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Analytical Methods

Dispersive liquid–liquid microextraction combined with field-amplified sample stacking in capillary electrophoresis for the determination of non-steroidal anti-inflammatory drugs in milk and dairy products

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ABSTRACT

Dispersive liquid–liquid microextraction (DLLME) was coupled with field-amplified sample stacking in capillary electrophoresis (FASS) for the determination of five non-steroidal anti-inflammatory drugs (NSAIDs) in bovine milk and dairy products. After extraction, the enriched analytes were back-extracted into a basic aqueous solution for injection into CE. Under optimum conditions, enrichment factors were in the range 46–229. Limits of detection of the analytes ranged from 3.0 to 13.1 μg kg^{-1} for all matrices analysed. Calibration graphs showed good linearity with coefficients of determination (R^2) ⩾ 0.9915 and relative standard deviations (RSD%) of the analyses in the range of 0.6–6.2% (n = 5). Recoveries of all NSAIDs from bottled milk, raw milk, yogurt and white cheese samples were in the ranges of 86.6–109.3%, 84.3–100.5%, 77.4–107.3%, and 90.9–101.6%, respectively. DLLME–FASS-CE was demonstrated to be a rapid and convenient method for the determination of NSAIDs in milk and dairy products.

1. Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs), including etodolac (ET), naproxen (NAX), ketoprofen (KTP), flurbiprofen (FBP) and diclofenac (DIC), are one of the most frequently used pharmaceutically active compounds in veterinary medicine (Arroyo, Ortiz, & Sarabia, 2011). Their effectiveness in reducing pain, preventing inflammation, treating allergies and respiratory diseases, and lowering fever has resulted in their widespread use in food-producing animals, in conjunction with antibiotics (Gallo et al., 2010; Malone, Dowling, Elliott, Kennedy, & Regan, 2009). NSAIDs are also administered to cattle to improve some quality characteristics of the product such as production of pale meats (Gallo et al., 2008) and for reduction of edible fat (Gallo et al., 2006). These uses, however, are not without side effects which include gastrointestinal bleeding, intestinal ulceration, aplastic anaemia and inhibition of platelet aggregation (Gallo et al., 2006). Moreover, long-term exposure to some NSAIDs has been reported to induce kidney tumors in rats and liver tumors in mice (Dowling, Gallo, Malone, & Regan, 2009; Hu et al., 2012). As such, the use of NSAIDs in food-producing animals might create public health problems and more so where international trade of milk and dairy products is concerned. The widespread use of NSAIDs presents a potential risk to the consumer if their residues enter the food chain so there is a need for the development of analytical methods to monitor compliance with legislations.

The analysis of complex samples such as milk and dairy products and quantitation of analytes at trace levels are nowadays two of the main analytical problems (Payan, Lopez, Torres, Navarro, & Mochon, 2011). The analytical complexity increases in this case since both problems are present. Milk and dairy products have high concentrations of lipids, carbohydrates, proteins and variable concentrations of vitamins and minerals. Thus, sample pretreatment and preconcentration are prerequisites in the determination of NSAIDs in such samples. Liquid–liquid extraction (LLE) (Botello, Borrull, Calull, & Aguilar, 2011; Dubreil-Cheneau, Pirotais, Bessiral, Roudaut, & Verdon, 2011; Gallo et al., 2010) and solid-phase extraction (SPE) (Gallo et al., 2010; Stolker et al., 2008) have been the main techniques used to extract and/or preconcentrate NSAIDs from milk along with gas chromatography-mass spectrometry (GC–MS) (Arroyo et al., 2011), liquid chromatography–tandem mass spectrometry (LC–MS–MS) (Dubreil-Cheneau et al., 2011; Noche, Laespada, Pavon, Cordero, & Lorenzo, 2011), high-performance liquid chromatography (HPLC) with fluorescence detection (FLD) (Gallo et al., 2010) and ultra performance liquid chromatography-time-of-flight-mass spectrometry (UPLC–ToF-MS) (Stolker et al., 2008). Shortcomings associated with LLE such as emulsion formation, use of large sample volumes and toxic organic solvents make it labour-intensive, expensive, time-consuming and environmentally-unfriendly. Although SPE uses much less solvent than LLE, it can still be considered significant, and normally an extra step is needed to preconcentrate the

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analytes further into smaller volumes. SPE is also time-consuming and relatively expensive (Rezaee, Yamini, & Faraji, 2010).

The drive for "green" methods to overcome these inherent problems of conventional LLE and SPE has led to the development of solventless and solvent-minimized microextraction techniques such as solid-phase microextraction (SPME) and liquid-phase microextraction (LPME). SPME with a derivatization step has been used prior to GC–MS to preconcentrate seven NSAIDs in bovine milk (Arroyo et al., 2011). Despite the advantages provided by this technique, most commercial fibres used in SPME are relatively expensive, fragile and have limited lifetime. Moreover, sample carry-over is a possible problem (Fontanals, Marce, & Borrull, 2007). LPME with its various modes such as dispersive liquid–liquid microextraction (DLLME) (Rezaee et al., 2006), single drop microextraction (SDME) (Jain & Verma, 2011), hollow fibre–liquid phase microextraction (HF-LPME) (Pedersen-Bjergaard & Rasmussen, 2008) and solventbar microextraction (SBME) (Guo & Lee, 2012), among others, has emerged to overcome such problems of SPME.

DLLME, proposed by Assadi in 2006 (Rezaee et al., 2006), has gained increased prominence for its rapidity, simplicity, cheapness, environmental friendliness and ability to provide high extraction efficiencies. The heart of this method lies in the formation of a ternary solvent system composed of the aqueous solution containing the analytes, a water-immiscible extraction solvent and a water-miscible disperser solvent. DLLME has been successfully applied for preconcentration of several trace analytes in water and environmental samples (Herrera-Herrera, Hernandez-Borges, Borges-Miquel, & Rodriguez-Delgado, 2010; Rezaee, Yamini, Shariati, Esrafili, & Shamsipur, 2009). However, its application to complex matrices such as milk and dairy products is still very limited (Farajzadeh, Djozan, Mogaddam, & Bamorowat, 2011; Farajzadeh, Djozan, Reza, Mogaddam, & Norouzi, 2012; Liu et al., 2011).

