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Recovery, enrichment and selectivity in liquid-phase microextraction Comparison with conventional liquid–liquid extraction

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Abstract

Mathematical descriptions for extraction recovery and enrichment were applied for liquid-phase microextraction (LPME) and comparison with conventional two- and three-phase liquid–liquid extraction techniques (LLE) was made. The LPME theoretical calculations were verified by experimental determination of actual partition coefficients and by data obtained with LPME in a robust hollow fibre formate. With hollow fibre LPME operated in the two-phase mode, analytes were extracted from 1 to 4 ml aqueous samples into 25–50 μl of an organic solvent present in the pores and in the lumen of the porous hollow fibres. Compared with conventional two-phase LLE, two-phase LPME provided substantially higher enrichments for compounds with relatively large partition coefficients ($K_{org/d}$>500). In contrast, because of the large volume of organic solvent relative to the sample volume, LLE provided high recovery and moderate enrichment even for compounds with relatively low partition coefficients ($K_{org/d}$>5). Thus, two-phase LPME may be used for substantially enhanced extraction selectivity and enrichment of relatively hydrophobic analytes as compared with LLE whereas conventional two-phase LLE is superior for more hydrophilic analytes. Similar results were found for three-phase LPME where analytes where extracted from 1 to 4 ml aqueous samples through approximately 20 μl organic solvent immobilized within the pores of the hollow fibre and into 25 μl of an aqueous acceptor solution inside the lumen of the hollow fibre. The fundamental differences of LPME and LLE were further demonstrated with practical experiments on extraction of the basic drugs promethazine, methadone, and haloperidol from human plasma and urine.

Keywords: Liquid-phase microextraction; Liquid–liquid extraction; Extraction methods; Selectivity; Mathematical modelling; Partition coefficients; Hollow fibres; Promethazine; Methadone; Haloperidol

1. Introduction

Recently, some interest has been devoted to liquid-phase microextraction (LPME) as a sample preparation technique for chromatography and electrophoresis. In LPME, the principles of liquid–liquid extraction and the miniaturized nature of solid-phase microextraction are combined to realize the advantages of both techniques. LPME has been accomplished either by extraction into small water immiscible drops of organic solvents [1–11] (microdrop) or into small volumes of acceptor solution present inside the lumen of porous hollow fibres [12–16]. In both the microdrop concept and in the hollow fibre formate, the analytes of interest are extracted and preconcentrated into volumes of typically 25–50 μl. Because of this, LPME may be very effective for analyte enrichment and may result in major reduction in the use of organic solvents.

With the microdrop approach, several different concepts have been reported. In one system, a 1–2 μl drop of an organic solvent immiscible with water was suspended from the tip of a microsyringe needle

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and into a stirred sample solution [3–5,8,11]. The analytes were extracted from the aqueous sample and into the microdrop, the microdrop was retracted into the needle, and finally the microdrop was injected directly into a gas chromatography (GC) system. Alternatively, static LPME has been accomplished with a 8 μl organic microdrop located at the end of a PTFE rod, and after extraction, 1 μl of this was sampled with a microsyringe and transferred directly to a GC system [2]. The microdrop approach has also been reported in dynamic LPME systems; in one system 1–2 μl solvent was withdrawed into a microsyringe and subsequently, this was filled with 3–5 μl aqueous sample for a few seconds. The sample was then pushed out and a new portion of sample was withdrawed; this procedure was repeated several times (typically 20 times). Finally, 1 μl of the organic solvent was injected into a GC system. Dynamic LPME with organic microdrops have also been reported in different flow systems [1–10].

Although most LPME procedures with microdrops have involved two-phase extractions from aqueous samples and into organic solvents, also three-phase extractions have been reported [7,9]. In these reports, basic compounds were extracted from 0.5 to 1 ml aqueous samples (where pH was adjusted into the alkaline region), through a 40–80 μl organic phase held within a PTFE ring, and into a 0.1–0.2 ml aqueous phosphate buffer (pH 2.1). One major advantage of this concept was the compatibility with high-performance liquid chromatography (HPLC) and capillary electrophoresis (CE); the receiving phases were directly injected into HPLC and CE systems as these were aqueous solutions.

