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.

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

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.

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

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 CO₂ to assist the dispersion of the extraction solvent, removing a need for the dispersive solvent. The CO₂ 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 μ L) 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 μ g·L⁻¹ and the LOD was 0.01 μ g·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 μ L 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 μ g·L⁻¹ for penconazole and hexaconazole and the lowest recorded LOD value was 4 μ g·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 μ L) 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 ($\mu\text{g/L}$)	Reference
<i>DLLME</i>							
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 ($\mu\text{g/kg}$): 4.8–13 Yogurt ($\mu\text{g/kg}$): 3.0–9.7 Cheese ($\mu\text{g/kg}$): 6.1–7.7	[17]
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	[18]
Milk	Centrifugation, protein precipitation by ACN	Carbon tetrachloride (35 μL)/ethanol (800 μL)	Cholesterol	Not specified	HPLC-UV	0.01	[19]
Milk	Protein precipitation by ACN and NaCl	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	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	[22]
Milk	QuEChERS	Chloroform (200 μL)/10% acetic acid-ACN (1 mL)	Fluoroquinolones	Not specified	RP-HPLC-UV	0.8–5.0 $\mu\text{g/kg}$	[23]
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	[24]
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	[25]

TABLE 1: Continued.

Sample	Sample preparation	Extraction/dispersive solvent	Analytes extracted	Enrichment factor	Analytical method	LOD ($\mu\text{g/L}$)	Reference
<i>Ultrasound assisted DLLME</i>							
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: BI, MI	BI: 30, MI: 30	RP-HPLC-FL	BI: 0.1 $\mu\text{g/kg}$ MI: 0.01 $\mu\text{g/kg}$	[28]
<i>UA-IL/IL-DLLME</i>							
Infant formula	Dilution	$[\text{C}_6\text{MIM}][\text{PF}_6]$ (70 μL)/ $[\text{C}_4\text{MIM}][\text{BF}_4]$ (100 μL)	Sulfonamides	Not specified	RP-HPLC-PDA	2.94–16.7 $\mu\text{g/kg}$	[29]
<i>DLLME with back extraction</i>							
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]
<i>USA-RM-DLLME</i>							
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]
<i>USA-IL-DLLME</i>							
Milk	Microwave digestion with HNO_3 and H_2O_2 , chelation with 1-Phenylthiosemicarbazide	$[\text{C}_6\text{MIM}][\text{TF}_2\text{N}]$ (100 μL)	Selenium	150	Graphite furnace atomic absorption spectrometry (GFAAS)	12	[32]
<i>LDS-DLLME</i>							
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]
<i>VA-DLLME</i>							
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]
<i>IL-DLLME</i>							
Breast milk	Protein precipitation by HClO_4 , H_3PO_4 , MeOH	NFX: $[\text{C}_8\text{C}_1\text{im}][\text{PF}_6]$ (42 μL)/MeOH (80 μL) BNZ: $[\text{C}_8\text{C}_1\text{im}][\text{PF}_6]$ (42 μL)/MeOH (101 μL)	NFX, BNZ	NFX: 33.8, 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 μ L ACN/30 μ L tetrachloroethylene) was combined with 30 μ L 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 μ g L⁻¹ for both analytes and low LODs (BPA: 60.0 μ g L⁻¹, BPB: 30 μ g L⁻¹) were obtained corresponding to high enrichment factors (BPA: 237, BPB: 220). The method repeatability was $\leq 7\%$ when the analytes were at a concentration of 0.2 μ g 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 μ L 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 μ L 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 μ 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 μ L 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 μ g L⁻¹, DLLME: 0.5–3 μ 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 μ L) as dispersive solvent, tetrachloroethene (90 μ L) 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 μ g L⁻¹, and linearity between 0.5 and 10 μ 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 μ L) 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 M₁ (AFM₁) 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 AFM₁. The second method investigated used NaCl and ACN to simultaneously precipitate proteins and extract AFM₁ 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 $\mu\text{g L}^{-1}$ 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 $\mu\text{g L}^{-1}$) than QuEChERS (1.15–2.73 $\mu\text{g L}^{-1}$) and higher recoveries (92.9%–104.7% compared to 83.6%–97.1%, when samples were spiked with sulphonamides at 150 $\mu\text{g L}^{-1}$). 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 $\mu\text{g kg}^{-1}$ for DAN and below 15 $\mu\text{g kg}^{-1}$ 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 $\mu\text{g L}^{-1}$, 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 $\mu\text{g L}^{-1}$ 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 $\mu\text{g/kg}$ and 0.01 $\mu\text{g/kg}$, 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, H₂SO₄, 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 $\mu\text{g L}^{-1}$, sorbate: 0.15 $\mu\text{g L}^{-1}$) were much lower than those found in other methods (benzoate: 1.22–900 $\mu\text{g L}^{-1}$, sorbate: 2–500 $\mu\text{g L}^{-1}$). 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 g^{-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 $\mu\text{g L}^{-1}$, a linear range between 0.02 and 200 $\mu\text{g L}^{-1}$, 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 $\mu\text{g L}^{-1}$, 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 $[\text{C}_6\text{C}_1\text{im}][\text{PF}_6]$, 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 $[\text{C}_6\text{C}_1\text{im}][\text{PF}_6]$ (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 $\mu\text{g L}^{-1}$, a linear range from 300 to 34,400 $\mu\text{g L}^{-1}$, and an enrichment factor of 33.8. The BNZ procedure had a LOD of 180 $\mu\text{g L}^{-1}$, a linear range from 200 to 29,160 $\mu\text{g L}^{-1}$, 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, $[\text{C}_6\text{MIM}][\text{TF}_2\text{N}]$ (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 $\mu\text{g L}^{-1}$, a linear range between 0.04 and 3.0 $\mu\text{g L}^{-1}$, 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 $[\text{C}_6\text{MIM}][\text{BF}_4]$ (70 μL), as extraction solvent, were added to the sample and intensely shaken for 5 min. When complete, $[\text{C}_4\text{MIM}][\text{BF}_4]$ (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 $\mu\text{g kg}^{-1}$. 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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