Capillary electrophoresis (CE) is a powerful complementary technique to LC and GC. It has rapidly spread into a wide array of analytical areas as it is considered a green analytical technique, due to its low consumption of samples and reagents, extremely high separation efficiency, short analysis time, high versatility in terms of multiple separation modes and excellent biocompatibility (Xie & He, 2010). Nonetheless, most commercial CE instruments are equipped with online UV detectors, which suffer from low concentration sensitivity. To overcome this sensitivity problem, several online preconcentration strategies, such as stacking (Mala, Gebauer, & Bocek, 2011) and sweeping (Aranas, Guidote, & Quirino, 2009) have been developed. The simplest and most commonly used sample stacking technique is field-amplified sample stacking (FASS) (Osbourn, Weiss, & Lunte, 2000). It is based on the concept that ions electrophoretically migrating through a low-conductivity solution (sample plug) into a high-conductivity background electrolyte (BGE) slow down dramatically at the boundary of the two solutions.

The aim of this study was to develop a simple and rapid method for extraction, enrichment, and determination of the aforementioned five NSAIDs in bottled and raw bovine milk, yogurt and white cheese samples by the combination of DLLME with FASS in CE. NSAIDs were extracted into acetonitrile (ACN) which was then used as the disperser solvent in DLLME. Back-extraction of the analytes from the organic extract after DLLME into a basic aqueous solution facilitated their direct injection into CE. Influential factors on stacking and extraction efficiency were investigated and optimised.

2. Experimental

2.1. Chemicals and reagents

ET (log$P$ 3.44, pK_a 4.73) and KTP (log$P$ 3.61, pK_a 3.88) were kindly provided by Nobel İlaç (Istanbul, Turkey). NAX (log$P$ 2.99, pK_a 4.19), FBP (log$P$ 3.94, pK_a 4.42) and DIC (log$P$ 4.26, pK_a 4.00) were kindly provided by Abdi İbrahim İlaç Sanayi ve Tic. A. Ş. (Istanbul, Turkey). Sodium tetraborate ($Na_2B_4O_7{\cdot}10H_2O$) were obtained from Sigma–Aldrich (Munich, Germany). HPLC-grade ACN was purchased from Sigma–Aldrich (St. Louis, MO, USA). Acetone, propan-2-ol, sodium chloride, magnesium sulfate, calcium chloride, chloroform (CF, log$P$ 1.8), carbon tetrachloride (CTC, log$P$ 3.0), sodium hydroxide and hydrochloric acid were acquired from Merck (Darmstadt, Germany). 1-Undecanol (1-UN, 99.0%, log$P$ 3.9) and 1-dodecanol (1-DO, 98.0%, log$P$ 4.4) were from Sigma–Aldrich (Steinheim, Germany). All reagents were at least of analytical grade unless otherwise stated. Deionized (DI) water (18.2 MΩ cm) treated with Millipore (Simplicity, 185 water purification system) Milli-Q water purification apparatus was used for all aqueous solutions.

2.2. NSAIDs standard solutions

Individual stock solutions of NSAIDs at concentrations of 1000 mg L^{-1} were prepared in ACN and stored at −20 °C. Mixed standard solutions were freshly prepared at each working session from the stock solutions by proper dilution with DI water. All solutions and samples were degassed using a sonicator (Sonorex Bandelin Electronic, Walldorf, Germany) and filtered through 0.20 μm filters (Econofilters, Agilent Technologies, Darmstadt, Germany) before use. Stock solutions were stable for at least 6 months when stored at −20 °C. Working standard solutions at 50–250 mg kg^{-1} for conventional capillary zone electrophoresis (CZE) were prepared from the mixed standard solution by dilution with the BGE; and at 1.6–25 mg kg^{-1} in DI water, pH of which had been adjusted to 11.3 using 1.0 M NaOH (hereafter referred to as the back-extraction solution, BES) for calibration of FASS.

2.3. CE apparatus and conditions

The experiments were carried out on an HP^{3D} CE (Agilent Technologies, Waldbronn, Germany) equipped with an online UV diode-array detector (DAD) operated at a wavelength of 210 nm. Optimum wavelengths for the target analytes were determined using 'Isoabsorbance' and '3D' plots in the instrument's 'Data Analysis' software (Agilent Technologies, Waldbronn, Germany). Conventional CZE and FASS were performed using uncoated fused-silica capillaries (Postnova Analytics, Landsberg, Germany) of 75 μm i.d. and 38.5 cm length with effective length to the detector of 30 cm. Pressure injection was employed throughout the work. Injection was done at the anodic while detection was performed at the cathodic end of the capillary.

New capillaries were successively flushed with DI water (10 min), 1.0 M sodium hydroxide (20 min), DI water (10 min) and finally with the background electrolyte (BGE) for 20 min. To assure reproducibility, the capillary was successively flushed with DI water (1 min), 1.0 M sodium hydroxide (1 min), DI water (2 min) and the BGE (2 min) at the end of each run.

In conventional CZE, the capillary was conditioned with a BGE (30 mM acetate buffer at pH 4.0 containing 25% ACN, v/v); the sample, prepared in this BGE, was injected for 5 s at 50 mbar and a positive voltage of 25 kV was applied. With this BGE composition, the electroosmotic flow was minimized and a short capillary was sufficient to obtain a baseline resolution within acceptable analysis time. The analytes migrated in a homogeneous conductivity medium and were separated by the CZE mode.

In FASS, the capillary was conditioned with the aforementioned BGE; the sample present in a low-conductivity medium was hydrodynamically injected for 5 s at 50 mbar; the analytes stacked at the boundary between the low-conductivity sample plug and the

high-conductivity BGE. The following separation occurred at 25 kV by the CZE mode.

2.4. Sample clean-up by salting-out extraction (SOE)

Bottled (pasteurised) bovine milk, white cheese and yogurt samples were purchased from a local market, and raw (unpasteurised) bovine milk was obtained from a dairy farm seller (Ankara). The samples were freshly analysed as received. Fat content was reported by the producer on the containers except for raw milk.

Two grams (±0.01 g) of homogenised NSAID-free milk were transferred into 10 mL glass centrifuge tubes and spiked with prescribed concentrations of the NSAIDs. The samples were vortex-mixed for 1 min and allowed to rest for 10 min in a dark place. Then, 100 μL concentrated phosphoric acid, 0.6 g sodium chloride and 4.0 mL ACN were added, respectively. The mixtures were centrifuged (5000 rpm, 10 min) and 3.5 mL ACN (upper phase), containing the NSAIDs, were transferred into another test tube. One milliliter of n-hexane was used to defatten this extract. The n-hexane layer was discarded and an aliquot of ACN (2.0 mL) was used in the subsequent DLLME procedure.

For yogurt and white cheese samples, 2.0 g (±0.01 g) were homogenised in 2.0 mL DI water and the same procedure mentioned above for milk was followed.

2.5. DLLME procedure

The DLLME procedure involved transferring 2.0 mL of the ACN extract (disperser solvent) into a glass test tube and mixing it with 150 μL CF (extraction solvent) followed by rapid injection of this mixture into 8.0 mL of DI water in a screw-cap conical-bottomed glass test tube. The mixture was then vortex-mixed for 1 min which resulted into the formation of a cloudy solution. The dispersed fine droplets of the extraction solvent sedimented at the bottom of the test tube upon centrifugation (5000 rpm, 3 min) and were quantitatively transferred into a 1.0-mL snaplock microtube using a 100 μL HPLC syringe (Hamilton, USA). NSAIDs were back-extracted into 70 μL of BES upon vortex-mixing for 1 min and centrifugation (5000 rpm, 3 min) for direct injection into CE.

3. Results and discussion

The determination of NSAIDs at trace concentration levels in milk and dairy products such as yogurt and cheese necessitates a sample clean-up step owing to the complexity of these matrices, followed by a further purification of the extracts and/or preconcentration of the analytes to increase the specificity and achieve the required low levels of detection. In this study, SOE and DLLME were used for sample clean-up and analyte preconcentration, respectively. The solvent used in the SOE step, containing the target analytes, acted as the disperser solvent in the following DLLME step. Optimization of SOE and DLLME conditions were performed using spiked sample matrix.

3.1. Sample clean-up by SOE

Variables affecting extraction efficiency of SOE include acidity of sample solution, type and volume of extraction solvent, type and amount of salt, and extraction (vortex-mixing) time. It was reported that acidifying the samples with phosphoric acid before extraction with acetonitrile increased the extraction efficiency of NSAIDs (Hu et al., 2012). In this study, the addition of 100 μL of concentrated phosphoric acid per 2.0 g sample gave the highest extraction efficiency.

As well as ACN, acetone and propan-2-ol were also assayed as the extraction solvents in SOE. ACN showed the highest extraction efficiency of the target analytes. Different volumes of ACN (2.0–6.0 mL) were studied. The use of 4.0 mL ACN for 2.0 g milk resulted in the salt-induced phase separation of ca. 3.7 mL ACN, 2.0 mL of which were used for further experiments and gave the highest extraction efficiency among the other volumes investigated. The amounts of salts (calcium chloride, magnesium sulphate and sodium chloride) and the corresponding signals obtained after extraction were compared. The largest peak areas for the five analytes were obtained with 0.6 g of sodium chloride, such that this value was selected. Finally, extraction time of 1 min was chosen because no significant increase in the analytical signals occurred upon increasing the vortex-mixing time.

3.2. Optimization of DLLME conditions

The most influential parameters in DLLME including the type and volume of extraction and disperser solvents, ionic strength and extraction time were studied in detail and optimised. Enrichment factor (EF) was used to evaluate the impact of each parameter on the extraction efficiency of the method using the one-factor-at-a-time approach.

3.2.1. Type and volume of the extraction solvent

Solvents with higher densities than water are generally used in DLLME. However, some applications of lower-density solvents including ones with melting points near room temperature have been proposed (Leong & Huang, 2008). In the latter case, the organic solvent drop can be easily collected by solidifying it at low temperature. Based on these criteria, the most frequently used solvents of CF (d: 1.483 g mL^{-1}), CTC (d: 1.587 g mL^{-1}), 1-UN (mp: 13–20 °C; d: 0.830 g mL^{-1}) and 1-DO (mp: 24–27 °C; d: 0.833 g mL^{-1}) were investigated. The experiments for the selection of the extraction solvent were performed using 1.5 mL of ACN as the disperser solvent and a volume of 200 μL of the extraction solvent. For all of the four extraction solvents used, a stable cloudy solution formed and acceptable recoveries were observed (Fig. 1a). CF gave the highest extraction efficiency among the other investigated solvents for the five NSAIDs. Therefore, CF was selected as the extraction solvent in the present study.

In order to evaluate the effect of extraction solvent volume, different volumes of CF (100, 150, 200 and 250 μL) dissolved in a constant volume of ACN (1.5 mL) were subjected to the same DLLME procedure. Fig. 1b shows the curves of EF versus extraction solvent volume. As can be seen, by increasing the CF volume from 100 to 150 μL, the EF increased, and then decreased upon further increase of the CF volume for all the analytes, which was due to increase of the sedimented phase volume. On the basis of these results, 150 μL of CF was selected as the optimal solvent extraction volume.

3.2.2. Type and volume of the disperser solvent

Miscibility of the disperser solvent with the extraction solvent and aqueous phase is a crucial factor in DLLME affecting the selection of the disperser solvent. The experiments were performed using 1.5 mL of each disperser solvent resulting from the SOE step. ACN was found to provide the highest extraction efficiency (Fig. 1c). This may be attributed to the synergic effect of good compatibility of ACN with aqueous solutions and low distributive ratio of analytes in mixtures of ACN and water (Xiong, Ruan, Cai, & Tang, 2009). Hence, ACN was chosen as the disperser solvent for the following experiments.

Scrutinizing the influence of the disperser solvent volume on the extraction efficiency was not straightforward. Since the ACN extract from the SOE step which contained the analytes was used as the disperser solvent in DLLME, variation of its volume not only

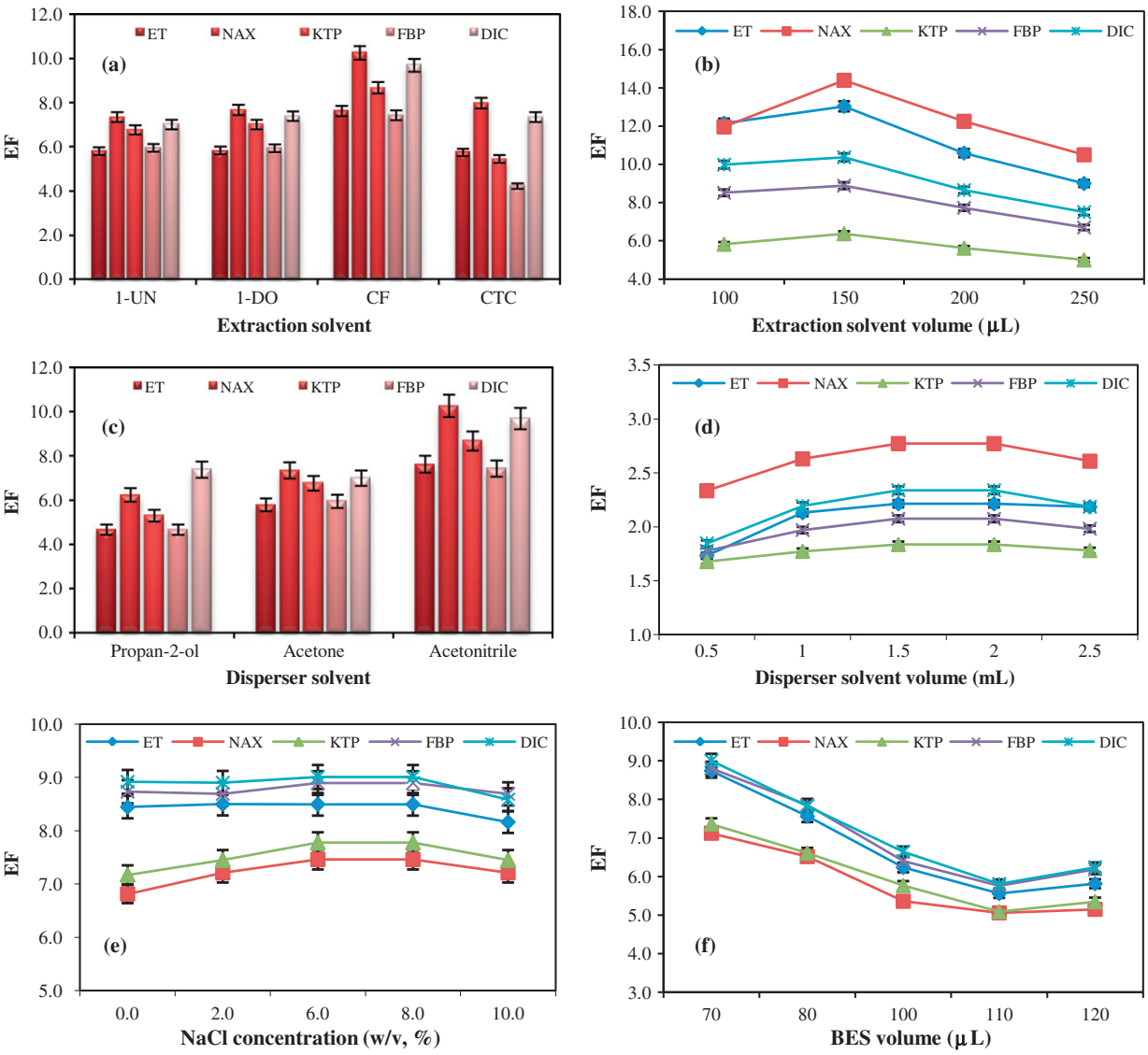

Fig. 1. Effect of experimental parameters on extraction efficiency of DLLME: (a) extraction solvent type, (b) extraction solvent volume, (c) disperser solvent type, (d) disperser solvent volume, (e) salt addition and (f) BES volume.

affected the extractability of the analytes but also changed their concentrations. To avoid the overlap of the two parameters, constant volumes of 0.5 mL of the extract were taken after SOE and completed to the desired ACN volume with the pure reagent. As can be seen from Fig. 1d, extraction efficiency increased from 0.5 mL to 1.5 mL and remained constant up to 2.0 mL. Beyond this point, it started to decrease. This observation was imputed to the poor dispersion of CF at low ACN volumes, which resulted in poor extraction efficiency of the analytes. Whereas, at a high volume of ACN, solubility of the target analytes and the extraction solvent (CF) in water increased accordingly, thereby, the extraction efficiency also decreased. In subsequent studies, 2.0 mL of ACN was chosen as the optimal disperser volume.

3.2.3. Ionic strength

In LLE, sodium chloride is generally added into sample solutions to increase their ionic strength and polarity, which can improve the partition of hydrophobic analytes between the aqueous phase and the organic phase due to the salting-out effect. However, no effect or even controversy results have been observed when DLLME was applied to analyses of milk depending on physicochemical properties of analytes under investigation (Farajzadeh et al., 2011). The experimental results shown in Fig. 1e, indicate that extraction efficiency of NAX and KTP increased with increasing sodium chloride concentration from 0% to 6.0% (w/v), and then decreased slightly when its concentration exceeded 8.0%. Whereas, salt addition had no noticeable effect on extraction efficiency of ET, FBP and DIC up to 8.0% where it started to decrease. Taking these observations into consideration, the concentration of sodium chloride was set at 6.0% in subsequent experiments.

3.2.4. Extraction time

In DLLME, extraction time is defined as the time interval between the injection of the mixture of the disperser solvent and the extraction solvent and the time at which the sample is centrifuged (Berijani, Assadi, Anbia, Hosseini, & Aghaee, 2006) which corresponded to the time of vortex-mixing in this study. The effect of extraction time was examined in the range of 0–5 min with the other experimental conditions kept constant. The results indicated that extraction time did not have any significant influence on the signals of the analytes (data not shown). An extraction time of

1 min was used in subsequent experiments to ensure good reproducibility.

3.3. Optimization of back-extraction conditions

The effect of BES (pH 11.3) volume on the extraction efficiency was studied over the range 70–120 μL. It can be seen from Fig. 1f, that extraction efficiency decreased gradually upon increasing BES volume due to dilution. Lower volumes than 70 μL could not be used due to loss of phase separation. Hence, subsequent experiments were performed using 70 μL as the BES volume.

The effect of extraction time on back-extraction efficiency was also investigated and was found to have no effect. This was thought to be due to the formation of microemulsion in the BES due to the presence of trace amount of ACN in CF which greatly accelerated the transfer of the analytes back into the BES. Therefore, a back-extraction time of 1 min was chosen in further experiments.

3.4. Analytical performance

Standard calibration graphs without extraction were obtained by plotting peak areas versus concentrations of the analytes in working standard solutions (prepared by diluting the mixed standard solutions in the BGE) for conventional CZE and in the BES for FASS. Analytical performance parameters of conventional CZE and FASS are listed in Table 1. EFs with FASS were compared with respect to CZE from the ratio of the calibration slopes obtained with FASS to those obtained with conventional CZE. As shown in Table 1, using FASS as the online preconcentration method lowered limits of detection (LOD, calculated based on a signal-to-noise (S/N) ratio of 3; N: noise of the baseline calculated for eleven noise peaks chosen at different places of the baseline void of analytical peaks) by 4.5–9.4 times.

In order to evaluate the performance of the proposed DLLME–FASS-CE method, matrix-matched calibration graphs were constructed using 2.0 g of homogenised samples (milk, yogurt or white cheese), which were free of the NSAIDs spiked with appropriate amounts of mixed standard solution of the target analytes. A series of samples containing each of the NSAIDs at seven concentration levels of 0.2, 0.5, 1.0, 1.5, 2.0 and 2.5 and 5.0 mg kg^{-1} were used. The samples were then subjected to the DLLME procedure optimised above. For each level, three replicate extractions were performed and peak areas of each analyte were used for quantification. Regression equations, coefficients of determination (R^2), linear dynamic ranges (LDR), LODs, relative standard deviations (RSD) and EFs are summarized in Table 2.