As an alternative to the microdrop concept, we recently introduced an inexpensive and disposable hollow fibre based device for static LPME both in a two- or three-phase system [12–16]. The LPME technique is a further development of the supported liquid membrane technique (SLM) and of microporous membrane liquid–liquid extraction (MMLLE) [17–23]. In the two-phase LPME system, the analytes were extracted from 1 to 4 ml of aqueous samples through a water immiscible organic solvent immobilized in the pores of a porous hollow fibre (20 μl) and into the same organic solvent (25 μl) present in the lumen of the hollow fibre. In the three-phase LPME system, the analytes were extracted through the organic solvent in the pores of the hollow fibre and into a new aqueous solution (25 μl) inside the fibre. Because the extracting medium was adsorbed by capillary forces in a hollow fibre, the hollow fibre approach to LPME is highly robust [24]. The hollow fibre based LPME device has been used for extraction of several basic and acidic drugs present in humane urine, plasma, and whole blood. With both GC coupled with nitrogen–phosphorus detection (NPD) and CE coupled with UV detection, drugs have been detected down to the 1–10 ng/ml level in 0.5–4 ml volumes of biological sample owing to an efficient analyte enrichment. In addition, the hollow fibre base LPME device was found to provide excellent clean-up from the most important biological samples [12–16]. In conclusion, initial LPME reports have indicated a high potential of the technique.

As small volumes of organic solvents are emulsified in biological fluids such as whole blood, serum or plasma, conventional LLE procedures for drugs are normally based on an excess of organic solvent relative to the sample. The phase ratios between the solvent and sample (V_{org}/V_d) are normally in the range of 1–5 as demonstrated by recent procedures [25–39]. The phase ratios (V_{org}/V_d) used in published LPME procedures have been in the range of 0.005–0.05 [12–16].

Although several papers have emerged on LPME as discussed above, very little fundamental information is available on similarities and differences between LPME and conventional LLE with respect to analyte recovery, enrichment, and extraction selectivity. This type of information is crucial for further development of the different formates of LPME and for this technique to be generally accepted. Therefore, in the present work, mathematical descriptions were applied for the calculation of recoveries and enrichment in LPME and LLE, and theoretical values for both parameters in LPME and LLE were compared. In addition, the calculations were verified experimentally by the determination of actual partition coefficients and by comparison of theoretical and experimental LPME results. Finally, the practical consequences of the inherent characteristics of LPME and LLE were illustrated by extraction of some basic drugs from human urine and plasma. All experimental and theoretically derived LPME data

were generated with focus on the hollow fibre based LPME recently introduced [12–16], but the majority of the conclusions are valid also for other formates of LPME.

2. Theory

2.1. Basic principle of LPME device

Fig. 1 illustrate the basic principle of the hollow fiber based device for LPME. Extractions were performed from aqueous samples present in either 1 or 4 ml vials. Prior to extraction, pH in the sample was adjusted in cases of ionizable analytes to ensure deionization and followingly to reduce their solubility within the sample solution; for LPME of basic substances, pH was adjusted into the alkaline region while the sample was acidified for LPME of acidic compounds. Extraction was accomplished by a porous hollow fibre of polypropylene placed directly into the sample solution. For two-phase LPME, both the pores and the lumen of the hollow fibres were filled with an organic solvent immiscible with water. The analytes were extracted from the sample solution and into this solvent through the pores of the hollow fibre. Normally, approximately 20 μl organic solvent was immobilized within the pores of the hollow fibre, while the volume of organic solvent inside the lumen was 25 μl. After extraction, the 25 μl volume of organic solvent inside the lumen was easily transferred to a micro insert by application of a small head pressure on the inlet tube for the hollow fibre. The microextract was directly compatible with GC.

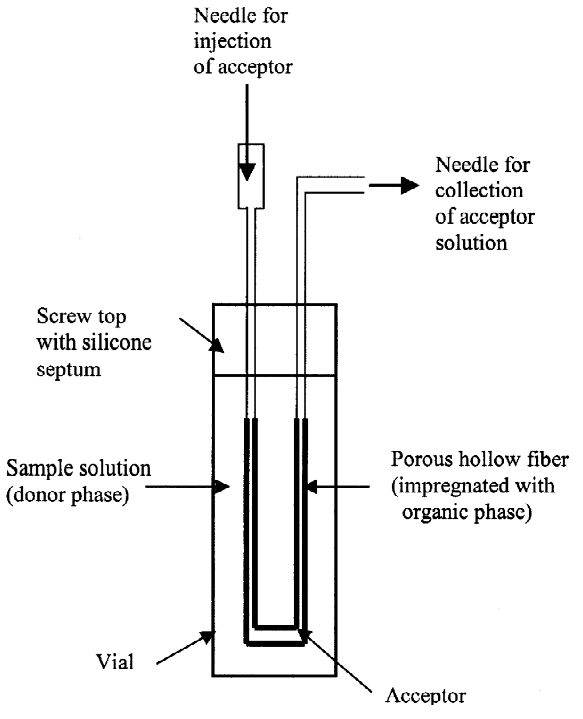

Fig. 1. Diagram of the disposable LPME device.

For three-phase LPME, an aqueous phase was filled inside the lumen of the hollow fibre. Thus, the analytes of interest were extracted from 1 to 4 ml aqueous samples, where pH adjustment served to deionize the analytes, into the immobilized organic solvent within the pores of the hollow fibre, and further into the aqueous acceptor phase. The organic phase served as a barrier between the sample solution and the acceptor phase. For the extraction of basic compounds, an aqueous solution of an acid was utilized as acceptor phase, whereas an alkaline solution was used for three-phase LPME of acidic components. Since the analytes became ionized following extraction into the acceptor phase, they were prevented from re-entering the organic solvent in the pores of the hollow fibre. The aqueous acceptor phase was easily collected into a micro-insert and analyzed directly by HPLC or CE.

Provided the analytes were efficiently transferred to the acceptor solution, LPME both in the two- and three-phase modes resulted in substantial analyte enrichment. From 4 ml samples and with a 25 μl acceptor phase volume, analytes may theoretically be enriched by a factor of 160 following 100% recovery. In addition to enrichment, substantial sample clean-up was achieved by LPME both in the two- and three-phase modes as will be discussed in detail below.

2.2. Calculation of recovery and enrichment in two-phase LPME and LLE

In two-phase LPME, the analytes are extracted from the aqueous sample solution (donor phase) and into the organic solvent (acceptor phase) present in the pores and inside the lumen of the hollow fibre as

discussed above. This process may be illustrated with the following equation:

$$\text{A (donor phase)} \leftarrow\rightarrow \text{A (acceptor phase)} \quad (1)$$

where A represents the analyte of interest. The partition coefficient $K_{org/d}$ is:

$$K_{org/d} = \frac{C_{eq,org}}{C_{eq,d}} \quad (2)$$

where $C_{eq,org}$ is the concentration of A in the acceptor phase at equilibrium and $C_{eq,d}$ is the concentration of A in the donor phase at equilibrium. The initial amount of analyte n_i is equal to the sum of the individual amounts of analyte present in the two phases during the whole extraction process:

$$n_i = n_d + n_{org} \quad (3)$$

where n_d is the amount of analyte present in the donor phase and n_{org} is the amount of analyte present in the acceptor phase. At equilibrium, Eq. (3) can also be written as:

$$C_i V_d = C_{eq,d} V_d + C_{eq,org} V_{org} \quad (4)$$

where C_i is the initial analyte concentration in the sample, and V_d and V_{org} are the sample volume (donor phase) and acceptor phase volume, respectively. At equilibrium, the amount of analyte extracted into the acceptor phase $n_{eq,org}$ of the system can be expressed by [40–42]:

$$n_{eq,org} = \frac{K_{org/d} V_{org} C_i V_d}{K_{org/d} V_{org} + V_d} \quad (5)$$

The recovery (R) of the analyte is calculate by the equation:

$$R = \frac{100 n_{eq,org}}{C_i V_d} = \frac{K_{org/d} V_{org}}{K_{org/d} V_{org} + V_d} \cdot 100 \quad (6)$$

The enrichment (E) of the analyte can be calculated by the formula:

$$E = \frac{C_{org}}{C_i} = \frac{V_d R}{100 V_{org}} \quad (7)$$

C_{org} is the concentration of A in the acceptor phase at the end of extraction. Eqs. (6) and (7) may also be used for two-phase LLE. For two-phase LPME, the actual recovery is lower than calculated by Eq. (6) because the fraction of the organic solvent which is immobilized in the pores of the hollow fibre is not available for further analysis; only the fraction present in the lumen may be collected into a micro insert.

2.3. Calculation of recovery and enrichment in three-phase LPME and LLE

In three-phase LPME, the analytes are extracted from the aqueous sample solution (donor phase), through the organic solvent immobilized in the pores of the hollow fibre (organic phase), and further into the acceptor solution (acceptor phase) present inside the lumen of the hollow fibre. This process may be illustrated with the following equation:

$$\text{A (donor phase)} \leftarrow\rightarrow \text{A[3(organic phase)} \leftarrow\rightarrow \text{A (acceptor phase)} \quad (8)$$

In the three-phase system, the initial amount of analyte n_i is equal to the sum of the individual amounts of analyte present in the three phases during the whole extraction process:

$$n_i = n_d + n_{org} + n_a \quad (9)$$

n_d is the amount of analyte present in the donor phase (the sample), n_{org} is the amount of analyte present in the organic phase, and n_a is the amount of analyte present in the acceptor phase at any time during extraction process. At equilibrium, Eq. (9) can be expressed:

$$C_i V_d = C_{eq,d} V_d + C_{eq,org} V_{org} + C_{eq,a} V_a \quad (10)$$

C_i is the initial concentration in the sample. $C_{eq,d}$, $C_{eq,org}$, and $C_{eq,a}$ are the analyte concentrations in the donor phase, organic phase, and acceptor phase at equilibrium, respectively. V_d, V_{org}, and V_a are the volumes of sample (donor phase), organic phase, and acceptor phase, respectively.

In the three-phase LPME system, partition coefficients both between the organic phase and the donor phase as well as between the acceptor phase and the organic phase have to be considered:

$$K_{org/d} = \frac{C_{eq,org}}{C_{eq,d}} \quad (11)$$

$$K_{a/org} = \frac{C_{eq,a}}{C_{eq,org}} \quad (12)$$

The partition coefficient between the acceptor phase and the donor phase $K_{a/d}$ can be written as:

$$K_{a/d} = = \frac{C_{eq,a}}{C_{eq,d}} = K_{org/d}K_{a/org} \quad (13)$$

The amount of analyte extracted into the acceptor phase of the system can be calculated by substituting $K_{a/d}$ $C_{eq,d}$ for $C_{eq,a}$ and by rearrangement of Eq. (9). At equilibrium, the amount of analyte present in the acceptor phase $n_{eq,a}$ may be calculated by [40–42]:

$$n_{eq,a} = \frac{K_{a/d}V_aC_iV_d}{K_{a/d}V_a + K_{org/d}V_{org} + V_d} \quad (14)$$

The recovery (R) can be expressed as:

$$R = \frac{100n_{eq,a}}{C_iV_d} = \frac{100K_{a/d}V_a}{K_{a/d}V_a + K_{org/d}V_{org} + V_d} \quad (15)$$

The enrichment (E) can be calculated by the formula:

$$E = \frac{C_a}{C_i} = \frac{V_dR}{100V_a} \quad (16)$$

Eqs. (15) and (16) may be used to calculate recovery and enrichment for three-phase LPME. In the case of three-phase LPME, the whole volume of acceptor phase is available for further analysis, and the recovery is therefore directly calculated from Eq. (15).

Eqs. (15) and (16) may also be used for calculation of recoveries and enrichment in three-phase LLE, where analytes in a first step are extracted from an aqueous sample into an organic solvent immiscible with water, and secondly back extracted in a separate step into a new aqueous phase.

3. Experimental section

3.1. Capillary gas chromatography

The GC system was an 8000 series Fisons Instruments (Fisons Instruments, Rodano, Italy) with a flame ionisation detection (FID) system and a SPB-1 fused-silica capillary column (30 m×0.25 mm I.D., 0.25 μm) (Supelco, Bellefonte, PA, USA) coated with 100% polydimethylsiloxane. Helium of 99.998% purity (AGA, Oslo, Norway) was utilized as carrier gas at 1.0 ml/min. The extracts (1 μl) were injected manually in the split mode (split ratio 1:10). The injector and the detector were operated at 250 °C. The GC oven was maintained at 175 °C for 1 min following injection and subsequently programmed at 25 °C/min to 300 °C (held for 5 min).

3.2. Capillary electrophoresis

Capillary electrophoresis was performed with a MDQ instrument (Beckman, Fullerton, CA, USA) equipped with a UV detector. The analyses were accomplished in a 75 μm I.D. fused-silica capillary (BGB Analyk, Anwil, Switzerland) with an effective length of 30 cm and utilizing a 25 m*M* phosphate buffer adjusted to pH 2.75 as the running buffer. The instrument was operated at 15 kV. All samples were introduced by hydrodynamic injection at 0.5 p.s.i. for 5 s (1 p.s.i.=6894.76 Pa). Detection was accomplished at 200 nm utilizing a 800×100 μm slit.

3.3. LPME equipment

LPME was carried out in conventional 4 ml sample vials (Supelco) equipped with screw caps containing a silicon septum. Two conventional 0.8 mm O.D. medical syringe needles were inserted through the silicon septum in the screw top, and the two ends were connected to each other by a 8 cm piece of Q3/2 Accurel KM polypropylene hollow fibre (Membrana, Wuppertal, Germany). The inner diameter of the hollow fibre was 600 μm, the thickness of the wall was 200 μm, and the pore size was 0.2 μm.

3.4. Chemicals

Methadone and promethazine were obtained from Norsk Medisinaldepot (Oslo, Norway), and haloperidol was obtained from Sigma (St. Louis, MO, USA). Sodium hydroxide, hydrochloric acid, orthophosphoric acid, disodium hydrogenphosphate, methanol, and *tert.*-butyl methyl ether were obtained from Merck (Darmstadt, Germany). Dihexyl ether was obtained from Sigma.

3.5. Standard solutions and biological samples

Working solutions of promethazine, methadone, and haloperidol in urine and plasma were prepared by dilution from 2 μg/ml and 5 μg/ml standard solutions in water. These standard solutions were prepared from 1 mg/ml stock solutions of promethazine, metadone, and haloperidol in methanol. Standard solutions and stock solutions were stored at 5 °C protected from light.

3.6. LLE procedure

One ml of urine or plasma samples was pipetted into a glass centrifugated tube. The samples were spiked with promethazine, methadone, and haloperidol to 100 ng/ml and 2.5 μg/ml for LLE with back extraction and LLE, respectively. All the samples were extracted according to the following procedure; the samples (including blank samples) were made alkaline with 250 μl 2 *M* NaOH and diluted with pure water to a total volume of 2 ml. The samples were extracted with 5 ml of *tert*.-butyl methyl ether by shaking at 60 rpm for 15 min. After phase separation, which was obtained by centrifugation at 500 rpm for 15 min, the upper organic layer was transferred and evaporated to dryness at 40 °C under a light stream of pure nitrogen. The residue was redissolved in 75 μl of *tert*.-butyl methyl ether and a 1 μl aliquot of each extract was injected into the GC.

For LLE with back extraction, the upper organic layer was transferred into a new centrifugated glass tube which contained 150 μl 0.01 *M* HCl. The sample mixture was shaked at 60 rpm for 15 min and followed by centrifugation at 500 rpm for 15 min. The organic layer was discarded and the aqueous phase (0.01 *M* HCl) was put into a 200 μl microinsert for the CE instrument.

3.7. LPME procedure

LPME was carried out according to the following procedure; a 1 ml sample solution (urine or plasma spiked with 100 ng/ml and 2.5 μg/ml of each of the model compounds for three-phase LPME and two-phase LPME, respectively) was filled into a 4 ml sample vial. The sample was made alkaline with 250 μl 2 *M* NaOH and diluted with pure water to a total volume of 4 ml.

For two-phase LPME, the hollow fibre was dipped for 5 s in dihexyl ether followed by ultrasonification for 15 s in a water bath to remove excess of solvent. Subsequently, 25 μl of dihexyl ether (acceptor phase) was injected into the lumen of the hollow fibre with a microsyringe. This activated fibre was placed in the sample solution present in a 4 ml vial. During extraction, the samples were vibrated at 1500 rpm (maximum speed of the vibrator) for a period of 45 min by using a Vibramax 100 (Heidolph, Kelheim, Germany) in order to reach equilibrium. After extraction, the acceptor phase was flushed into a 200 μl glass vial, and 1 μl was injected into the GC system for the final analysis.

For three-phase LPME, the hollow fibre was dipped for 5 s in dihexyl ether followed by ultrasonification for 15 s in a water bath to remove excess of solvent. Subsequently, 25 μl of 10 m*M* HCl (acceptor phase) was injected into the lumen of the hollow fibre with a microsyringe. This activated fibre was placed in the sample solution present in a 4 ml vial. Also in this case, the samples were vibrated at 1500 rpm for a period of 45 min in order to reach equilibrium. After extraction, the acceptor phase was flushed into a 200 μl micro insert (Beckman) for the CE instrument, followed by CE analysis.

3.8. