisation was optimised by monitoring increases in the ratio of peak areas between heptadecanoic acid methyl ester (C17:0) and nonanoic acid methyl ester. As the concentration of nonanoic acid methyl ester was constant, any increase in peak ratio was due to an increase in heptadecanoic acid methyl ester peak area. Heptadecanoic acid methyl ester was derivatised using acid catalysis from glyceryl triheptadecanoate, a triglyceride that contains three molecules of heptadecanoic acid. The structure of glyceryl triheptadecanoate and heptadecanoic acid methyl ester can be seen below in Figure 3.19. Glyceryl triheptadecanoate was used as the model compound to determine the appropriate derivatisation procedure in the following sections.

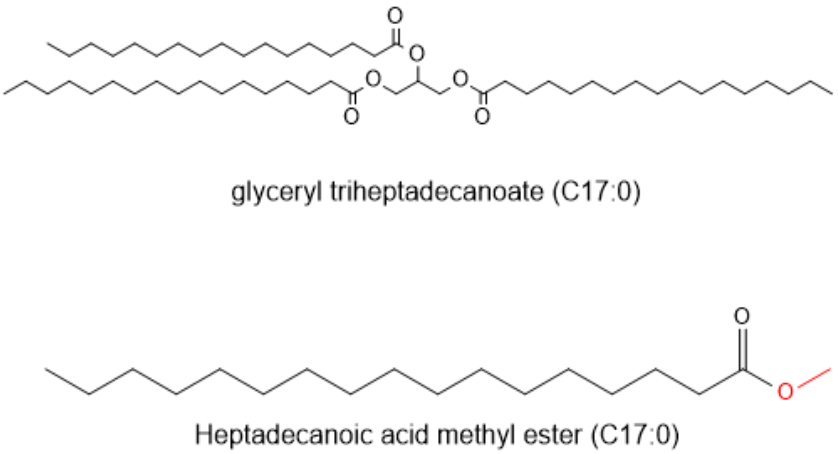

Figure 3.19: Structures of glyceryl triheptadecanoate and heptadecanoic acid methyl ester.

3.4.5.1. Determination of acid catalyst

Acid catalysed transesterification has already been shown to be superior when compared to base catalysed transesterification (Section 3.2.1). Base catalysed derivatisation followed by acid catalysed derivatisation was not explored as acid catalysis resulted in

the complete derivatisation of all fatty acids without the formation of side products, as reported by others [112]. Several acid type catalysts (BF_3, H_2SO_4, HCl) in methanol have been used in the formation of FAMEs. The optimum derivatisation conditions for BF_3 and H_2SO_4 have been compared, below.

Derivatisation rates have been shown to be affected by the temperature at which the derivatisation was carried out, and the length of time that analytes were derivatised [113]. As these experimental factors are interlinked, a 2^2 factorial design was constructed to determine which factors, if any, have a significant impact on derivatisation. The response measured was the ratio of peak areas between nonanoic acid methyl ester (the reference standard) and heptadecanoic acid methyl ester, with a bigger ratio indicating a more efficient derivatisation. Centre points were added to the experimental design to detect curvature. The same experimental design was used for both H_2SO_4 and BF_3. The experimental table that was generated by Minitab 16 is shown below in Table 3.3

Table 3.3: 2^2 factorial screening for acid catalysed derivatisation.

Factor	**Minimum level (-1)**	**Centre point (0)**	**Max. level (+1)**
Time (min)	15	22.5	30
Temperature (°C)	50	75	90

3.4.5.2. Derivatisation using H_2SO_4 1% v/v in methanol

Table 3.4: Experimental table for 2^2 factorial screening of H_2SO_4 catalysis.

Standard order	Run order	Centre point	Block	Time	Temperature
2	1	1	1	1	-1
4	2	1	1	1	1
6	3	0	1	0	0
3	4	1	1	-1	1
7	5	0	1	0	0
1	6	1	1	-1	-1
5	7	0	1	0	0

Glyceryl triheptadecanoate was derivatised using H_2SO_4 1% v/v in methanol using the method outlined below. The concentration of H_2SO_4 in methanol was taken from literature [112].

Nonanoic acid methyl ester (200 µL; 1.12 mg/mL) in hexane, as internal standard, glyceryl triheptadecanoate (200 µL; 1.03 mg/mL) in hexane, and H_2SO_4 (1 mL; 1% v/v) in methanol were added to a micro reaction vial. The samples were then derivatised according to the experimental design. After cooling, hexane (1 mL) and water (1 mL)

were added. The vials were agitated for 2 minutes, after phase separation the supernatant was transferred to a GC vial for analysis.

Analysis of the resulting data by Minitab 16, showed that the only significant factor was temperature. As the results of the 2^2 factorial screening experiment indicated that a higher temperature (90 °C) was the only significant factor (of those investigated). The optimum derivatisation time at this temperature was investigated. This was done using the method outlined below:

Nonanoic acid methyl ester (100 µL; 1.12 mg/mL) in hexane, as internal standard, glyceryl triheptadecanoate (100 µL; 1.03 mg/mL) in hexane, and H_2SO_4 (1 mL; 1% v/v) in methanol were added to a micro reaction vial. The samples were then derivatised at 90 °C for 10, 20, 30, 40, 50, or 60 minutes. After cooling, hexane (1 mL) and water (1 mL) were added. The vials were agitated for 2 minutes, after phase separation the supernatant was transferred to a GC vial for analysis.

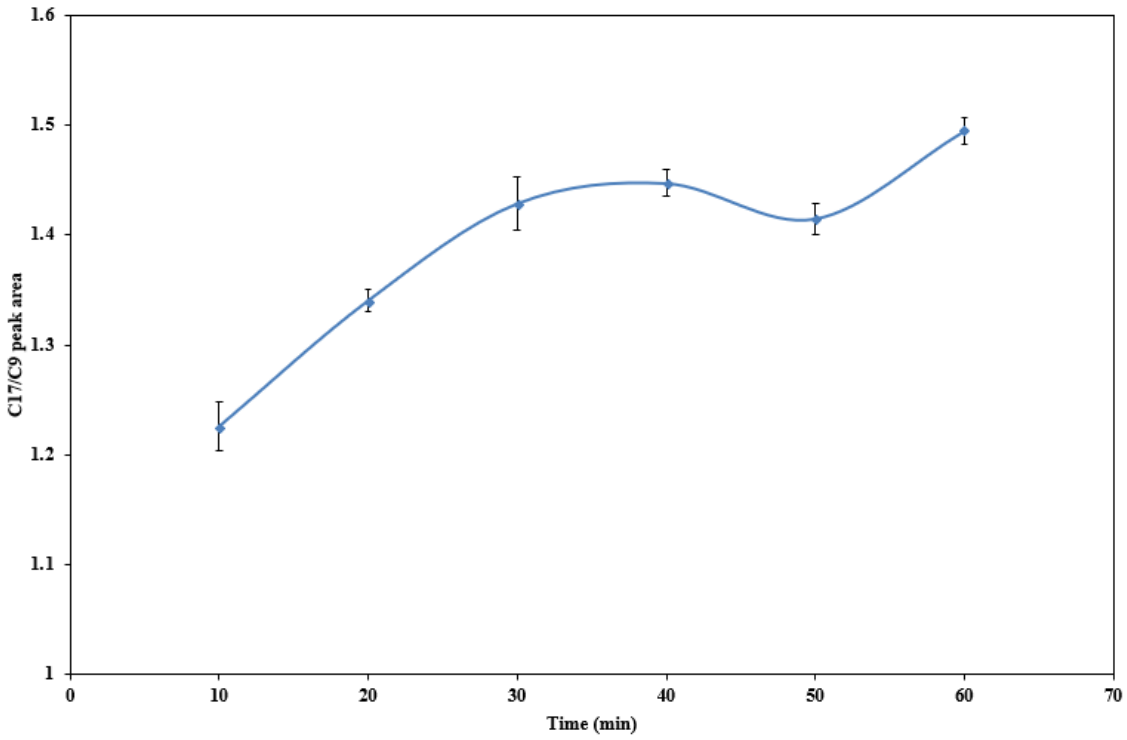

Figure 3.20: Acid catalysed derivatisation by 1% H_2SO_4 in methanol (n=3).

As can be seen from the above figure, the derivatisation reaction was still ongoing at 60 minutes. The error bars were calculated using the standard deviation of repeat injections (n=3) of the sample. It was assumed that peak ratios would plateau, indicating that glyceryl triheptadecanoate was fully derivatised. From the above plot it can be seen that derivatisation was still ongoing at 60 minutes. Derivatisation times longer than this were not explored as the longer derivatisation times would result in excessively long total analysis times.

3.4.5.3. Derivatisation using BF_3 (12% w/v) in methanol

Glyceryl triheptadecanoate was derivatised using BF_3 12 % w/v in methanol using the method outlined below.

Nonanoic acid methyl ester (200 µL; 1.12 mg/mL) in hexane, as internal standard, glyceryl triheptadecanoate (200 µL; 1.03 mg/mL) in hexane, and BF_3 (1 mL; 14% v/v) in methanol were added to a micro reaction vial. The samples were then derivatised according to the experimental design. After cooling, hexane (1 mL) and water (1 mL) were added. The vials were shaken by hand for 2 minutes, after phase separation the supernatant was transferred to a GC vial for analysis.

As with derivatisation using H_2SO_4, temperature was found to be the only significant factor in this experimental design.

As the results of the 2^2 factorial screening experiment, for BF_3 catalysis, indicated that a higher temperature (90 °C) was the only significant factor, the optimum derivatisation time at this temperature was investigated. This was done using the method outlined below:

Nonanoic acid methyl ester (100 µL; 1.12 mg/mL) in hexane, as internal standard, glyceryl triheptadecanoate (100 µL; 1.03 mg/mL) in hexane, and BF_3 (1 mL; 12% w/v) in methanol were added to a micro reaction vial. The samples were then derivatised at 90 °C for 10, 20, 30, 40, 50, or 60 minutes. After cooling, hexane (1 mL) and water (1 mL)

were added. The vials were shaken by hand for 2 minutes, after phase separation the supernatant was transferred to a GC vial for analysis.

As was expected, peak ratios plateaued after 30 minutes of derivatisation. The derivatisation reaction proceeded at least twice as fast when BF_3 was used as the catalyst compared to H_2SO_4.

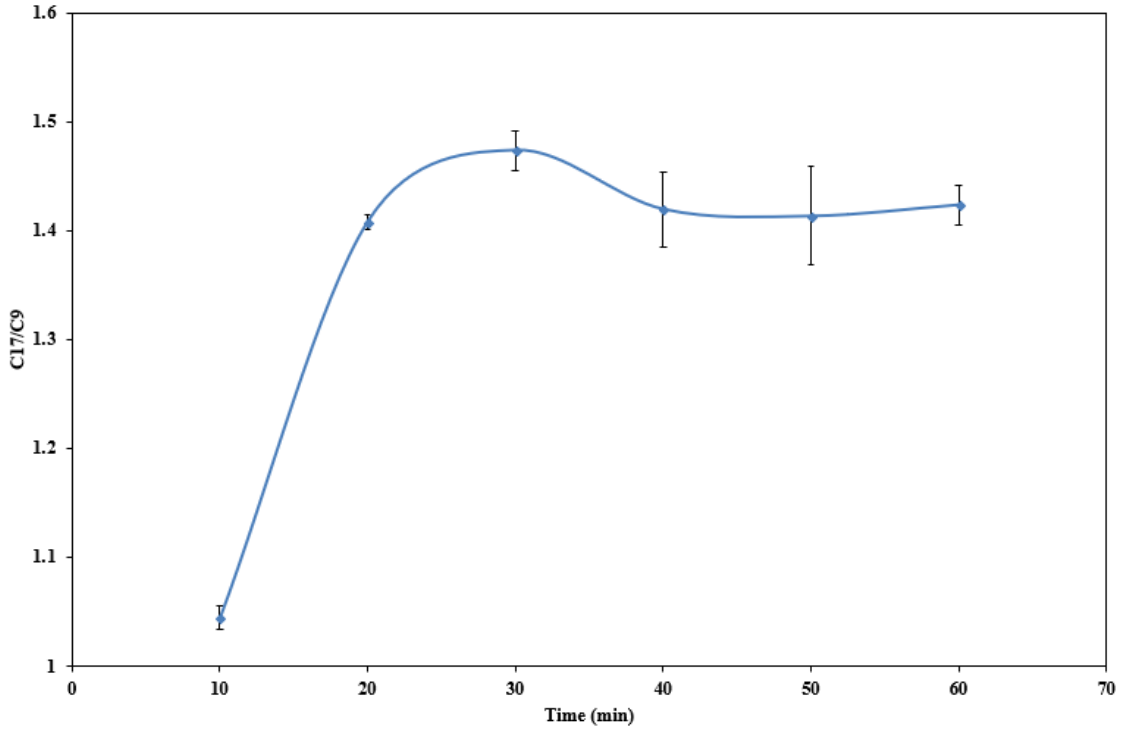

Figure 3.21: Acid catalysed derivatisation by BF_3 in methanol.

The decrease in derivatisation time when using BF_3 (12% w/v) in methanol could be due to the increased amount of catalyst compared to H_2SO_4 (1% v/v) in methanol. The stated concentration of H_2SO_4 in methanol is the concentration cited in literature [112]. The addition of the BF_3 to the carbonyl oxygen results in a greater polarisation of the carbonyl bond than proton transfer from H_2SO_4 to the carbonyl oxygen. This facilitated faster derivatisation times using BF_3 compared to H_2SO_4, as BF_3 rendered the carbonyl more electrophilic.

Although H_2SO_4 resulted in higher absolute magnitude of C17/C9, BF_3 (12% w/v) in methanol gave complete derivatisation in less time than H_2SO_4 (1% v/v) in methanol, it was chosen as the catalyst for the subsequent method development.

3.4.6. Optimisation and validation of DLLME protocol

Before optimisation of the DLLME process began, the optimum extraction solvent for DLLME was determined. Previously, hexane:MTBE (1:1) was chosen as the extraction solvent but hexane is not miscible with methanol (dispersive solvent) and so could not be considered for use as the final extraction solvent. Folch reagent (chloroform:methanol 2:1) replaced hexane:MTBE as the extraction solvent. Folch reagent was chosen as a replacement extraction solvent as it provides better extraction efficiency than other solvents for FAME analysis [109].

Extraction of FAMEs were compared using both Folch and hexane: MTBE reagents to ensure equivalent, or better, extraction was achieved with Folch reagent. The method used to compare the extractions is outlined below.

Nonanoic acid methyl ester (100 µL; 2.16 mg/mL) in hexane, as internal standard, glyceryl triheptadecanoate (200 µL; 1.03 mg/mL) in hexane, and BF_3 (1 mL; 12% w/v) in methanol were added to a micro reaction vial. The samples were then derivatised at 90 °C for 30 minutes. After cooling, hexane: MTBE (1 mL) or chloroform (1 mL) and water (1 mL) were added. The vials were shaken by hand for 2 minutes, after phase separation the organic phase was transferred to a GC vial for analysis.

Response was measured as the ratio between C17 and C9 peak areas, the results of which can be seen below (Figure 3.22). The results indicated that chloroform extracted FAMEs significantly better than hexane, thus chloroform was used as the extraction solvent in the development of a DLLME procedure.

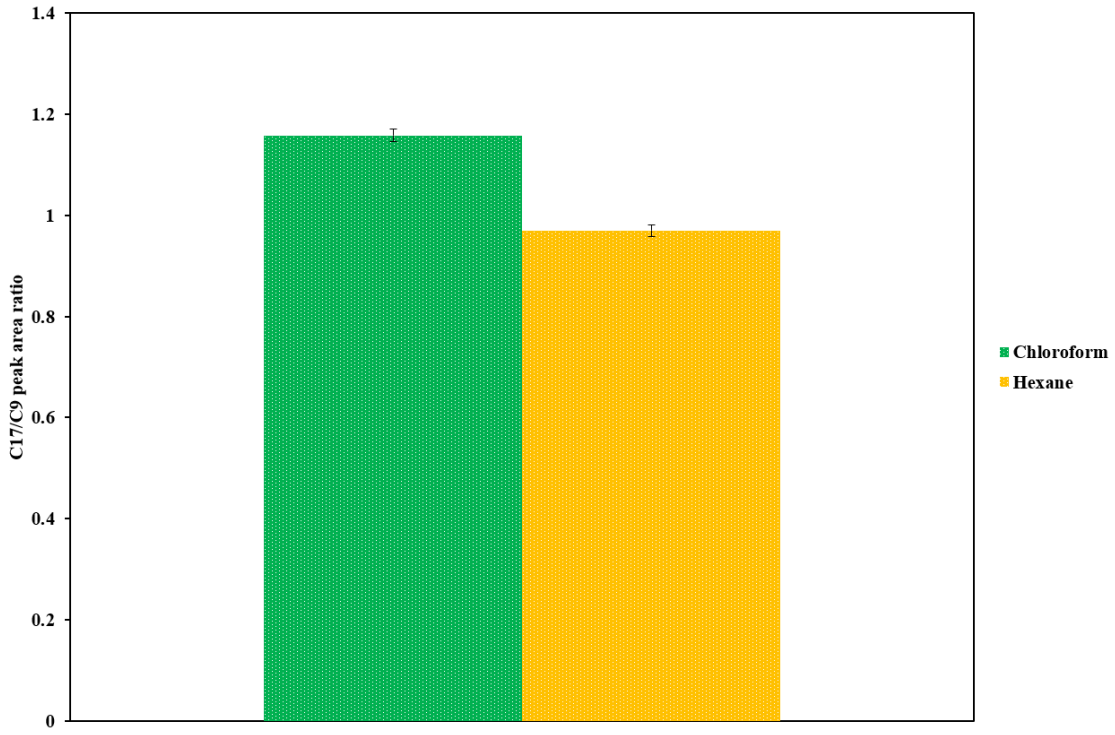

Figure 3.22: Comparison of extraction solvents for FAMEs (n=9).

3.4.6.1. Screening of experimental factors in DLLME

A list of experimental factors and levels that were screened in a 2^7 1/8 factorial design can be seen in Table 3.5. This resolution prevented confounding between all main effects and two factor interactions. Main effects were confounded with three factor effects and higher. A 2^7 1/8 factorial design was chosen as it offered a compromise between the number of runs and resolution between main and three factor effects. A full 2^7 factorial design would require 128 experiments; a 2^7 1/8 factorial design reduces this to 16 experiments. When significant factors are found the experimental design will be augmented to a central composite design to generate a response surface. This cannot be done if centre points are included in the initial screening experiments. It is for this reason that centre points were not included.

During preliminary experiments, it was noticed that pre-concentration of FAMEs from real milk samples from both Holstein and Rotbunt cows resulted in peak fronting, even

after 10:1 split injection. To enable accurate peak integration, the final DLLME extract was diluted further diluted by a factor of 10.

Table 3.5: Screening factors for DLLME procedure.

Factor	Minimum level (-1)	Maximum level (1)
Phosphoric acid (µL)	30	200
NaCl solution (2 M) (µL)	400	1000
Shake time (minutes)	0.5	2
Extraction solvent (µL)	50	200
Aqueous phase (mL)	5	10
Dispersive solvent (µL)	0	400
Extraction time (minutes)	0	2
Sonication (minutes)	0	5

Preliminary experiments were carried out to ensure that the levels chosen sufficiently precipitated proteins, and provided a stable cloudy solution. During these preliminary experiments, a sodium chloride solution (2 M) was used as the aqueous phase for DLLME. This resulted in an unstable cloudy solution where chloroform droplets collected at the bottom of the centrifuge tube, therefore only water was considered for the aqueous phase in the experimental screening. The optimum extraction was defined as the

extraction that provided the largest total peak area for caprylic, capric, palmitic, stearic, and oleic acid methyl esters.

The results of the screening experiment can be seen below in the ANOVA table. The only significant factor was the volume of extraction solvent used. The figures below show that the addition of more extraction solvent did not increase peak areas. The addition of extraction solvent before rapid injection could result in a dilution of the analytes in the final extraction phase, due to the increased volume of extraction phase. This would lower the concentration of analyte in the final extraction phase.

Analysis of Variance for Response (coded units)

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Main Effects	8	637614630	637614630	79701829	3.98	0.043
Phosphoric acid	1	22120199	22120199	22120199	1.10	0.328
NaCl 2M	1	38795065	38795065	38795065	1.94	0.207
Shake time	1	68359065	68359065	68359065	3.41	0.107
Extract. solv. vol.	1	460918007	460918007	460918007	23.01	0.002
Aqueous volume	1	7387390	7387390	7387390	0.37	0.563
Disp. solvent vol.	1	18114280	18114280	18114280	0.90	0.373
Extraction time	1	18690258	18690258	18690258	0.93	0.366
Sonication	1	3230367	3230367	3230367	0.16	0.700
Residual Error	7	140226373	140226373	20032339		
Total	15	777841003				

Figure 3.23: ANOVA table for screening experiment.

As there was only one significant factor (volume of extraction solvent), it was not necessary to use a central composite design and a response surface. The surface plot is shown below in Figure 3.24. The optimised extraction procedure is outlined below.

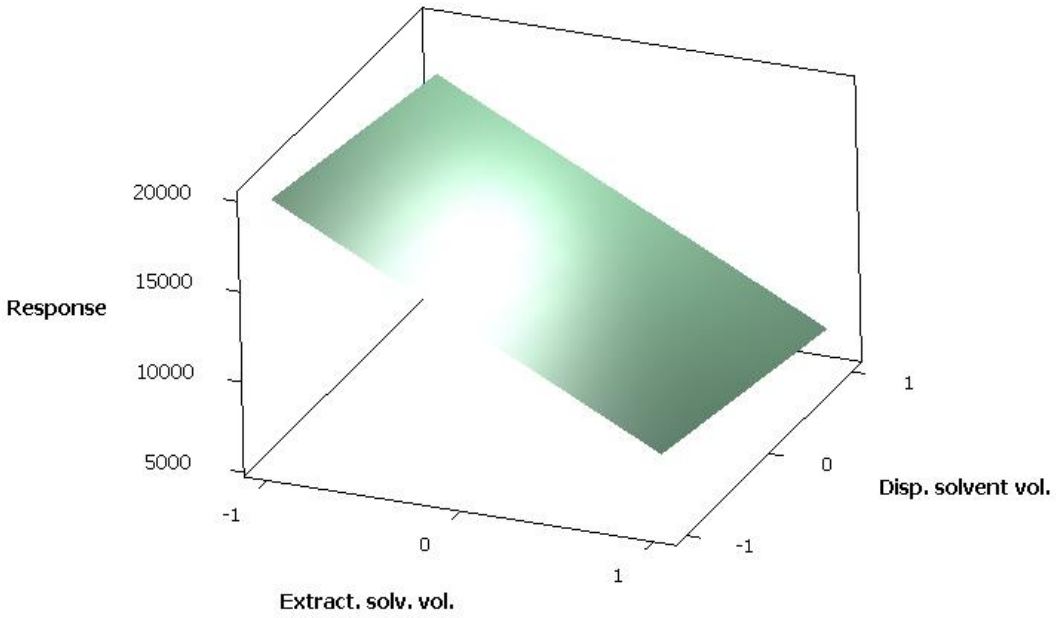

Figure 3.24: Surface plot of response from experimental design.

Milk (1 mL), conc. phosphoric acid (30 µL), sodium chloride solution (1 mL; 2 M), and Folch reagent (750 µL) were placed in a centrifuge tube. The samples were shaken by hand for 30 seconds and centrifuged for 5 minutes at 4500 rpm. The aqueous supernatant was discarded and the sedimented chloroform phase (250 µL) was transferred to a micro reaction vial. BF_3 in methanol (1 mL; 12% w/v) was added and the samples were heated for 30 minutes at 90 °C. After cooling, the reaction mixture (1 mL) was rapidly injected into water (5 mL). The resulting cloudy solution was centrifuged for 5 minutes at 4500 rpm. The aqueous phase was discarded and the sedimented chloroform phase was transferred to a GC vial where it was diluted 1:10 with chloroform.

3.4.7. Comparison between optimised DLLME and LLE procedures

The optimised DLLME method was compared against two liquid-liquid extraction methods. The first used chloroform as an extraction solvent, the second used hexane. Extraction solvent volume was kept constant (500 µL) for all three methods. This was to

ensure that the pre-concentration factors were due to the presence of a stable emulsion of micro-droplets, allowing rapid mass transfer of analytes into the extraction phase, and not simply a result of reduced extraction solvent volume. Hexane was chosen as a third extraction solvent as it is a commonly used in the extraction of FAMEs.

DLLME: Milk (1 mL), conc. phosphoric acid (30 µL), sodium chloride solution (1 mL; 2 M), and Folch reagent (750 µL) were placed in a centrifuge tube. The Folch reagent contained nonanoic acid methyl ester as internal standard (0.25 mg/mL). The samples were shaken by hand for 30 seconds and centrifuged for 5 minutes at 4500 rpm. The aqueous supernatant was discarded and the sedimented chloroform phase (250 µL) was transferred to a micro reaction vial. BF_3 in methanol (1 mL; 12% w/v) was added and the samples were heated for 30 minutes at 90 °C. After cooling, the reaction mixture (1 mL) was rapidly injected into water (5 mL). The resulting cloudy solution was centrifuged for 5 minutes at 4500 rpm. The aqueous phase was discarded and the sedimented chloroform phase was transferred to a GC vial where it was diluted 1:10 with chloroform.

Liquid-liquid extraction: Milk (1 mL), conc. phosphoric acid (30 µL), sodium chloride solution (1 mL; 2 M), and Folch reagent (750 µL), (or 500 µL of hexane) were placed in a centrifuge tube. The samples were shaken by hand for 30 seconds and centrifuged for 5 minutes at 4500 rpm. The aqueous supernatant was discarded and the sedimented chloroform phase (250 µL) was transferred to a micro reaction vial. BF_3 in methanol (1 mL; 12% w/v) was added and the samples were heated for 30 minutes at 90 °C. After cooling, phase separation was induced by the addition of water (1 mL). Without the appropriate ratios of aqueous, dispersive, and extraction solvents; a stable emulsion consisting of micro-droplets of extraction solvent could not form. The aqueous phase was discarded and the sedimented chloroform phase was transferred to a GC vial where it was diluted 1:10 with chloroform.

The preconcentration factors (Section 1.1.1) obtained from each method are shown below. The preconcentration factors of DLLME, chloroform liquid-liquid extraction, and hexane liquid-liquid extraction were 5.9, 2.0, and 1.4, respectively. Extraction of FAMEs using the optimised DLLME method resulted in an increase in preconcentration factors of approximately 3. Figure 3.25 shows an average of preconcentration factors of all analytes.

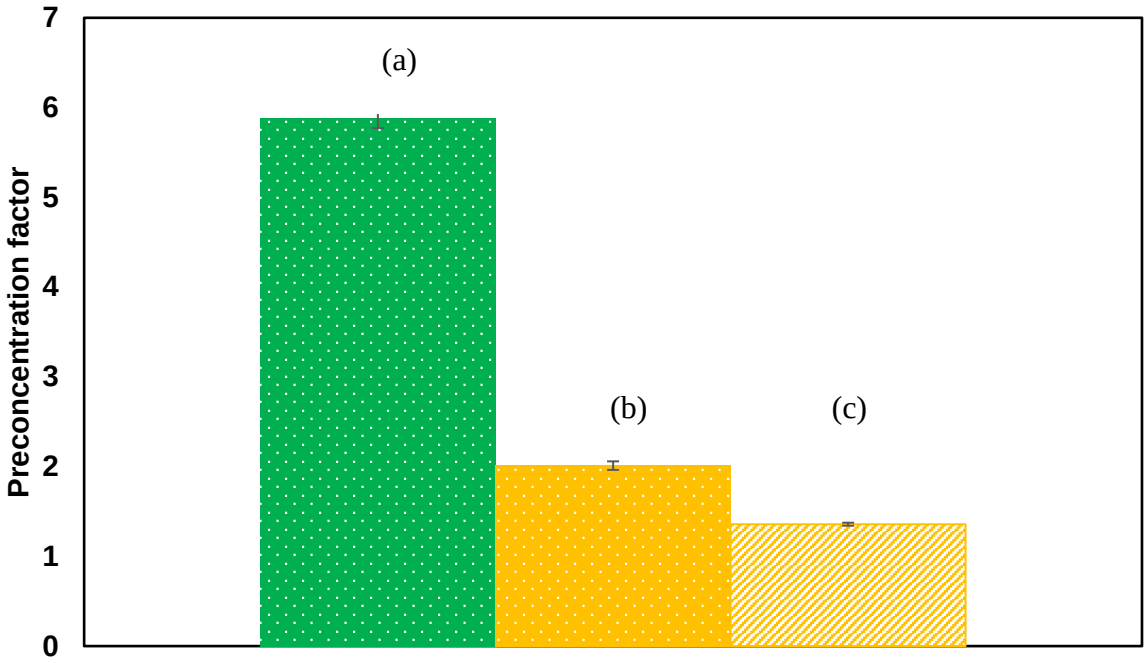

Figure 3.25: Comparison of DLLME and liquid-liquid extractions (n=9). (a) DLLME, (b) chloroform liquid-liquid extraction, (c) hexane liquid-liquid extraction.

The results of this experiment confirmed that it was the presence of micro-droplets of extraction solvent that resulted in the increased preconcentration factors when DLLME is used. There was also a significant ($p<0.05$) increase in preconcentration factors when chloroform was used in liquid-liquid extraction.

The optimised method then underwent validation before being used to analyse milk samples from Holstein and Rotbunt cows.

3.4.8. Method validation

The analytical method was validated according to the following parameters: linearity, recovery, repeatability, limit of detection (LOD), and limit of quantification (LOQ).

The method was found to be linear for all analytes between the 0.04-0.09 µg/mL range. Recoveries for each analyte were as follows: octanoic (70%), capric (80%), palmitic

(84%), stearic (73%), and oleic (89%). Repeated (n=6) DLLME extractions yielded a repeatability of 7%. The LOD was 0.04 mg/mL, while the LOQ was 0.1 mg/mL. Figures of merit for the method can be seen in Table 3.6. The calculated LOD was confirmed and an example of the chromatography for palmitic acid can be seen in the

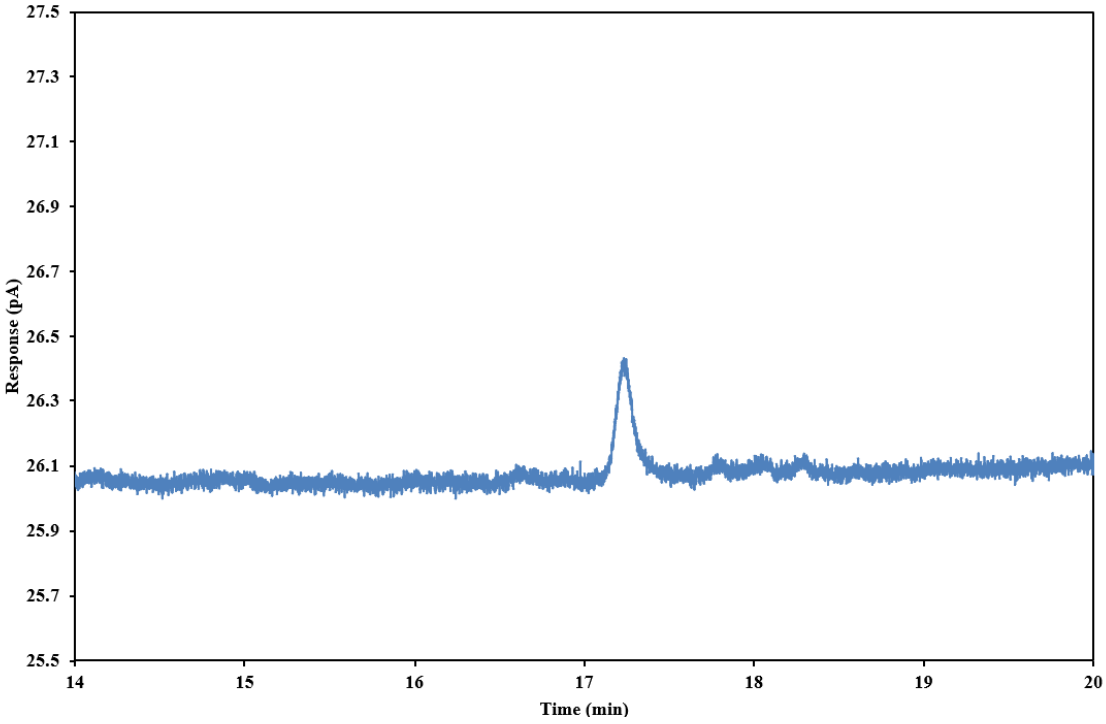

Figure 3.26: LOD chromatogram for palmitic acid.

Table 3.6: Figures of method for DLLME method for FAME analysis.

Analyte	Linearity (R^2)	LOD (µg/mL)	Reproducibility (%RSD) (n=6)	Recovery (%) (n=6)	EF
Caprylic acid (8:0)	0.994	0.08	4.2	70	11
Capric acid (10:0)	0.998	0.04	6.9	80	15
Palmitic acid (16:0)	0.994	0.04	6.3	84	8
Stearic acid (18:0)	0.997	0.09	4.1	73	10
Oleic acid (18:1 cis 9)	0.994	0.07	5.2	89	9

3.4.9. Comparison with recently published methods

Fatty acid analysis in milk products is an important area of research and as such there have been many published methods relating to this field. The newly developed method has been compared to recently published methods to ensure this method adds to the current scientific knowledge. The comparison can be seen in Table 3.7. The newly presented method displayed faster derivatisation times and/or lower LOD than recently published methods for the same analytes.

Table 3.7: Comparison with recently published methods.

Sample	Analyte	Derivatisation agent	Derivatisation time (min)	Analytical method	LOD (µg/mL)	Ref
Milk powder	10:0, 16:0, 18:0, 18:1	DMPP	120	UHPLC-MS/MS	0.00086 - .00172	[114]
Bovine milk	16:0, 18:0, 18:1	BF_3-MeOH	15	GC-FID	24.66-30.17	[115]
Breast milk	8:0, 10:0, 16:0, 18:0	HCl-MeOH	60	GC-FID	10	[116]
Bovine milk	8:0, 10:0, 16:0, 18:0, 18:1	BF_3-MeOH	20	GC-FID	0.04-0.09	This work [51]

3.5. Discussion

3.5.1. Body condition score

The milk samples from each cow were analysed according to the optimised DLLME method outlined in the results section. A ratio of the FAME and C9:0 internal standard peak areas can be seen below in Figure 3.27. The values shown in the below bar charts below represent the ratio between the internal standard (C9:0) and the respective FAME. Each cow milk sample was aliquoted and analysed in triplicate. The peak ratios for each injection can be seen in Table 3.8 and Table 3.9.

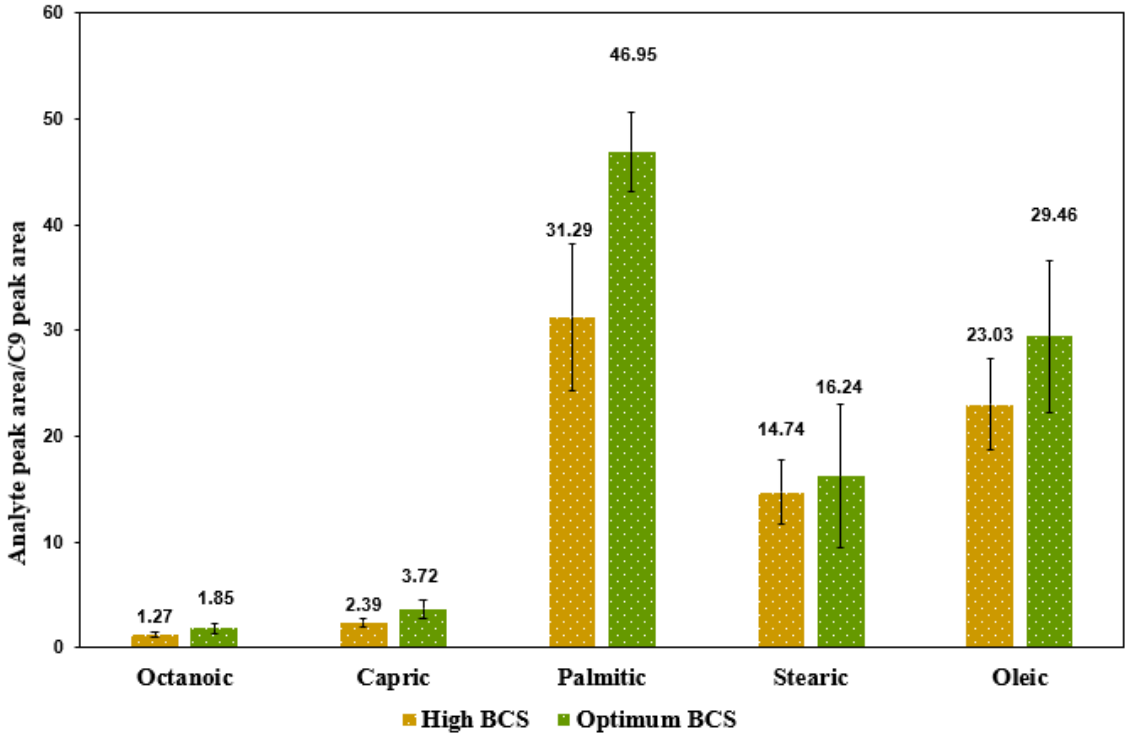

Figure 3.27: Fatty acid levels in cows (n=3) with high BCS and Optimum BCS.

As can be seen from Figure 3.27, variation in both the high and optimum body condition score groups appeared to be quite large. The validation data showed that the method provides an accurate, precise, and repeatable extraction of FAMEs from milk samples. It is believed that the small number of cows (n=2) in each group is the source of the variation. Although cows in each group were controlled for age and lactation cycle, the effects can still vary from cow to cow due to the complexity of the biological system. While the results point to a significant difference, a larger sample size would need to be examined to further explore this trend

As can be seen from Table 3.8 and Table 3.9 , all results had a relative standard deviation of less than 6%. This again suggests that the variation came from the small sample size of cows studied and not the analytical method itself.

Table 3.8: Peak ratios and relative standard deviations for peak ratios between selected FAMEs and internal standard for cows with an optimum body condition score.

Optimum body condition score				
Cow number	**FAME/C9 peak area**	**Sample 1 (n=3) (%RSD)**	**Sample 2 (n=3) (%RSD)**	**Sample 3 (n=3) (%RSD)**
Cow 1	Octanoic	1.44 (0.38%)	1.49 (0.16%)	1.39 (0.85%)
	Capric	2.91 (0.08%)	2.98 (0.23%)	2.82 (0.40%)
	Palmitic	44.42 (0.14%)	44.48 (0.42 %)	43.22 (1.81%)
	Stearic	9.71 (0.21%)	9.54 (0.45%)	9.76 (3.39%)
	Oleic	22.98 (0.12%)	22.81 (0.30%)	22.31 (1.91%)
Cow 2	Octanoic	2.48 (0.37%)	2.18 (0.29%)	2.14 (0.50%)
	Capric	4.96 (0.20%)	4.38 (0.14%)	4.27 (0.19%)
	Palmitic	54.02 (0.73)	48.27 (0.66%)	47.33 (0.42%)
	Stearic	24.46 (1.01%)	22.15 (0.75)	21.79 (0.57%)
	Oleic	39.81 (0.36%)	35.06 (0.36%)	33.77 (0.64%)

Table 3.9: Peak ratios and relative standard deviations for peak ratios between selected FAMEs and internal standard for cows with a high body condition score.

High body condition score				
Cow number	**FAME/C9 peak area**	**Sample 1 (n=3) (%RSD)**	**Sample 2 (n=3) (%RSD)**	**Sample 3 (n=3) (%RSD)**
Cow 3	Octanoic	1.21 (0.13%)	1.07 (0.24%)	1.18 (0.13%)
	Capric	2.24 (0.43%)	1.99 (0.17%)	2.17 (0.06%)
	Palmitic	27.15 (1.20%)	23.52 (0.21%)	25.16 (0.14%)
	Stearic	13.18 (0.68%)	11.33 (0.18%)	11.86 (0.33%)
	Oleic	21.34 (5.50%)	18.08 (0.57%)	19.46 (3.61%)
Cow 4	Octanoic	1.60 (0.32%)	1.26 (0.18%)	1.25 (0.45%)
	Capric	3.06 (0.13%)	2.41 (0.14%)	2.46 (0.43%)
	Palmitic	43.12 (0.24%)	33.96 (0.81%)	37.83 (0.61%)
	Stearic	19.98 (0.28%)	15.85 (0.87%)	16.26 (0.59%)
	Oleic	30.69 (0.34%)	23.93 (1.10%)	24.67 (0.48%)

Although variation between samples was present, important trends can be discerned from the data. As can be seen from Figure 3.22, cows had significantly more long chain fatty acids (palmitic, stearic, and oleic) than short chain fatty acids (octanoic and capric), which was in agreement with current literature [102].

Using the equation of the line from the calibration curves, the concentration of each FAME was calculated. The average concentration of the selected FAME from the optimum and high BCS cows were then subjected to a one tailed, 2 sample t-test, where the variance of the group is not known. This variation of t-test was chosen as a decrease in concentration of FAMEs was expected when comparing the optimum and high body condition score cows. The measurements were carried out on two different populations and so a paired t-test could not be used. The null hypothesis proposed in this study was: fatty acid concentration remains the same for cows that have different body condition scores. The confidence interval was set to 95%. The results of the t-test can be seen in Table 3.10 below.

Table 3.10: FAME concentrations and p values of cows in optimum BCS and high BCS groups.

FAME	Optimum BCS (mg/mL)	High BCS (mg/mL)	P value
Octanoic	Cow 1: 0.52 Cow 2: 0.71	Cow 3: 0.39 Cow 4: 0.45	0.12
Capric	Cow 1: 0.98 Cow 2: 1.34	Cow 3: 0.67 Cow 4: 0.81	0.11
Palmitic	Cow 1: 10.98 Cow 2: 10.72	Cow 3: 5.47 Cow 4: 5.98	0.005
Stearic	Cow 1: 2.61 Cow 2: 4.72	Cow 3: 2.61 Cow 4: 3.64	0.35
Oleic	Cow 1: 6.06 Cow 2: 6.77	Cow 3: 4.22 Cow 4: 5.63	0.12

As seen in Table 3.10 above, the t-test identified the change in palmitic acid methyl ester as the only significant difference in the two groups, and so the null hypothesis was rejected. Palmitic acid is the most common fatty acid in dairy cows [102] and the method developed can be used to distinguish cows that have a high body condition score from those in the optimum range based on the levels of palmitic acid methyl ester detected.

A second trend emerged when the levels of palmitic, stearic, and oleic are analysed. It can be seen from Figure 3.22 and Table 3.8, that the concentrations of these FAMEs showed the largest difference when comparing cows that had optimum and high body condition scores. As outlined in the introduction, cows that have a high body condition score (BCS > 3.5) undergo significant lipolysis of adipose tissue after calving. This can result in a greater drop in body condition score compared to cows whose body condition score is more effectively managed. Importantly, this drop in body condition score can also prevent the cow from cycling in the next calving season.

The results show that the newly optimised DLLME method can be used to distinguish fatty acid profiles in cows that have different body condition scores.

3.5.2. Lactation cycle

In the period after calving, fatty acid production will increase until approximately week 7 in the lactation cycle. After this point, fatty acid production decreases to the end of the lactation cycle. Thus, it was expected that there would be a significant decrease in fatty acid concentration over the lactation period.

The milk samples from each cow were analysed according to the optimised DLLME method in the results section. Milk samples were taken from British Friesian cows at the beginning and end of each cow's lactation cycle. The samples were analysed in triplicate and the concentration of selected fatty acids were determined. The ratio of the selected FAME and the internal standard (C9:0) can be seen in the below bar chart, while the concentrations of each FAME can be found in Table 3.11.

Although the error bars on Figure 3.28 showed some variation in the results, it was believed that this was a result of a small number of cows tested (n=3). When each analyses was compared separately, it was evident that the optimized DLLME method provided accurate and repeatable results with relative standard deviations of less than 4%.

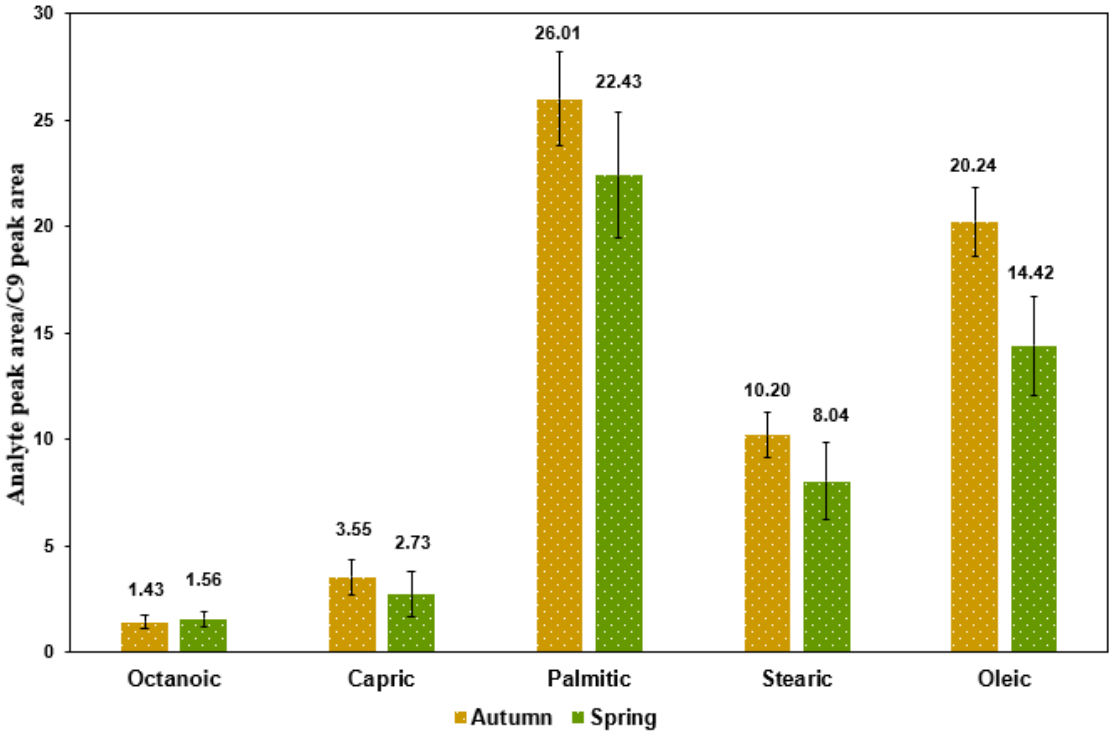

Figure 3.28: Fatty acid levels cows (n=3) across lactation cycles.

The ratios of selected FAMEs and internal standard for both cows in the early and late stage of the lactation cycle can be seen in Table 3.11 and Table 3.12 respectively.

It was clear from Figure 3.28 that longer chain fatty acids showed a larger decrease than short chain fatty acids, and this effect seemed to be more pronounced as carbon chain length increased. To investigate if this decrease was statistically significant, the concentrations of the selected FAMEs were calculated using the equation of the line obtained from calibration curves in method validation.

Table 3.11: Peak ratios and relative standard deviations for peak ratios between selected FAMEs and internal standard for cows in early lactation.

Early lactation cycle

Cow number	FAME/C9 peak area	Sample 1 (n=3) (%RSD)	Sample 2 (n=3) (%RSD)	Sample 3 (n=3) (%RSD)
Cow A (1363)	Octanoic	1.19 (0.48%)	1.16 (0.12%)	1.26 (0.14%)
	Capric	2.83 (0.29%)	2.76 (0.08%)	2.76 (0.39%)
	Palmitic	24.32 (0.71%)	22.43 (0.59%)	23.84 (0.76%)
	Stearic	9.63 (3.79%)	8.99 (0.64%)	9.19 (0.66%)
	Oleic	20.30 (1.34%)	18.56 (0.17%)	20.09 (1.25%)
Cow B (1183)	Octanoic	1.30 (0.17%)	1.36 (0.09%)	1.30 (0.35%)
	Capric	3.38 (0.26%)	3.56 (0.13%)	3.39 (0.27%)
	Palmitic	23.14 (0.77%)	25.16 (0.34%)	24.16 (0.40%)
	Stearic	8.78 (0.82%)	9.83 (0.16%)	9.55 (0.59%)
	Oleic	17.69 (0.99%)	19.44 (0.58%)	18.78 (0.56%)
Cow C (910)	Octanoic	1.88 (0.35%)	1.69 (0.18%)	1.81 (0.18%)
	Capric	4.62 (0.20%)	4.16 (0.07%)	4.46 (0.08%)
	Palmitic	30.55 (0.46%)	27.62(0.45%)	29.88 (0.53%)

	Stearic	11.84 (0.58%)	10.70 (0.63%)	11.69 (0.58%)
	Oleic	23.06 (3.31%)	20.36 (0.52%)	22.15 (1.15%)

Table 3.12: Peak ratios and relative standard deviations for peak ratios between selected FAMEs and internal standard for cows in late lactation.

Late lactation cycle				
Cow number	**FAME/C9 peak area**	**Sample 1 (n=3) (%RSD)**	**Sample 2 (n=3) (%RSD)**	**Sample 3 (n=3) (%RSD)**
Cow A (1363)	Octanoic	1.16 (0.08%)	1.15 (0.18%)	1.19 (0.06%)
	Capric	2.86 (0.19%)	2.83 (0.39%)	2.90 (0.19%)
	Palmitic	24.19 (0.75%)	23.68 (0.60%)	24.02 (0.32%)
	Stearic	8.44 (0.75%)	8.24 (0.34%)	8.34 (0.35%)
	Oleic	13.78 (1.06%)	13.36 (0.28%)	12.74 (0.67%)
Cow B (1183)	Octanoic	0.72 (0.13%)	0.68 (0.36%)	0.70 (0.02%)
	Capric	1.67 (0.25%)	1.57 (0.13%)	1.63 (0.09%)
	Palmitic	12.35 (0.73%)	11.61 (0.07%)	11.96 (0.30%)

	Stearic	6.27 (0.69%)	5.88 (0.18%)	6.10 (0.34%)
	Oleic	13.31 (0.07%)	12.49 (0.71%)	12.81 (0.67%)
Cow C (910)	Octanoic	2.18 (0.26%)	1.79 (0.32%)	1.88 (0.18%)
	Capric	4.98 (0.11%)	4.17 (0.47%)	4.35 (0.17%)
	Palmitic	32.93 (0.61%)	29.49 (0.67%)	29.51 (0.19%)
	Stearic	10.04 (3.10%)	9.75 (0.71%)	9.34 (0.29%)
	Oleic	18.40 (1.16%)	16.86 (1.24%)	16.03 (1.36%)

To determine if the decrease in fatty acid concentration was statistically significant, a paired t-test with one tail was carried out. In contrast to Section 4.1, a paired t-test was used in this analysis as the same population was being tested at two different time points. The null hypothesis in this test was that fatty acid concentration would not change over the lactation cycle. A one tail test was used as it was predicted that the concentrations of fatty acids would decrease with time. The concentrations of each fatty acid and the p values can be seen in Table 3.11.

Table 3.13: FAME concentrations and p values of cows early and late lactation cycles.

FAME	Cow number	Early lactation (mg/mL)	Late lactation (mg/mL)	P value
Octanoic	Cow A	0.40	0.39	0.28
	Cow B	0.44	0.26	
	Cow C	0.57	0.62	
Capric	Cow A	0.85	0.87	0.28
	Cow B	0.44	0.26	
	Cow C	1.31	1.33	
Palmitic	Cow A	5.10	5.19	0.25
	Cow B	5.23	2.63	
	Cow C	6.34	6.62	
Stearic	Cow A	2.02	1.83	0.05
	Cow B	2.04	1.38	
	Cow C	2.45	2.11	
Oleic	Cow A	4.23	2.92	0.003

	Cow B	4.02	2.83	
	Cow C	4.68	3.7	

The mean (n=3) from each cow at each time point was calculated and used in the paired t-test. As can be seen from Table 3.11, only stearic and oleic acid methyl esters and p values less than 0.5 (95% confidence interval). Octanoic, capric, and palmitic levels were not significantly different from early lactation levels compared to late lactation.

This results confirm the null hypothesis for octanoic, capric, and palmitic acids as $p<0.05$; their concentration does not change over the duration of the lactation cycle. This was not the case for stearic and oleic acids, which had a p value of 0.05 and 0.003, respectively. The null hypothesis was rejected for both stearic and oleic acid. Indicating that the fatty acid profile is significantly different when compared across the lactation cycle.

These findings suggest that the stage in lactation cycle has a significant impact on long chain fatty acids compared to shorter ones. As explained in the introduction, short chain fatty acids are synthesized *de novo* in the mammary gland and so any negative energy balance would not impact shorter chain fatty acids. This is in contrast to long chain fatty acids which primarily obtained through feed and are stored in adipose tissue. During times of negative energy balance, during lactation, the adipose tissue is one of the first energy stores utilized to compensate for the negative energy balance. This loss in adipose tissue would also impact the levels of long chain fatty acids present in the milk, as was observed in the finding of this study.

3.6. Conclusion

A temperature gradient GC separation was developed on a PEG column for the separation target analytes from 37 methyl esters. All 37 FAMEs were not baseline resolved but the method provided resolution of the target FAMEs from other FAMEs in the mix. A novel

DLLME method was developed and optimised using a 2^7 factorial experimental design. The optimised method was validated according to ICH guidelines showing lower LOD and LOQ values than traditional liquid-liquid extraction procedures. This highlights the strength of the novel extraction procedure developed above. The optimised DLLME procedure was then used to analyse milk samples from cows that had different body condition scores and milk samples from cows at the beginning and end of their lactation cycle.

A significant difference was found in the levels of palmitic acid in samples from cows with different body condition score. A significant difference for other selected fatty acids was not observed in these samples. Milk samples taken from the same cows at the beginning and end of their respective lactation cycles showed a significant increase in the levels of stearic and oleic acids.

The ability of the newly developed DLLME method to detect significant differences in selected fatty acid levels in a complex biological sample is important for the agricultural industry. This method may be used for the screening of the fatty acid profile in milk. This can give indications to the quality of feed that a cow is given as milk lipids are predominantly obtained by the diet [117].

Chapter 4

Determination of amino acid profile of commercial milk samples

4. Determination of free amino acid profile of commercial milk samples

4.1. Introduction

Free amino acid (FAA) concentrations in physiological fluids can reflect the metabolic health of the organism in question [118]. They can be altered by a range of factors including: nutrition, environmental conditions, and genetic effects [119]. FAA also provide the building blocks for proteins and are the precursors for nucleic acid production [120]. While important for monitoring metabolic health, FAA play a role in human nutrition. There are 9 essential amino acids that cannot be synthesised by the human body and so must be obtained from the diet, these are: histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine. Their structures can be seen in Figure 4.1. FAA present in milk are used as human supplements as they are easier to digest than protein [121]. The concentration of FAA present in milk samples can be indicative of milk quality as increased levels are a result of proteolysis [122]. This is a process which negatively affects milk processability and reduces the economic value of milk [123].

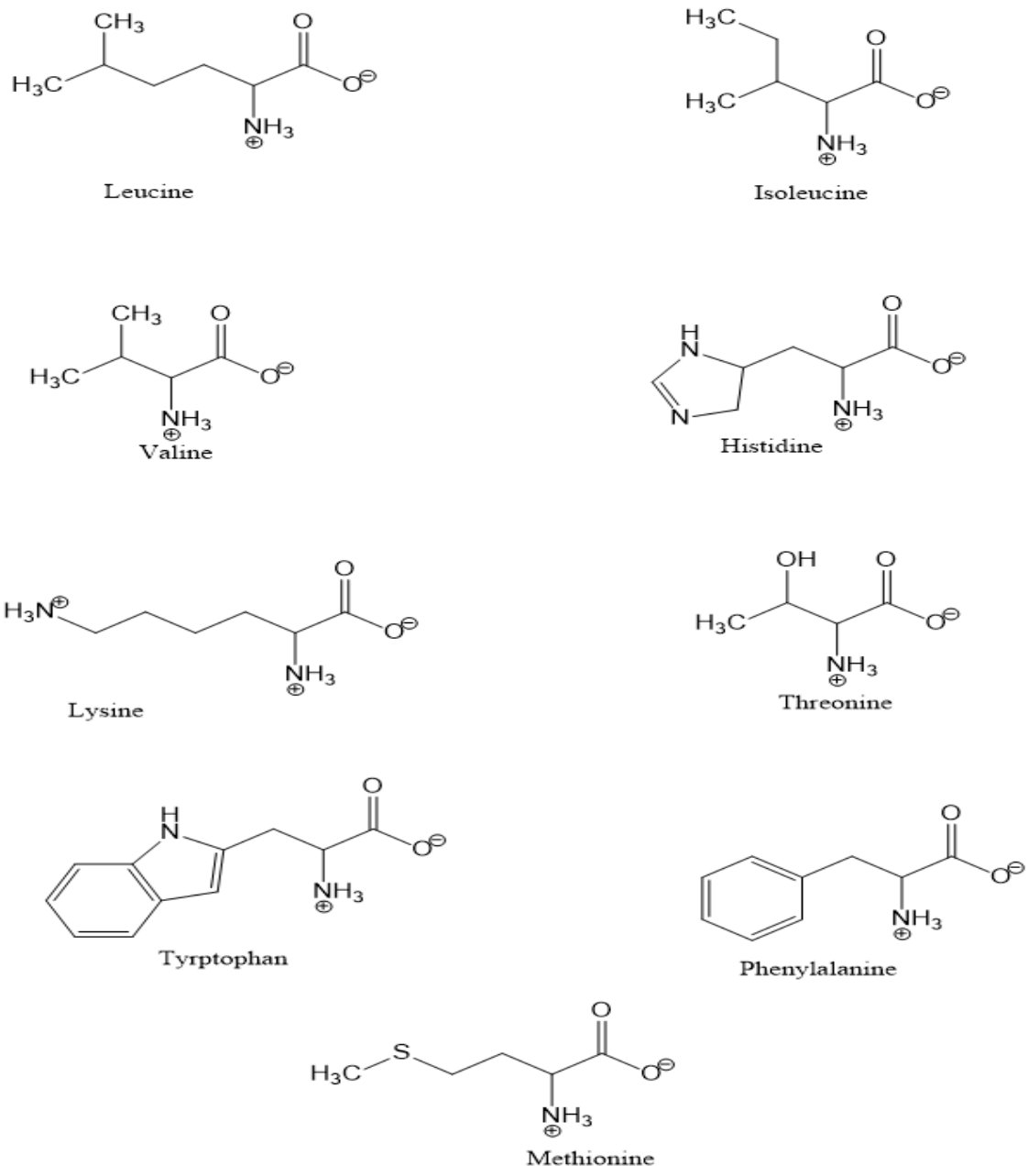

Figure 4.1: Structures of essential amino acids.

4.1.1. Current methods of analysing amino acids

Amino acids are a challenging analyte to study. They are characterised as zwitterions which contain both positive and negative charges on the same molecule. At their isoelectric point, amino acids have a net charge of 0. Varying the pH of the solution allows

for manipulation of the net charge to either overall positive or negative, depending on whether the carboxylate or amino group is protonated. Amino acids can be loosely grouped into different categories depending on the R groups attached to the amino acid. The different groups of amino acids are: nonpolar aliphatic R groups, nonpolar aromatic R groups, polar uncharged R groups, positively charged R groups, and negatively charged R groups. Methods to analyse amino acids are varied. They include gas chromatography (GC) with flame ionisation (FID) [124] or mass spectrometry detection (MS) [125,126], capillary electrophoresis with conductivity detection [127], high performance liquid chromatography (HPLC) with UV [128], ion-pair liquid chromatography [129], fluorescence [130], or MS detection [131,132]. Specialised instruments dedicated to amino acid analysis are also available [133].

4.1.2. Amino acid derivatisation methods

Amino acids generally require pre or post column derivatisation to enable detection by UV or fluorescence detectors for HPLC analysis, or to increase the volatility of the amino acids for GC analysis. Derivatisation for HPLC is required to increase sensitivity for UV detection. The majority of amino acids do not possess a chromophore, with the exception of phenylalanine, tyrosine, and tryptophan. Gatti *et al.* have used 2,5-dimethyl-1H-pyrrole-3,4-dicarbaldehyde to derivatise amino acids for UV detection [134]. This derivatisation reaction takes place at ambient temperature but requires a long reaction time of 10 minutes. Similarly amino acids lack a strong fluorophore and are unsuitable for fluorescence detection without derivatisation. Several derivatisation reagents are available for the addition of a fluorophore to the amino acid of interest. They include: *o*-pthaldialdehyde (OPA) [135], fluorenylmethyloxycarbonyl chloride (FMOC) [136], 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) [137]. OPA and FMOC present an interesting option for fluorometric detection of amino acids and can also be used for UV detection. The reaction takes place takes in less than 2 minutes at ambient temperatures and produces no toxic side products. Unfortunately there have been some concerns over the stability of the derivatives formed [138,139]. Recently efforts have been made to overcome this issue by the development of derivatisation protocols which use the injector programme function on HPLC autosamplers to carry out the derivatisation

reaction [140]. The automated methods do have some limitations however. Injecting the reaction mixture directly onto a RP-HPLC column can result in reduced column lifetime due to the high pH involved (pH 9). Furthermore, there is an increased risk of sample carryover without careful washing of the needle.

AQC is another derivatisation agent to add a fluorophore to amino acids for HPLC-fluorescence detection. It is available as a commercial kit from Waters (trade name: AccQ-Tag). While the convenience of a commercial kit for amino acid analysis is beneficial, the derivatisation reaction still requires heating at 55 °C for 10 minutes. This has an obvious impact on lab productivity and sample throughput.

It must be noted that the use of a MS detector coupled to HPLC negates the need for derivatisation. Several reports have analysed amino acids using MS detection as noted in Section 4.1.1. While this potentially reduces sample preparation time, the cost of MS detector is high and all laboratories may not have the capability to perform MS analysis. Method development and transfer are also more complicated as MS methods require volatile buffers.

Like HPLC-UV/FLD, analysis of amino acids by GC also requires derivatisation. Pentafluorobenzyl bromide has been used in the analysis of amino acids in wheat flour [141]. This derivatisation reaction increases the volatility of the amino acids by the addition of a pentafluorobenzyl group on both the amine and carboxylic acid groups [142].

By contrast, Hušek developed a rapid method for derivatisation of amino acids, at ambient temperature and in aqueous solution, for GC analysis in 1991 using ethyl chloroformate [143–145]. Since then several different alkylchloroformates have been used, these include: methyl chloroformate [146] isobutyl chloroformate [147], and propyl chloroformate [147]. Hušek has authored a review on the use of alkylchloroformates which highlights the properties and applications of the different alkylchloroformates [148].

Ethyl chloroformate converts both the carboxylic and amine groups to ethyl esters using pyridine as a catalyst. A proposed reaction mechanism can be seen in Figure 4.2. Pyridine deprotonates the carboxylic hydrogen resulting in a negative charge on the oxygen. This increases the nucleophilic properties of the carboxylic oxygen which leads to nucleophilic

substitution on the ethyl chloroformate (electrophile). As a result of the nucleophilic substitution reaction, the chlorine acts as the leaving group and an anhydride type functional group is formed. This undergoes an intramolecular decarboxylation which produced an ethyl ester, liberating carbon dioxide in the process. The amine group then acts as a nucleophile in a second nucleophilic attack on ECF. Chlorine again acts as the leaving group in this reaction. In the last step of the derivatisation reaction, pyridine acts as a base and deprotonates the positively charged amine, forming a N(O,S) – ethoxycarbonyl ethyl ester

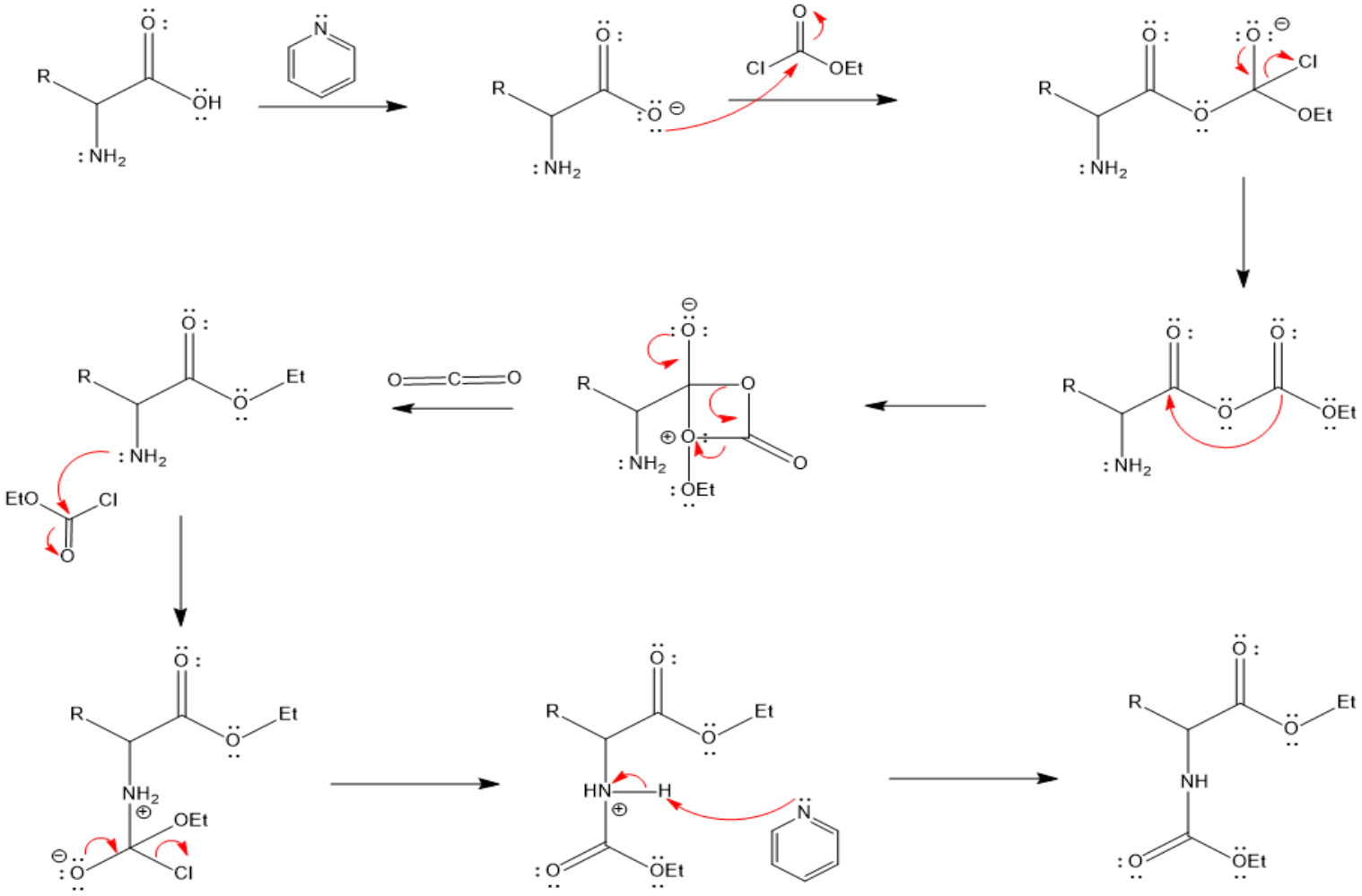

Figure 4.2: Proposed reaction mechanism for amino acid derivatisation by ethyl chloroformate.

4.1.3. Overcoming matrix interferences

Derivatisation is not the only challenge in amino acid analysis. Biological fluids (including milk) are complex samples and require significant pre-treatment to remove matrix interferences. Sample pre-treatment methods include: pulsed electromembrane extraction, solid-phase extraction, and microwave assisted extraction. Pulsed electromembrane extraction has been used for the determination of amino acids in gelatin from animal origins [149]. The membrane extraction was used in conjunction with HPLC-UV detection. Before pulsed electromembrane extraction could take place, the samples were hydrolysed using sodium hydroxide and derivatised using OPA. The derivatised sample solution was then placed in the pulsed electromembrane device where a voltage was passed through the sample for 20 minutes to allow analytes to migrate into the acceptor phase, across the supported liquid membrane. The membrane provides a physical barrier to some components but any components that are ionised in the solution (pH 3.0) have the potential to migrate across the barrier. In addition the long extraction times (20 minutes) are not conducive to high throughput analysis.

Solid-phase extraction (SPE) has also been used to analyse amino acids [150]. SPE has been used extensively for concentration of analytes, Vidal *et al.* has authored a review of SPE [151]. While SPE is an effective technique for concentrating analytes and removing matrix intereferences, the cartridges are expensive and extensive method development is needed to ensure that loss of analyte does not occur during loading and washing steps.

Microwave assisted extraction have been used to both remove matrix interferences and preconcentrate amino acids [152]. Cai *et al.* employed microwave assisted extraction to extract amino acids from tobacco leaves. The increased temperature and pressure of closed extraction vessel microwave extraction allowed the amino acids to partition to into the extraction phase quicker than conventional solid-liquid extraction. This process still took 10 minutes at 60 °C under 600 W microwave power. Microwave assisted extraction suffers from some drawbacks: the target analytes and extraction solvent must be polar, and the analytes must be non-volatile [153].

4.1.4. Dispersive liquid-liquid microextraction and amino acid analysis

Dispersive liquid-liquid microextraction, first developed in 2006 [76] and described in detail in Section 1.1.1, has been used for amino analysis in previous studies by a number of research groups. SFO-DLLME hase been used to analyse amino acids found in tobacco leaves [154]. The amino acids were derivatised by isobutyl chloroformate and extracted into 2-dodecanol before analysis by GC-MS. In this work, Li *et al*. analysed 11 different amino acids (alanine, glycine, valine, leucine, isoleucine, proline, asparagine, methionine, phenylalanine, cysteine, and tyrosine). The lowest LOD reported was 0.18 µg/mL for both leucine and proline, while the highest was tyrosine at 2.82 µg/mL. Mudiam *et al.* used UA-DLLME for the simultaneous determination of 20 amino acids in complex matrices such as hair, urine, and soybean seeds [155]. Trichloroethylene was used as the extraction solvent after derivatisation with ethylchloroformate. The amino acids were analysed by GC-MS. Mudiam *et al.* achieved an LOD of 0.38 µg/L for leucine and 0.51 µg/L for proline. While both amino acid derivatives were analysed by GC-MS, the MS detectors were different. Mudiam *et al.* used a triple quadrupole MS compared to Li *et al.* who only had access to a single quadrupole MS. Triple quad MS allows for lower LOD due to reduced noise and a wider linear dynamic range.

Interestingly, Mudiam *et al.* performed simultaneous extraction and derivatisation of amino acids from the complex samples studied [155]. This was achieved by rapidly injecting a mixture of the extraction solvent (trichloroethylene), dispersive solvent (ACN), and the derivatisation reagent (ECF) into the previously digested samples. This technique was possible as the volume of derivatisation agent was smaller (60 µL) than the volume of extraction solvent 80 µL, which minimised the dilution effect.

From the papers mentioned above it is clear that derivatisation of amino acids by alkyl chloroformates are compatible with DLLME. This is due to their ability to rapidly derivatise both the amine and carboxylic groups of the amino acid. Furthermore, the derivatisation reaction can take place in aqueous solutions which is particularly useful when dealing with biological samples. The presented chapter describes rapid derivatisation of amino acids combined with DLLME for the analysis of amino acids in a complex sample such as milk.

4.2. Overarching aims for this chapter

The overarching aims for this chapter were to develop a GC-MS method for the separation and identification of derivatised free amino acids from commercial milk samples. The amino acid profile of the samples will be determined at two time points. These time points are: the date the samples were purchased (t_0), and the sell by date of the samples that was printed on the packaging (t_1). Free amino acids will be extracted and preconcentrated using a DLLME method that has been optimised by a design of experiments approach. Significance of results will be determined by the appropriate statistical test.

4.3. Materials and methods

4.3.1. Chemicals and materials

Ethyl chloroformate (97%), phenylethyl acetate (IS) (99%), and BD Precisionglide syringe needles gauge 30 L 1.0 inch were purchased from Sigma-Aldrich (Dublin, Ireland). Ethanol (99%), pyridine (98%), chloroform (HPLC grade), dichloromethane (DCM) (HPLC grade), ethyl acetate (97%), and acetonitrile (HPLC grade) were purchased from Lennox (Dublin, Ireland). Alanine (99%), asparagine (99%), aspartic acid (99%), cysteine (98%), GABA (99%), glutamic acid (99%), glutamine, glycine (99%), histidine (99%), isoleucine (98%), leucine (99%), lysine (98%), methionine (99%), phenylalanine (98%), proline (99%), serine (99%), threonine (99%), tryptophan (98.5%), tyrosine (98.5%), and valine (98%) were purchased from Tokyo Chemical Industries (TCI) (Oxford, United Kingdom).

4.3.2. Instrumentation and chromatographic conditions

Analysis was performed on a Varian CP-3800 coupled to a Saturn 2000 MS. Chromatography was performed using VF-5MS (5% phenyl 95% di-methyl

polysiloxane) column (30 m x 0.25 mm x 0.25 µm). The final method conditions used were as follows. The injector temperature was 280 °C, and the injection volume was 1 µL in splitless mode. Helium was used as the carrier gas at a flow rate of 1 mL/min. The column temperature was programmed as follows: the initial temperature was 40 °C, increased to 125 °C at 10°C/min and held for 5 min. The temperature was then increased to 280 °C at 10°C/min and held for 10 min. The internal standard calibration method with peak was used for quantification of selected amino acids. Statistical analysis was carried out using Minitab 18.

4.3.3. Stock standard preparation

Stock standard solutions were prepared by dissolving the amino acids in 0.1 M HCl to the following concentrations: alanine (2.17 mg/mL), asparagine (1.28 mg/mL), aspartic acid (1.62 mg/mL), cysteine (1.57 mg/mL), glutamic acid (1.45 mg/mL), glutamine (2.00 mg/mL), glycine (1.43 mg/mL), histidine (1.32 mg/mL), isoleucine (1.29 mg/mL), leucine (1.34 mg/mL), lysine (1.72 mg/mL), methionine (1.78 mg/mL), phenylalanine (1.24 mg/mL), proline (1.36 mg/mL), serine (2.05 mg/mL), threonine (1.74 mg/mL), tryptophan (1.51 mg/mL), tyrosine (1.44 mg/mL), valine (1.36 mg/mL).

4.3.4. Derivatisation and DLLME procedure

Milk (1 mL), ACN (3 mL), and phenylethyl acetate (IS) were combined and centrifuged at 4,500 rpm for 5 minutes. An aliquot of the supernatant (2 mL) was removed and placed and mixed with pyridine (600 µL), ethanol (5 µL), and ECF (600 µL). The reaction was agitated. Chloroform (100 µL) was added and this mixture was rapidly injected into water (5 mL). The resulting cloudy solution was centrifuged at 4,500 rpm for 5 minutes. The sedimented phase was transferred to a GC vial and analysed by GC-MS. Samples were prepared in triplicate. Values shown are average values of the triplicate injection of each sample

4.3.5. Analytical curves

Amino acids in samples were identified and quantified by comparison to amino acid standard retention times and spectra. Analytical curves were based on the internal standardisation method. The internal standard used was phenylethyl acetate at a concentration of 0.2 mg/mL.

4.4. Results and discussion

4.4.1. Selecting a stationary phase

As the derivatised amino acids were analysed using GC-MS, a stationary phase was required that exhibited low bleed. Column bleed is a result of normal stationary phase degradation via the mechanism shown in Figure 4.3, where the stationary phase has degraded producing siloxanes, in a process known as backbiting. This contributed to baseline noise and ultimately impacted signal to noise ratio. Mass spectrometry detectors are sensitive to contamination from these siloxanes and so specially designed MS columns that have reduced bleed are used in conjunction with MS detectors. An adequate separation was obtained using the VF-5MS column and so other columns were not screened. The selectivity of MS detectors in EIC or SIM mode can also potentially circumvent the issues with co-elution. The presence of phenyl groups in the siloxane backbone of the VF-5MS column helped prevent stationary phase degradation as outlined in Figure 4.3.

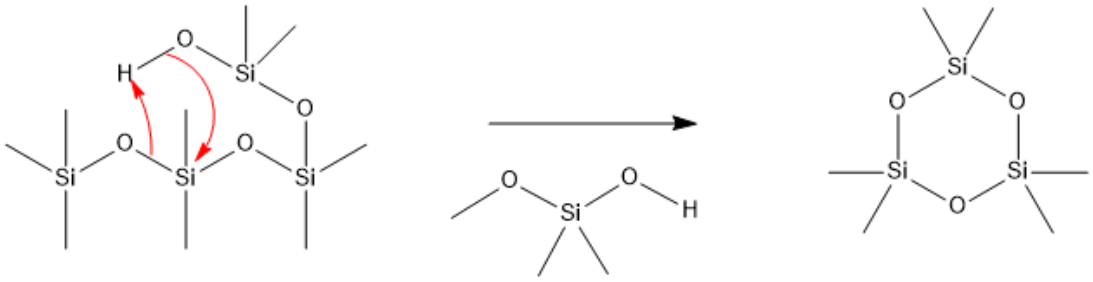

Figure 4.3: Stationary phase degradation producing column bleed.

Initially a scouting gradient of 40-280 °C was used. The temperature was increased by 10 °C/min. Peaks that co-eluted were resolved using Giddings approximation (outlined in Section 3.4.1.2). The optimised separation consisted of a starting temperature of 40 °C which was ramped to 125 °C at 10 °C/min and held for 5 minutes. Then ramped to the final temperature of 280 °C at 10 °C/min with an isothermal hold at this temperature to ensure all analytes had eluted and any matrix compounds were also eluted prior to the next injection. The upper temperature limit of the column is 320 °C but the front valve on the GC-MS was unable to be heated beyond 280 °C, so this was the maximum temperature chosen for this analysis. The optimised separation can be seen in Figure 4.4.

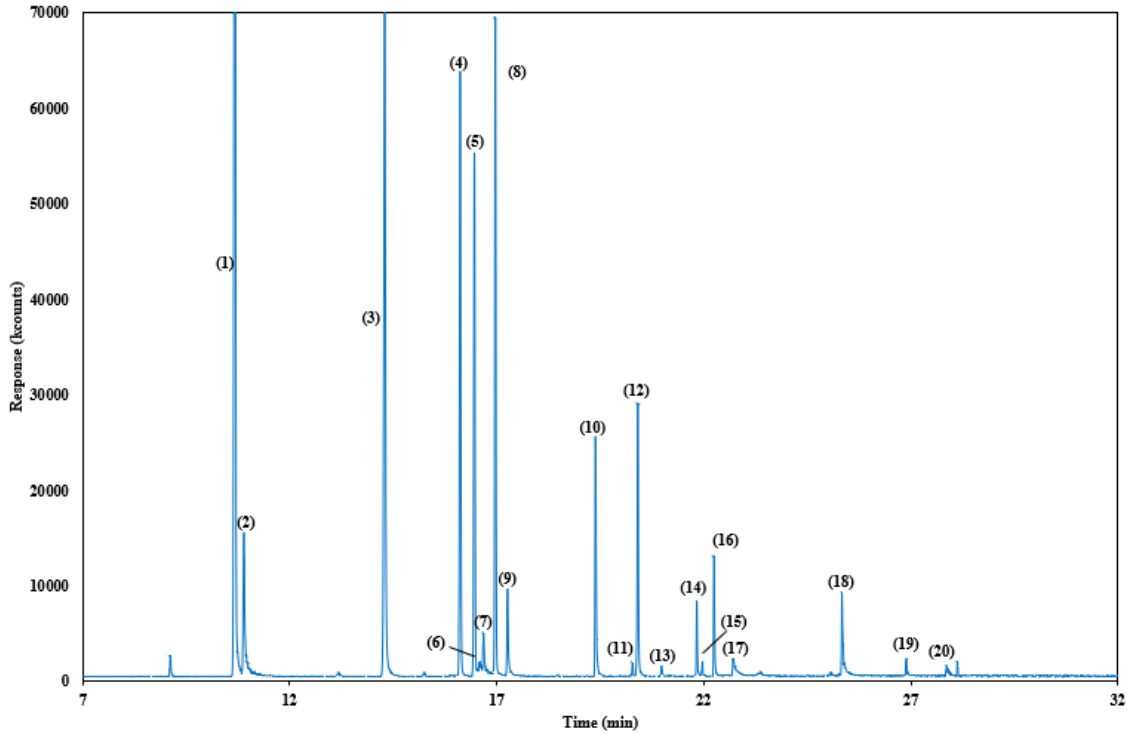

Figure 4.4: Optimised separation of amino acids (all amino acids at approx. 0.2 mg/mL). Amino acid identity can be found in Table 4.1. Chromatographic conditions: stationary phase: VF-5MS (5% phenyl-methyl, 30 m x 0.25 mm x 0.25 µm), carrier gas: helium, flow rate: 1 mL/min, initial temperature: 40 °C, ramped to 125 °C at 10 °C/min and held for 5 minutes, then ramped to 280 °C at 10 °C/min. Final temperature: 280 °C, inlet temperature: 280 °C, scan range TIC m/z 40-600.

4.4.2. Selecting an internal standard

Isotopically labelled analogous of the analyte of interest are the most appropriate internal standard to use. They exhibit the same chemical characteristics as the analyte in question which means it will partition into the extraction phase to the same extent as the analyte, allowing for more accurate quantification. As the chemical characteristics are the same the isotopically labelled standard and analyte will co-elute but the use of MS detection will allow for both the standard and analyte to be quantified.

Although they are considered the most accurate internal standard to use, they do have some drawbacks. Isotopically labelled standards are expensive and the method requires

an MS detector to differentiate between the standard and analyte. This prevents the method from being transferred to another instrument that does not have MS capabilities.

Due to the constraints described above compounds that exhibited similar chemical characteristics were investigated as potential internal standards. The purpose of internal standards are explained in Section 3.4.4.2. For this work, two compounds were evaluated to determine their suitability for use as an internal standard: benzylamine and phenylethyl acetate. As benzylamine contains a free amine group, this will also be derivatised using ethyl chloroformate to ethyl benzylcarbamate. Both compounds have similar chemical characteristics which enables their use as internal standards. The structures of phenylethyl acetate, benzylamine, and ethyl benzylcarbamate can be seen in Figure 4.5.

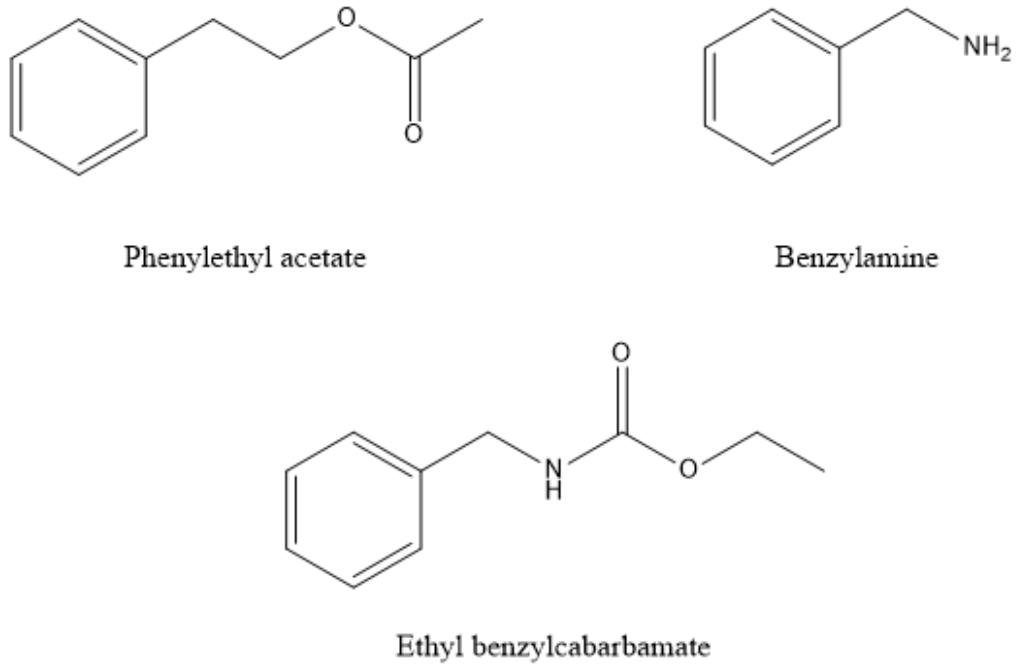

Figure 4.5: Structures of proposed internal standards.

Ethyl benzylcarbamate co-eluted with other amino acids and so was not used as an internal standard. As mentioned previously, this would prevent the method being transferred to an instrument without MS capabilities such as GC-FID. Phenylethyl acetate

eluted with a retention time of 9.1 minutes. It was resolved from other peaks in the chromatogram and it was used as the internal standard for the remainder of the analysis.

4.4.3. Identification by mass spectra

Derivatised amino acids were identified by retention time and mass spectra with derivatised standards.. A list of exact masses and base peak ions for each derivatised amino acid can be found in Table 4.1. The base peak ion will be used as the quan ion and chromatograms were analysed in Extracted Ion Chromatography (EIC) mode. In all cases, the base peak was used as the quan ion for each amino acid derivative

Table 4.1: Derivatised amino acid exact mass, retention times, and characteristic ions.

Analyte	**Derivatised exact mass (Da)**	**Retention time (min)**	**Base peak (m/z)**	**Other ions (m/z)**
Phenylethyl acetate (IS)	164.08	9.1	122	108, 107
Alanine	189.10	10.7	116	44, 190
Glycine	175.18	10.9	176	102
Valine	217.13	14.3	144	116, 218
Leucine	231.29	16.1	158	102, 232
Serine	205.10	16.9	206	132, 60
Isoleucine	231.29	16.4	158	102, 232
Threonine	219.11	16.6	128	101, 175, 220
GABA	203.12	16.6	116	86, 122, 130, 158
Proline	215.12	16.9	142	70
Asparagine	232.11	17.2	69	141, 215
Aspartic acid	233.09	19.4	188	74, 116, 142, 262

Glutamine	246.12	20.3	128	100, 129, 175
Methionine	249.10	20.4	176	101, 204, 248
Glutamic acid	247.11	20.9	128	202
Phenylalanine	265.13	21.8	176	120, 148, 192, 266
Lysine	246.16	22.2	294	102, 132, 220
Cysteine	221.07	25.3	156	N/A
Histidine	255.12	25.9	328	254
Tyrosine	281.13	26.9	107	192, 280, 354
Tyrptophan	304.14	27.8	130	N/A

In some cases the molecular ion for the derivatised amino acid was detected, e.g. for derivatised valine. Unfortunately the derivatised amino acids were not available in the mass spectral library and so identifications were confirmed by mass spectral fragmentation analysis and comparison with retention time of derivatised standards. An example of a mass spectra used for the identification of valine, can be seen in Figure 4.6. A proposed fragmentation analysis for valine is shown in Figure 4.7. A similar approach was used in the analysis of other derivatised amino acid spectra.

Although serine was detected when derivatised individually, it was not detected when all amino acids were combined. The reasons for its absence are yet to be determined. Hydrogen bonding between the carboxylate and hydroxy group may prevent derivatisation, however this does not provide an explanation of why serine is detected when derivatised individually. Experiments were conducted that varied the concentration of derivatisation reagents but serine was not detected when all amino acids were derivatised.

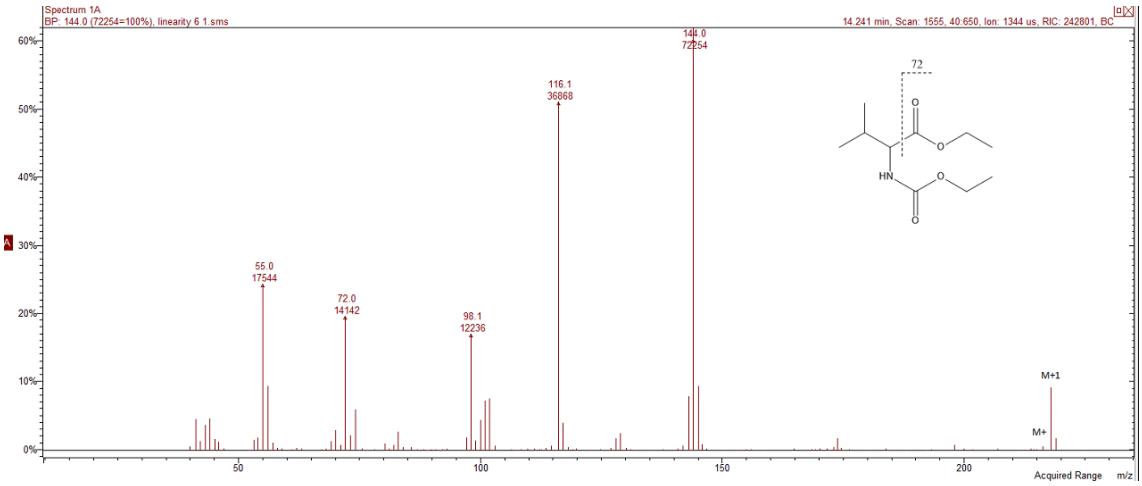

Figure 4.6: Mass spectra used in the identification of valine.

The proposed fragmentation analysis shows the formation of the ions listed in Table 4.1 for valine. The base peak had an m/z value of 144, which resulted from a loss of CO_2Et (m/z 72). The resulting carbocation is resonance stabilised so the positive charge resides on the nitrogen atom. A McLafferty rearrangement results in the loss of an ethylene molecule giving a fragment with m/z 116. A McLafferty rearrangement involves the transfer of a γ hydrogen to a double bonded atom through a six membered ring [156]. The ethylene fragment may be observed but at a molar mass of 28.05 g/mol, its use as a characteristic ion for identification of valine is limited. A second hydrogen transfer results in a fragment with m/z 98 and the loss of water. This carbocation has two potential routes for resonance stabilisation, denoted as (a) and (b) in Figure 4.7. It is likely that resonance structure (b) will be observed more frequently than (a) as carbocations would be less stable than a positively charged oxygen. Carbocations are generally unstable but the structure in (a) will suffer from even greater electron deficiency due to the electronegative nitrogen. Although oxygen also has a positive charge in (b), it is more electronegative than the adjoining carbon and so will pull electrons towards itself; partially stabilising the positive charge.

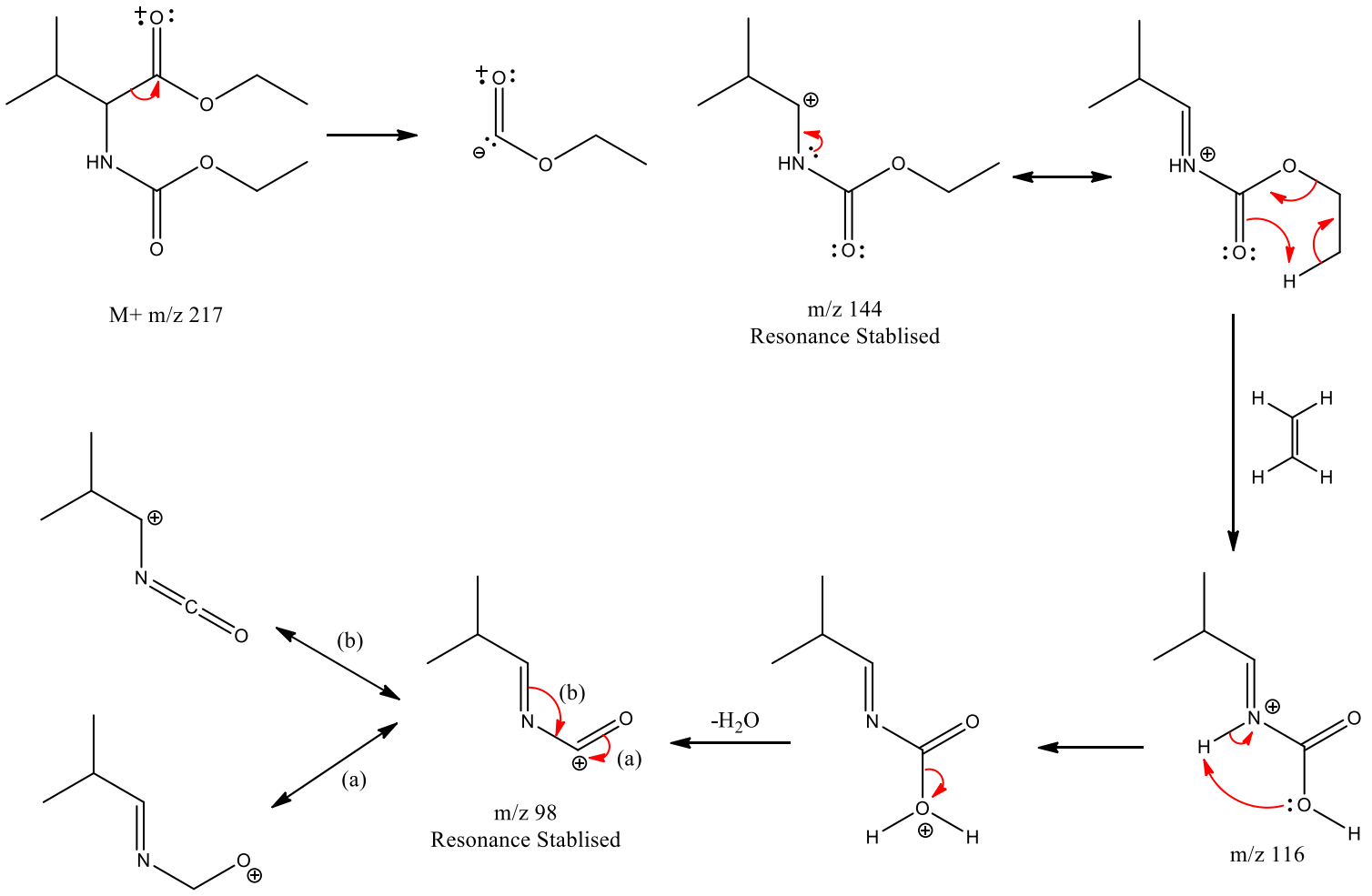

Figure 4.7: Proposed valine fragmentation pattern.

4.4.3.1. Arginine derivatisation

While arginine is also an amino acid, it was not included in this study. Arginine has a side chain consisting of a guanidine group which will not be derivatised by the mechanism described in Figure 4.2 . The structure of arginine can be seen in Figure 4.8.

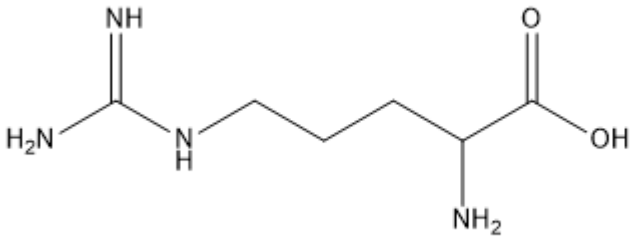

Figure 4.8: Structure of arginine.

Although ECF derivatises the α-amine group it fails to derivatise the guanidine side chain. The pka of guanidine side group is approximately 13.6 [157], meaning it is ionised in milk (pH 6.5). The positive charge is delocalised through resonance, possibly preventing the lone pair of electrons on each nitrogen from taking part in nucleophilic attack on ECF. Additionally, ECF may not be electrophilic enough for this reaction to take place. No partial derivatisation of arginine was detected even though both the α-amino and α-carboxylic groups would have been derivatised. Evidence from literature suggests that the guanidine side group does not elute into the MS due to absorption to the GC column [158].

Efforts to derivatise arginine included the use of glyoxal [159], isovalerylacetone [160], and hexafluoroacetylacetone [161]. Both isovaleroylacetone and hexafluoroacetylacetone have been used in conjunction with ethyl chloroformate and so it was hoped that the combination of one of these derivatisation agents and ECF would allow for detection on all amino acids. Arginine derivatisation using isovalerylacetone and ECF seemed to produce a peak, although with very low intensity.

To investigate if this peak was the derivatised form of arginine and the instability of the fragments produced in electron impact were responsible for the low intensity, chemical ionisation (CI) was used. This is a soft ionisation technique that involves the ionisation of a reagent gas, in this case ACN. The ionised ACN collides with the analyte molecules, ionising them through proton transfer. In CI mode, fragmentation of the analyte molecule is greatly reduced and so should allow for detection of the molecular ion. The product of arginine derivatisation with isovalerylacetone is shown in Figure 4.9. The product as a molecular weight of 382 g/mol. After CI through proton transfer, the expected molecular ion would have an m/z 383.

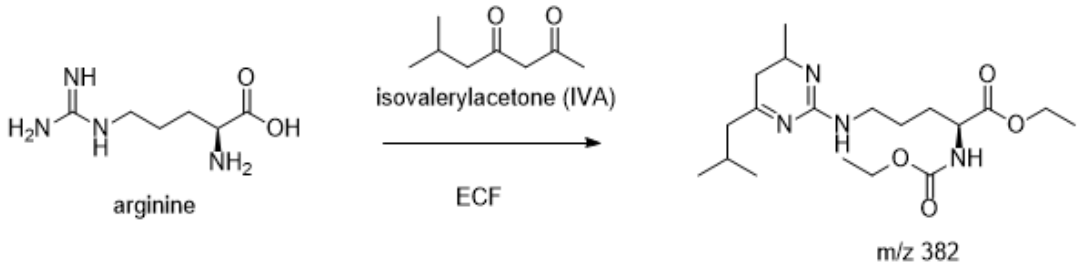

Figure 4.9: Schematic of arginine derivatisation using IVA and ECF.

After performing derivatisation using both IVA and ECF according to Zounr *et al.* [160], the reaction mixture was analysed by GC-MS in CI mode. The overlay of the reaction mixture and the blank show that no peak at m/z 382 was detected. The peak at approximately 29 minutes was found to also be in the blank. All reasonable efforts to include arginine in this study have been explored. Several derivatisation reactions have been explored and the results have been examined in both EI and CI modes. The derivatised form of arginine was not detected. As a result arginine analysis was not included in subsequent studies.

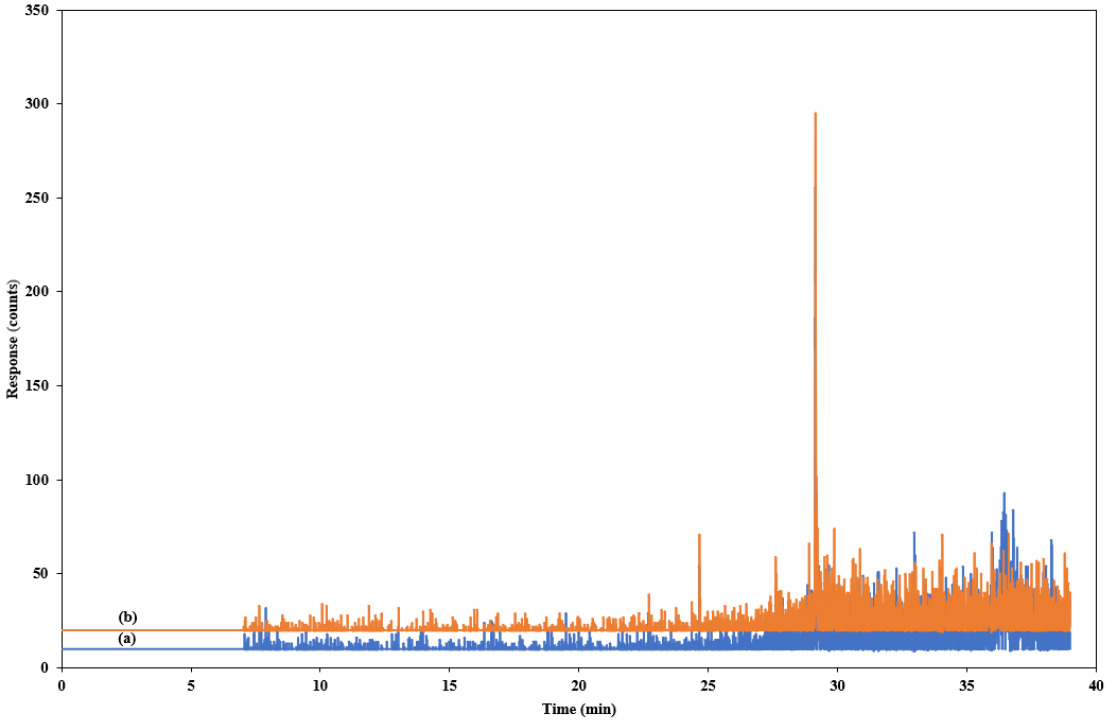

Figure 4.10: Arginine derivatisation in CI mode. (a) blank, (b) arginine derivatisation. Chromatographic conditions Figure 4.4.

4.4.4. Optimisation of derivatisation conditions

The derivatisation process was optimised using Design of Experiments (DoE). The following parameters were examined: ECF concentration, pyridine concentration, and ethanol concentration. As outlined in Section 4.1.4, alkyl chloroformates are seen to be the most conducive derivatisation agents for DLLME. Ethyl chloroformate has been found to be most effective of the alkyl chloroformates tested in literature. Methyl and ethyl chloroformates have generally faster reaction rates due to less steric hindrance compared to bulkier isobutyl groups. Ethyl derivatives would also be more hydrophobic, allowing for more exhaustive extraction into the extraction solvent, than their methyl equivalents. For these reasons ethyl chloroformate was selected as the derivatisation reagent. These factors underwent a full factorial design as a screening experiment to determine which factors were significant for maximising total chromatographic peak area which would be indicative of a more efficient derivatisation. Selecting levels for each factor requires some prior knowledge of the derivatisation reaction. The levels are

selected at the extremes of the experimental space. In the case of derivatisation reactions, it must be ensured that enough reagents are present in all experimental runs to ensure full derivatisation of the analytes. The total concentration of all amino acids used in the optimisation was 0.012 mM. The concentration of ECF at the -1 level was 0.063 mM, which gives an approximate 2.5 fold excess of the derivatisation reagent compared to the total amino acid concentration. This ensures that optimisation would not be skewed by incomplete derivatisation of amino acids. The levels for each factor can be seen in Table 4.2.

Table 4.2: Factors and levels used for screening design.

Factor	**-1**	**+1**
Pyridine (µL)	6	600
Ethanol (µL)	5	500
ECF (µL)	6	600

The resulting pareto chart (Figure 4.11) indicated that the only significant factor was the interaction between pyridine and ECF.

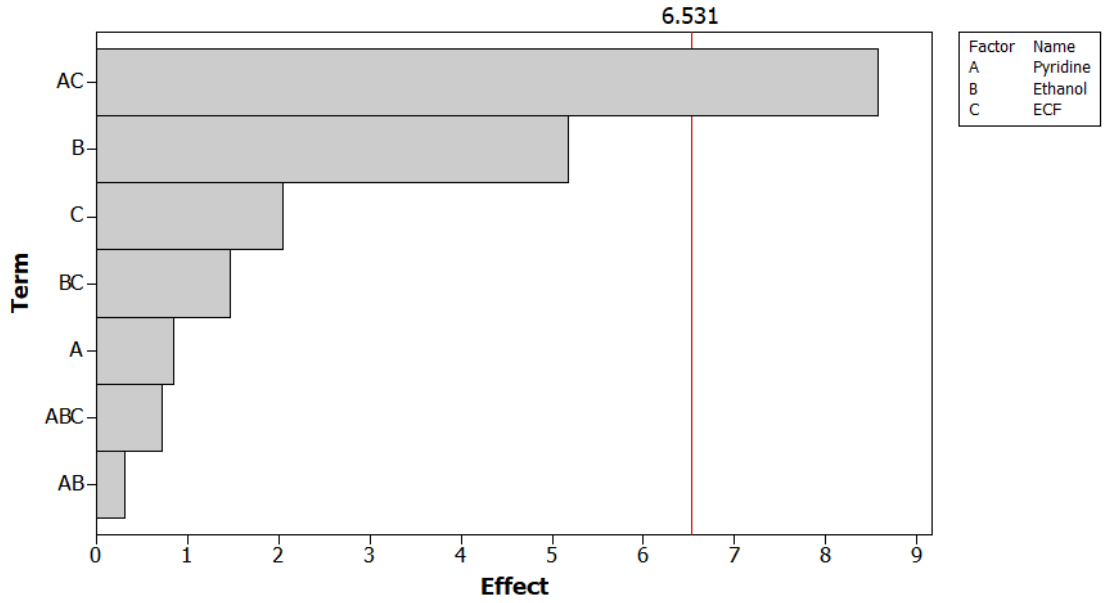

Figure 4.11: Pareto chart showing significant factors found in screening experiment for optimisation of derivatisation conditions.

A central composite design was used to model the interaction between these two factors. The levels used for each factor can be seen in Table 4.3.

Table 4.3: Factors and levels for derivatisation response surface.

Factor	**-α**	**-1**	**0**	**+1**	**+α**
Pyridine (µL)	68.6	100	350	600	848
ECF (µL)	68.6	100	350	600	848

The ANOVA table showed that only ECF volume was significant (p-value = 0.012) while the volume of pyridine used was insignificant (p-value = 0.184). Interestingly, the interaction between ECF and pyridine was determined to be highly significant (p-value =

0.001). As pyridine acts as a catalyst for the derivatisation reaction between ECF and the amino acids, it is appropriate that the concentration of both the catalyst and derivatisation reagent are the most significant factors in this experimental design. The ANOVA table can be seen in Figure 4.12.

Term	Coef	SE Coef	T	P
Constant	13.9324	0.8007	17.400	0.000
Pyridine	0.9323	0.6330	1.473	0.184
ECF	2.1374	0.6330	3.377	0.012
Pyridine*Pyridine	-3.4970	0.6788	-5.152	0.001
ECF*ECF	-2.4592	0.6788	-3.623	0.008
Pyridine*ECF	5.1822	0.8952	5.789	0.001

Figure 4.12: ANOVA table for amino acid derivatisation response surface.

The response surface produced indicated that maximum response was achieved when values for ECF and pyridine were selected at the +1 level. As illustrated in Figure 4.13 both ECF and pyridine need to be selected at their maximum level to ensure complete derivatisation.

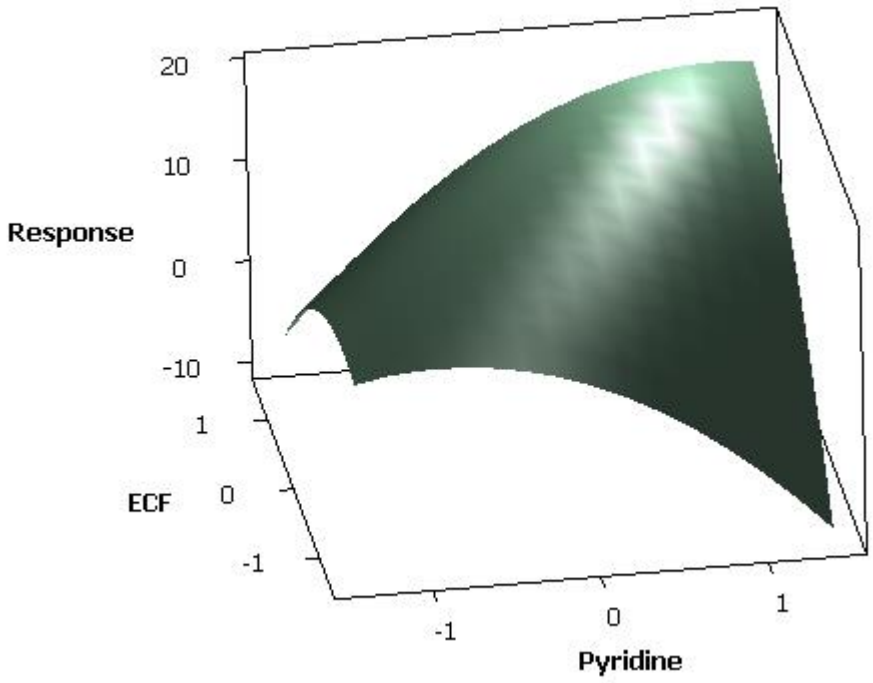

Figure 4.13*:* Response surface for derivatisation optimisation.

4.4.5. Optimisation of DLLME procedure

4.4.5.1. Selection of extraction solvent

Chloroform, DCM, and ethyl acetate were trialled as potential extraction solvents. LDS-DLLME was not evaluated in this study as typical extraction solvents for this technique have high boiling points, which would be unsuitable for GC analysis. A comparison of potential extraction solvent boiling points can be seen in Table 4.4.

Table 4.4: Boiling points of potential extraction solvents.

Solvent	Boiling point (°C)	Density (g/cm^3)
Dichloromethane	39	1.33
Chloroform	69	1.49
Heptanol	175	0.82
Octanol	194	0.82

To evaluate the effect of each solvent, analytes were derivatised and then extracted with 200 µL of the selected solvent and the ratio of IS and peak areas were compared. Ethyl acetate did not produce phase separation and so was discarded. Chloroform and DCM extracts were prepared in triplicate and each extract was then injected in triplicate, giving n=9. The sum ratio of analyte area and IS area for each extract was calculated and used in a t-test to determine if there was any significant difference between extraction solvents. The null hypothesis stated that there was no significant difference between the extraction solvents. The t-test returned a value of 0.470 which indicated that no significant difference

existed in the extraction efficiencies between chloroform and DCM ($\alpha = 0.05$). The ratio of each analyte and IS peak areas did not differ significantly as shown in Figure 4.14. Chloroform was used as the extraction solvent for the remainder of method development due to its greater density (1.49 g/cm^3) compared to DCM (1.33 g/cm^3). The greater density of chloroform could result in better phase separation during centrifugation; leading to more quantitative analyte extraction.

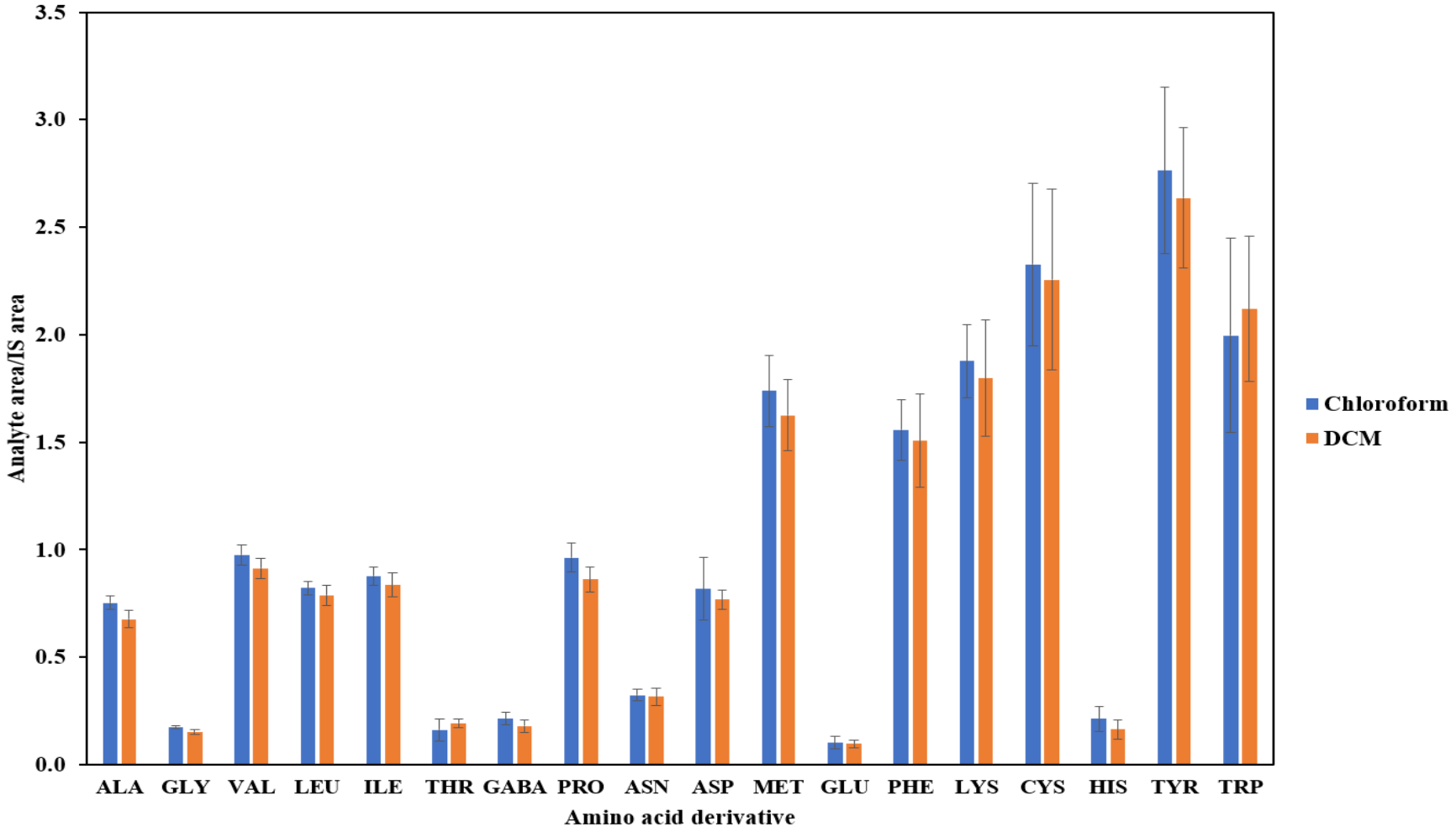

Figure 4.14: Comparison of extraction solvents.

4.4.5.2. Factorial screening

The DLLME process was optimised by DOE. A ½ factorial design with 3 centre points was used to screen for significant factors. The list of factors and levels can be found in Table 4.5. The levels chosen were based on preliminary experiments to determine what combination of factors led to a stable cloudy solution. Values outside these ranges did not produce a stable emulsion or the volume of extraction solvent was too low to allow for practical recovery GC analysis. Acetonitrile was selected as the dispersive solvent as it was used to precipitate the proteins.

Table 4.5: List of factors and levels for DLLME screening.

Factor	**-1**	**0**	**+1**
Chloroform (µL)	100	300	500
ACN (mL)	2	2.75	3.5
H_2O (mL)	5	7.5	10
Sonication time (min)	0	2.5	5

The results of the screening experiment showed that the volume of ACN, the sonication time, and the 2-way interaction between the volume of chloroform used and sonication time were significant. The pareto chart from the screening experiment can be seen in Figure 4.15. The volume of dispersive solvent (ACN) was determined to be the most significant factor (factor B). As the dispersive solvent is soluble in both the extraction and aqueous phases the volumes of this solvent used has a great effect on the stability of the cloudy solution. Interestingly the volume of extraction solvent (factor A) by itself was

not significant. It is only the higher interactions between the extraction solvent and sonication time that are shown to be significant. It is likely that the ultrasonic waves produce a greater number of micro droplets allowing for faster partitioning of analytes into the extraction phase. This effect has been termed ultrasonic assisted DLLME and examples of this phenomenon can be seen in Section 1.2.2. The ability of DoE experiments to detect higher interactions between factors is likely the most important application of this technique. Screening factors using a one-at-a-time approach may have resulted in this interaction not being detected.

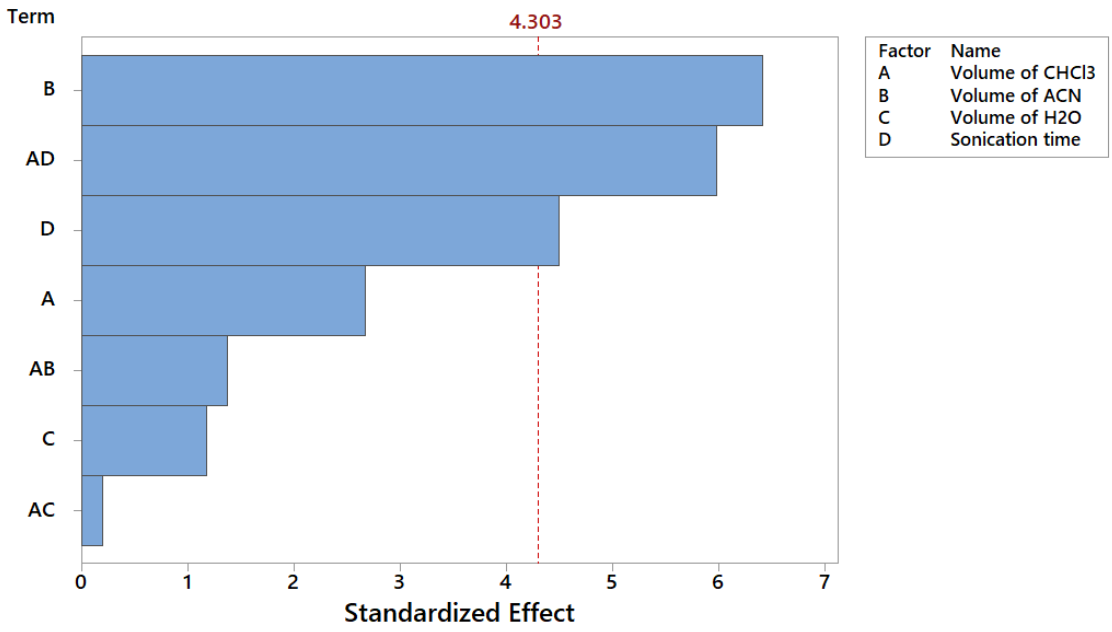

Figure 4.15: Pareto chart for DLLME screening showing significant factors.

The p-values for each factor and interaction between factors can be seen in Figure 4.16. These values mirror the results from the Pareto Chart Figure 4.15, showing only volume of ACN, sonication time, and volume of chloroform*sonication time as the significant factors. Additionally, the ANOVA table provides information on the linearity of the relationships between factors. The inclusion of centre points in the screening design allows for the detection of curvature. As the p-value for curvature is greater than 0.05, it is concluded that all relationships between factors are linear.

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	8	72.4397	9.0550	13.65	0.070
Linear	4	46.4270	11.6067	17.50	0.055
Volume of CHCl3	1	4.7447	4.7447	7.15	0.116
Volume of ACN	1	27.3393	27.3393	41.22	0.023
Volume of H2O	1	0.9241	0.9241	1.39	0.359
Sonication time	1	13.4188	13.4188	20.23	0.046
2-Way Interactions	3	25.0280	8.3427	12.58	0.075
Volume of CHCl3*Volume of ACN	1	1.2427	1.2427	1.87	0.305
Volume of CHCl3*Volume of H2O	1	0.0252	0.0252	0.04	0.863
Volume of CHCl3*Sonication time	1	23.7602	23.7602	35.82	0.027
Curvature	1	0.9847	0.9847	1.48	0.347
Error	2	1.3266	0.6633		
Total	10	73.7663			

Figure 4.16: ANOVA table of screening experiment.

4.4.5.3. Response surface

The significant factors that were determined in the screening experiment were further investigated by response surface design. The response surface design that was used was a Central Composite Design. The list of factors and levels that were used can be seen in Table 4.6

Table 4.6: Factors and levels for response surface.

Factor	**-α**	**-1**	**0**	**+1**	**+α**
ACN (mL)	0.89	1.5	1.88	2.26	3.8
Chloroform (µL)	100	168	334	500	840
Sonication (min)	0	1	3	5	8.4

Upon statistical analysis of the response surface data, it was found that the sonication time was not significant in the central composite design, and so it was removed along with any higher interactions involving sonication time. At times, factors can appear significant in the initial screening experiments due to confounding factors. Once the experimental space had been explored in detail using a response surface methodology, it appeared that sonication time was not significant. The analysis from Minitab can be seen in Figure 4.17. The highlighted row in this figure shows sonication time with a p value greater than 0.05, which indicated that it was not a significant factor.

Coded Coefficients

Term	Effect	Coef	SE Coef	T-Value	P-Value	VIF
Constant		10.759	0.753	14.29	0.000	
ACN volume	-4.453	-2.227	0.499	-4.46	0.001	1.00
CHCl3 volume	3.592	1.796	0.499	3.60	0.005	1.00
Sonication time	-1.546	-0.773	0.499	-1.55	0.153	1.00
ACN volume*ACN volume	-1.305	-0.653	0.486	-1.34	0.209	1.02
CHCl3 volume*CHCl3 volume	0.267	0.133	0.486	0.27	0.789	1.02
Sonication time*Sonication time	-0.754	-0.377	0.486	-0.78	0.456	1.02
ACN volume*CHCl3 volume	4.009	2.005	0.653	3.07	0.012	1.00
ACN volume*Sonication time	-2.565	-1.282	0.653	-1.96	0.078	1.00
CHCl3 volume*Sonication time	1.047	0.523	0.653	0.80	0.441	1.00

Figure 4.17: Minitab analysis for 3 factor central composite design.

Sonication time and any higher order factor that included sonication time was removed from the analysis, which left only ACN and chloroform volumes as the significant factors. The experimental data was once more analysed by Minitab and the ANOVA table can be seen in Figure 4.18. The ANOVA table shows that both ACN and chloroform factors are significant, along with ACN volume*$CHCl_3$ volume also showing significance. The Lack-of-Fit test, which determines if the generated response surface methodology accurately maps the experimental space between the limits described. As the Lack-of-Fit is insignificant (p value = 0.518) it can be assumed that the response surface describes the experimental space accurately.

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	5	149.545	29.9089	7.01	0.002
Linear	2	111.301	55.6506	13.05	0.001
ACN volume	1	67.262	67.2616	15.77	0.001
CHCl3 volume	1	44.040	44.0397	10.33	0.006
Square	2	6.091	3.0456	0.71	0.507
ACN volume*ACN volume	1	5.357	5.3575	1.26	0.281
The CHCl3 volume*CHCl3 volume	1	0.415	0.4148	0.10	0.760
2-Way Interaction	1	32.152	32.1522	7.54	0.016
ACN volume*CHCl3 volume	1	32.152	32.1522	7.54	0.016
Error	14	59.707	4.2648		
Lack-of-Fit	9	38.747	4.3052	1.03	0.518
Pure Error	5	20.960	4.1920		
Total	19	209.251			

Figure 4.18: ANOVA table for response surface design.

The resulting response surface plot for chloroform and ACN volume can be seen in Figure 4.19. The response surface plot indicated that the response was maximised when the factors were set at their minimum values.

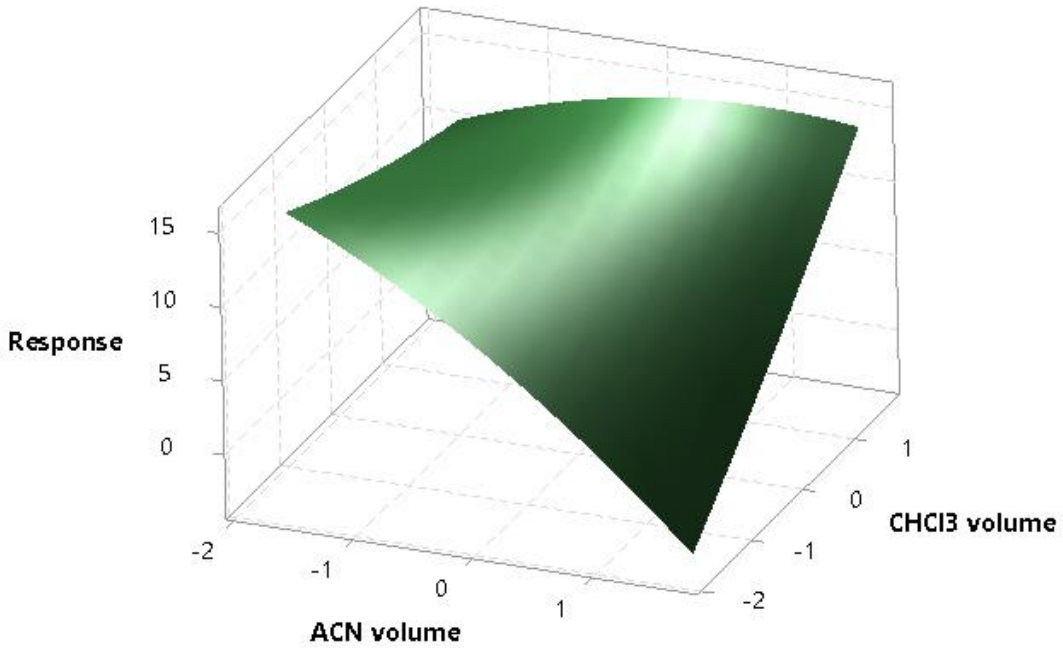

Figure 4.19*:* Response surface plot for DLLME optimisation.

The regression equation for this response surface can be used to determine what the response would be if the factor values were different than those selected in Table 4.6. The regression equation can be seen in Figure 4.20. This equation can be used to predict the response once the values chosen are inside the experimental limits.

```
Response = 10.449 - 2.219 ACN volume + 1.796 CHCl3 volume - 0.607 ACN volume*ACN volume
           + 0.169 CHCl3 volume*CHCl3 volume + 2.005 ACN volume*CHCl3 volume
```

Figure 4.20: Regression equation for response surface.

4.4.6. Linearity, repeatability, LOD, LOQ, and EF

The analytical method was validated by assessing linearity, repeatability, limit of detection (LOD), and limit of quantification (LOQ), according to ICH guidelines for these parameters. The LOD and LOQ were calculated by multiplying the ratio of standard deviation of the response and slope of the calibration curves by 3.3 and 10 [162], respectively.

The method response was found to be linear for all analytes between 0.50 and 9.12 ppm. Recoveries for analytes ranged from 56 – 108%. Interday reproducibility ranged from 5.72 – 10.27% RSD. The LOD ranged from 0.37 – 0.84 ppm while the LOQ ranged from 1.02 – 2.56 ppm.

Table 4.7: Linearity, repeatability, LOD, LOQ, and reproducibility.

Analyte	Recovery (%)	LOD (ppm)	LOQ (ppm)	Linearity (R^2)	Reproducibility (RSD)	EF
Alanine	95.95	0.84	2.56	0.9883	9.5	15
Glycine	108.04	0.56	1.71	0.9889	10.3	19

Analyte	Recovery (%)	LOD (ppm)	LOQ (ppm)	Linearity (R^2)	Reproducibility (RSD)	EF
Valine	56.58	0.72	2.18	0.9766	9.3	22
Leucine	83.46	0.45	1.37	0.983	9.0	17
Isoleucine	68.82	0.31	0.95	0.9919	10.1	15
Threonine	104.63	0.82	2.54	0.9899	8.9	24
GABA	107.56	0.34	1.06	0.9846	9.1	8
Proline	72.18	0.82	2.54	0.9819	9.3	14
Asparagine	102.73	0.66	1.99	0.9822	10.4	20
Aspartic acid	89.63	0.33	1.02	0.9977	12.8	19
Glutamine	91.49	0.51	1.51	0.9859	10.1	21
Methionine	75.43	0.81	2.4	0.9864	6.9	16
Glutamic acid	82.63	0.49	1.49	0.9946	7.8	20
Phenylalanine	100.97	0.36	1.11	0.9894	9.4	18
Lysine	93.44	0.59	1.79	0.9861	7.7	10
Cysteine	115.17	0.52	1.58	0.9859	10.3	19

Analyte	Recovery (%)	LOD (ppm)	LOQ (ppm)	Linearity (R^2)	Reproducibility (RSD)	EF
Histidine	88.74	0.41	1.25	0.9891	6.6	25
Tyrosine	77.64	0.21	0.65	0.9983	9.6	11
Tyrptophan	97.74	0.63	1.91	0.9889	10.9	14

The LODs were calculated from the calibration curves. An example of the chromatography for phenylalanine can be seen in the below Figure 4.21

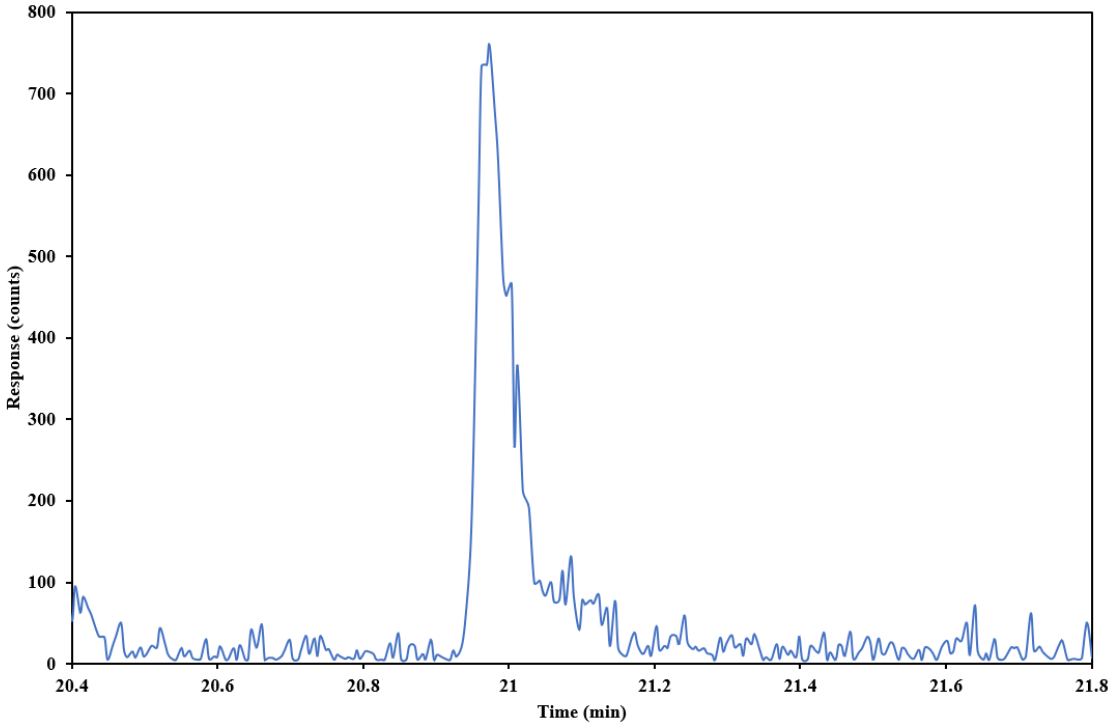

Figure 4.21: LOD of phenylalanine. Chromatographic conditions as outlined in Figure 4.4.

4.4.7. Comparison with published methods

The validated and optimised method has been compared to recently published methods for the analysis of amino acids in dairy products. The comparison can be seen in Table 4.8. Li *et al*. used SFO-DLLME to quantify amino acids found in tobacco leaves and analysed them by GC-MS [154]. The DLLME procedure was optimised using a "one factor at a time approach". The extraction solvent used was 2-dodecanol, this solvent has a boiling point of 252 °C. To avoid the solvent peak masking the analytes eluting at the same temperature, the MS was in selected ion monitoring (SIM) mode. While this is a useful detection mode, it also poses a risk of damaging the sensitive ionisation filaments in the MS instrument. Only the ions selected (which are chosen to represent specific analytes/derivatives) will be observed in the mass spectrum, however all ions are produced; potentially allowing large concentrations of ions to saturate the filament in the ionisation source in the MS. This is even more critical when analysing complex biological fluids, like milk, where there are many unknown compounds that could damage the MS.

Ehling and Reddy used limited sample pre-treatment to preconcentrate and extract leucine from breast milk [163]. This group opted for methanolic protein precipitation before transferring the acidified supernatant for analysis by HPLC-MS, specifically a triple quad MS. Although using a sensitive detector, the LOD obtained was relatively high. This may be due to the lack of preconcentration of leucine. Ehling and Reddy did not use DLLME to preconcentrate and extract leucine from breast milk [163]. This group opted for methanolic protein precipitation before transferring the acidified supernatant for analysis by HPLC-MS, specifically a triple quad MS. Although using a sensitive detector, the LOD obtained was 1 mg/L. This may be due to the lack of preconcentration of leucine.

By contrast, Mudiam and Ratnasekhar, combined DLLME and triple quad MS detection for analysis of amino acids in hair, soybean, and urine samples [164]. The DLLME method was optimised using a DoE approach. The factors were screened using a Placket-Burman design followed by a Central Composite Design. It was determined that 80 µL of TCE (extraction solvent), 0.25 mL of ACN (dispersive solvent), and a pH of 10 were found to produce maximum peak area. The combination of DLLME, optimised by DoE,

and a triple quad MS detector resulted in 0.36 – 3.68 µg/L LOD values for 20 amino acids. The use of a triple quad MS resulted in lower LOD than was achieved in this work.

The work presented in this chapter analysed amino acids in commercial bovine milk using GC-MS. This process was optimised using a factorial screening design and a central composite design to determine the significant factors that resulted in the most efficient extraction. The critical factors determined were ACN volume (dispersive solvent) and chloroform volume (extraction solvent). In conducting screening experiments for this work, it was found that pH did not significantly affect the derivatisation reaction.

Table 4.8: Comparison with published methods for free amino acid analysis.

Sample	Analyte	Derivatisation agent	Derivatisation time (min)	LOD (ppm)	Reference
Tobacco	11 amino acids	Isobutyl chloroformate	<1	0.12-2.82	[154]
Breast milk	Leucine	None	N/A	1	[163]
Hair, soybean seeds, urine	20 amino acids	Ethyl chloroformate	<1	0.00036-0.0037	[155]
Bovine milk	20 amino acids	Ethyl chloroformate	<1	0.37-0.84	Presented work

4.5. Sample analysis

Commercial milk samples were purchased in a local shop and analysed according to the method detailed in Section 4.3.4. Commercial milk samples included: protein milk (full fat milk with added whey and casein protein), full fat milk, and slimline milk (0% fat). Each milk sample was analysed, on the day of purchase (t_0) to determine if the storage of

bovine milk resulted in a significant difference of the amino acid profile. The commercial milk samples were then placed at 4 °C and the amino acid profile determined on the sample expiration date (t_1), 10 days later. The results of the analysis can be seen in Table 4.9 and typical sample chromatograms can be seen in Figure 4.22. The peaks eluting after phenylalanine were identified using the mass spectral library. They were identified as various siloxanes, whose formation has been described in Section 4.4.1.

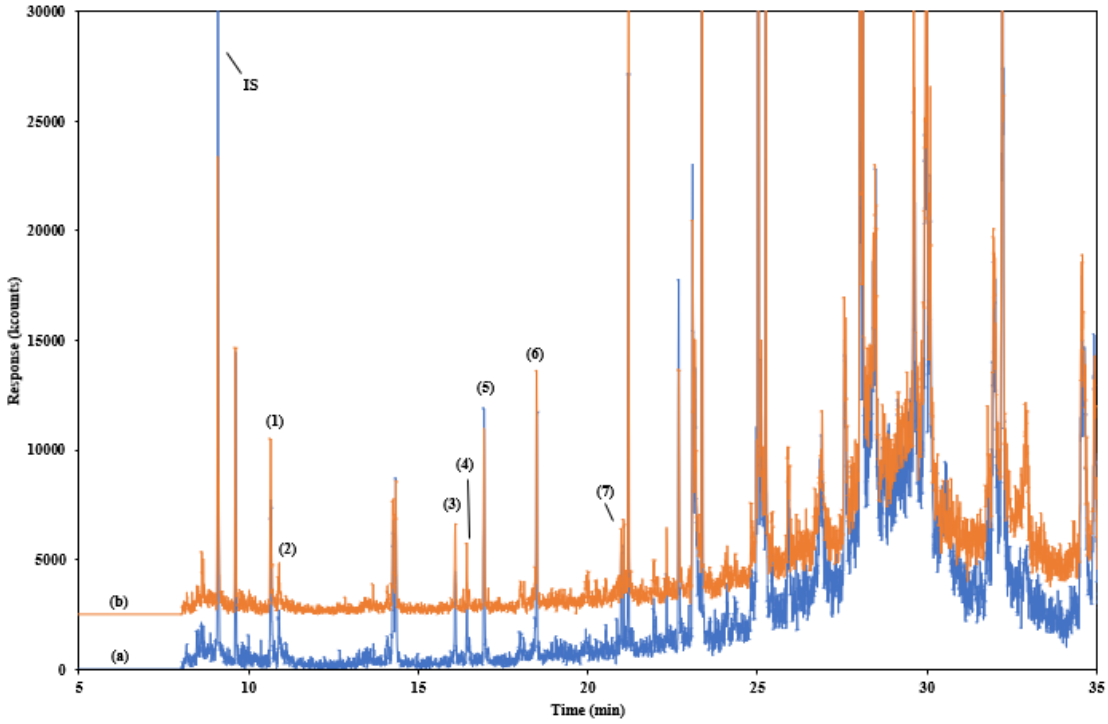

Figure 4.22: Sample chromatograms in EIC mode (a) full fat milk t_0, (b) full fat milk t_1. Peak identification: (IS) internal standard, (1) alanine, (2) glycine, (3) leucine, (4) isoleucine, (5) proline, (6) glutamic acid, (7) phenylalanine. Chromatographic conditions as outlined in Figure 4.4

Significant differences were determined by a t-test with the confidence interval set at 95%. At t_0, there was no significant difference in the selected amino acid concentrations between the samples. Significant differences started to emerge at t_1. Concentrations of alanine showed a significant increase in protein milk, full fat milk, and slimline milk after storage , which could possibly be due to casein and whey proteins undergoing proteolysis. Glycine concentrations appeared to decrease but a p value could not be determined as

glycine levels at t_1 were below the LOQ. Glutamic acid showed a significant increase in the protein milk sample and the full fat milk sample. All other amino acids showed no significant differences from t_0.

Table 4.9: Selected free amino acids in commercial milk products.

Sample	Alanine (ppm)			Glycine (ppm)			Leucine (ppm)			Isoleucine (ppm)			Proline (ppm)			Glutamic acid (ppm)			Phenylalanine (ppm)		
	t_0	t_1	p-value	t_0	t_1	p-value	t_0	t_1	p-value	t_0	t_1	p-value	t_0	t_1	p-value	t_0	t_1	p-value	t_0	t_1	p-value
Protein milk (n=9)	1.48	2.59	0.01	0.98	Below LOQ	N/A	1.68	1.62	0.13	1.32	1.37	0.67	1.92	2.01	0.12	0.94	1.19	0.003	0.89	0.94	0.07
Full fat milk (n=9)	1.42	1.51	0.02	1.05	Below LOQ	N/A	1.67	1.63	0.09	1.38	1.34	0.42	1.97	2.10	0.09	0.99	1.16	0.04	0.95	0.98	0.20
Slimline milk (n=9)	1.43	1.52	0.04	1.09	Below LOQ	N/A	1.68	1.67	0.10	1.29	1.32	0.34	1.82	1.99	0.11	1.02	1.11	0.55	0.91	0.94	0.12

4.6. Conclusion

A rapid derivatisation method coupled with DLLME was developed for the analysis of free amino acids in bovine milk. Both the derivatisation and DLLME processes were optimised using DoE. For the derivatisation of amino acids using ECF, pyridine, and ethanol; only ECF and pyridine were found to be significant. Values at the maximum levels (600 µL) of the experimental design were determined to give maximum peak area. DLLME was also optimised using DoE, and volumes of extraction solvent and dispersive solvent were found to give greater peak area when dispersive solvent was at its maximum (2 mL) and extraction solvent at its minimum (100 µL). The analytes were separated and identified using GC-MS. The newly developed method was compared to previously published methods for free amino acid analysis and has been shown to offer faster derivatisation times and/or lower LOD values than using isobutyl chloroformate and lower LOD than HPLC-MS.

The method was used to determine the free amino acid profile in commercial milk samples and the effect of proteolysis on storage of these samples. While no significant differences were found between samples at initial testing (t_0), differences were detected for 3 amino acids (alanine, glycine, and glutamic acid).

5. Future work

In the short term, the work presented in Chapter 2 and 3 could be further strengthened by increasing the sample size in each study. The focus of this thesis was the development of a DLLME procedure for the extraction and preconcentration of selected compounds from bovine milk. The number of cows used provided adequate statistical significance for the purpose of showing DLLME applicability to real world samples.

Chapter 2 investigated the effect of supplementation of cow feed with seaweed on the tocopherol content in milk. The study was conducted on 12 cows: 6 cows had their feed supplemented with seaweed, while another group of 6 cows were used as the control. While a positive significant difference was detected in tocopherol content between groups, it would be important to see if the results were replicated over a larger population size. Also of interest would be the seasonal variability of the seaweed due to climate conditions and weather. This would have an effect on the tocopherol content found in bovine milk. In addition, other possible studies include: investigating the level of supplementation with different quantities of seaweed. Chapter 3 investigated the relationship between the fatty acid profile of bovine milk and the cow's body condition score and lactation cycle. Again, while the sample size was adequate to prove DLLME could be applied to real world samples; the sample size was not large enough to be considered as robust animal trial.

The methods developed in Chapter 4 could be further developed to include arginine and serine derivatisation. Derivatisation by alkyl chloroformates still presents an attractive derivatisation method. However, sensitivity could be increased further by developing similar halogenated derivatisation reagents. Analysis could then be carried out in EIC or SIM mode for the detection of the halogenated derivative.

In the longer term, DLLME has the potential to be applied to the interface between chemistry and biology. Ionic liquids have been used to extract and quantify DNA from complex matrices using real-time PCR, and have been proven to be quicker than traditional methods. [165,166]. Traditional methods to purify DNA are time consuming and labour intensive. These methods include: phenol-chloroform liquid-liquid extraction, sonication, and enzyme degradation. Ionic liquids allow for a selective DNA extraction which do not interfere with real-time PCR analysis. In 2013, Li *et al.* used IL-DLLME

for the extraction and quantification of DNA from DNA solutions [167]. This preliminary work was carried out using a model solution consisting of an aqueous solution of DNA spiked with albumin and various metal ions to asses any matrix interferences.

An optimised IL-DLLME extraction method for DNA analysis from real samples presents an interesting application to the techniques discussed in this thesis. An interdisciplinary project could involve the development of a rapid DNA extraction protocol and then apply it to areas such as biocatalysis or ecology. The method may have the potential to allow rapid identification of genes that show promising enantioselectivity for enantiomers of pharmaceutical value. While in ecology, the method may aid in identifying what species has been active in the area through analysis of faeces, for example.

While DLLME by itself has the potential to reduce analysis time, there is scope to automate the process. The development of an automated IL-DLLME protocol and its application to an area other than analytical chemistry could provide an interesting avenue for further research. The automation of such processes has already been demonstrated in similar areas, e.g. the automation of derivatisation procedures for gas and liquid chromatographic applications [168–170]

For example, Duong *et al.* have shown that a microfluidic platform can be used for the rapid derivatisation of lipids into FAMEs [171]. The lipids are derivatised using methanolic HCl and the reaction took place in 6 minutes. This method has yet to be applied to a milk sample but presents an interesting avenue for further exploration. Some pretreatment would still need to occur, to remove proteins for example.

Sensors have been used to analyse compounds in a variety of food and biological matrices [172,173]. An *in-situ* bismuth-film electrode has been used for the determination of endocrine disrupters in skimmed milk [174]. The sensor was placed directly into the sample and the endocrine disruptor was analysed at an elevated concentration. There is potential to expand this technique to include the analyse of amino acids in milk.

Automated sample preparation techniques have been developed which use solvent terminated DLLME. Guo *et al.* have used the autosampler on a GC-MS for the analysis of phthalate esters in water samples [175]. Phthalates have also been the subject of considerable analysis in dairy products [176–178]. The development of an automated

DLLME protocol for phthalates in milk sample would greatly reduce sample preparation times which would be of benefit to quality control laboratories

Moving away from DLLME, other interesting microextraction techniques are emerging that could be applicable analysis of trace compounds in complex matrices. One such example is headspace water-based liquid-phase microextraction. This is a green extraction technique, that is completely solvent free and has previously been used for the analysis of organic acids in wastewater [179,180]. As this is a headspace extraction technique it has the potential to eliminate any matrix interferences, such as proteins. This technique could be applied to volatile organic acids present in dairy samples. As the extraction "solvent" is sodium hydroxide, this technique could be easily coupled to an ion chromatography system using a conductivity detection system. It is hoped that this process could also be automated.

6. References

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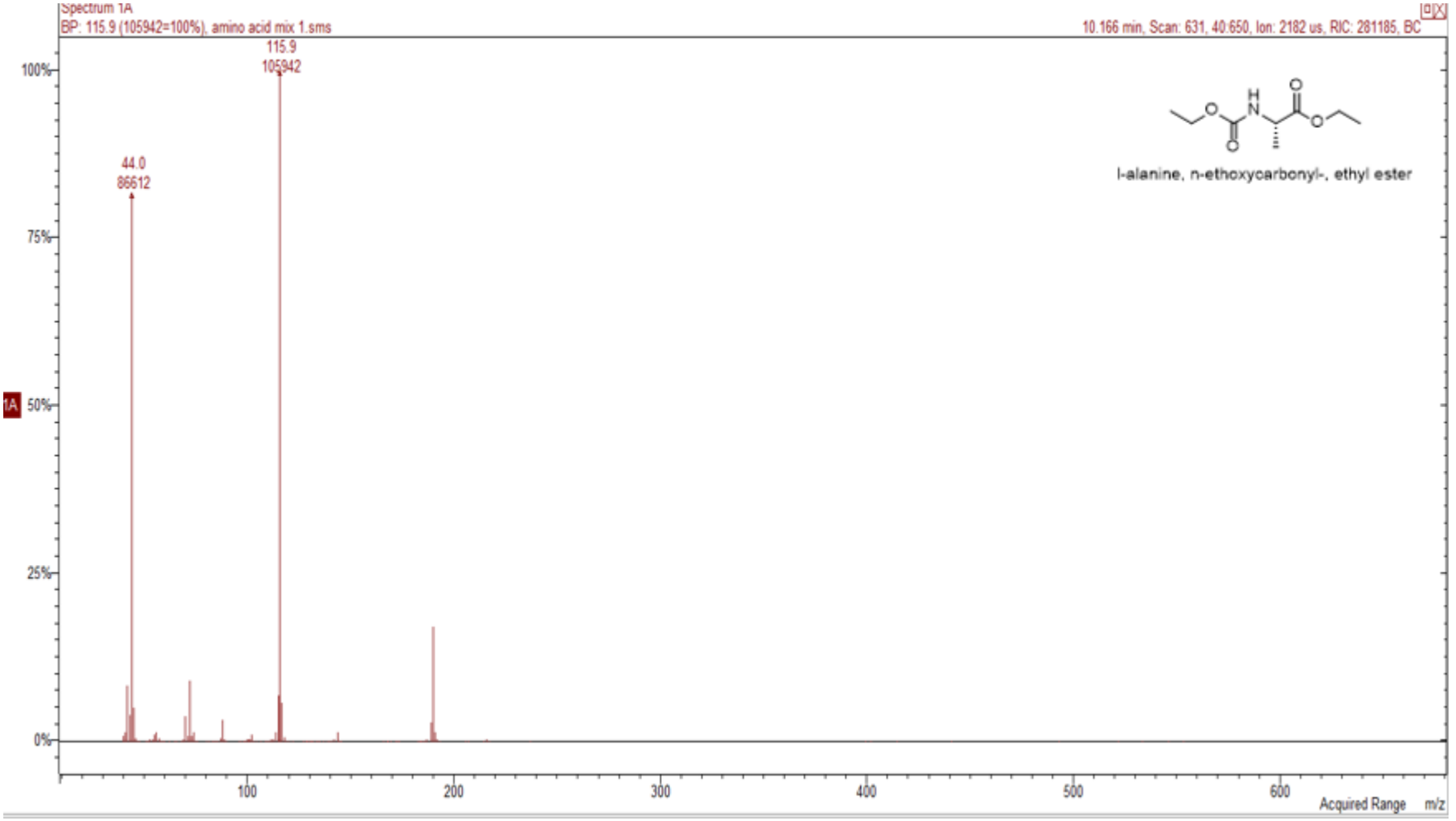
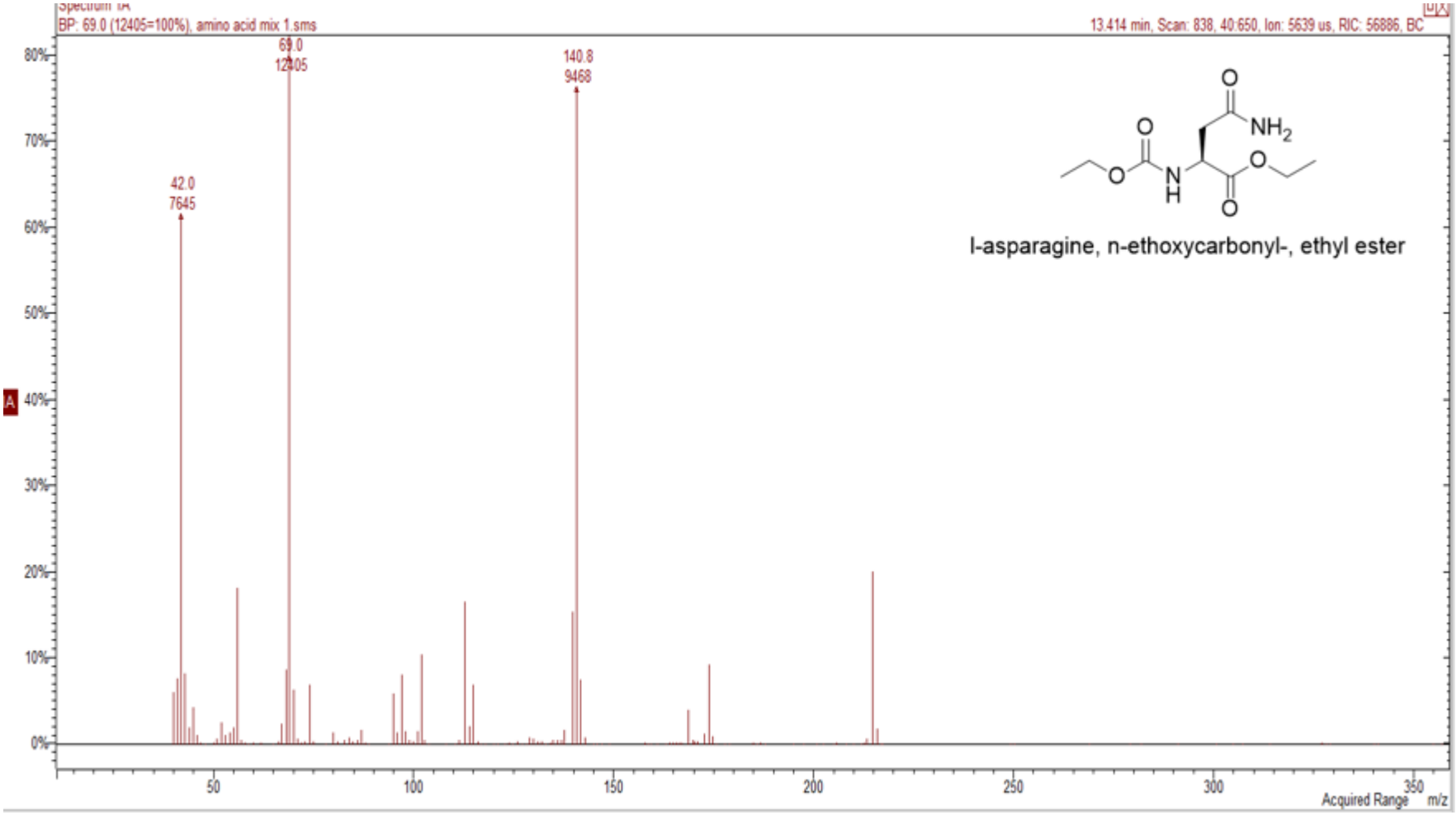
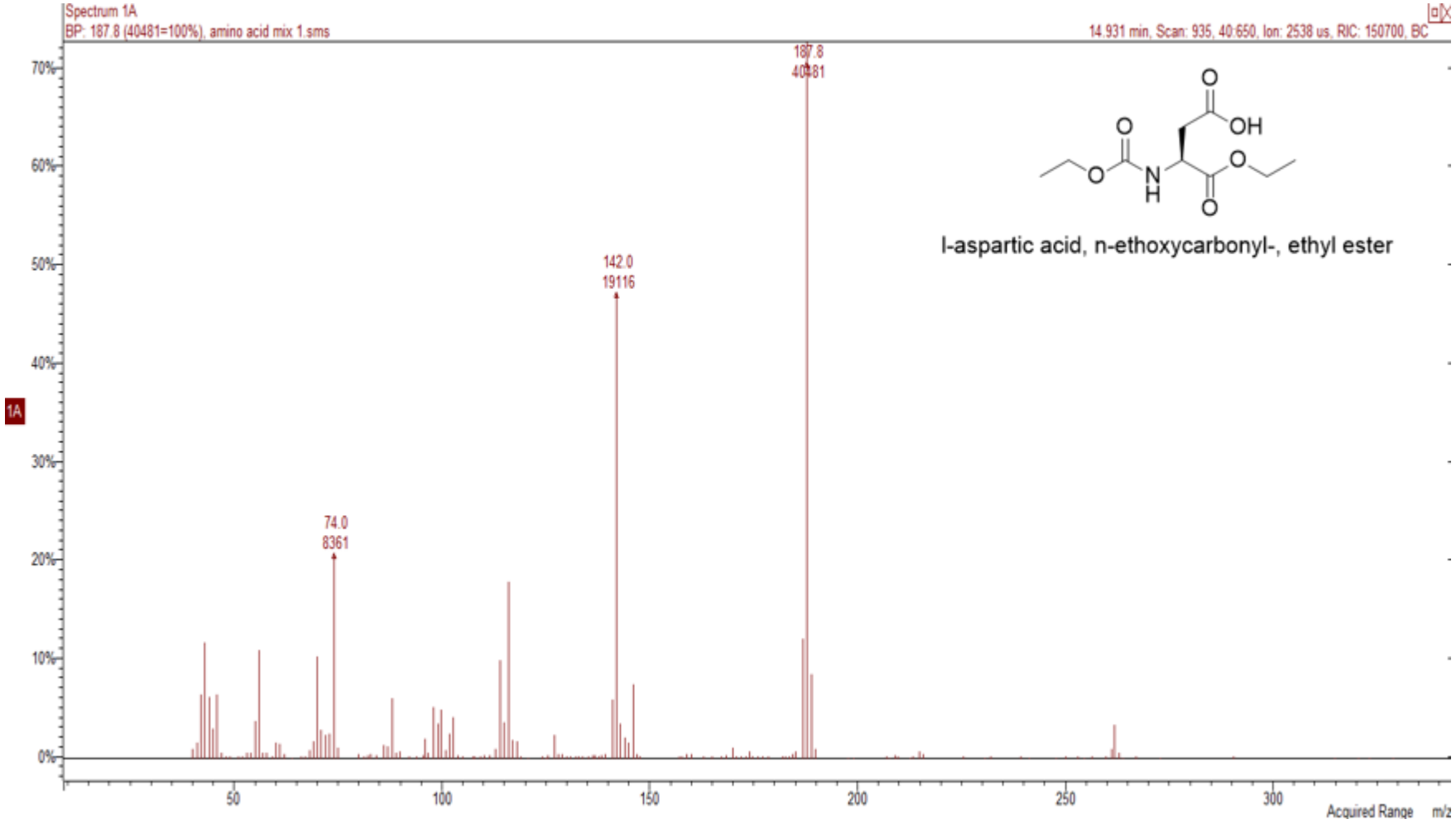
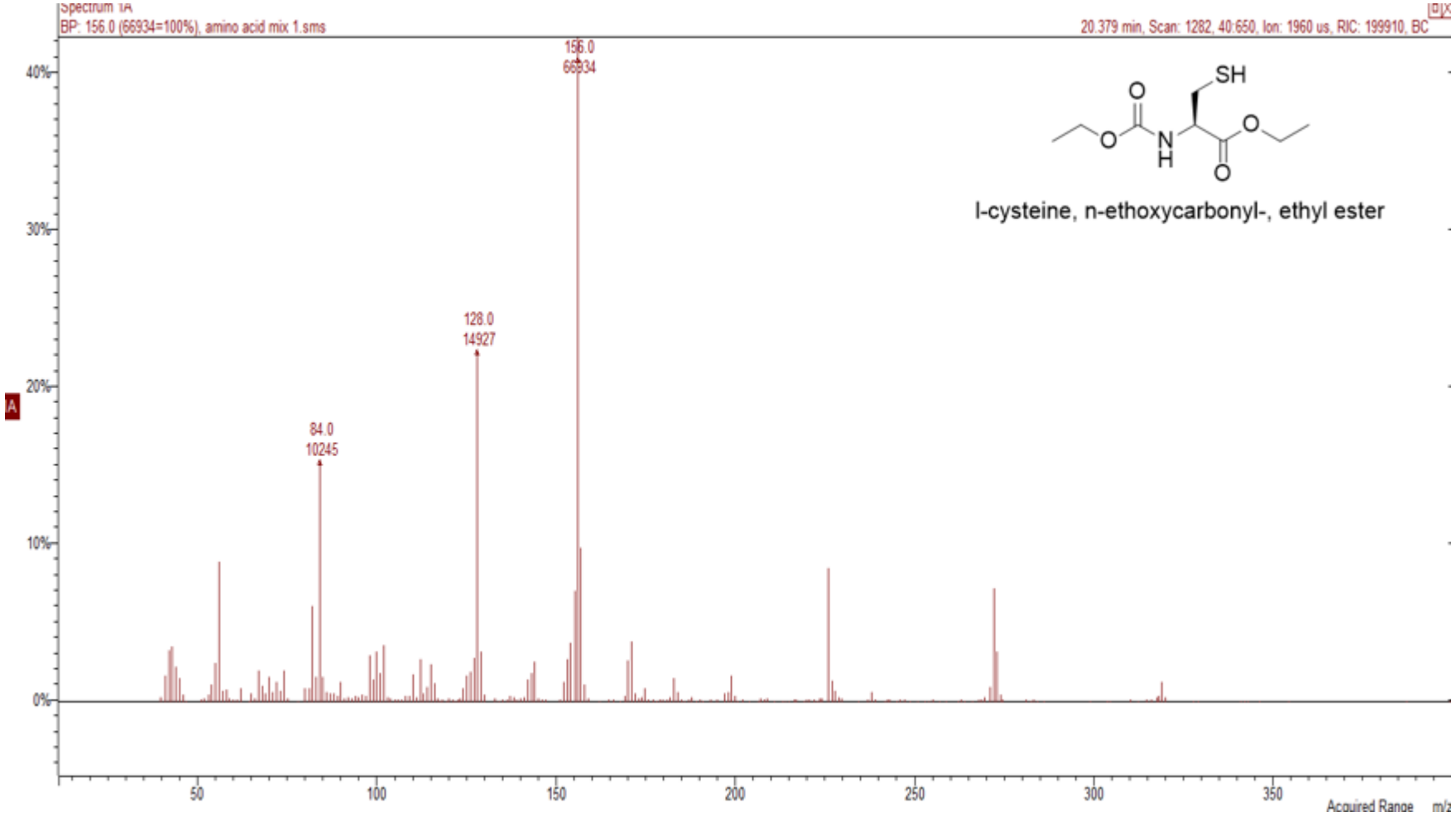
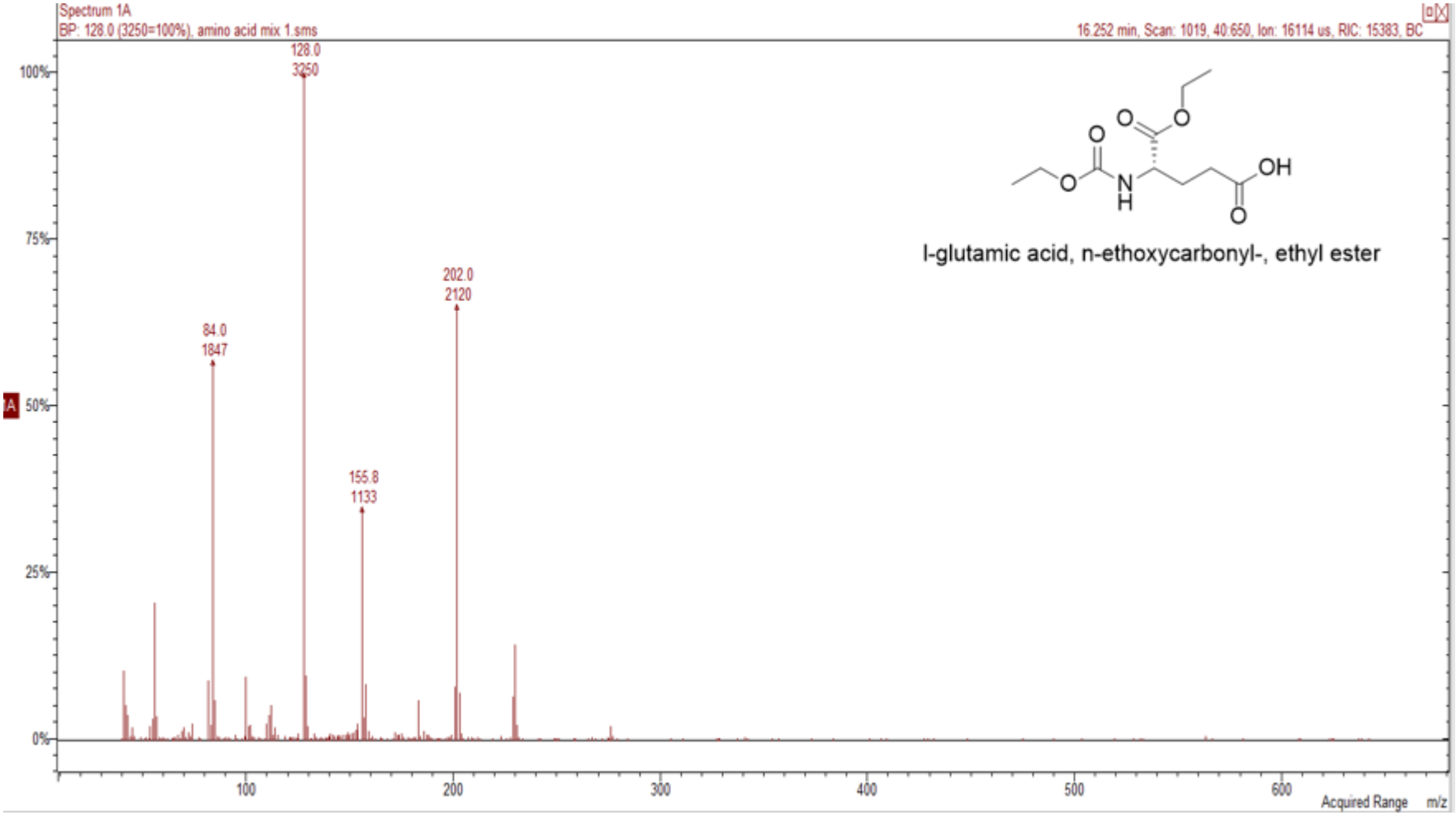
Appendix 1: Mass spectra of derivatised amino acids

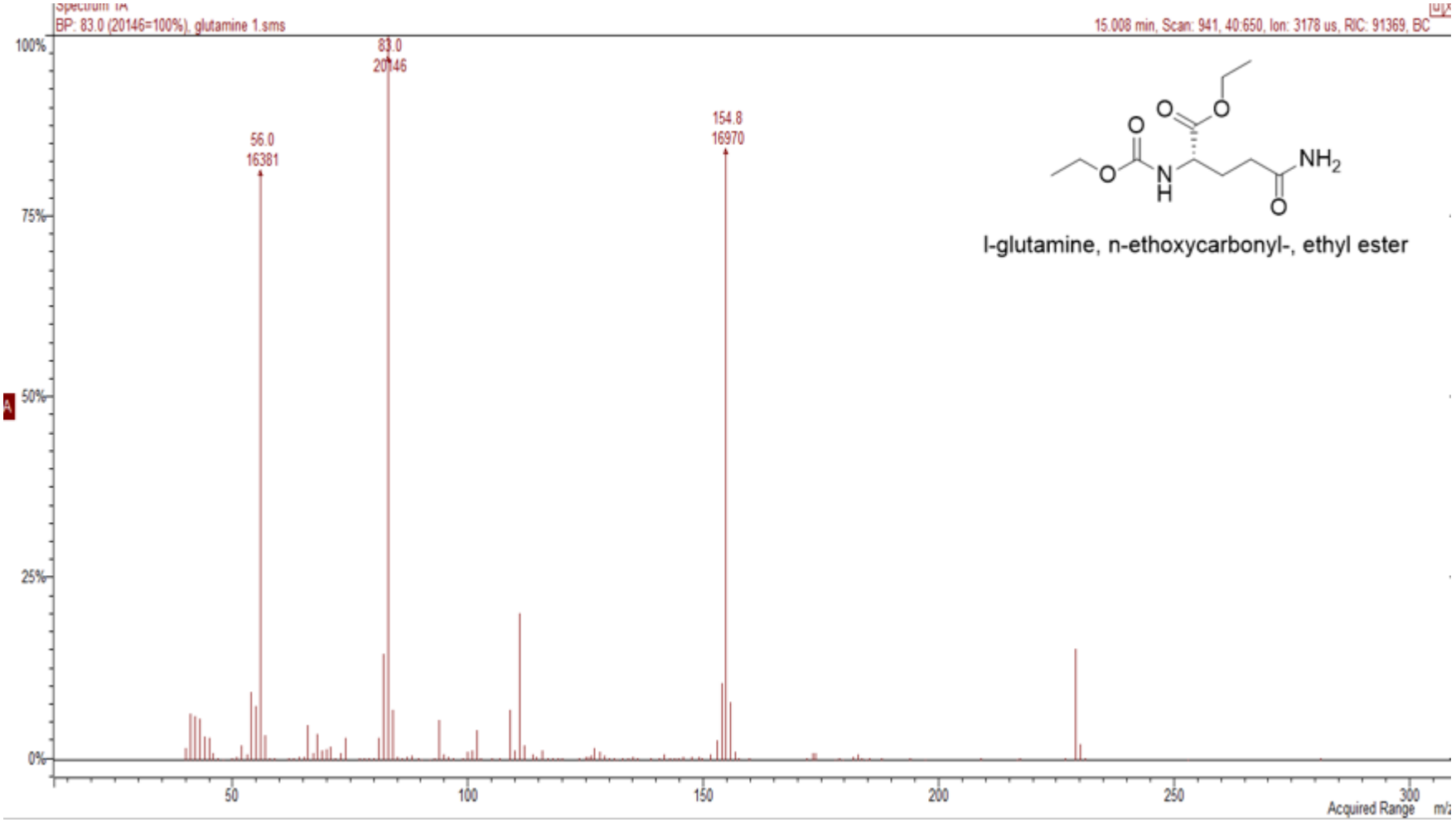

Spectrum 1A
BP: 115.9 (105942=100%), amino acid mix 1.sms
10.166 min, Scan: 631, 40:650, Ion: 2182 us, RIC: 281185, BC
115.9
105942
44.0
86612
100%
75%
50%
25%
0%
1A
100
200
300
400
500
600
Acquired Range
m/z
l-alanine, n-ethoxycarbonyl-, ethyl ester

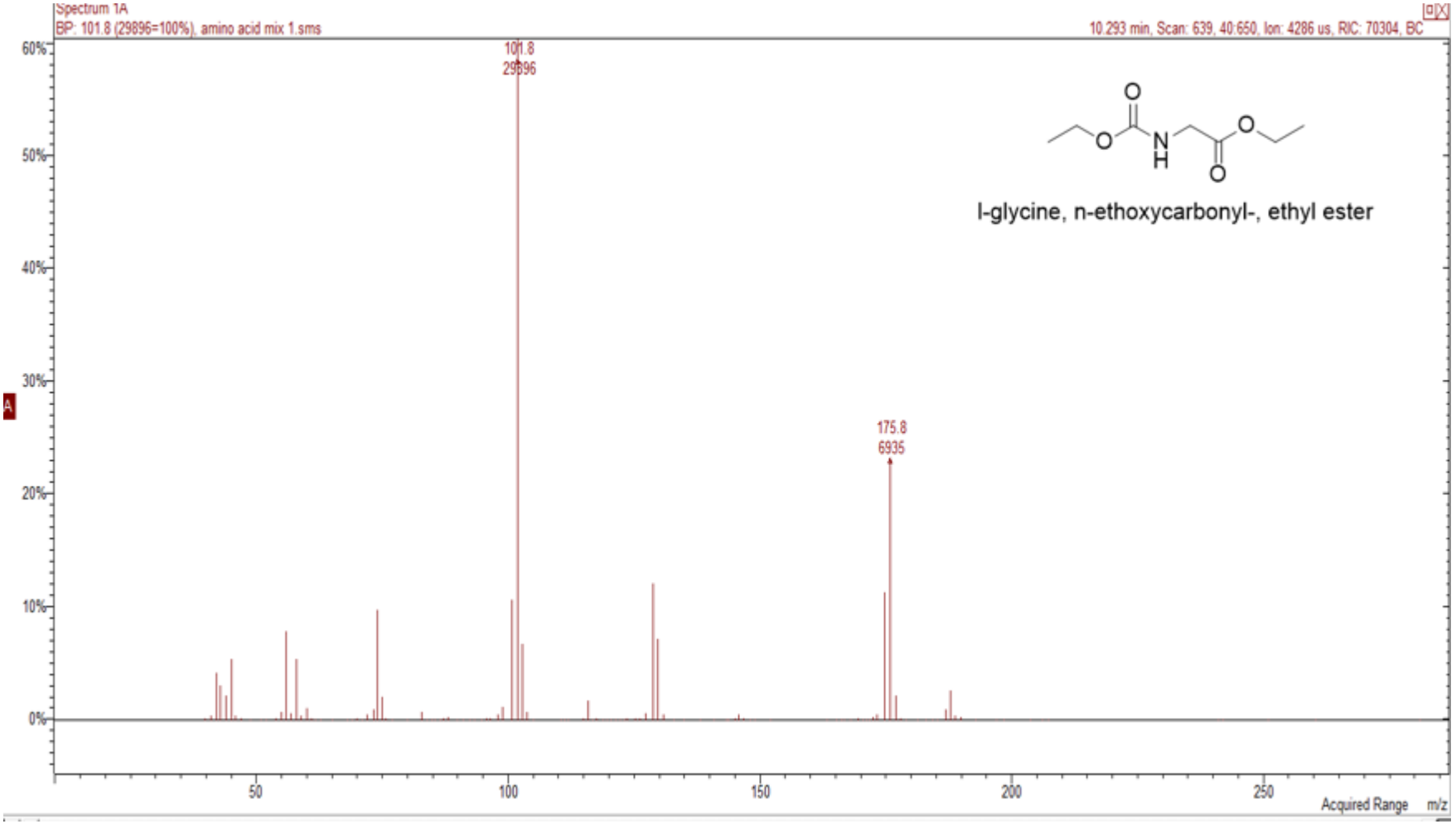

BP: 69.0 (12405=100%), amino acid mix 1.sms
13.414 min, Scan: 838, 40:650, Ion: 5639 us, RIC: 56886, BC
69.0
12405
140.8
9468
42.0
7645
80%
70%
60%
50%
40%
30%
20%
10%
0%
A
50
100
150
200
250
300
350
Acquired Range
m/z
O
NH2
O
N
H
O
O
O
l-asparagine, n-ethoxycarbonyl-, ethyl ester

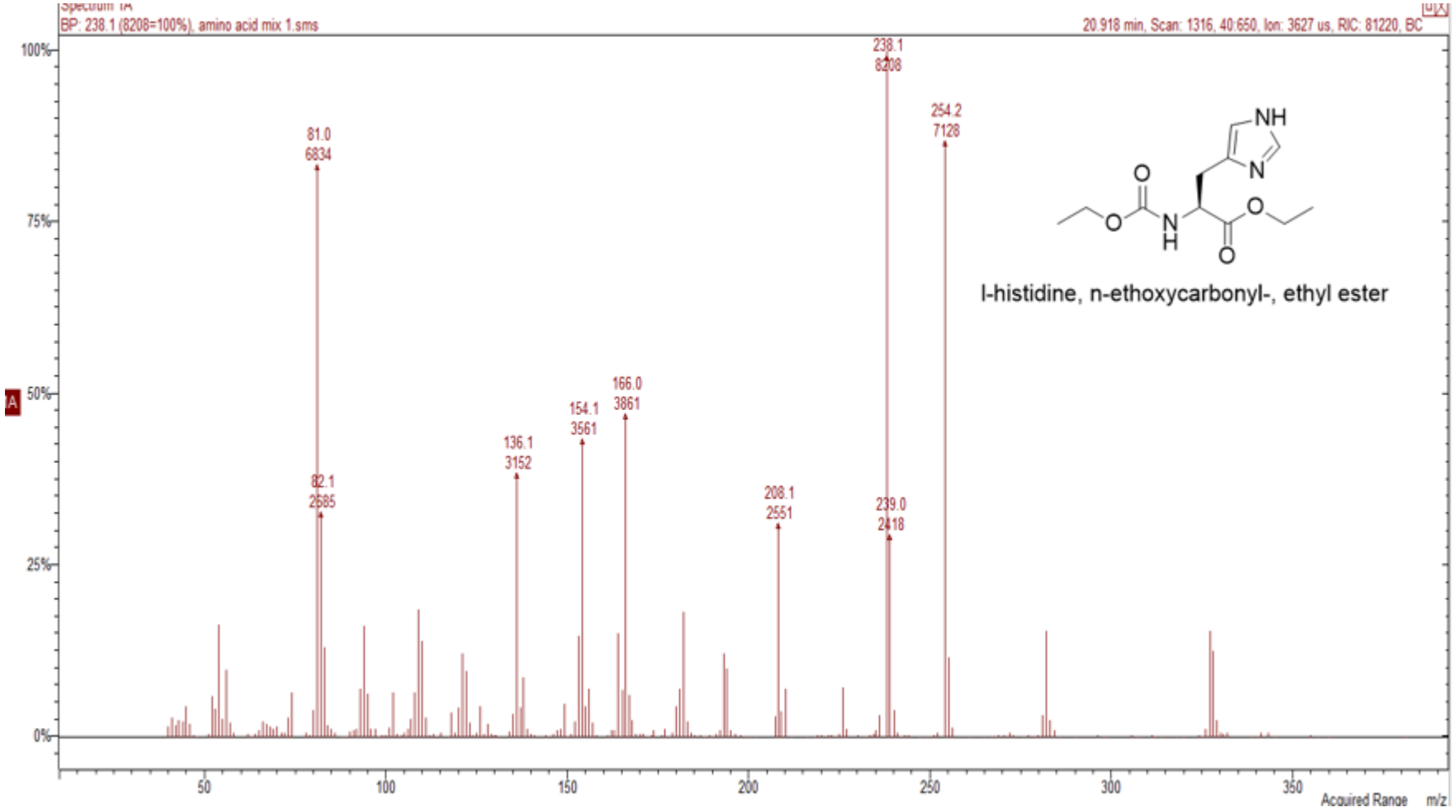

Spectrum 1A
BP: 187.8 (40481=100%), amino acid mix 1.sms
14.931 min, Scan: 935, 40:650, Ion: 2538 us, RIC: 150700, BC
187.8
40481
142.0
19116
74.0
8361
1A
70%
60%
50%
40%
30%
20%
10%
0%
50
100
150
200
250
300
Acquired Range m/z
O
OH
O
N
H
O
O
O
l-aspartic acid, n-ethoxycarbonyl-, ethyl ester

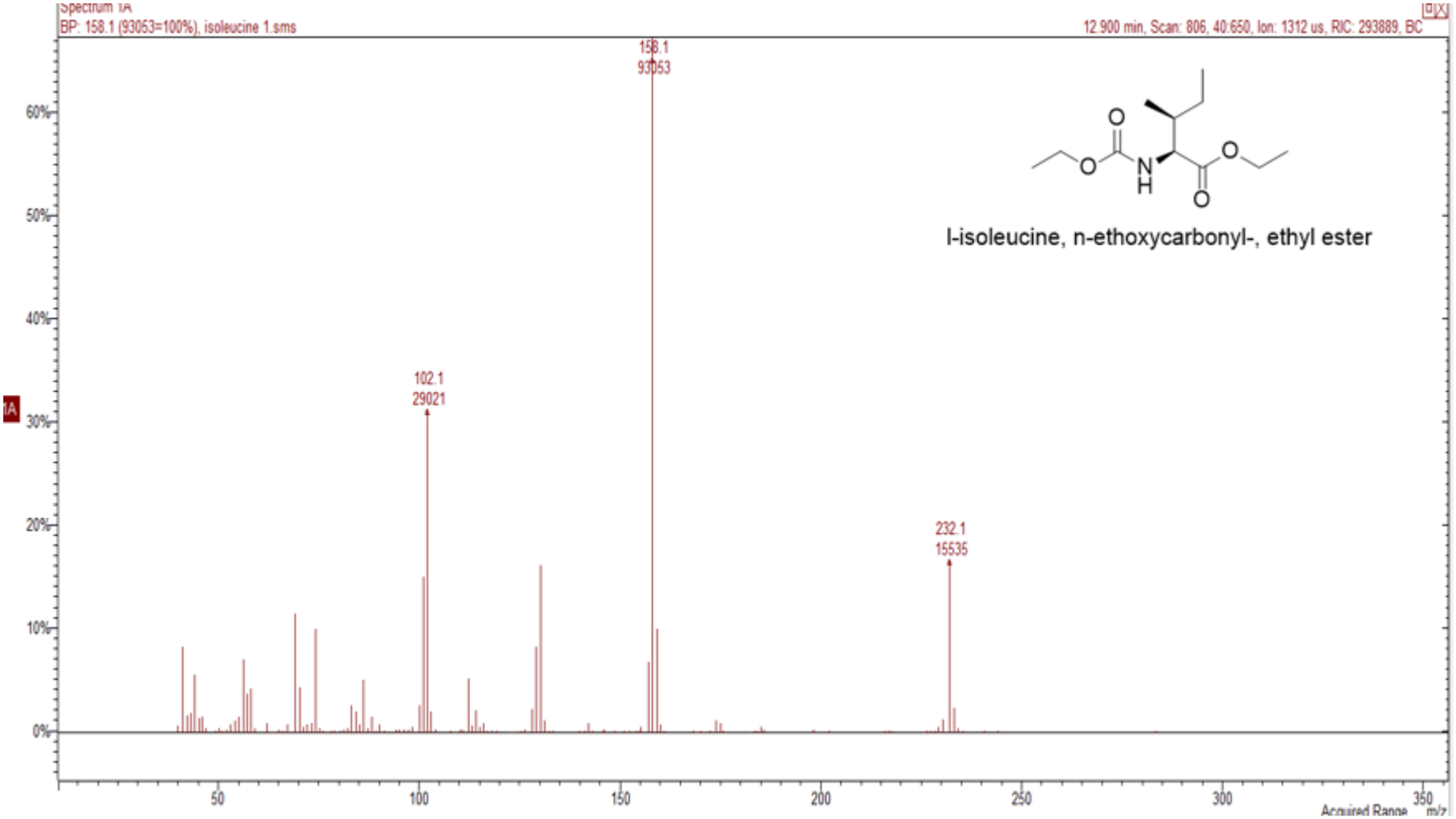

Spectrum 1A
BP: 156.0 (66934=100%), amino acid mix 1.sms
20.379 min, Scan: 1282, 40:650, Ion: 1960 us, RIC: 199910, BC
156.0
66934
128.0
14927
84.0
10245
40%
30%
20%
10%
0%
A
50
100
150
200
250
300
350
Acquired Range
m/z
SH
O
O
N
H
O
O
l-cysteine, n-ethoxycarbonyl-, ethyl ester

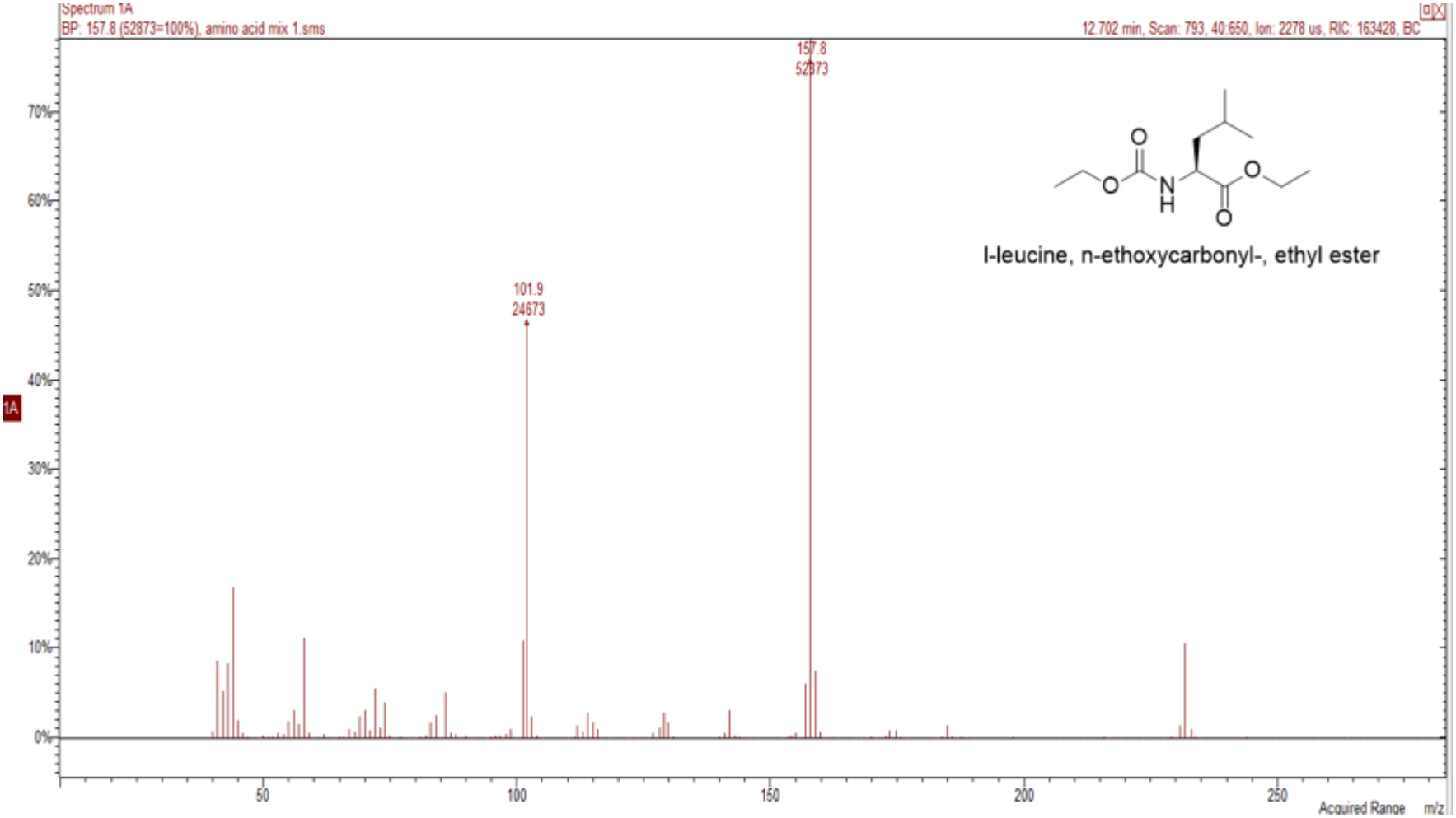

Spectrum 1A
BP: 128.0 (3250=100%), amino acid mix 1.sms
16.252 min, Scan: 1019, 40:650, Ion: 16114 us, RIC: 15383, BC
128.0
3250
84.0
1847
155.8
1133
202.0
2120
100%
75%
50%
25%
0%
100
200
300
400
500
600
Acquired Range
m/z
l-glutamic acid, n-ethoxycarbonyl-, ethyl ester

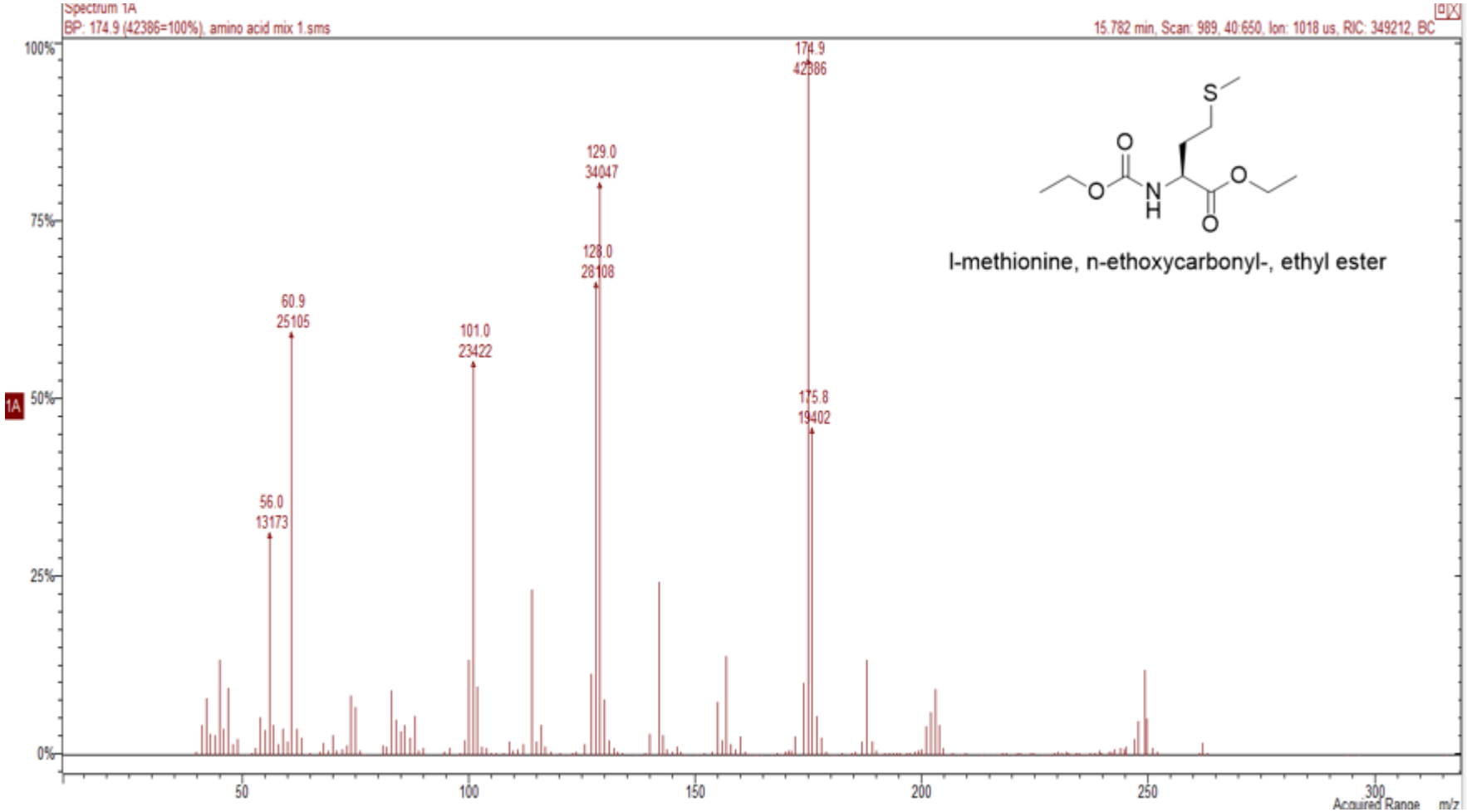

BP: 83.0 (20146=100%), glutamine 1.sms
15.008 min, Scan: 941, 40:650, Ion: 3178 us, RIC: 91369, BC
100%
75%
50%
25%
0%
56.0
16381
83.0
20146
154.8
16970
50
100
150
200
250
300
Acquired Range
l-glutamine, n-ethoxycarbonyl-, ethyl ester

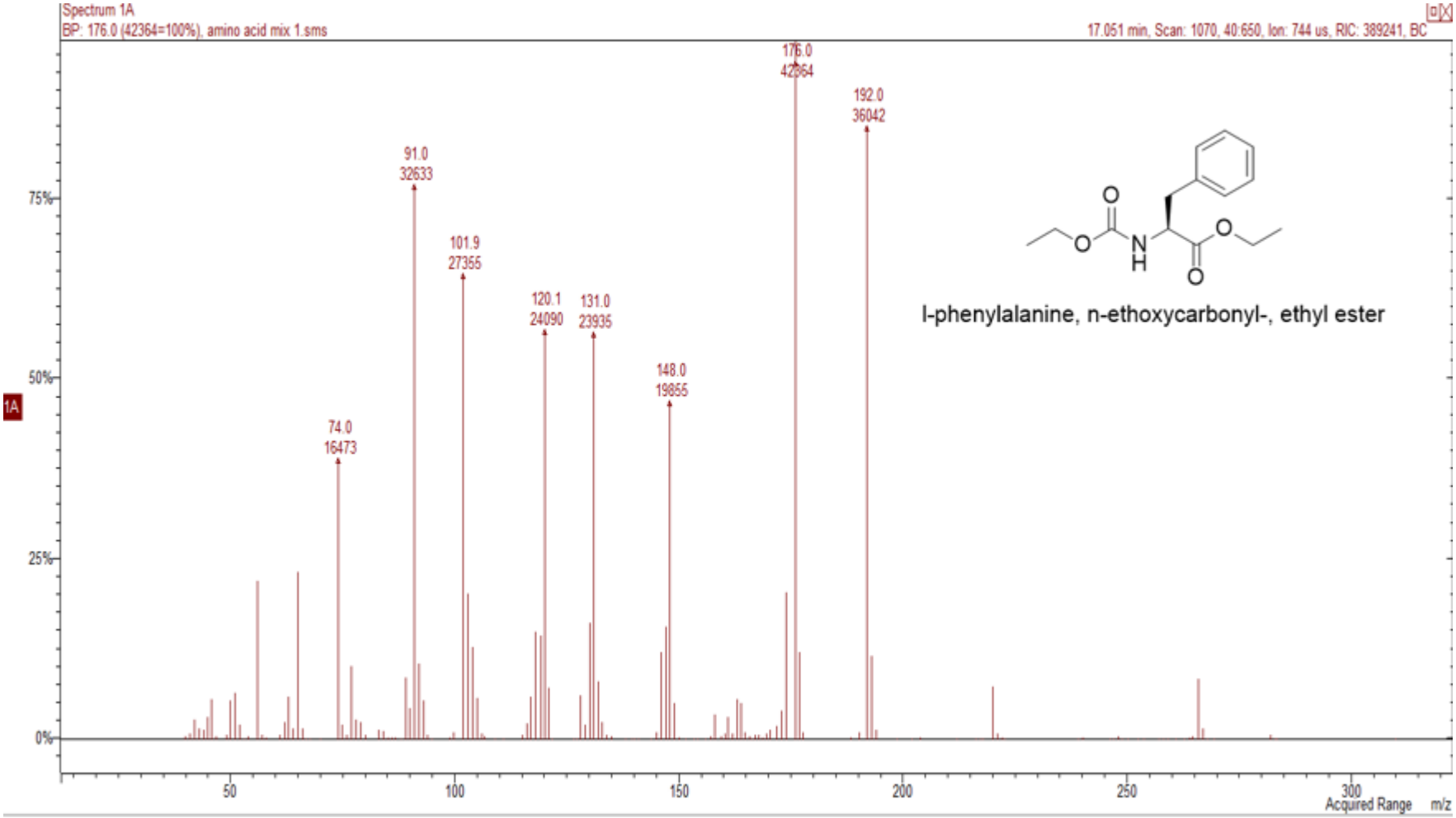

Spectrum 1A
BP: 101.8 (29896=100%), amino acid mix 1.sms
10.293 min, Scan: 639, 40:650, Ion: 4286 us, RIC: 70304, BC
101.8
29896
175.8
6935
l-glycine, n-ethoxycarbonyl-, ethyl ester
60%
50%
40%
30%
20%
10%
0%
A
50
100
150
200
250
Acquired Range
m/z

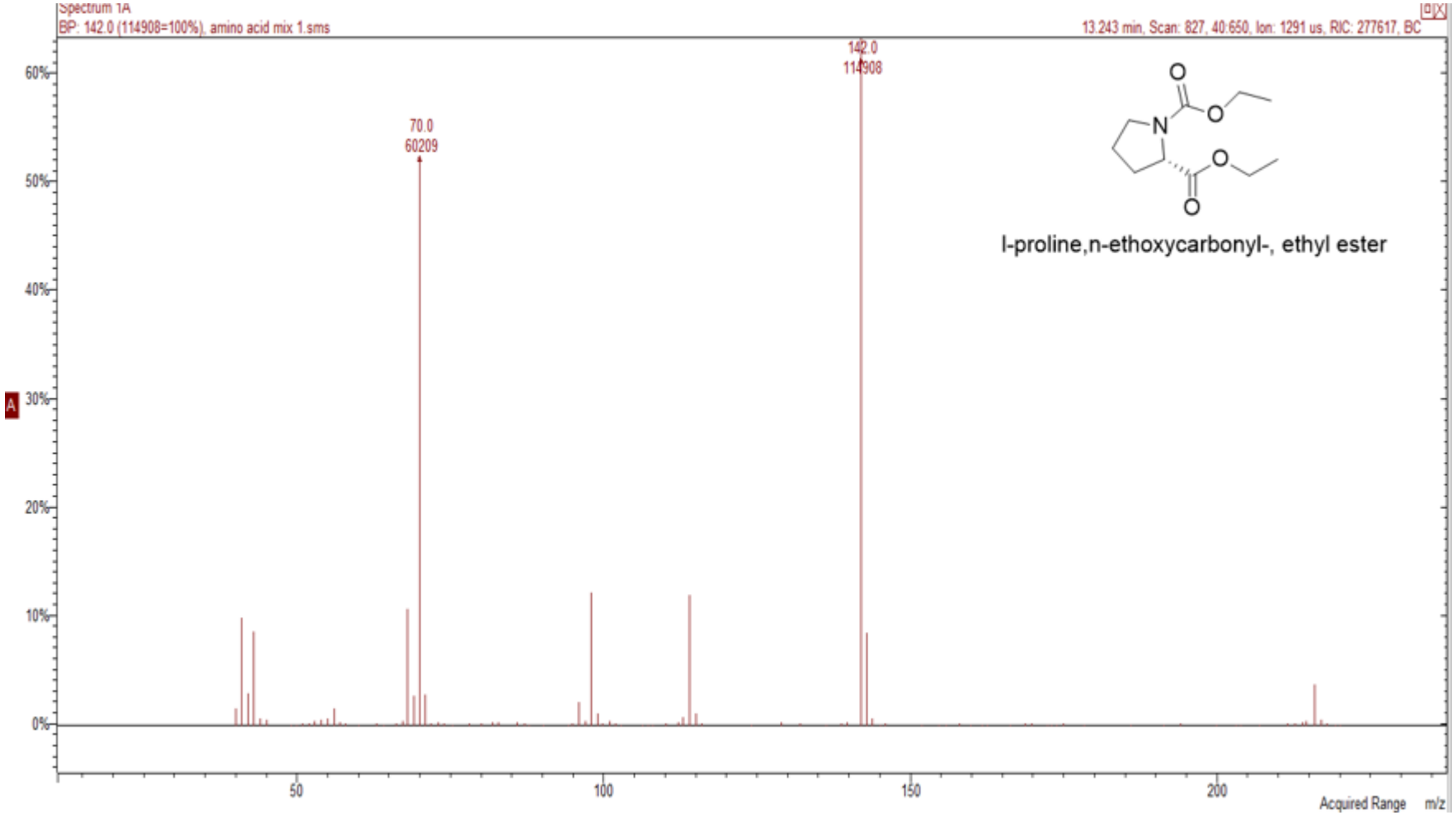

BP: 238.1 (8208=100%), amino acid mix 1.sms
20.918 min, Scan: 1316, 40:650, Ion: 3627 us, RIC: 81220, BC
100%
75%
50%
25%
0%
238.1
8208
254.2
7128
81.0
6834
166.0
3861
154.1
3561
136.1
3152
82.1
2585
208.1
2551
239.0
2418
NH
N
O
O
N
H
O
O
l-histidine, n-ethoxycarbonyl-, ethyl ester
50
100
150
200
250
300
350
Acquired Range
m/z

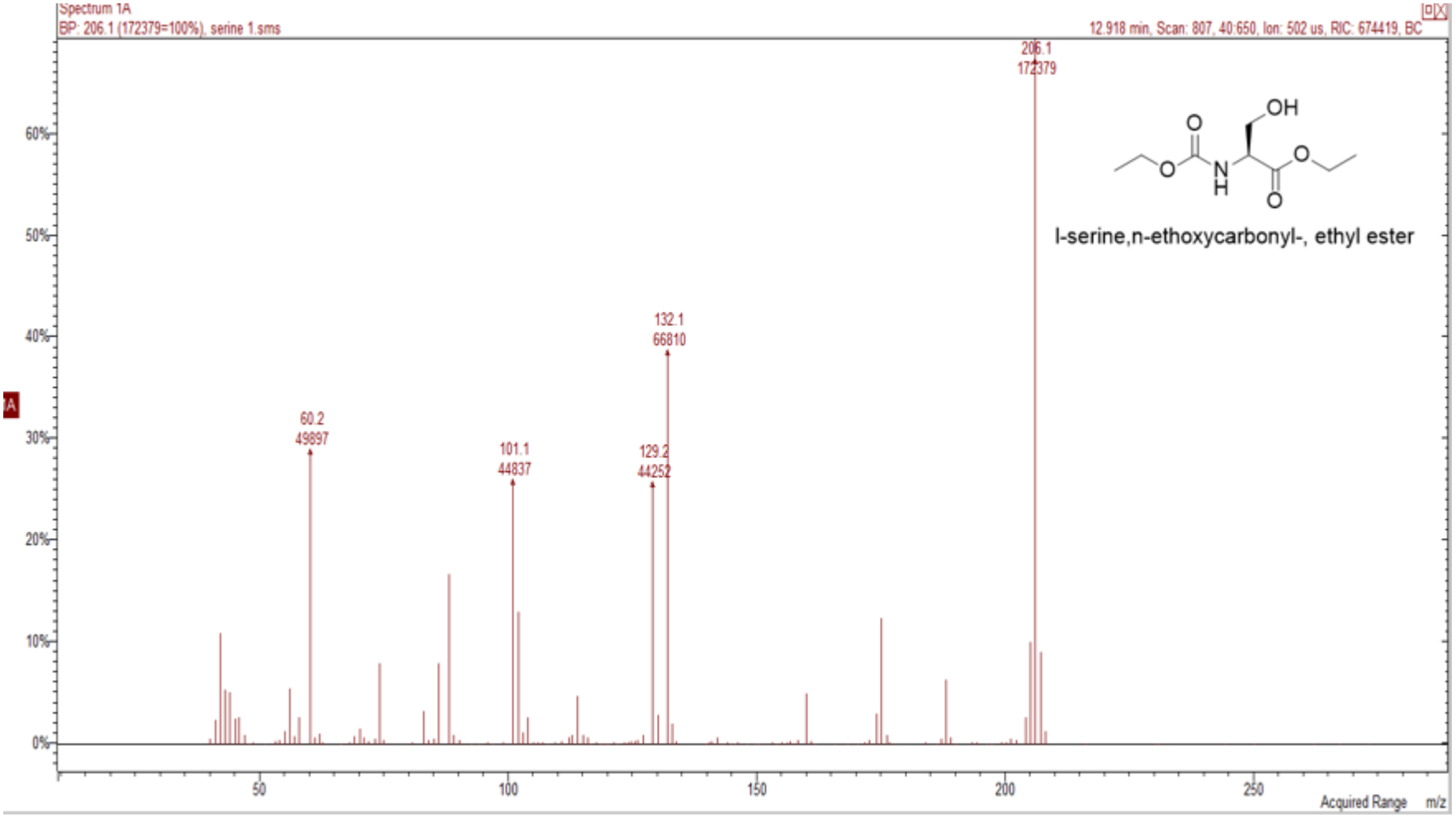

Spectrum 1A
BP: 158.1 (93053=100%), isoleucine 1.sms
12.900 min, Scan: 806, 40:650, Ion: 1312 us, RIC: 293889, BC
158.1
93053
102.1
29021
232.1
15535
l-isoleucine, n-ethoxycarbonyl-, ethyl ester
60%
50%
40%
30%
20%
10%
0%
50
100
150
200
250
300
350
Acquired Range m/z

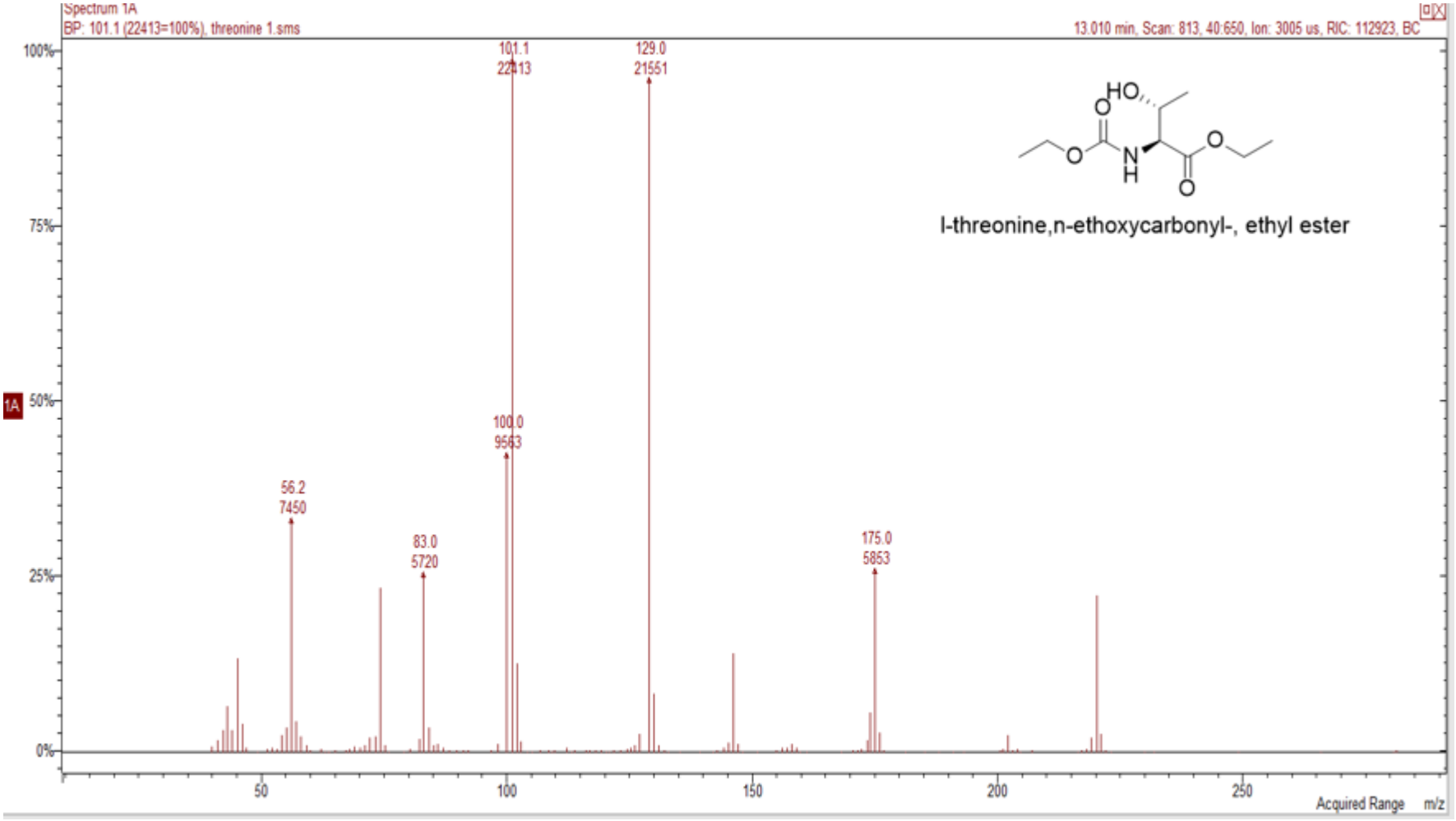

Spectrum 1A
BP: 157.8 (52873=100%), amino acid mix 1.sms
12.702 min, Scan: 793, 40:650, Ion: 2278 us, RIC: 163428, BC
157.8
52873
101.9
24673
l-leucine, n-ethoxycarbonyl-, ethyl ester
70%
60%
50%
40%
30%
20%
10%
0%
50
100
150
200
250
Acquired Range m/z

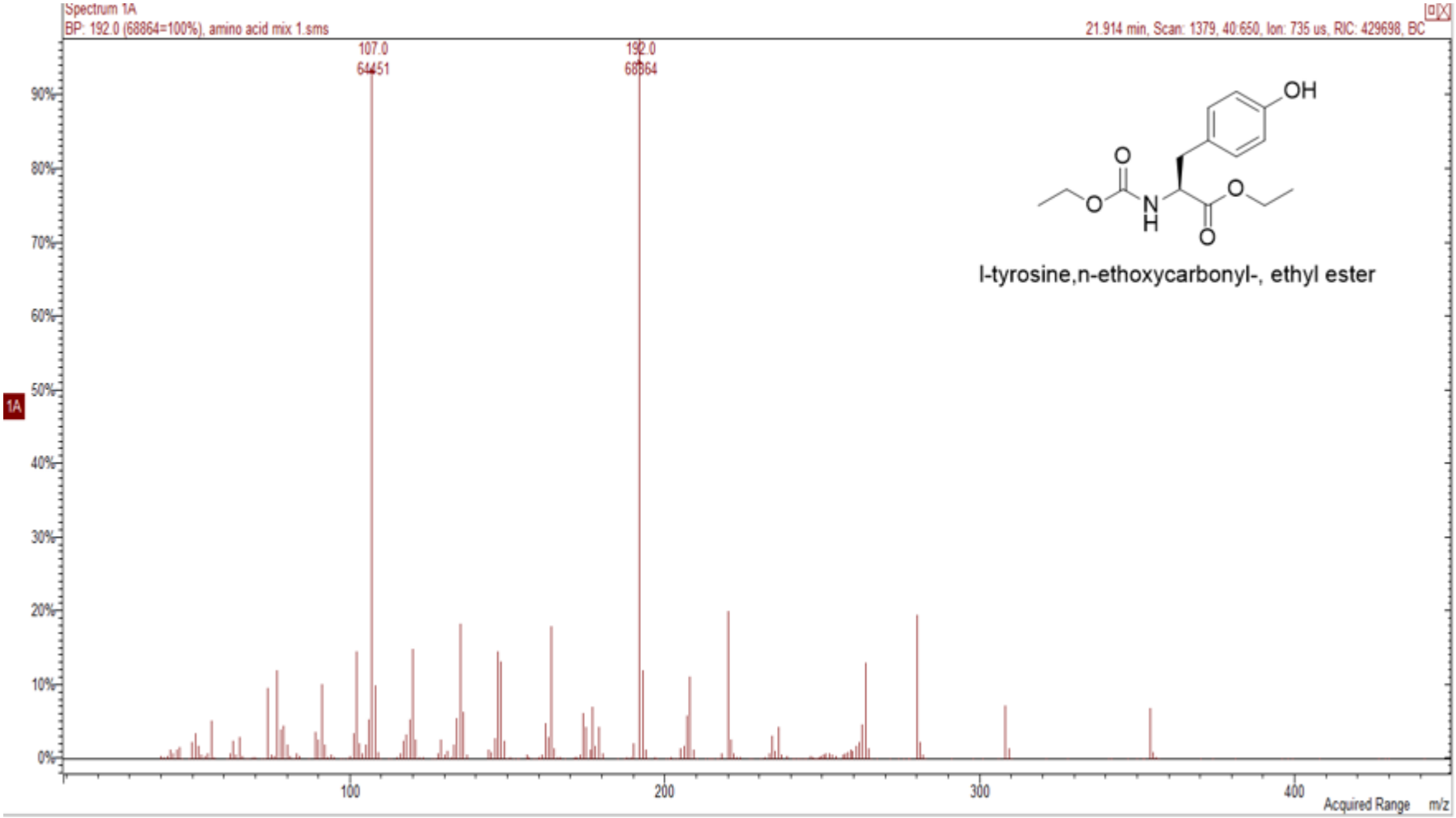

Spectrum 1A
BP: 174.9 (42386=100%), amino acid mix 1.sms
15.782 min, Scan: 989, 40:650, Ion: 1018 us, RIC: 349212, BC
100%
75%
50%
25%
0%
1A
174.9
42386
129.0
34047
128.0
28108
60.9
25105
101.0
23422
175.8
19402
56.0
13173
50
100
150
200
250
300
Acquired Range
m/z
l-methionine, n-ethoxycarbonyl-, ethyl ester

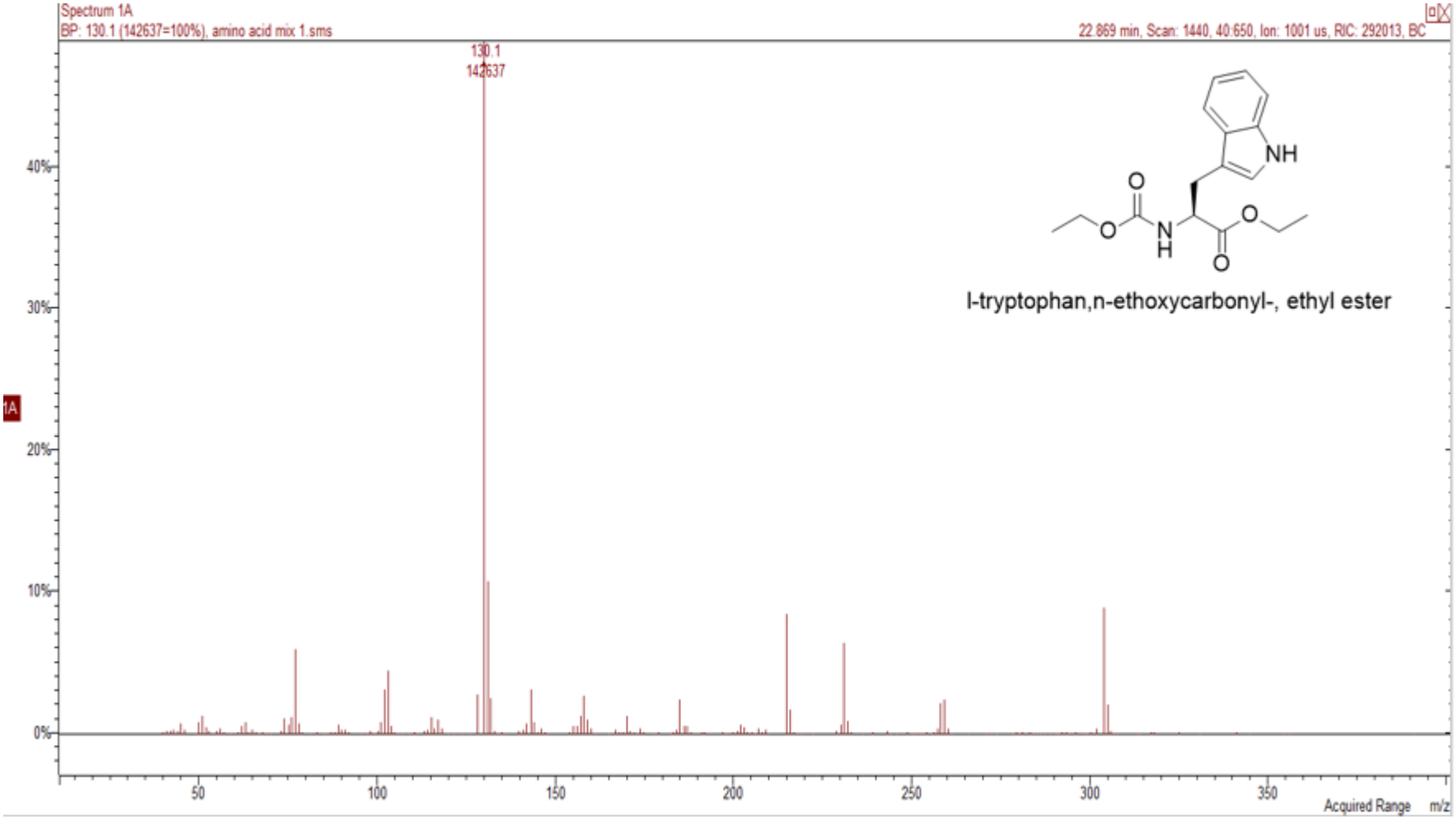

Spectrum 1A
BP: 176.0 (42364=100%), amino acid mix 1.sms
17.051 min, Scan: 1070, 40:650, Ion: 744 us, RIC: 389241, BC
176.0
42364
192.0
36042
91.0
32633
101.9
27355
120.1
24090
131.0
23935
148.0
19855
74.0
16473
75%
50%
25%
0%
1A
50
100
150
200
250
300
Acquired Range
m/z
O
O
N
H
O
O
l-phenylalanine, n-ethoxycarbonyl-, ethyl ester


Spectrum 1A
BP: 142.0 (114908=100%), amino acid mix 1.sms
13.243 min, Scan: 827, 40:650, Ion: 1291 us, RIC: 277617, BC
142.0
114908
70.0
60209
l-proline,n-ethoxycarbonyl-, ethyl ester
60%
50%
40%
30%
20%
10%
0%
A
50
100
150
200
Acquired Range
m/z


Spectrum 1A
BP: 206.1 (172379=100%), serine 1.sms
12.918 min, Scan: 807, 40:650, Ion: 502 us, RIC: 674419, BC
206.1
172379
132.1
66810
60.2
49897
101.1
44837
129.2
44252
OH
O
O
N
H
O
O
l-serine,n-ethoxycarbonyl-, ethyl ester
60%
50%
40%
30%
20%
10%
0%
50
100
150
200
250
Acquired Range
m/z


Spectrum 1A
BP: 101.1 (22413=100%), threonine 1.sms
13.010 min, Scan: 813, 40:650, Ion: 3005 us, RIC: 112923, BC
101.1
22413
129.0
21551
100.0
9563
56.2
7450
83.0
5720
175.0
5853
HO
O
O
N
H
O
O
l-threonine,n-ethoxycarbonyl-, ethyl ester
100%
75%
50%
25%
0%
1A
50
100
150
200
250
Acquired Range m/z


Spectrum 1A
BP: 192.0 (68864=100%), amino acid mix 1.sms
21.914 min, Scan: 1379, 40:650, Ion: 735 us, RIC: 429698, BC
107.0
64451
192.0
68864
OH
O
O
N
H
O
O
l-tyrosine,n-ethoxycarbonyl-, ethyl ester
1A
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
100
200
300
400
Acquired Range
m/z


Spectrum 1A
BP: 130.1 (142637=100%), amino acid mix 1.sms
22.869 min, Scan: 1440, 40:650, Ion: 1001 us, RIC: 292013, BC
130.1
142637
40%
30%
20%
10%
0%
50
100
150
200
250
300
350
Acquired Range
m/z
NH
O
O
N
H
O
O
l-tryptophan,n-ethoxycarbonyl-, ethyl ester

Appendix 2: Publications

Hindawi Publishing Corporation
Journal of Chemistry
Volume 2016, Article ID 4040165, 12 pages
http://dx.doi.org/10.1155/2016/4040165

Review Article

Dispersive Liquid-Liquid Microextraction in the Analysis of Milk and Dairy Products: A Review

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Dispersive liquid-liquid microextraction (DLLME) is an extraction technique developed within the last decade, which involves the dispersion of fine droplets of extraction solvent in an aqueous sample. Partitioning of analytes into the extraction phase is instantaneous due to the very high collective surface area of the droplets. This leads to very high enrichment factors and very low solvent consumption, relative to other liquid or solid phase extraction methods. A comprehensive review of the various modes of DLLME in the analysis of organic and inorganic analytes in dairy products (milk, cheese, infant formula, yogurt, and breast milk) is presented here. Dairy products present a complex sample matrix and the removal of interfering matrix components can prove troublesome. This review focuses on sample pretreatment prior to the appropriate DLLME procedure, the extraction and dispersive solvents chosen, derivatisation methods, and analytical figures of merit. Where possible, a critical comparison of DLLME methods has been undertaken. The overall suitability, and limitations, of DLLME as a sample preparation technique for dairy products has been assessed.

1. Introduction

One of the most important steps in any analytical procedure is the extraction and clean-up of the sample in question. There are a variety of methods that perform these tasks, such as liquid-liquid extraction (LLE) [1] and solid phase extraction (SPE) [2]. While these methods perform the above tasks adequately, they also suffer a number of drawbacks. Both LLE and SPE are environmentally unfriendly due to the large amounts of organic solvents used, they are slow, and labour intensive. The use of an SPE method also requires the purchase of solid phase extraction cartridges.

The development of microextraction techniques has gone some way to resolving some of these problems. Solid phase microextraction (SPME) was first developed in 1990 [3] and has been used extensively for a range of analytes (triazines from water [4], cephalosporins from milk [5], and short chain fatty acids from rat faeces [6]). Although SPME is more environmentally friendly than LLE and SPE as the technique does not require solvents, it still presents considerable disadvantages. The SPME fibres have a limited lifetime and are expensive and sample carryover can be an issue.

Liquid phase microextraction (LPME) offers an alternative to SPME. LPME can be divided into three classes: single drop liquid phase microextraction (SD-LPME) [7], hollow fibre liquid phase microextraction (HF-LPME) [8], and dispersive liquid-liquid microextraction (DLLME) [9]. All three forms of LPME are environmentally friendly since the volume of organic solvent used is typically in the microliter range. These methods do not have the high cost and sample carryover problems associated with SPME. Even though SD-LPME vastly reduces the volume of organic solvent used, there are other intrinsic problems with this method. Excessive stirring tends to break up the droplet, the extraction is time consuming, and reaching equilibrium can often prove challenging [10]. The development of HF-LPME [8] provides a way to stabilise the extraction droplet in SD-LPME by placing it in a hollow fibre but in general the method still

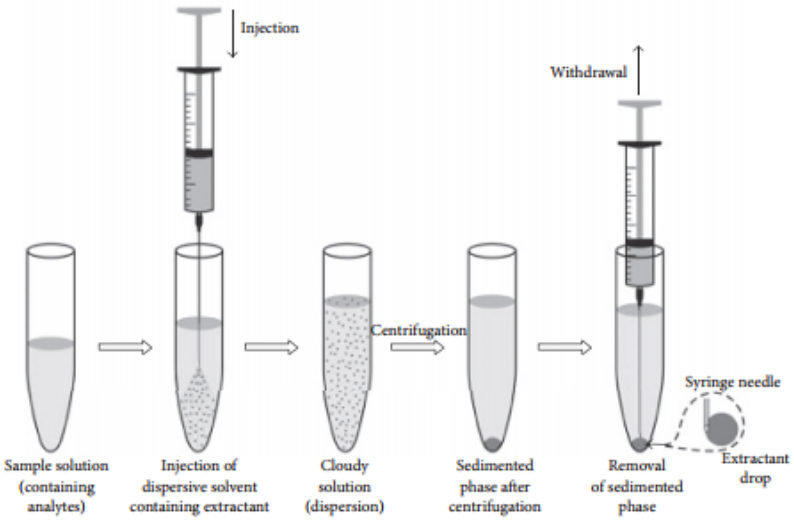

FIGURE 1: Schematic of DLLME technique [9].

requires long extraction times of at least 20 minutes [11] although methods have been reported using extraction times as low as eight minutes [12]. DLLME is the latest development in LPME and is discussed in more detail below.

2. Principles of DLLME

In a typical DLLME protocol, an extraction solvent is mixed with a dispersive solvent and this solvent mixture is then rapidly injected into the aqueous sample. The rapid injection of the extraction-dispersive solvent mixture produces a cloudy solution, formed of microdroplets of extraction solvent dispersed in the aqueous sample as shown in Figure 1. The formation of a cloudy solution/emulsion allows for the instantaneous partitioning of analytes from the aqueous sample into the extraction phase (a major advantage of this technique). This is achieved by the large surface area relative to LLE created by the numerous microdroplets. The cloudy solution is then centrifuged which breaks the emulsion into a two-phase system allowing for easy recovery of the extraction solvent for analysis.

There are several requirements that must be fulfilled in order for DLLME to be successful. The extraction solvent must be immiscible with water and miscible with the dispersive solvent and show a high affinity for the target analytes. In what will be referred to hereafter as *"traditional DLLME,"* the extraction solvent is typically denser than water such that it will form a *"sedimented phase"* upon centrifugation for easy collection with a fine syringe needle. Conversely, the dispersive solvent has to be miscible with both the extraction solvent and the aqueous sample. Ideally, the extraction solvent will be compatible with the analytical technique being used; otherwise evaporation of the extraction solvent and reconstitution in an appropriate solvent is required. Alternatively, *in-syringe* back extraction could be used to extract the analytes into a compatible solvent [38]. Prior to analysis, the volume and type of extraction and dispersive solvent, ionic strength, pH of the aqueous phase, extraction time, and centrifugation time must be optimised to ensure quantitative extraction of analytes.

An efficient DLLME method is characterised by a high enrichment factor (EF) and high relative recovery (RR). Enrichment factor (EF) is calculated as shown in (1), where C_0 represents the concentration of the analyte in the original sample and C_{sed} represents the concentration of the analyte in the sedimented extraction solvent.

$$\text{Enrichment factor} = \frac{C_{sed}}{C_0}. \quad (1)$$

The RR is calculated according to (2), where C_{found} shows total amount of analyte found after addition of standard, C_{real} is the original concentration of analyte in the sample, and C_{add} is the amount of standard that was spiked into the original sample.

$$\text{Relative recovery} = \frac{C_{found} - C_{real}}{C_{add}} \times 100. \quad (2)$$

3. Alternative Modes of DLLME

Recently, low-density solvents have been used as extraction solvents in DLLME in order to increase the range of extraction solvents compatible with the method. This mode is called low-density solvent based DLLME (LDS-DLLME) [39] and the extraction solvent (including toluene, xylene, hexane, and

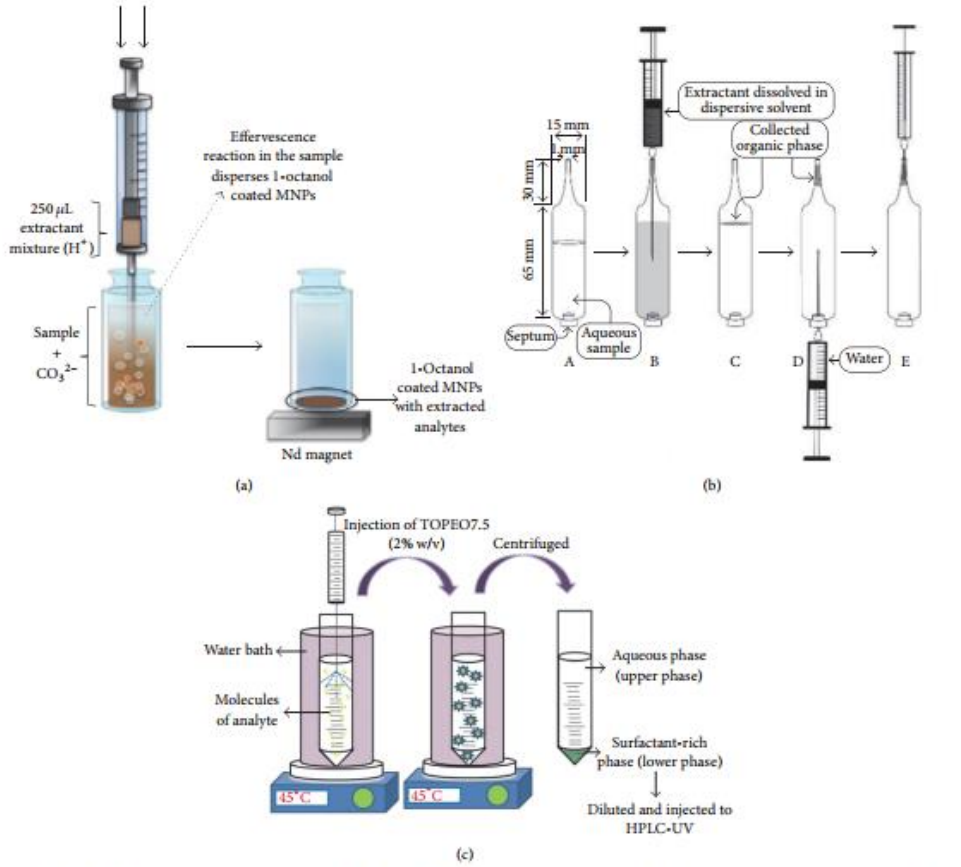

FIGURE 2: Schematic diagram of (a) effervescence DLLME [46], (b) LDS-DLLME using specialist glassware [52], and (c) CP-DLLME [48].

heptane) floats on the surface of the aqueous phase after phase separation is induced. The solvent is recovered using a fine needle and this process is simplified when specialist glassware or other vessels are used to trap the floating solvent in a narrow restriction in the vessel [40] as shown in Figure 2(b). An advantage of LDS-DLLME is that, after centrifugation, any matrix components will be sedimented at the bottom of the extraction vessel while the extraction solvent will be floating on top. This will lead to a cleaner extract and potentially cleaner chromatography [41].

Solidified floating organic drop DLLME (SFO-DLLME) was developed by Melwanki et al. [38, 39] and involves the use of low-density extraction solvents having a melting point close to room temperature (typically 1-undecanol or 1-dodecanol). After phase separation the floating extraction solvent is frozen by placing the vessel on ice after which the frozen drop is easily collected into a separate vessel where it is usually diluted with a chromatographically suitable solvent prior to analysis [42]. While the use of a less toxic extraction solvent is advantageous, the choice of extraction solvent is limited to those that have a melting point at approximately room temperature [43].

Additional modifications to DLLME methods include the elimination of time-consuming centrifugation steps via the use of a deemulsification solvent which causes phase separation of the emulsion upon its addition [41]. Seebunrueng et al. have reported a similar method whereby the addition of a salt ($AlCl_3$) is used to induce phase separation due to a disruption of the interfacial tension at the droplet surface [44]. Alternative methods have also been developed to enhance the dispersion of the extraction solvent throughout the aqueous sample. The use of ultrasound, vortex, or manual shaking will

increase the number of microdroplets of extraction solvent resulting in an even larger surface area [45]. Effervescence assisted DLLME involves the *in situ* generation of bubbles of CO_2 to assist the dispersion of the extraction solvent, removing a need for the dispersive solvent. The CO_2 is produced by adding a mixture of sodium carbonate and a weak acid (citric acid), usually in the form of a pressed tablet [44, 46]. This technique allows for the reduction in the use of organic solvents, potentially lowering the cost of the overall analysis.

Air assisted dispersive liquid-liquid microextraction (AA-DLLME) removes the need for a dispersive solvent by repeatedly aspirating the aqueous phase and the extraction solvent into a glass syringe until a cloudy solution is formed [40]. Methods to allow easier recovery of the extraction solvent have also been developed. Hydrophobic magnetic nanoparticles interact with the extraction phase and can be sedimented by applying a magnet; this eliminates the centrifugation step [47]. The use of magnetic nanoparticles has also been combined with effervescence assisted dispersion, mentioned above [46]. A schematic of this method can be seen in Figure 2(a).

Surfactant assisted DLLME (SA-DLLME) uses surfactants as dispersive solvents [34] whereas cloud point DLLME (CP-DLLME) uses surfactants as an extraction solvent to produce a surfactant rich sedimented phase after centrifugation [48]. Specifically, it involves heating the sample solution containing the appropriate surfactant past its cloud point. The cloud point is defined as the temperature at which phase separation occurs and the analytes are extracted into the surfactant rich phase as shown in Figure 2(c). Ionic liquids have been used as an alternative to traditional organic extraction solvents in ionic liquid DLLME (IL-DLLME) because they have tuneable physicochemical properties. For example, ionic liquid miscibility in either water or organic solvents can be controlled by selecting the appropriate anion/cation combination and by incorporating the proper functional group within the IL. In addition, they exhibit lower toxicity than organic extraction solvents [49]. Ionic liquids have also been used as both dispersive and extraction solvents in combination with ultrasound assisted dispersion, referred to as ultrasound assisted ionic liquid/ionic liquid DLLME (UA-IL/IL-DLLME) [29]. In an effort to improve selectivity for polar or acidic/basic analytes, pH-controlled DLLME (pH-DLLME) has also been developed [50]. By performing two DLLME procedures it is possible to remove matrix interferences in the first extraction step, followed by a back extraction after appropriate pH adjustment.

4. Modes of DLLME Used in Dairy Analysis

4.1. Traditional DLLME. Prior to a DLLME procedure on a complex matrix such as milk, lipids and proteins must be eliminated since they can act like surfactants and disrupt the interfacial tension at the droplet surface, hindering phase separation [51]. A list of sample pretreatment procedures, extraction solvent type and volume, dispersive solvent type and volume, analytical method used, and analytical figures of merit can be found in Table 1. One of the first reports of traditional DLLME used to extract analytes from dairy products was in 2009 by Daneshfar et al. [19] who extracted cholesterol in several food samples (egg yolk, milk, and olive oil). Previously centrifuged milk samples were subjected to acetonitrile precipitation to eliminate proteins and the aqueous supernatant (after further centrifugation) was subjected to a DLLME protocol. Acetone, ethanol, and acetonitrile were trialled as dispersive solvents using carbon tetrachloride as extraction solvent. Ethanol (0.8 mL) resulted in highest recoveries for cholesterol; lower and higher volumes resulted in either unstable emulsions or higher solubility of cholesterol in water, respectively. Four extraction solvents (carbon disulphide, dichloromethane, chloroform, and carbon tetrachloride) were tested but only carbon tetrachloride (35 μL) yielded stable suspensions with ethanol. An extraction pH of 8.5 maximised recovery and partition of cholesterol was also deemed instantaneous upon generation of the stable emulsion (i.e., extraction time was several seconds). Non-aqueous reversed phase HPLC was used to quantify the analyte; because of poor chromatographic behaviour carbon tetrachloride extracts were evaporated to dryness and reconstituted in ethanol for injection. The method proved linear in the range 0.03–10 $\mu g \cdot L^{-1}$ and the LOD was 0.01 $\mu g \cdot L^{-1}$ representing detection limits 100 times lower than previously reported methods for cholesterol determination in milk.

Later in 2011 Farajzadeh et al. used DLLME for the extraction and preconcentration of triazole pesticides from milk samples [20], using GC-FID and GC-MS to quantify the analytes. Proteins were precipitated using both acetonitrile precipitation and NaCl salting out and the pesticides were preconcentrated from 1.0 mL of the ACN supernatant by adding 40 μL of chloroform and rapidly injecting the mixture into 5 mL of deionized water. After a 5-minute centrifugation at 4,000 rpm, enrichment factors of 156 (penconazole), 166 (hexaconazole), 180 (tebuconazole), 243 (triticonazole), and 387 (difenconazole) were achieved. The linear range was as wide as 20–80,000 $\mu g \cdot L^{-1}$ for penconazole and hexaconazole and the lowest recorded LOD value was 4 $\mu g \cdot L^{-1}$ for hexaconazole.

That same year, Liu et al. combined SPE and DLLME to enable the determination of 14 different polychlorinated diphenyls (PCDEs) and polybrominated diphenyl ethers (PBDEs) in milk using GC-MS [24]. To precipitate proteins, 50% NaOH and acetone were added and the samples were heated at 70°C in a water bath. Afterwards, the analytes were extracted into 5 mL of hexane, dried over anhydrous sodium sulphate, and concentrated to 2 mL by evaporation before loading onto the SPE column. The resulting fractions from SPE were dried and reconstituted in 1 mL of acetone, which was used as the dispersive solvent in the optimised DLLME procedure. Chlorobenzene (19 μL) was mixed with the dispersive solvent and rapidly injected into 5 mL of Milli-Q water. The developed SPE-DLLME procedure proved to be effective since the sample matrix did not have a significant impact on extraction efficiencies. The method provided good recoveries and %RSD values for both polychlorinated diphenyls (recovery: 100.0–131.8%; precision: 3.20–10.20%) and polybrominated diphenyl ethers (recovery: 74.0–93.6%; 1.12–12.34%).

Table 1: Modes of DLLME used in dairy analysis.

Sample	Sample preparation	Extraction/dispersive solvent	Analytes extracted	Enrichment factor	Analytical method	LOD (μg/L)	Reference
DLLME							
Infant formula, fermented milk	Acid hydrolysis, enzymatic hydrolysis and derivatisation	Tetrachloroethene (90 μL)/acetonitrile (0.5 mL)	Thiamine	Not specified	Reversed phase HPLC (RP-HPLC)	0.09	[13]
Soybean milk	Liquid-liquid extraction	Carbon tetrachloride (40 μL)/acetonitrile (1 mL)	Phthalate acid esters	200–260	GC-MS	0.57–0.79 ng/g	[14]
Full fat milk, half fat milk, skimmed milk, follow-on formula	Dilution, protein precipitation by TCA	Chloroform (200 μL)/acetonitrile (2 mL)	Macrocyclic lactones	65–200	HPLC-DAD coupled to APCI-IT-MS/MS	HPLC-DAD: 0.3–1.4 ng/g LC-MS/MS: 0.03–0.72 ng/g	[15]
Milk	Protein precipitation by TCA, pH adjustment, derivatisation by fluorescamine	Chloroform (1 mL)/ACN (1.9 mL)	Sulfonamides	2.2	RP-HPLC-FL	0.6–1.03	[16]
Milk, white cheese, yogurt, unpasteurised milk	Milks: protein precipitation, removal of fat; yogurt and cheese: dilution, removal of fat	Chloroform (150 μL)/ACN (2 mL)	Nonsteroidal anti-inflammatory drugs	Milk: 81.1–141, yogurt: 45.9–81 cheese: 145–229	Field amplified sample stacking in CE (FASS)	Milk (μg/kg): 4.8–13 Yogurt (μg/kg): 3.0–9.7 Cheese (μg/kg): 6.1–7.7	[17]
Whole milk, skimmed milk, powdered milk	Whole milk: centrifugation, powdered milk: reconstitution with ultrapure water	Chloroform (1.5 mL)/ACN (2.4 mL)	Aflatoxin M1	33	UHPLC-MS/MS	0.6 ng/kg	[18]
Milk	Protein precipitation by ACN and NaCl	Carbon tetrachloride (35 μL)/ethanol (800 μL)	Cholesterol	Not specified	HPLC-UV	0.01	[19]
Milk	Centrifugation, protein precipitation by ACN	Chloroform (40 μL)/ACN (1 mL)	Pesticides	176–435	GC-FID, GC-MS	4–58	[20]
Milk	Protein precipitation by ACN and NaCl	1,2-Dibromoethane (20 μL)/ACN (800 μL)	Phthalates	397–499	GC-FID, GC-MS	1.0–3.0	[21]
Milk	Protein precipitation by ACN and NaCl	1,1,2,2-Tetrachloroethane (22 μL)/ACN (1 mL)	Polybrominated diphenyl ethers	271–307	GC-MS	0.012–0.29	[22]
Milk	Saponification, in-tube LLE	Chloroform (200 μL)/10% acetic acid-ACN (1 mL)	Fluoroquinolones	Not specified	RP-HPLC-UV	0.8–5.0 μg/kg	[23]
Milk	QuEChERS	Chlorobenzene (19 μL)/acetone (1 mL)	Polychlorinated biphenyls, polybrominated diphenyl ethers	Not specified	GC-MS	Polychlorinated biphenyls: 0.01–0.04 polybrominated biphenyls: 0.2–0.4	[24]
Infant formula	Protein precipitation and fat removal by NaOH and acetone, SPE	Tetrachloroethylene (30 μL)/ACN (440 μL)	BPA, BPB	BPA: 237, BPB: 220	GC-MS	BPA: 0.06, BPB: 0.03	[25]
Infant formula	Protein precipitation by TCA, pH adjustment						

Table 1: Continued.

Sample	Sample preparation	Extraction/dispersive solvent	Analytes extracted	Enrichment factor	Analytical method	LOD (μg/L)	Reference
Ultrasound assisted DLLME							
Bottled milk	Protein precipitation by TCA and lead acetate	Carbon tetrachloride (40 μL)/methanol (0.8 μL)	Phthalate acid esters (PAEs), butyl benzyl ester (BBP), di-isooctyl phthalate (DIOP)	PAEs: 226–258, BBP: 270, DIOP: 220–229	GC-FID	0.75–0.79 ng/g, 0.66 ng/g 0.64–0.76 ng/g	[26]
Skimmed milk	Protein precipitation by ACN, derivatisation by HFBI	Chloroform (100 μL)/ACN (2 mL)	Chloropropanols	Not specified	GC-MS/MS	0.9–3.6	[27]
Milk, cheese	QuEChERS	Chloroform (500 μL)/ACN (3 mL)	Aflatoxins: B1, M1	B1: 30, M1: 30	RP-HPLC-FL	B1: 0.1 μg/kg M1: 0.01 μg/kg	[28]
UA-IL/IL-DLLME							
Infant formula	Dilution	$[C_8MIM][PF_6]$ (70 μL)/$[C_4MIM][BF_4]$ (100 μL)	Sulfonamides	Not specified	RP-HPLC-PDA	2.94–16.7 μg/kg	[29]
DLLME with back extraction							
Breast milk, ice cream	Protein precipitation by salting out with NaCl, phosphoric acid, ACN	Chloroform (200 μL)/ACN (1 mL)	Parabens	4.6–9.2	CE-DAD	300	[30]
USA-RM-DLLME							
Butter	Melting for 5 min at 40°C	Triton X-100 (1.25% w/v)/water (400 μL)	Acetoin	245	RP-HPLC-UV	200,000	[31]
USA-IL-DLLME							
Milk	Microwave digestion with HNO_3 and H_2O_2, chelation with 1-Phenylthiosemicarbazide	$[C_8MIM][Tf_2N]$ (100 μL)	Selenium	150	Graphite furnace atomic absorption spectrometry (GFAAS)	12	[32]
LDS-DLLME							
Cheese	Protein precipitation, filtration	1-octanol (60 μL)/acetone (475 μL)	Sorbic and benzoic acids	Cheese: sorbate (143), benzoate (170)	GC-FID	Cheese sorbate 150 ng/g, benzoate: 140 ng/g	[33]
Milk	Fat removal by centrifugation	1-Heptanol (320 μL)/MeOH : water (80 : 20) (3 mL)	Aflatoxin M1	Not specified	Fluorescence spectrophotometer	0.013	[34]
Yoghurt drinks	Protein precipitation, filtration	1-Octanol (60 μL)/ethanol (450 μL)	Benzoate, sorbate	Benzoate: 162, sorbate: 181	RP-HPLC-UV	Benzoate: 0.2, sorbate: 0.5	[35]
VA-DLLME							
Whole milk, skimmed milk, semiskimmed goat milk, yogurt	Protein precipitation by ACN and acetic acid, fat removal by hexane and centrifugation	Chloroform (110 μL)/ACN (500 μL)	Estrogenic compounds	Not specified	MEKC-MS	Whole milk: 6–71, skimmed milk: 3–64, semiskimmed goat milk: 4–220, yogurt: 12–140	[36]
IL-DLLME							
Breast milk	Protein precipitation by $HClO_4$, H_3PO_4, MeOH	NFX: $[C_8C_1im][PF_6]$ (42 μL)/MeOH (80 μL) BNZ: $[C_8C_1im][PF_6]$ (42 μL)/MeOH (101 μL)	NFX, BNZ	NFX: 338, BNZ: 28.8	RP-HPLC-UV	NFX: 90, BNZ: 60	[37]

Cunha et al. expanded the range of dairy samples from milk to infant formula, while analysing bisphenol A (BPA) and bisphenol B (BPB) content using heart-cutting GC-MS [25]. The authors developed an optimised DLLME method coupled with *in situ* derivatisation using acetic anhydride in the presence of potassium carbonate (K_2CO_3). After protein precipitation using trichloroacetic acid, K_2CO_3 was added until the pH was greater than 10; this mixture was then used as the aqueous phase in the DLLME procedure. The dispersive-extraction solvent mixture (440 μL ACN/30 μL tetrachloroethylene) was combined with 30 μL of acetic acid as derivatisation agent and rapidly injected into the aqueous phase and the resulting cloudy suspension was allowed to react for 1 minute. Using deuterated BPA as an internal standard, recovery of BPA and BPB was found to be 114% and 68%, respectively. The method was linear between 0.5 and 10 $\mu g\,L^{-1}$ for both analytes and low LODs (BPA: 60.0 $\mu g\,L^{-1}$, BPB: 30 $\mu g\,L^{-1}$) were obtained corresponding to high enrichment factors (BPA: 237, BPB: 220). The method repeatability was ≤7% when the analytes were at a concentration of 0.2 $\mu g{\cdot}L^{-1}$.

In contrast with Liu et al. [24], Han et al. combined saponification, LLE, and DLLME in the determination of PBDEs in milk using GC-MS [22]. Saponification was carried out by adding a sample of milk to 50% NaOH and ethanol; this mixture was heated to 70°C under reflux for one hour. The saponified mixture was cooled and rinsed five times with petroleum ether. The washings were collected and centrifuged. The supernatant was dried over anhydrous sodium sulphate and evaporated to dryness under nitrogen.

The residue was reconstituted in 2 mL of ACN. To carry out the DLLME procedure, 1 mL of the ACN solution (dispersive solvent) and 22 μL of 1,1,2,2-tetrachloroethane (extraction solvent) were combined and then rapidly injected into 5 mL of deionized water. The cloudy solution was centrifuged and the sedimented phase was removed and dried under nitrogen. The resulting residue was dissolved in 15 μL of hexane and used for GC-MS analysis. The combination of saponification, LLE, and DLLME resulted in effective matrix removal, lower LODs (0.012–0.29 $\mu g\,L^{-1}$), and higher recoveries (83–120%) than were reported by Liu et al. The above method also had high enrichment factors (270–307) and a short extraction time of 15 min. This method has the potential to be applied to the analysis of other organic compounds in fatty foods.

In 2012, traditional DLLME was coupled with GC-FID and GC-MS for the analysis of several phthalate esters found in milk [21]. Proteins were precipitated and phthalate esters (dimethyl phthalate (DMP), diethyl phthalate (DEP), di-isobutyl phthalate (DIBP), di-*n*-butyl phthalate (DNBP), and di-2-ethylhexyl phthalate (DEHP)) were extracted using NaCl and ACN. It was found that maximum peak area for all analytes was obtained when 0.8 mL of ACN (from the previous extraction step) was mixed with 20 μL of 1,2-dibromoethane and then rapidly injected into a 8% NaCl solution. Identification of analyte peaks found in GC-FID chromatograms was confirmed by GC-MS. Enrichment factors were very high for all analytes, 397–499. This optimised DLLME method was compared to other methods in the literature for the analysis of phthalate esters in milk. Although a reported LLE-LC-MS/MS method had a much lower LOD (LLE-LC-MS/MS: 0.01–0.5 $\mu g\,L^{-1}$, DLLME: 0.5–3 $\mu g\,L^{-1}$) the extraction time was much longer (LLE-LC-MS/MS: 100 min, DLLME: 15 min).

Viñas et al. determined the concentration of thiamine in infant formula and fermented milk using traditional DLLME with HPLC fluorometric detection [13]. All samples underwent a derivatisation reaction to differentiate between thiamine and its esters. The maximum peak area was achieved by selecting ACN (500 μL) as dispersive solvent, tetrachloroethene (90 μL) as extraction solvent, an aqueous phase with an ionic strength (NaCl) of 24%, and centrifugation for 1 minute at 4,000 rpm. The results indicated that DLLME was time-independent, as equilibrium was reached almost instantaneously. The optimised DLLME procedure resulted in lower extraction times (a few seconds) compared to a LPME method (30 min), better extraction efficiency, an LOD of 0.09 $\mu g\,L^{-1}$, and linearity between 0.5 and 10 $\mu g\,L^{-1}$. Recovery of thiamine in infant formula was found to be 98.7% with an RSD of 5.4%.

It was 2013 before DLLME was coupled with field-amplified sample stacking in CE, in the determination of five different nonsteroidal anti-inflammatory drugs (NSAIDs) in milk, yogurt, and cheese [17]. As with other milk samples previously mentioned, proteins were precipitated using phosphoric acid, NaCl, and ACN and centrifugation. Hexane was added to the supernatant to remove any fat present; the hexane was then discarded and the ACN layer was used in the DLLME procedure. For cheese and yogurt, the samples were homogenised with 2 mL of deionized water and the same procedure was followed as outlined above. The results from the optimised DLLME procedure were compared to other preconcentration techniques used in the extraction of NSAIDs. The extraction time was at least five times faster than other reported methods and used at least half the amount of organic solvents.

Campillo et al. analysed several macrocyclic lactones in milk using HPLC-DAD coupled to atmospheric pressure chemical ionization in negative ion mode ion-trap tandem mass spectrometry (APCI-IT-MS/MS) [15]. Prior to DLLME, the proteins were precipitated using TCA. The maximum peak area was achieved when ACN (2 mL) as dispersive solvent and chloroform (200 μL) as extraction solvent were used. The optimum ionic strength of the aqueous phase was obtained by adding NaCl to achieve a concentration of 24% w/v. Using DAD detection, the widest linearity was 5–2500 $ng\,g^{-1}$ (DOR) while the lowest LOD was 0.3 $ng\,g^{-1}$ (MOX and DOR). The lowest LOD achieved by MS/MS was 0.03 $ng\,g^{-1}$. LC-MS/MS detection produced higher selectivity and improved sensitivity.

Campone et al. used a Box-Behnken experimental design to optimise the DLLME procedure used to determine aflatoxin M_1 (AFM_1) in whole, skimmed, and powdered milk with UHPLC-MS/MS detection [18]. The authors also compared two different methods for protein precipitation. Firstly, acetic acid was added and then the sample was heated to 100°C for 3 min and centrifuged and aqueous supernatant was used in the DLLME procedure. This method resulted in a

recovery of only 42.7%, possibly due to proteins binding with AFM_1. The second method investigated used NaCl and ACN to simultaneously precipitate proteins and extract AFM_1 into the ACN. After centrifugation, the ACN supernatant was used as the dispersive solvent in the following DLLME procedure. The volumes of chloroform and ACN that resulted in highest recovery were 1.5 mL and 3.8 mL, respectively. The mixture of extraction and dispersive solvent was rapidly injected into 5 mL of water. Recovery for whole, skimmed, and powdered milk was 75.3%, 74.2%, and 73.3% with precision ranging from 1.6% to 7.6%. The method was linear from 0.25 to 25 $\mu g\,L^{-1}$ and had a LOD of 0.6 $ng\,kg^{-1}$, which is lower than regulations (50 $ng\,kg^{-1}$ [53]).

In 2014, Arroyo-Manzanares et al. used traditional DLLME for the determination of several sulphonamides in milk; the analytes were detected by HPLC with fluorescence detection [16]. The authors also compared their optimised DLLME procedure to QuEChERS. Proteins were precipitated using TCA and then filtered. The DLLME extraction procedure was optimised using a central composite design. The optimum volumes for the extraction solvent (chloroform) and dispersive solvent (ACN) were 1 mL and 1.9 mL, respectively. DLLME resulted in lower LODs (0.73–1.21 $\mu g\,L^{-1}$) than QuEChERS (1.15–2.73 $\mu g\,L^{-1}$) and higher recoveries (92.9%–104.7% compared to 83.6%–97.1%, when samples were spiked with sulphonamides at 150 $\mu g\,L^{-1}$). QuEChERS did prove to be more reproducible than DLLME with lower %RSD values of 2.9%–7.1% and 3.0%–9.7%, respectively.

DLLME was coupled to QuEChERS in 2014 for the determination of six antibiotic fluoroquinolones with HPLC-UV detection [23]. The dried supernatant from the QuEChERS method was resuspended in 1.0 mL of a 10% acetic acid-ACN mixture, combined with 200 μL of chloroform and rapidly injected into 4 mL of deionized water. The cloudy solution was centrifuged for 5 min at 4,500 rpm. By coupling QuEChERS to DLLME, the authors have removed matrix interference, which is common problem with the detection of fluoroquinolones. The method shows good recovery (74.1–101.4% for all analytes) and low LOQs (below 2.5 $\mu g\,kg^{-1}$ for DAN and below 15 $\mu g\,kg^{-1}$ for all other analytes).

In 2015, Alshana et al. determined the concentration of parabens in breast milk and ice cream using DLLME with back extraction before being analysed by CE [30]. Phosphoric acid (100 μL), ACN (1.5 mL), and NaCl solution (0.5 mL) were added to samples prior to vortex mixing for 1 minute and centrifugation for 3 minutes at 4,000 rpm. The ACN supernatant (1 mL) was then used as the dispersive solvent in the DLLME step. Chloroform (200 μL) was added as the extraction solvent before the sample was made up to 8 mL with deionized water. The sample was vortexed for 1 minute which resulted in the formation of a cloudy solution. After centrifugation, the sedimented chloroform phase was transferred to a microtube where the analytes were back extracted into 80 μL of BES for direct injection into CE. Enrichment factors for each paraben ranged from 7.0 to 10.7 and LOD values were between 100 and 200 $\mu g\,L^{-1}$, while RSD values were from 0.6% to 2.3%.

4.1.1. Ultrasound Assisted DLLME. Ultrasound assisted DLLME was first used on a dairy product for the determination of phthalate esters (DMP, DEP, DBP, BBP, DNOP, and DIOP) in milk using GC-FID [26]. Before UA-DLLME could take place, TCA and lead acetate were added to the milk samples. A mixture of MeOH (800 μL) and carbon tetrachloride (40 μL) was used as the dispersive and extraction solvent, respectively. Once the cloudy solution had formed, it was placed in an ultrasonic bath for two minutes. The UA-DLLME method resulted in low LODs (0.64–0.79 $ng\,g^{-1}$), high enrichment factors (220–270), and %RSD values from 2.8 to 4.0%.

In 2013, simultaneous derivatisation and UA-DLLME were developed for the determination of chlorophenols (1,3-DCP, 2,3-DCP, and 3-MCPD) in milk using GC-MS [27]. Proteins were precipitated by ACN (2 mL), which was also used as the dispersive solvent. Both the extraction solvent, chloroform (100 μL), and the derivatisation reagent, HFBI (50 μL), were mixed with ACN. After the formation of the cloudy solution, the sample was placed in an ultrasonic bath heated to 30°C for five minutes. This was to aid emulsion formation and to ensure that derivatisation was complete. The extraction parameters were optimised by experimental design. LODs as low as 0.9–3.6 $\mu g\,L^{-1}$ were achieved along with recoveries ranging from 99% to 102%.

Karaseva et al. coupled QuEChERS to UA-DLLME for the determination of aflatoxins B1 and M1 in milk and cheese samples using HPLC with fluorescence detection [28]. QuEChERS was used as a sample pretreatment protocol and to initially extract the aflatoxins from the milk samples. ACN (3 mL) and chloroform (500 μL) were used as dispersive and extraction solvents, respectively. Once a cloudy solution had formed, it was placed in an ultrasonic bath for two minutes. The sedimented phase that was produced after centrifugation was dried under nitrogen. The residue was then reconstituted in ACN and subjected to HPLC analysis. The limits of detection for both B1 and M1 were 0.1 $\mu g/kg$ and 0.01 $\mu g/kg$, respectively. Recoveries for B1 for all samples were between 51.2% and 75.4%, while M1 had recoveries between 52.5% and 72.2%. Total sample preparation time was approximately 1.5 hours.

4.1.2. Low-Density Solvent DLLME. Solvents that have a density lower than water were used as extraction solvents in the determination of benzoate and sorbate in yogurt drinks [35]. Sample preparation involved protein precipitation by NaOH, H_2SO_4, potassium hexaferrocyanide, and zinc acetate. The supernatant from the previous step was used as the aqueous phase for LDS-DLLME. Ethanol (450 μL) and 1-octanol (60 μL) were used as the dispersive and extraction solvents, respectively. After centrifugation of the cloudy solution, the 1-octanol was removed and injected into HPLC-UV system for analysis. The LDS-DLLME parameters were optimised by a central composite experimental design. This method was compared to several other procedures reported in the literature for the analysis of benzoate and sorbate. LODs for this method (benzoate: 0.06 $\mu g\,L^{-1}$, sorbate: 0.15 $\mu g\,L^{-1}$) were much lower than those found in other methods (benzoate: 1.22–900 $\mu g\,L^{-1}$, sorbate: 2–500 $\mu g\,L^{-1}$). The method also

provided good recovery of both benzoate (91.25%) and sorbate (106%).

Abedi et al. also determined benzoate and sorbate concentration in milk, cheese, and yogurt drinks by LDS-DLLME, this time using GC-FID as the detection method [33]. Many aspects of the papers are the same: both methods are optimised by central composite design, both use similar sample pretreatment procedures, and both have found that the optimum extraction solvent is 60 μL of 1-octanol. Abedi et al. have found that 475 μL of acetone is the optimum dispersive solvent. The newly developed LDS-DLLME-GC-FID method showed recoveries of benzoate (103.7%) and sorbate (88%) that differ from the previous paper. LODs were 140 ng g^{-1} and 150 ng^{-1} for benzoate and sorbate, respectively.

In 2015, Amoli-Diva et al. coupled LDS-DLLME with vortex-assisted dispersive solid phase extraction (VA-D-SPE) for the analysis of aflatoxin M1 in milk samples [34]. Once the optimised LDS-DLLME emulsion had been formed (extraction solvent: 1-heptanol; 320 μL, dispersive solvent : MeOH/water (80 : 20); 3 mL), 500 μL of adsorbent (containing acid modified magnetic nanoparticles (MNPs)) was added and the sample was agitated on a vortex. An external magnet was applied which allowed the safe removal of supernatant. The analyte was desorbed from the adsorbent by the addition of 2 mL of ACN. Finally, the analyte was separated from the MNPs by magnetic decantation. The ACN eluent was evaporated to dryness and the residue was reconstituted in Triton X-100 before analysis by fluorescence spectrophotometer. The method had an LOD for aflatoxin M1 of 0.013 $\mu g\,L^{-1}$, a linear range between 0.02 and 200 $\mu g\,L^{-1}$, and an extraction time of 20 min.

4.1.3. UA-RM-DLLME. Previously, all analytes mentioned have largely been nonpolar, hydrophobic compounds. Roosta et al. have developed a method using a surfactant that forms reverse micelles (Triton X-100) for the determination of acetoin, a polar compound, in butter using an ultrasound assisted-reverse micelle-DLLME procedure coupled with HPLC-UV detection [31]. The butter samples (2 g) were melted by heating at 40°C for 5 minutes before dilution with 2 mL of hexane and adding Triton X-100 (1.25% w/v). The sample was mixed by vortex for 1 minute. Distilled water (400 μL) was added as a modifier and the formation of a cloudy solution was produced by placing the sample in an ultrasonic bath for 4 minutes followed by centrifugation. The extraction process was optimised by a Box-Behnken experimental design. The LOD for the developed method was found to be 200 $\mu g\,L^{-1}$, while extraction recovery and repeatability were 96.40% and 2.86%, respectively.

4.1.4. IL-DLLME. Room temperature ionic liquids are another alternative green extraction solvent. Recently, in 2015, an IL-DLLME procedure was developed for the determination of nifurtimox (NFX) and benznidazole (BNZ) in breast milk coupled to HPLC-UV [37]. Proteins and lipids were removed by the addition of a precipitation mixture ($HClO_4$, H_3PO_4, and methanol) followed by incubation at 80°C for 60 minutes. After centrifugation, the supernatant was separated from the solid material (proteins and lipids). This process was repeated and the supernatants combined. Analysis of NFX and BNZ was carried out separately using two different IL-DLLME procedures. For NFX, a mixture of NaOH (50 μL; 2 M) and KCl (150 μL; 30% w/v) was added to the supernatant. Then 42 μL of $[C_6C_1im][PF_6]$, as extraction solvent, and 80 μL of MeOH, as dispersive solvent, were rapidly injected into the above supernatant. For BNZ, a mixture of NaOH (45 μL; 2 M) and KCl (100 μL; 30% w/v) was added to the supernatant obtained from the pretreatment step. Both $[C_6C_1im][PF_6]$ (42 μL) and MeOH (101 μL) were mixed and rapidly injected into the above supernatant. The NFX and BNZ samples were shaken on a vortex for 6 minutes and centrifuged at 10,000 rpm for 20 min. The extraction solvent was sedimented at the bottom of the centrifuge tube. The supernatant was removed and the extraction solvent was injected for analysis. The NFX procedure had an LOD of 290 $\mu g\,L^{-1}$, a linear range from 300 to 34,400 $\mu g\,L^{-1}$, and an enrichment factor of 33.8. The BNZ procedure had a LOD of 180 $\mu g\,L^{-1}$, a linear range from 200 to 29,160 $\mu g\,L^{-1}$, and an enrichment factor of 28.8.

4.1.5. UA-IL-DLLME. The use of ionic liquids as extraction solvents has been combined with US-DLLME in technique termed: UA-IL-DLLME. Tuzen and Pekiner developed an US-IL-DLLME method for the determination of selenium in milk using graphite furnace atomic absorption spectrometric detection [32]. Prior to microextraction, the pH of the sample was lowered to pH 2 with dilute HCl. Chelation of selenium was achieved through adding 0.1% 1-phenylthiosemicarbazide (1 mL). The extraction solvent, $[C_6MIM][Tf_2N]$ (100 μL), was added and the sample was placed in an ultrasonic bath for 10 minutes. The resulting cloudy solution was centrifuged and, afterwards, placed on ice to increase the viscosity of the now sedimented extraction solvent. The aqueous phase was removed by simple decantation. A mixture of HNO_3 and ethanol (1 : 1 v/v) was added to the extraction solvent to decrease viscosity and allow for easier retrieval. The authors found that, without the use of ultrasound, recovery of selenium was below 25% and quantitative recovery was achieved when the sample was sonicated for 10 minutes. The UA-IL-DLLME method had an LOD of 0.012 $\mu g\,L^{-1}$, a linear range between 0.04 and 3.0 $\mu g\,L^{-1}$, a %RSD value of 4.2%, and an enrichment factor of 150.

4.1.6. UA-IL/IL-DLLME . Ionic liquids have also been used as both dispersive (hydrophilic IL) and extraction (hydrophobic IL) solvents in the same method. Gao et al. have developed a UA-IL/IL-DLLME method to determine the concentration of sulphonamides in infant formula using HPLC-PDA detection [29]. A sample of milk powder was weighed and dissolved in distilled water (50°C); the ratio of infant formula to water was 1 : 8. Orthophosphoric acid (20 μL) and $[C_6MIM][BF_4]$ (70 μL), as extraction solvent, were added to the sample and intensely shaken for 5 min. When complete, $[C_4MIM][BF_4]$ (100 μL), as dispersive solvent, was added and the sample was transferred to an ultrasonic bath for 2 minutes. The resulting cloudy solution was then centrifuged and the sedimented extraction phase was collected. The IL was diluted

with ACN and 0.1% formic acid to 200 μL before being filtered and injected into HPLC for analysis. The optimised method was used to determine the concentration of six different sulphonamides: sulfamerazine (SMI), sulfamethizole (SMT), sulfachlopyridazine (SCP), sulfamonomethoxine (SMM), sulfamethoxazole (SMX), and sulfisoxazole (SIA). The LODs for each sulphonamide ranged from 2.94 to 16.7 μg kg^{-1}. Recovery for all the sulphonamides was all above 95% with RSD values less than 6.5%.

4.1.7. VA-DLLME. D'Orazio et al. developed a VA-DLLME method to determine estrogenic compounds in milk and yogurt coupled to micellar electrokinetic chromatography with mass spectrometry [36]. The removal of proteins and fats was achieved by adding ACN (4 mL) and acetic acid (100 μL). The sample was vortexed for 2 minutes and left in the dark for 15 minutes before centrifugation for 10 minutes at 4400 rpm. The supernatant was treated with 2 mL of hexane and the above vortex and centrifugation process was repeated. The aqueous layer was evaporated to 1.5 mL using a rotavapor (40°C; 180 mbar). The extract was diluted to 7.5 mL with Milli-Q water and NaCl was added (30% w/v). After filtration, a mixture of dispersive solvent (ACN; 500 μL) and extraction solvent (chloroform; 110 μL) was added and the sample was vortexed for 2 minutes. After centrifugation, the sedimented chloroform phase was collected and evaporated to dryness, before being reconstituted in 75 μL of the sample medium (11.25 mM APFO, pH 9 containing 10% v/v MeOH) and injected into the MEKC-MS system.

5. Conclusion

This is the first review of the use of DLLME in dairy samples. It can be seen that the various modes of DLLME can be applied to a range of analytes in different samples, while being coupled to various analytical techniques. The review also highlights the importance of the sample pretreatment step in carrying out a successful DLLME method. With the correct sample pretreatment, DLLME can be a powerful tool in the analysis of analytes in dairy products, affording high enrichment factors while using minimal organic solvents. The technique allows the use of different analytical techniques which increases the number of potential analytes that can be tested. In general, the above modes of DLLME are both quick and easy to use, but they do have some drawbacks. Each sample can require: pH adjustment, filtration, or centrifugation, depending on the sample pretreatment required. This can increase total sample preparation time.

Competing Interests

The authors declare that they have no competing interests.

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Effect of seaweed supplementation on tocopherol concentrations in bovine milk using dispersive liquid-liquid microextraction

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ABSTRACT

A dispersive liquid-liquid microextraction (DLLME) method, combined with HPLC-UV detection, was developed for the extraction and preconcentration of δ-tocopherol from bovine milk. This method was used to study the effect of supplementing cow feed with the seaweed *Ascophyllum nodosum* on vitamin content in milk. The optimal experimental conditions were determined: 200 µL of chloroform (extraction solvent), 1.0 mL of ethanol (dispersive solvent), 5 mL of water (aqueous phase). Under these optimal conditions the DLLME method provided linearity in the range 0.01 µg/mL to 8 µg/mL with R^2 values of 0.998. Limit of detection (LOD) was 0.01 µg/mL, while the enrichment factor was 89. Cow feed that was supplemented with *Ascophyllum nodosum* was shown to increase δ-tocopherol levels from 3.82 µg/mL to 5.96 µg/mL.

1. Introduction

Milk is considered one of the most important sources of protein, lipids, vitamins, and minerals for humans [1]. The composition of bovine milk is well characterised and a detailed breakdown for each constituent has been provided by Meurant [2]. In terms of fat soluble vitamins, bovine milk is a particularly good source of tocopherols [3]. Tocopherols, which belong to the vitamin E family, exist as α, β, γ, and δ forms; each exhibiting lipid antioxidant properties which protect polyunsaturated fatty acids from oxidation [4]. Importantly, δ-tocopherol shows the highest antioxidant activity in foods [5]. Other health benefits of increased vitamin E intake include: improving reproductive health in females [6], preventing progression of muscle skeletal disorders (e.g. sarcopenia) [7], and reducing the build-up of amyloid-β plaques linked to Alzheimer's disease [8].

Recently, efforts have been made to alter the composition of bovine milk by supplementing cow feed to increase the nutritional benefits of the milk while also improving their immunological health [9–11]. For example, supplementation of cow feed with various vegetable fats, fish oils, or seed oils has been shown to increase CLA content in milk [12]. Conjugated linoleic acid (CLA) and other polyunsaturated fatty acids have been shown to have many potential health benefits for human health [13]. Furthermore, other studies have investigated similar effects using marine microalgae [14–17]. Several studies have examined the relationship between diet supplementation with *Ascophyllum nodosum* and the mineral content of milk, for example iodine [15, 18], toxic trace elements such as arsenic [17], and more recently polyunsaturated fatty acid content [16]; all of which can increase or decrease the nutritional properties of the milk.

The use of *A. nodosum* as a vitamin supplement is relatively unexplored. Kidane et al. supplemented cow feed with approximately 35% of a commercial dried *A. nodosum* supplement and found that there was no significant increase in milk tocopherol levels [4]. *A. nodosum* is the most common seaweed found along North Atlantic coasts [19] and this is reflected in the anecdotal evidence for the benefits of *A. nodosum* as a supplement for cow feed [20].

Given that bovine milk is a complex biological fluid, sample preparation is a crucial step in its analysis. Tocopherol is present in trace amounts so any sample preparation step must not only selectively extract the analyte in question, but also preconcentrate it to allow for accurate quantification. Traditional methods to preconcentrate and extract fat soluble vitamins in milk include solid phase extraction (SPE) [21], super critical fluid extraction [22], and liquid-liquid extraction [23]. The aforementioned techniques are time consuming, require high volumes of solvent and also require expensive consumables. In this study the use of dispersive liquid-liquid microextraction (DLLME) [24] was investigated. This technique uses low volumes of organic solvents, enables rapid extraction, and offers significant preconcentration of analytes. Elsewhere, DLLME has been used in the analysis of foodstuffs such as fruit juices [25], plant based foods [26], and urine [27] among others.

Therefore, the objective of this study was to optimise a DLLME

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method using Design of Experiments (DoE) and to investigate the effect of *A. nodosum* supplementation on tocopherol content in milk.

2. Materials and methods

2.1. Chemicals and materials

HPLC grade methanol (MeOH), acetonitrile (ACN), and ethanol were purchased from Lennox (Dublin, Ireland). BD Precision glide syringe needles gauge 30 L 1.0 in., δ-tocopherol, and ascorbic acid, were purchased from Sigma Aldrich (Dublin, Ireland). Ultrapure water was provided by a Whitewater purification system (Dublin, Ireland).

2.2. Apparatus

Chromatography was performed on an Agilent 1100 HPLC system equipped with an Agilent Zorbax Eclipse Plus C_{18} column (50 × 4.6 mm; 1.8 µm). ACN was used as an isocratic mobile phase at a flow rate of 1.0 mL/min. The injection volume was 1 µL. The separation was carried out at 30 °C. Detection was carried out at a wavelength of 327 nm. Statistical analysis was carried out using Minitab (v18.0). A separation of δ-tocopherol can be seen in Fig. 1.

2.3. Stock standard preparation

A stock standard solution was prepared by dissolving δ-tocopherol (1.32 mg/mL) in MeOH.

2.4. Experimental design and diet supplementation

A total of 12 British-Friesian dairy cows were enrolled in this study and were balanced for age, parity, calving date, average lactation milk yield and average body condition score. Control ($n = 6$) and experimental ($n = 6$) animals were housed indoors on slats in separate pens for the duration of the experiment and were fed a diet consisting of grass silage supplemented with a standard dairy cow ration and pre-calver minerals. A pre-trial period was enforced to ensure intake of *A. nodosum* by the experimental group prior to trial commencement. Diet supplementation began approximately day 80 precalving until day 15 postcalving. *A. nodosum* was harvested and offered to the experimental group daily.

2.5. Analytical procedure

The milk samples were obtained from cows and frozen at −20 °C until analysis. The samples were thawed and shaken before extraction. The samples were prepared as follows: 1.0 mL of milk, 9.0 mL of ethanol (containing ascorbic acid; 5 g/L) were added together. The samples were heated at 78 °C for 30 min and shaken at 10 min intervals. Post heating, samples were cooled on ice and centrifuged for 5 min at 4500 rpm. 1.0 mL of supernatant was mixed with 200 µL of chloroform and rapidly injected into 5 mL of ultrapure water. The resulting cloudy solution was centrifuged for 5 min at 4500 rpm. The organic phase was again centrifuged for 10 min at 13,500 rpm before injection on HPLC system.

2.6. Calculation of enrichment factor

The enrichment factor (EF) for each of the selected vitamins was calculated using the following equation

$$EF = \frac{C_{final}}{C_{initial}} \quad (1)$$

where C_{final} is the concentration of the analyte in the extraction solvent, and $C_{initial}$ is the concentration of the analyte in the sample solution.

3. Results

3.1. Optimisation of DLLME procedure

In the present work, parameters such as the protein precipitation solvent, extraction solvent, volume of extraction solvent, volume of dispersive solvent, and volume of aqueous phase were optimised. The protein precipitation and extraction solvent were optimised first. The

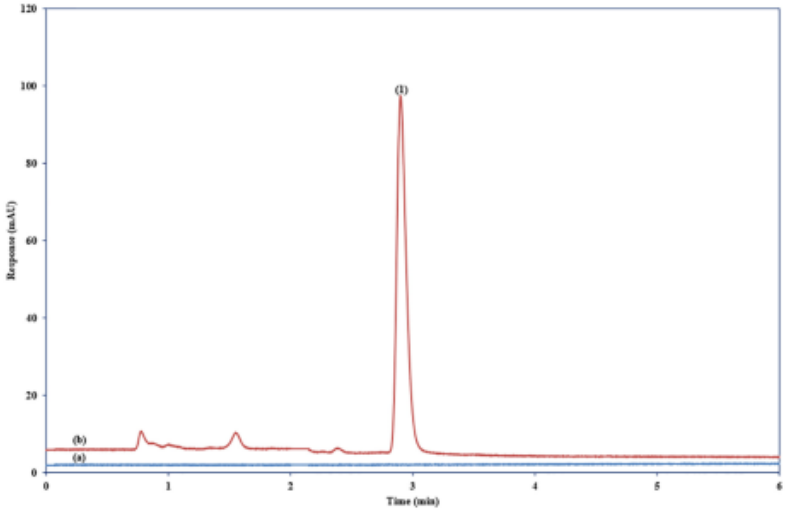

Fig. 1. Chromatogram of δ-tocopherol. Peak identification: (1) δ tocopherol (20 µg/mL). Chromatographic conditions: stationary phase: C_{18} (50 × 4.6 mm; 1.8 µm), mobile phase: ACN, flow rate: 1.0 mL/min, temperature: 30 °C, detection wavelength: 327 nm.

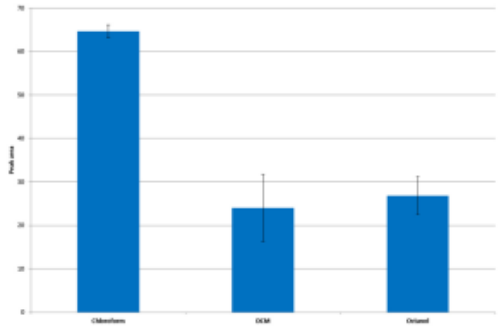

Fig. 2. Evaluation of extraction solvents ($n = 3$). Analyte concentration and chromatographic conditions as in Fig. 1.

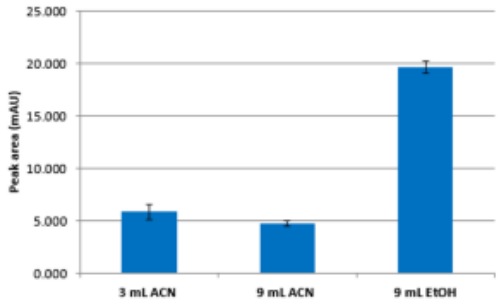

Fig. 3. Screening of potential protein precipitation solvents ($n = 3$). Analyte concentration and chromatographic conditions as in Fig. 1.

Table 1
Screened factors and levels.

Factor	−1	+1
Dispersive solvent volume (mL)	0.5	1.0
Extraction solvent (μL)	200	400
Aqueous phase volume (mL)	5	10

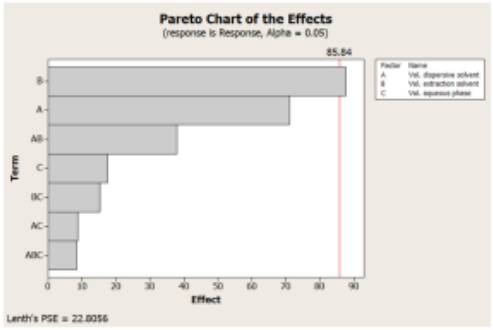

Fig. 4. Pareto chart of factorial screening experiment ($p < 0.05$).

Table 2
Factors and levels in central composite design.

Factor	−α	−1	0	+1	+α
Dispersive solvent (mL)	0.293	0.5	0.75	1.0	1.41
Extraction solvent (μL)	117.16	200	300	400	565.68

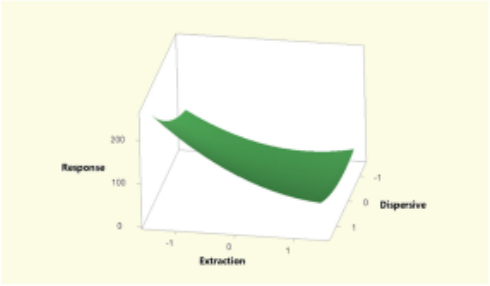

Fig. 5. Response surface generated from the central composite design.

Table 3
Figures of merit for novel DLLME method.

Analyte	Linearity (R^2)	LOD (μg/mL)	Reproducibility (% RSD) ($n = 6$)	Recovery (%) ($n = 6$)	EF
δ-tocopherol	0.998	0.10	2.9	81	89

Table 4
Comparison with published methods.

Sample	Analyte	Mode of analysis	LOD (μg/mL)	Reference
Milk	δ-tocopherol	HPLC-UV	0.5	2014 [31]
Multivitamin syrup	α-tocopherol acetate	HPLC-UV	76.1	2015 [21]
Milk	α-tocopherol	HPLC-FLD	0.1	2017 [32]
Milk	δ-tocopherol	HPLC-UV	0.1	Presented work

volumes of the solvents used were then optimised by design of experiments (DoE) using a factorial screening and a central composite design approach. The optimisation process was carried out using milk purchased from a local shop before applying the method to supplemented milk samples. Parameters such as ascorbic acid concentration, heating time and temperature were previously optimised [28] and adapted for this work.

3.1.1. Selection of organic extraction solvent

Any extraction solvent for DLLME must fulfil the criteria outlined by Rezaee et al. [24]. Solvents that had both higher and lower densities than water were examined as potential extraction solvents. The screened solvents were chloroform ($CHCl_3$), dichloromethane (DCM) (CH_2Cl_2), and octanol. As Fig. 2 shows, chloroform displayed the highest extraction efficiency and thus was selected as the optimum extraction solvent.

3.1.2. Selection of protein precipitation solvent

The method of protein precipitation must be compatible with DLLME. Simultaneous protein precipitation and analyte extraction using organic solvents was trialled, as protein precipitation without

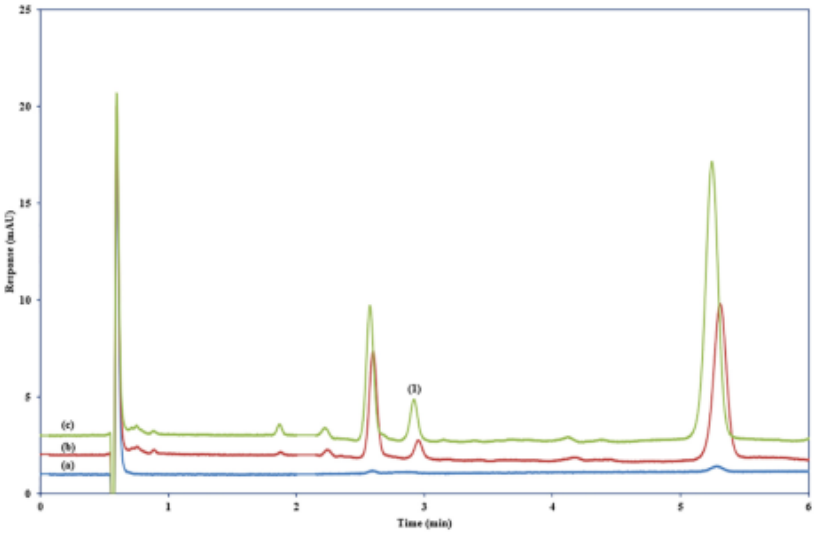

Fig. 6. Chromatograms obtained from sample analysis. Chromatogram (a) blank, (b) no seaweed supplementation, (c) seaweed supplementation. Peak identification: (1) tocopherol. Chromatographic conditions as outlined in Fig. 1.

efficient extraction would result in poor recovery and higher LOD. Acetonitrile (ACN) and ethanol were screened as potential protein precipitation and extraction solvents. These solvents are common dispersive solvents in DLLME methods and also commonly used in protein precipitation applications [29, 30]. Fig. 3 shows that ethanol had greater extraction efficiency than ACN and so ethanol was used as the dispersive solvent in the DoE. The possible explanation for the better extraction efficiency when ethanol is used compared to ACN is two-fold. Firstly, ethanol produced a more stable cloudy solution upon rapid injection into the aqueous phase. This facilitates a more rapid transfer of the analytes into the extraction phase, resulting in a more efficient extraction. Secondly, the solubility of the analytes could be greater in ethanol than ACN; also resulting in a more efficient extraction.

3.1.3. Factorial screening

A 4 factor, 2 level factorial design (2^4) was used to screen for significant factors. A list of the factors and levels can be found in Table 1. Preliminary experiments determined the minimum and maximum levels for each factor. Values outside these ranges resulted in poor extractions due to an unstable cloudy solution. In the case of the minimum extraction solvent volume, when < 200 µL was used the resulting organic phase volume was too loo low to be analysed.

The resulting Pareto chart (Fig. 4) showed that only the volume of extraction solvent was significant. This is evident as the volume of dispersive solvent is the only factor that crosses the significance line. As the extraction efficiency of DLLME is dependent on the stability of the cloudy solution formed from the interaction of extraction and dispersive solvents, it was decided to further optimise the DLLME procedure.

3.1.4. Central composite design

A central composite design was chosen to further optimise the DLLME process by varying the volumes of dispersive and extraction solvents. The goal was to maximise the response, in this case response was analyte peak area. The central composite design consisted of a 2^2 full factorial design which was augmented with both star (± α) and centre points (0). The levels for each factor are given in Table 2 and the resulting response surface can be seen in Fig. 5.

The response was maximised when 200 µL of extraction volume and 1.0 mL of dispersive solvent was used.

3.2. Validation of DLLME procedure

To evaluate the applicability of the developed DLLME method to δ-tocopherol analysis in bovine milk, linearity, limit of detection (LOD), reproducibility, recovery, and enrichment factor were determined; these were evaluated with spiked samples. The figures of merit are shown in Table 3. Linearity for δ-tocopherol was obtained in the range 0.1 to 8 µg/mL. The analyte was spiked in at the following concentrations: 0.1, 2, 4, 6, and 8 µg/mL. The linearity was determined by plotting calibration curves of peak area versus the concentration of each analyte. The coefficients of the analyte was 0.998. The LOD was obtained from the slope of the linearity curve, according to Eq. 2 where σ is standard deviation of the calibration curve and s is the slope of the calibration curve. The relative standard deviation (%RSD) was 2.9% (n = 6). Recovery for δ-tocopherol was 81%, while the enrichment factor was 89.

$$\mathrm{LOD} = 3.3 \times (\sigma/s) \quad (2)$$

3.3. Comparison with published methods

The optimised method was compared against recently published methods for tocopherol analysis. The presented method has shown lower limits of detection or has obtained equal LOD values but without the use of extensive sample preparation techniques, such as SPE. The comparison can be seen in Table 4.

3.4. Sample analysis

3.4.1. Standard addition

Given the complexity of the sample, analytes were quantified using standard addition. This method was preferred over calibration in presence of matrix to account for any matrix interference in the analyte response. The standard addition curve was constructed from five points. The first point in the standard addition curve was obtained from analysing sample without added standards, while the remaining four points were obtained from adding increasing amounts of standard (2, 4, 6, 8 μg/mL).

3.4.2. Effect of seaweed supplementation

The effect of seaweed supplementation on the FSV content of bovine milk was investigated with the newly developed DLLME method. The analysis was carried out as detailed in Section 2.5. It was found that seaweed supplementation had a statistically significant ($p > 0.05$) effect on the concentrations of δ-tocopherol, increasing from 3.82 μg/mL to 5.96 μg/mL. An example of the chromatograms obtained can be seen in Fig. 6. As outlined in the introduction, an increase in the levels of tocopherol have numerous benefits. The use of a locally grown, renewable resource grown to increase the nutritional benefits of bovine milk could have a wide impact on the agricultural and food industries in Ireland.

4. Conclusion

An optimised DLLME sample preparation method combined with HPLC-UV was developed and used to investigate the effect of *A. nodosum* on the tocopherol content of British Friesian milk. The DLLME method was optimised by DoE which resulted in an environmentally friendly method which used minimal organic solvents. It afforded high enrichment factors, low detection limits, and good repeatability. The supplementation resulted in an increase in delta tocopherol content from 3.82 μg/mL to 5.96 μg/mL.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgment

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The application of dispersive liquid–liquid microextraction in the analyses of the fatty acid profile in bovine milk in response to changes in body condition score

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ABSTRACT

Dispersive liquid–liquid microextraction (DLLME) was used prior to gas chromatography flame ionization detection (GC-FID) for the extraction of five fatty acids from milk taken from cows with different body condition scores. Optimum extraction conditions were: 300 µL of chloroform (extraction solvent), and 1 mL methanol (dispersive solvent). The procedure was optimised using Design of Experiments (DoE). The analytes were separated on a GC capillary column containing a polyethylene glycol stationary phase (15 m × 0.53 mm × 1.2 µm). Enrichment factors were in the range of 8–15 and limit of detection (LOD) was 0.04 µg/mL. Calibration graphs showed good linearity with coefficients of determination higher than 0.994% and relative standard deviations lower than 7%. This method provided a simple and rapid derivatisation and extraction method for the determination of fatty acids in bovine milk. It showed that there was a significant difference in the palmitic acid content of milk from cows that had an optimum body condition score (10.85 mg/mL) compared to cows that had a high body condition score (5.73 mg/mL).

1. Introduction

Bovine milk is an important source of energy, protein, vitamins, and essential minerals for humans. The composition of milk has a direct influence on the nutritional quality and processability of any subsequent dairy products. In particular, different fatty acids have nutritional benefits [1]. Poly unsaturated fatty acids (PUFA) play a vital role in the prevention of coronary heart disease [2]. Although saturated fatty acids are structurally similar, they can exhibit remarkably different biological properties. For example, stearic acid (18:0) and myristic acid (14:0) only differ in chain length by 4 carbons but only myristic acid will increase total cholesterol levels [3]. Palmitic acid (16:0) can be converted to unsaturated forms through the action of SCD1 [3]. One such unsaturated form is palmitoleic acid (16:1 n-7), which has been shown to enhance insulin sensitivity and uptake of glucose [4]. Trans vaccenic acid (18:1 *trans*-11) is a monounsaturated fatty acid (MUFA) which makes up the majority (~70%) of total *trans* fatty acids which are found in ruminant lipids, such as milk fat [5]. Supplementing dairy foods with *trans* fatty acids can prevent adipose tissue lipogenesis and has anticarcinogen properties in some animal models. [5]. Like their longer chain counterparts, levels of shortchain fatty acids (SCFAs) are also found to be important in the analysis of several physiological conditions such as diarrhoea [6], and inflammatory bowel disease [7]. Given the important physiological functions of these compounds, a sensitive and selective extraction method is needed.

Selectively extracting and analysing fatty acids from a complex biological fluid such as milk presents a sizable challenge. In addition, there are over 400 different fatty acids present in bovine milk. The majority (98%) are found in triglyceride (TG) form, with the remaining 2% found as free fatty acids (FFA) [8].

For analysis by gas chromatography (GC), non-volatile fatty acids require derivatisation. Typically, fatty acids are derivatised to fatty acid methyl esters (FAMEs); which are volatile and compatible with GC analysis. This derivatisation is carried out by acid catalysed derivatisation [9], base catalysed derivatisation [10], or derivatisation by pyrolysis [11].

The body condition score (BCS) is a visual and tactile assessment of the proportion of body fat that a dairy cow possesses [12]. The BCS is generally scored on a scale ranging from 0 to 5, with 0.25 increments; although this varies from country to country. A low score reflects that a cow is emaciated while a higher score indicates obesity. The optimum BCS prepartum is between 3.0–3.50. Values outside this optimum range result in cows that will be immunocompromised. In addition to

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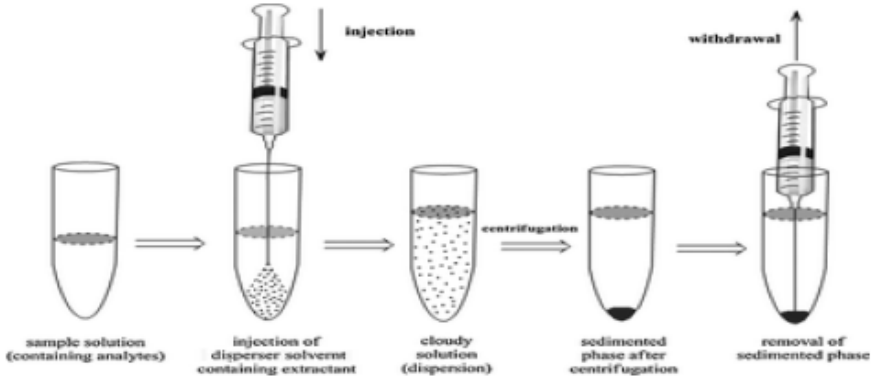

Fig. 1. Schematic of DLLME [20].

increased chances of infection, the cow may not cycle in time for the next calving. The body condition score of a cow can have a marked influence in milk quality, with a low BCS resulting in higher levels of cholesterol [13].

Dispersive liquid–liquid microextraction was developed in 2006 [14]. This new liquid–liquid extraction uses a ternary solvent system to create a cloudy solution created by rapid injection of extraction and dispersive solvents into an aqueous solution. This stable emulsion of micro-droplets of extraction solvent enables rapid mass transfer of analytes into the extraction solvent, while simultaneously pre-concentrating the analytes. Centrifugation of the cloudy solution sediments the extraction phase allowing for easy retrieval for analysis by the chosen method. A schematic of the process can be seen in Fig. 1.

DLLME has been applied to fatty acid analysis in other matrices [15,16] and in some dairy samples [17] but to the best of the author's knowledge, this is the first time DLLME has been used in the analysis of FAMEs from bovine milk. The successful development of a DLLME and derivatisation method, both optimised using DoE, for the analyses of fatty acids found in bovine milk is an exciting step forward. In addition, successfully linking changes in the fatty acid profile with changes in BCS will potentially be of great benefit to the dairy industry.

2. Experimental

2.1. Chemicals and reagents

BF_3-MeOH (14% w/v), phosphoric acid, sodium chloride, chloroform, methanol, supelco 37 component FAME mix (certified reference material), nonanoic acid methyl ester, and glyceryl triheptadecanoate were purchased from Sigma Aldrich (Ireland). Ultrapure water was provided by a Whitewater purification system.

2.2. Instrument and analytical conditions

The analytical instrument was an Agilent 6890 GC, coupled with an FID detector (Agilent, USA). The GC was equipped with a 6890 autosampler. The injector temperature was 300 °C, and the injection volume was 1 µL with a 10:1 split. Helium was used as the carrier gas at a flow rate of 4 mL/min. The separation of analytes was performed on an Alltech AT-1000 capillary column (stationary phase: polyethylene glycol, 15 m × 0.53 mm; 1.2 µm). The column temperature was programmed as follows: the initial temperature was 40 °C, increased to 114 °C at 10 °C/min and held for 1 min. The temperature was then ramped to 220 °C at 5 °C/min. The total run time was 50 min. The FID detector temperature was set 280 °C. The internal standard calibration method with peak area was used for quantification of selected fatty acids.

2.3. Derivatisation and DLLME procedure

Milk (1 mL), conc. phosphoric acid (30 µL), sodium chloride solution (1 mL; 2 M), and Folch solution (750 µL) were placed in a centrifuge tube. The Folch solution contained nonanoic acid methyl ester as internal standard (0.25 mg/mL). The samples were shaken by hand for 30 s and centrifuged for 5 min at 4500 rpm. The aqueous supernatant was discarded and the sedimented chloroform phase (250 µL) was transferred to a micro reaction vial. BF_3 in methanol (1 mL; 14% w/v) was added and the samples were heated for 30 min at 90 °C. After cooling, the reaction mixture (1 mL) was rapidly injected into water (5 mL). The resulting cloudy solution was centrifuged for 5 min at 4500 rpm. The aqueous phase was discarded and the sedimented chloroform phase was transferred to a GC vial. If analytes are present in higher concentrations, the extract was diluted by combining 1 part extract with 9 parts chloroform.

2.4. Analytical curves

FAMEs were retention time matched to peaks in the sample with those of standard compounds. Analytical curves were based on the internal standardisation method. The internal standard used was nonanoic acid (C9:0) at a concentration of 0.25 mg/mL. The concentrations for caprylic and capric acid ranged from 0.01 to 2.5 mg/mL. The concentrations for palmitic, oleic, and stearic 2–12 mg/mL.

2.5. Enrichment factors

Enrichment factor (EF) was defined as the ratio of analytes concentration in the organic phase, C_f, and the initial concentration of milk sample, C_o. The formula was as follows:

$$Enrichmentfactor = \frac{C_f}{C_o}$$

2.6. Method validation

The analytical method was validated by assessing linearity, recovery, repeatability, limit of detection (LOD), and limit of quantification (LOQ), according to ICH guidelines for these parameters. The LOD and LOQ were calculated by multiplying the ratio of standard

deviation of the response and slope of the calibration curves by 3.3 and 10, respectively.

The method was found to be linear for all analytes between 0.01–4000 μg/mL. Recoveries for each analyte were as follows: caprylic (70%), capric (80%), palmitic (84%), stearic (73%), and oleic (89%). Repeated (n = 6) DLLME extractions yielded a repeatability of 7%. The LOD was 0.04 μg/mL, while the LOQ was 0.1 μg/mL.

2.7. Body condition score

The BCS of Holstein Frieseian cows was determined by visual and tactile means, they were rated on 0–5 point scale with 0.25 increments. The measurement was carried out by a trained professional. The cows were divided into two groups: optimum BCS (n = 2) and high BCS (n = 2).

3. Results and discussion

3.1. Optimisation of GC-FID conditions

Optimisation of the GC-FID method was performed with a standard solution containing 37 FAMEs, purchased from Sigma-Aldrich. The standard solution was diluted with 9 parts hexane 1 part standard solution. An initial scouting gradient of 5 °C/min was used. The early eluting peaks (0–30 min) were well resolved and so the ramp rate was increased to 10 °C/min to shorten analysis times, after 30 min the ramp rate was reduced to 5 °C/min. Giddings approximation was used to further resolve the remaining critical peak pair, resulting in an isothermal hold at 114 °C for 1 min. The length of the capillary column used, meant that resolution of all 37 FAMEs was not possible in an acceptable analysis time. It is recommended that the injection temperature is approximately 50 °C higher than the last eluting analyte, and so the injection temperature was set at 300 °C. A chromatogram of the standard mix solution obtained under the above-mentioned conditions was shown in Fig. 2.

3.2. Optimisation of derivatisation conditions

FAMEs can be produced by both acid and base catalysis. Base catalysed reactions only produce FAMEs from fatty acids that are part of triglyceride molecule, leaving free fatty acids underivatised. Acid catalysed reactions derivatise both free fatty acids and fatty acids that were present in triglyceride form. Also preliminary DLLME studies using base catalysed derivatisations showed that phase separation after the formation of stable cloudy solution was not possible. It was thought that the presence of underivatised free fatty acids prevented the sedimentation of the extraction phase. It is for this reason that acid catalysed derivatisation methods were optimised.

Table 1
2^2 factorial screening for acid catalysed derivatisation.

Factor	Minimum level (−1)	Centre point (0)	Max. level (+1)
Time (min)	15	22.5	30
Temperature (°C)	50	75	90

3.2.1. Selection of acid catalyst

Derivatisation rates have been shown to be affected by the temperature at which the derivatisation was carried out, and the length of time that analytes were derivatised. As these experimental factors are interlinked, a 2^2 factorial design was constructed to determine which factors, if any, have a significant impact on derivatisation. The response was the reciprocal peak areas of nonanoic acid methyl ester and heptadecanoic acid methyl ester, with a bigger ratio indicating better derivatisation. Minimum and maximum levels for both derivatisation time and derivatisation temperature can be seen in Table 1 below. Centre points were added to the experimental design to detect curvature. The same experimental design was used for both H_2SO_4 and BF_3.

The comparison of both catalysts was carried out using the method detailed below.

Nonanoic acid methyl ester (200 μL; 1.12 mg/mL) in hexane, as internal standard, glyceryl triheptadecanoate (200 μL; 1.03 mg/mL) in hexane, and H_2SO_4 (1 mL; 1% v/v) in methanol were added to a micro reaction vial. The samples were then derivatised according to the experimental design. After cooling, chloroform (1 mL) and water (1 mL) were added. The vials were shaken by hand for 2 min, after phase separation the supernatant was transferred to a GC vial for analysis.

The results of the 2^2 screening experiment indicated that temperature was the only significant factor in the derivatisation of fatty acids to FAMEs within the restrictions of the factors screened. It indicated that the higher the temperature, the quicker the derivatisation was completed. As such, derivatisation time at 90 °C was optimised using the below method.

Nonanoic acid methyl ester (100 μL; 1.12 mg/mL) in hexane, as internal standard, glyceryl triheptadecanoate (100 μL; 1.03 mg/mL) in hexane, and H_2SO_4 (1 mL; 1% v/v), or BF_3 (14% w/v) in methanol were added to a micro reaction vial. The samples were then derivatised at 90 °C for 10, 20, 30, 40, 50, or 60 min. After cooling, chloroform (1 mL) and water (1 mL) were added. The vials were shaken by hand for 2 min, after phase separation the supernatant was transferred to a GC vial for

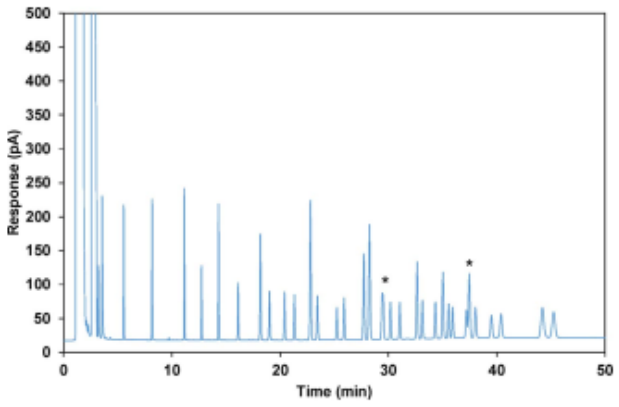

Fig. 2. Optimised separation of FAME mix. Peak identification: asterisk indicates critical peak pairs whose resolution could not be improved. Chromatographic conditions: stationary phase: AT-1000 (PEG 15 m × 0.53 mm; 1.2 μm), carrier gas: helium, flow rate: 4 mL/min, temperature programming: initial: 40 °C, ramp to 114 °C at 10 °C/min and held for 1 min, ramped to final temperature of 220 °C at 5 °C/min, inlet temperature: 300 °C, detector temperature:280 °C.

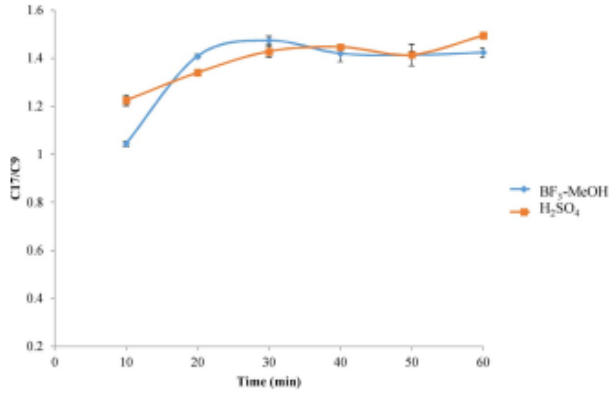

Fig. 3. Optimisation of derivatisation catalyst and time.

analysis.

As can be seen from Fig. 3, the derivatisation reaction was still ongoing at 60 min. It was assumed that peak ratios would plateau, indicating that glyceryl triheptadecanoate was fully derivatised. When BF_3 was used as derivatisation reagent, peak ratios plateaued after 30 min of derivatisation. This can be seen in Fig. 3. The derivatisation reaction proceeded at least twice as fast when BF_3 was used as the catalyst compared to H_2SO_4.

The decrease in derivatisation time when using BF_3 (14% w/v) in methanol could be due to the increased amount of catalyst compared to H_2SO_4 (1% v/v) in methanol. The stated concentration of H_2SO_4 in methanol is the concentration cited in literature [18]. The decrease in derivatisation time could also be due to the better orbital overlap of BF_3, a Lewis acid, with the respective fatty acid. This is in contrast to the derivatisation mechanism using H_2SO_4, where it is the protonation of the carbonyl that activates the fatty acid and not orbital overlap.

As BF_3 (14% w/v) in methanol gave complete derivatisation in less time than H_2SO_4 (1% v/v) in methanol, it was chosen as the catalyst for the remainder of method development. The derivatisation time and temperature was 30 min at 90 °C.

3.3. Optimisation of DLLME procedure

3.3.1. Selection of extraction solvent

As Folch solution [19] was used to initially extract lipids from milk, chloroform was also used as the extraction solvent for DLLME. The extraction efficiency of chloroform was compared to hexane. Hexane is a commonly used solvent for extraction of FAMEs, so it was vital that any novel DLLME extraction solvent obtained equivalent if not better extraction efficiency.

Nonanoic acid methyl ester (100 µL; 2.16 mg/mL) in hexane, as internal standard, glyceryl triheptadecanoate (200 µL; 1.03 mg/mL) in hexane, and BF_3 (1 mL; 14% w/v) in methanol were added to a micro reaction vial. The samples were then derivatised at 90 °C for 30 min. After cooling, hexane (1 mL) or chloroform (1 mL) and water (1 mL) were added. The vials were shaken by hand for 2 min, after phase separation the organic phase was transferred to a GC vial for analysis.

The ratio of triheptadecanoate and nonanoic acid methyl ester (IS) peak areas were compared, and the results presented in Fig. 4 The use of chloroform resulted in greater extractions of FAMEs. Chloroform was therefore selected as the extraction solvent for the novel DLLME method.

3.3.2. Selection of dispersive solvent

Methanol was selected as the dispersive solvent as it was already present in the derivatisation protocol. The combination of chloroform (extraction solvent) and methanol (dispersive solvent) produced a stable cloudy solution in preliminary trials.

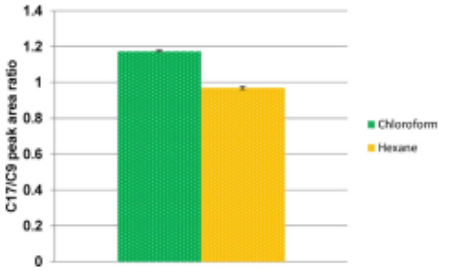

Fig. 4. Comparison of extraction solvents for FAMEs (n = 3).

3.4. Statistical analyses

A design of experiments (DoE) approach was used to optimise the DLLME protocol. A 2^7 1/8 factorial design was used to determine which factors had a significant impact on the preconcentration of analytes. A 2^7 1/8 factorial design was chosen as it reduced the number of experiments needed while still provided resolution between single and two factor interactions. A list of the factors and levels studied can be found in Table 2.

The results of the factorial design indicated that there was only one significant factor: extraction solvent volume. Specifically, FAME peak areas were maximised when the minimum extraction solvent volume was used. Unlike in traditional liquid–liquid extraction, the preconcentration effect in DLLME is not governed by a reduction in

Table 2
Screening factors and levels for DLLME procedure.

Factor	Minimum level (−1)	Maximum level (1)
Phosphoric acid (µL)	30	200
NaCl solution (2 M) (µL)	400	1000
Shake time (minutes)	0.5	2
Extraction solvent (µL)	50	200
Aqueous phase (mL)	5	10
Dispersive solvent (µL)	0	400
Extraction time (minutes)	0	2
Sonication (minutes)	0	5

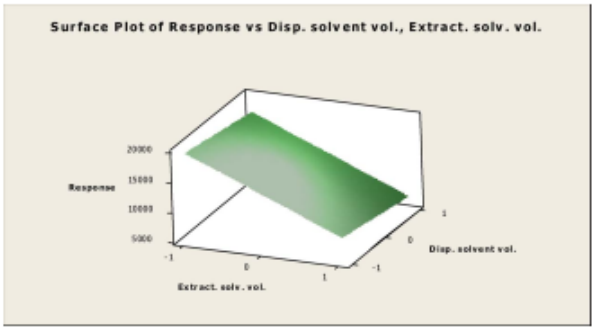

Fig. 5. Response surface resulting from factorial design.

Table 3
Figures of merit for the proposed method.

Analyte	Linearity	LOD (μg/mL)	Reproducibility (%RSD)	Recovery (%)	EF
Caprylic	0.994	0.08	4.2	70	11
Capric	0.998	0.04	6.9	80	15
Palmitic	0.994	0.04	6.3	84	8
Stearic	0.997	0.09	4.1	73	10
Oleic	0.994	0.07	5.2	89	9

extraction solvent volume alone. The stability of the cloudy solution determines the preconcentration of analytes and this stability can be maximised by determining the optimum ratio of extraction solvent, dispersive solvent, and aqueous phase. The response surface can be seen in Fig. 5. As there was only one significant factor, there was no need for further optimisation of experimental factors.

The method was validated according to ICH guidelines and the figures of merit can be seen in Table 3. Using the optimised method, enrichment factors for the selected FAMEs were measured. They ranged from 8 to 15 which allowed for a lower limit of detection (0.04 mg/mL).

3.5. Comparison with other methods

The sample, analyte, derivatisation method, derivatisation time, mode of analysis, and LOD for the proposed method was compared to recently published methods for fatty acid analysis in milk and milk based products. The proposed method offered lower LOD or quicker derivatisation times (or both) for selected fatty acids, which shows the importance in developing the proposed method. The comparison of methods is outlined in Table 4.

3.6. Fatty acid profile of cows with different body condition score

Milk samples were collected from Holstein Friesian cattle and frozen at −20 °C until analysis. The samples were subjected to the method outlined in Section 3.3. The concentrations of FAMEs found in milk can be seen in Table 5. The results were subjected to a *t*-test to determine any significant differences in fatty acid concentration between cows with an optimum BCS and those with a high BCS. Typical chromatograms obtained in the analysis can be seen in Fig. 6.

Table 5
Concentrations of selected FAMEs in bovine milk from cows with high and optimum body condition scores.

FAME	Optimum BCS (mg/mL)	High BCS (mg/mL)	P value
Caprylic	Cow 1: 0.52 ± 0.03 Cow 2: 0.71 ± 0.10	Cow 3: 0.39 ± 0.02 Cow 4: 0.45 ± 0.07	0.12
Capric	Cow 1: 0.98 ± 0.05 Cow 2: 1.34 ± 0.09	Cow 3: 0.67 ± 0.04 Cow 4: 0.81 ± 0.03	0.11
Palmitic	Cow 1: 10.98 ± 0.01 Cow 2: 10.72 ± 0.06	Cow 3: 5.47 ± 0.04 Cow 4: 5.98 ± 0.07	0.005
Stearic	Cow 1: 2.61 ± 0.08 Cow 2: 4.72 ± 0.05	Cow 3: 2.61 ± 0.01 Cow 4: 3.64 ± 0.06	0.35
Oleic	Cow 1: 6.06 ± 0.12 Cow 2: 6.77 ± 0.07	Cow 3: 4.22 ± 0.09 Cow 4: 5.63 ± 0.04	0.12

Notes: Values are means ± are standard deviatations (n = 3).

As seen in Table 5, the *t*-test identified the change in palmitic acid methyl ester as the only significant difference in the two groups, and so the null hypothesis was rejected. Palmitic acid is the most common fatty acid in dairy cows and the method developed can be used to distinguish cows that have a high body condition score from those in the optimum range based on the levels of palmitic acid methyl ester detected.

4. Conclusion

A novel and rapid derivatisation and DLLME protocol for the analysis of fatty acids in bovine milk has been developed. This method has been applied to Holstein Friesian cows with optimum and high BCS. The analysis revealed that concentrations of palmitic acid can be used to determine if cows are in the optimum BCS range. Although the sample size in this study is small, it is sufficient for showing the applicability of the newly developed DLLME method and the correlation between changing BCS and the fatty acid profile of bovine milk. The authors believe that this method can be used in a wider study of fatty acid content in bovine milk.

Table 4
Comparison of the proposed method and recently published methods.

Sample	Fatty acids extracted	Derivatisation agent	Derivatisation time (min)	Analytical method	LOD (μg/mL)	Reference
Milk powder	10:0, 16:0, 18:0, 18:1	DMPP	120	UHPLC–MS/MS	0.00086–0.00172	[21]
Bovine milk	16:0, 18:0, 18:1	BF_3-MeOH	15	GC-FID	24.66–30.17	[22]
Breast milk	8:0, 10:0, 16:0, 18:0	HCl-MeOH	60	GC-FID	10	[23]
Bovine milk	8:0, 10:0, 16:0, 18:0, 18:1	BF_3-MeOH	20	GC-FID	0.04–0.09	Presented work

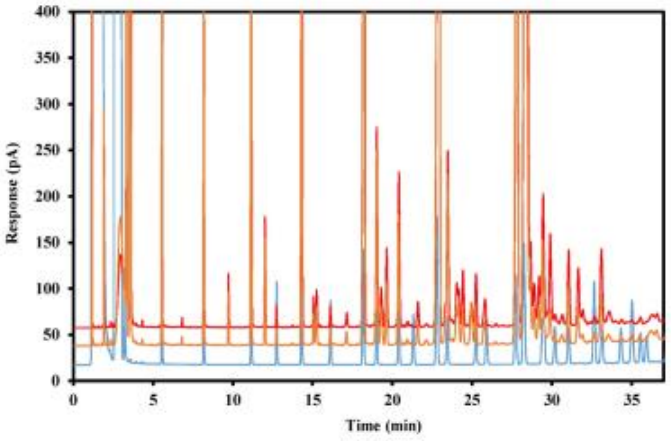

Fig. 6. Example of chromatograms obtained. FAME mix (blue), high body condition score (orange), optimum body condition score (red). Chromatographic conditions outlined in Fig. 2. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Conflict of interest

The authors declare that they have no conflict of interest.

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