The response was linear over the concentration range from their corresponding limits of quantitation (LOQ, S/N 10) to 5000 μg kg^{-1} for all analytes, with R^2 ranging from 0.9915 to 0.9997. LODs ranged between 3.0 and 13.1 μg kg^{-1}, and LOQs between 10 and 43.7 μg kg^{-1}. Reproducibility of the proposed method was evaluated in terms of intra-day and inter-day precision, by extracting

Table 1
Analytical performance parameters of conventional CZE and FASS.

NSAID	Conventional CZE				FASS				EF[d]
	Regression equation[a]	R^2	LDR[b] (mg kg^{-1})	LOD[c] (mg kg^{-1})	Regression equation[a]	R^2	LDR (mg kg^{-1})	LOD (mg kg^{-1})	
ET	$y = 0.7017x + 1.4905$	0.9991	26.3–250	7.9	$y = 4.6404x - 0.4114$	0.9999	3.7–25	1.1	6.6
NAX	$y = 0.8808x + 3.1786$	0.9976	44.0–250	13.2	$y = 7.0009x - 0.2343$	0.9999	5.3–25	1.6	7.9
KTP	$y = 0.4151x - 2.7506$	0.9954	70.0–250	21.0	$y = 3.8913x + 0.1029$	0.9999	1.7–25	0.5	9.4
FBP	$y = 0.9820x - 2.1354$	0.9962	63.7–250	19.1	$y = 4.4559x + 0.2943$	0.9998	6.3–25	1.9	4.5
DIC	$y = 0.7201x + 2.5905$	0.9974	45.7–250	13.7	$y = 5.2071x + 0.0800$	0.9996	8.7–25	2.6	7.2

[a] y (peak area = slope × [NSAID concentration (mg kg^{-1})] + intercept.
[b] LDR: linear dynamic range.
[c] LOD: limit of detection.
[d] Ratio of calibration slope with FASS to that with conventional CZE.

Table 2
Analytical performance parameters of DLLME–FASS-CE.

Matrix	NSAID	Regression equation[b]	R^2	LDR[c] (μg kg^{-1})	LOD[d] (μg kg^{-1})	RSD[e] (%)		EF[f]
						Intra-day	Inter-day	
Bovine milk (3.1)[a]	ET	$y = 71.3807x - 3.4552$	0.9980	43.7–5000	13.1	4.7	6.2	102
	NAX	$y = 99.9399x - 2.0218$	0.9983	39.7–5000	11.9	3.0	4.3	113
	KTP	$y = 52.3859x - 2.3546$	0.9982	41.3–5000	12.4	3.4	5.1	126
	FBP	$y = 79.6025x + 1.1272$	0.9988	33.3–5000	10.0	2.9	3.5	81.1
	DIC	$y = 101.6061x - 2.0110$	0.9997	16.0–5000	4.8	1.1	1.6	141
Yogurt (3.9)[a]	ET	$y = 40.6099x - 0.7036$	0.9963	21.0–5000	6.3	2.3	3.5	57.9
	NAX	$y = 62.2800x - 0.1100$	0.9994	10.0–5000	3.0	3.8	4.6	70.7
	KTP	$y = 33.9995x - 2.1079$	0.9946	27.0–5000	8.1	4.7	5.6	81.9
	FBP	$y = 45.0868x - 1.9836$	0.9930	25.7–5000	7.7	4.7	5.4	45.9
	DIC	$y = 63.2786x - 5.5093$	0.9915	32.3–5000	9.7	2.6	3.1	87.9
White cheese (21.4)[a]	ET	$y = 123.8000x - 5.3024$	0.9967	24.7–5000	7.4	1.5	2.4	176
	NAX	$y = 192.2000x - 6.5741$	0.9975	21.0–5000	6.3	0.6	0.8	218
	KTP	$y = 94.5957x - 3.8170$	0.9976	21.0–5000	6.3	2.2	3.1	228
	FBP	$y = 142.7800x - 5.8274$	0.9977	20.3–5000	6.1	1.5	2.3	145
	DIC	$y = 165.2500x - 8.1083$	0.9964	25.7–5000	7.7	2.1	3.0	229

[a] % Fat content.
[b] y (peak area = slope × [NSAID concentration (μg kg^{-1})] + intercept.
[c] LDR: linear dynamic range.
[d] LOD: limit of detection.
[e] % Relative standard deviation ($n = 5$).
[f] Ratio of calibration slope with DLLME–FASS-CE to that with conventional CZE.

the spiked samples at the seven concentration levels of the calibration graphs for each NSAID in the same day and in three consecutive days, respectively. The results, expressed as the average %RSD of peak areas, are presented in Table 2. An acceptable precision was obtained in all cases with %RSD values below 4.7% for intra-day and below 6.2% for inter-day experiments. EF with DLLME–FASS-CE as compared to conventional CZE (Table 1) were in the range of 45.9–229 (Table 2).

3.5. Application to real samples

In order to evaluate the applicability, recovery and possible matrix effect of the proposed DLLME–FASS-CE method, two types of milk samples: bottled (sample 1, sample 2 and sample 3) and raw milk, as well as yogurt and white cheese with varying fat contents (0.1–21.4%) were examined. Typical electropherograms of unspiked (blank) and spiked samples are shown in Fig. 2. As can also be seen from Fig. 2, no detectable signals from the target analytes were observed, nor any interfering peaks appeared at their migration times, indicating good selectivity of the method. The recoveries obtained for samples spiked at three concentration levels (0.5, 1.0 and 2.5 mg kg^{-1} of each NSAID) are listed in Table 3. According to the results, the recoveries obtained from matrix-matched calibrations were in the ranges of 86.6–109.3% (bottled milk), 84.3–100.5% (raw milk), 77.4–107.3% (yogurt) and 90.9–101.6% (white cheese). Matrix effect was evaluated by comparing the slopes of calibration graphs (Table 2). Different slopes indicated the presence of matrix effect which was linked to difference in fat content in the samples. Higher EF was obtained in white cheese (highest fat content). However, matrix effect was eliminated by

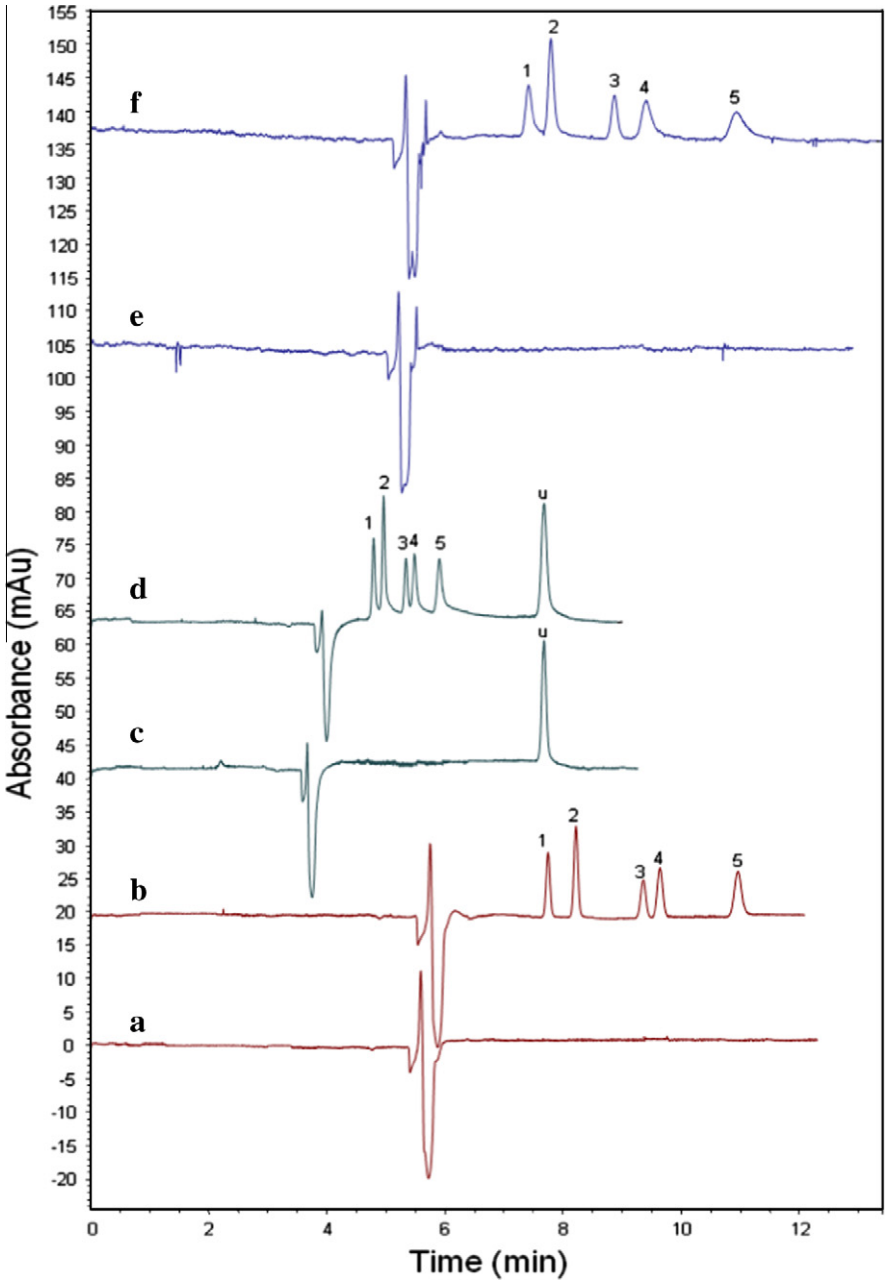

Fig. 2. Representative electropherograms of samples extracted and analysed under optimum DLLME–FASS-CE conditions: (a) blank bottled milk, (b) spiked bottled milk, (c) blank yogurt, (d) spiked yogurt, (e) blank white cheese and (f) spiked white cheese; spiked concentration level: 1.0 mg kg^{-1} of each NSAID in all samples. Peaks: 1, ET; 2, NAX; 3, KTP; 4, FBP; 5, DIC; and u, unknown peak.

using matrix-matched calibrations as can be inferred from good relative recovery (RR) values (Table 3).

3.6. Comparison with other preconcentration methods

Efficiency of the presented DLLME–FASS-CE method for the selected analytes was compared with those of other reported methods considering aspects such as extraction time, amount of sample used, total volume of organic solvents consumed per sample, LOD and LOQ. In comparison with other methods, the main advantages of this extraction method were rapidness, simplicity and cost effectiveness. As listed in Table 4, the extraction time was only 2 min in this study, which was much shorter than the other extraction methods due to the large surface area of contact between the extraction solvent and the sample solution during emulsion formation. The other methods required a longer time for equilibrium to be established. In addition, this method required the least amount of organic solvents for analysis. LODs and LOQs achieved were better than those of SPME–LC–UV. MS and MS–MS detectors are inherently more sensitive than UV hence the lower LODs or LOQs. Yet, these detectors are much more expensive and complicated. In SPME–GC–MS (Arroyo et al., 2011), a time-consuming derivatization step was required (15 min) in order to obtain desirable chromatographic characteristics and to improve the stability and detectability of the NSAIDs. Studies using conventional LLE and SPE are labour-intensive, expensive, and applied time-consuming procedures. Since DLLME–FASS-CE does not require special instrumentation, it is cheap, simple and minimizes contamination or sample carry-over during extraction.

4. Conclusion

In this study, the applicability of DLLME as an efficient preconcentration technique was successfully extended to the complex matrices of milk and dairy products. A novel combination of DLLME and FASS in CE for the determination of five NSAIDs in these

Table 3
Relative recoveries of NSAIDs from spiked bottled and raw bovine milk, yogurt and white cheese samples analysed using DLLME–FASS-CE.

NSAID	Spiked level (mg kg^{-1})	Bovine milk								Yogurt (3.9)[a]		White cheese (21.4)[a]	
		Bottled						Raw (unknown)[a]					
		Sample 1 (3.1)[a]		Sample 2 (0.1)[a]		Sample 3 (3.0)[a]							
		RR[b]	RSD[c]	RR[b]	RSD[c]	RR[b]	RSD[c]	RR[b]	RSD[c]	RR[b]	RSD[c]	RR[b]	RSD[c]
ET	0.5	102.4	4.5	99.5	1.8	100.9	1.1	95.4	1.5	100.6	2.0	101.3	1.4
	1.0	98.5	2.1	96.0	0.9	86.6	3.7	98.4	1.8	107.3	2.8	90.9	1.1
	2.5	93.4	4.4	100.9	2.9	102.1	1.8	100.3	1.0	103.2	1.3	101.5	0.5
NAX	0.5	106.6	2.9	102.3	2.5	101.5	2.1	93.9	2.1	101.3	3.6	101.6	0.6
	1.0	100.0	0.6	95.5	1.3	87.1	1.6	99.4	0.4	104.5	2.2	92.0	1.1
	2.5	94.1	2.2	100.7	1.5	102.0	2.5	100.1	4.6	99.3	2.2	101.3	0.9
KTP	0.5	101.1	3.2	104.9	5.1	101.9	2.3	91.4	1.9	95.0	3.5	100.7	1.9
	1.0	99.4	0.7	96.9	3.4	91.1	3.3	97.8	1.2	94.5	2.3	92.6	1.5
	2.5	93.7	3.0	100.4	1.0	101.4	2.4	100.5	7.1	104.6	0.3	101.2	1.1
FBP	0.5	109.3	2.9	102.5	3.9	98.8	1.6	84.3	1.9	95.2	3.5	100.4	1.4
	1.0	96.1	1.4	96.2	1.4	91.0	2.3	93.5	0.8	105.6	2.5	92.9	1.0
	2.5	95.2	2.8	102.2	1.7	101.3	2.6	100.5	2.9	104.2	8.3	101.2	2.3
DIC	0.5	99.8	1.1	104.3	1.6	102.1	2.9	96.4	1.7	77.4	1.3	101.1	1.9
	1.0	99.5	0.3	95.0	1.0	92.5	3.0	99.8	1.1	93.2	0.8	90.9	1.3
	2.5	97.6	2.2	101.5	2.2	101.2	1.1	100.1	4.8	102.6	0.9	101.5	2.9

[a] % Fat content.
[b] % Relative recovery, percentage value obtained considering extraction yields from matrix-matched calibrations.
[c] % Relative standard deviation (n = 3).

Table 4
Comparison of DLLME–FASS-CE with other reported methods for extraction and determination of NSAIDs.

Analyte	Matrix	Extraction method/ technique	Extraction time (min)	Amount of sample (g)	Total volume of organic solvents per sample (mL)	LOD (μg kg^{-1})	LOQ (μg kg^{-1})	Ref.
NAX, KTP, DIC	Bovine Milk	SPME–GC–MS[a]	~30	10	16	–	3.36–5.07	(Arroyo et al., 2011)
NAX, KTP, DIC	Milk	SPE–UPLC–ToF-MS[b]	30	2 (mL)	5	–	6.3–12.5 (μg L^{-1})	(Stolker et al., 2008)
NAX	Human urine	SPME–LC–UV[c]	30	1.0	–	30 (μg L^{-1})	200 (μg L^{-1})	(Aresta, Palmisano, & Zambonin, 2005)
NAX, KTP, DIC	Bovine Milk	LLE–LC–MS–MS[d]	10	2.0	8	0.1–3.43	–	(Dubreil-Cheneau et al., 2011)
NAX, FBP	Bovine Milk	SPE–HPLC–UV[e]	10	5.0	19.5	–	2.0, 4.0	(Gallo et al., 2010)
ET, NAX, KTP, FBP, DIC	Bovine Milk	DLLME–FASS-CE	2	2.0	4.15	4.8–13.1	16.0–43.7	This study
	Yogurt					3.0–9.7	10.0–32.3	
	White cheese					6.1–7.7	20.3–25.7	

[a] Solid-phase microextraction–gas chromatography–mass spectrometry.
[b] Solid-phase extraction–ultra performance liquid chromatography–time-of-flight-mass spectrometry.
[c] Solid-phase microextraction–liquid chromatography–ultraviolet.
[d] Liquid–liquid extraction–liquid chromatography–tandem mass spectrometry.
[e] Solid-phase extraction–high performance liquid chromatography–ultraviolet.

matrices was demonstrated to enhance the sensitivity of CE by 46–229 times resulting in LODs as low as 3.0–13.1 μg kg^{-1}. As compared with other conventional sample preparation techniques, this method offered numerous advantages, such as simplicity, low cost, ease of operation, use of less amounts of organic solvents and high enrichment factors within a very short analysis time. Despite the complexity of the matrices studied, good recoveries, high reproducibility and interference-free electropherograms were achieved. The results indicate that DLLME–FASS-CE could be of great interest, especially for determination of NSAIDs in foodstuff in routine analytical laboratories.

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References

Aranas, A. T., Guidote, A. M., & Quirino, J. P. (2009). Sweeping and new on-line sample preconcentration techniques in capillary electrophoresis. *Analytical and Bioanalytical Chemistry, 394*(1), 175–185.

Aresta, A., Palmisano, F., & Zambonin, C. G. (2005). Determination of naproxen in human urine by solid-phase microextraction coupled to liquid chromatography. *Journal of Pharmaceutical and Biomedical Analysis, 39*(3–4), 643–647.

Arroyo, D., Ortiz, M. C., & Sarabia, L. A. (2011). Optimization of the derivatization reaction and the solid-phase microextraction conditions using a D-optimal design and three-way calibration in the determination of non-steroidal anti-inflammatory drugs in bovine milk by gas chromatography–mass spectrometry. *Journal of Chromatography, A, 1218*(28), 4487–4497.

Berijani, S., Assadi, Y., Anbia, M., Hosseini, M. R. M., & Aghaee, E. (2006). Dispersive liquid–liquid microextraction combined with gas chromatography-flame photometric detection – Very simple, rapid and sensitive method for the determination of organophosphorus pesticides in water. *Journal of Chromatography, A, 1123*(1), 1–9.

Botello, I., Borrull, F., Calull, M., & Aguilar, C. (2011). Simultaneous determination of weakly ionizable analytes in urine and plasma samples by transient pseudo-isotachophoresis in capillary zone electrophoresis. *Analytical and Bioanalytical Chemistry, 400*(2), 527–534.

Dowling, G., Gallo, P., Malone, E., & Regan, L. (2009). Rapid confirmatory analysis of non-steroidal anti-inflammatory drugs in bovine milk by rapid resolution liquid chromatography tandem mass spectrometry. *Journal of Chromatography, A, 1216*(46), 8117–8131.

Dubreil-Cheneau, E., Pirotais, Y., Bessiral, M., Roudaut, B., & Verdon, E. (2011). Development and validation of a confirmatory method for the determination of 12 non steroidal anti-inflammatory drugs in milk using liquid chromatography–tandem mass spectrometry. *Journal of Chromatography, A, 1218*(37), 6292–6301.

Farajzadeh, M. A., Djozan, D., Mogaddam, M. R. A., & Bamorowat, M. (2011). Extraction and preconcentration technique for triazole pesticides from cow milk using dispersive liquid–liquid microextraction followed by GC–FID and GC–MS determinations. *Journal of Separation Science, 34*(11), 1309–1316.

Farajzadeh, M. A., Djozan, D., Reza, M., Mogaddam, A., & Norouzi, J. (2012). Determination of phthalate esters in cow milk samples using dispersive liquid–liquid microextraction coupled with gas chromatography followed by flame ionization and mass spectrometric detection. *Journal of Separation Science, 35*(5–6), 742–749.

Fontanals, N., Marce, R. M., & Borrull, F. (2007). New materials in sorptive extraction techniques for polar compounds. *Journal of Chromatography, A, 1152*(1–2), 14–31.

Gallo, P., Fabbrocino, S., Dowling, G., Salini, M., Fiori, M., Perretta, G., et al. (2010). Confirmatory analysis of non-steroidal anti-inflammatory drugs in bovine milk by high-performance liquid chromatography with fluorescence detection. *Journal of Chromatography, A, 1217*(17), 2832–2839.

Gallo, P., Fabbrocino, S., Vinci, F., Fiori, M., Danese, V., Nasi, A., et al. (2006). Multi-residue determination of non-steroidal anti-inflammatory drug residues in animal serum and plasma by HPLC and photo-diode array detection. *Journal of Chromatographic Science, 44*(10), 585–590.

Gallo, P., Fabbrocino, S., Vinci, F., Fiori, M., Danese, V., & Serpe, L. (2008). Confirmatory identification of sixteen non-steroidal anti-inflammatory drug residues in raw milk by liquid chromatography coupled with ion trap mass spectrometry. *Rapid Communications in Mass Spectrometry, 22*(6), 841–854.

Guo, L., & Lee, H. K. (2012). One step solvent bar microextraction and derivatization followed by gas chromatography–mass spectrometry for the determination of pharmaceutically active compounds in drain water samples. *Journal of Chromatography, A, 1235*, 26–33.

Herrera-Herrera, A. V., Hernandez-Borges, J., Borges-Miquel, T. M., & Rodriguez-Delgado, M. A. (2010). Dispersive liquid–liquid microextraction combined with nonaqueous capillary electrophoresis for the determination of fluoroquinolone antibiotics in waters. *Electrophoresis, 31*(20), 3457–3465.

Hu, T., Peng, T., Li, X. J., Chen, D. D., Dai, H. H., Deng, X. J., et al. (2012). Simultaneous determination of thirty non-steroidal anti-inflammatory drug residues in swine muscle by ultra-high-performance liquid chromatography with tandem mass spectrometry. *Journal of Chromatography, A, 1219*, 104–113.

Jain, A., & Verma, K. K. (2011). Recent advances in applications of single-drop microextraction: A review. *Analytica Chimica Acta, 706*(1), 37–65.

Leong, M. I., & Huang, S. D. (2008). Dispersive liquid–liquid microextraction method based on solidification of floating organic drop combined with gas chromatography with electron-capture or mass spectrometry detection. *Journal of Chromatography, A, 1211*(1–2), 8–12.

Liu, X. J., Zhao, A. J., Zhang, A. N., Liu, H. Q., Xiao, W. J., Wang, C. J., et al. (2011). Dispersive liquid–liquid microextraction and gas chromatography–mass spectrometry determination of polychlorinated biphenyls and polybrominated diphenyl ethers in milk. *Journal of Separation Science, 34*(9), 1084–1090.

Mala, Z., Gebauer, P., & Bocek, P. (2011). Contemporary sample stacking in analytical electrophoresis. *Electrophoresis, 32*(1), 116–126.

Malone, E. M., Dowling, G., Elliott, C. T., Kennedy, D. G., & Regan, L. (2009). Development of a rapid, multi-class method for the confirmatory analysis of anti-inflammatory drugs in bovine milk using liquid chromatography tandem mass spectrometry. *Journal of Chromatography, A, 1216*(46), 8132–8140.

Noche, G. G., Laespada, M. E. F., Pavon, J. L. P., Cordero, B. M., & Lorenzo, S. M. (2011). In situ aqueous derivatization and determination of non-steroidal anti-inflammatory drugs by salting-out-assisted liquid–liquid extraction and gas chromatography–mass spectrometry. *Journal of Chromatography, A, 1218*(37), 6240–6247.

Osbourn, D. M., Weiss, D. J., & Lunte, C. E. (2000). On-line preconcentration methods for capillary electrophoresis. *Electrophoresis, 21*(14), 2768–2779.

Payan, M. R., Lopez, M. A. B., Torres, R. F., Navarro, M. V., & Mochon, M. C. (2011). Electromembrane extraction (EME) and HPLC determination of non-steroidal anti-inflammatory drugs (NSAIDs) in wastewater samples. *Talanta, 85*(1), 394–399.

Pedersen-Bjergaard, S., & Rasmussen, K. E. (2008). Liquid-phase microextraction with porous hollow fibers, a miniaturized and highly flexible format for liquid–liquid extraction. *Journal of Chromatography, A, 1184*(1–2), 132–142.

Rezaee, M., Assadi, Y., Hosseinia, M. R. M., Aghaee, E., Ahmadi, F., & Berijani, S. (2006). Determination of organic compounds in water using dispersive liquid–liquid microextraction. *Journal of Chromatography, A, 1116*(1–2), 1–9.

Rezaee, M., Yamini, Y., & Faraji, M. (2010). Evolution of dispersive liquid–liquid microextraction method. *Journal of Chromatography, A, 1217*(16), 2342–2357.

Rezaee, M., Yamini, Y., Shariati, S., Esrafili, A., & Shamsipur, M. (2009). Dispersive liquid–liquid microextraction combined with high-performance liquid chromatography–UV detection as a very simple, rapid and sensitive method for the determination of bisphenol A in water samples. *Journal of Chromatography, A, 1216*(9), 1511–1514.

Stolker, A. A. M., Rutgers, P., Oosterink, E., Lasaroms, J. J. P., Peters, R. J. B., van Rhijn, J. A., et al. (2008). Comprehensive screening and quantification of veterinary drugs in milk using UPLC–ToF-MS. *Analytical and Bioanalytical Chemistry, 391*(6), 2309–2322.

Xie, H. Y., & He, Y. Z. (2010). Green analytical methodologies combining liquid-phase microextraction with capillary electrophoresis. *TrAc, Trends in Analytical Chemistry, 29*(7), 629–635.

Xiong, C. M., Ruan, J. L., Cai, Y. L., & Tang, Y. (2009). Extraction and determination of some psychotropic drugs in urine samples using dispersive liquid–liquid microextraction followed by high-performance liquid chromatography. *Journal of Pharmaceutical and Biomedical Analysis, 49*(2), 572–578.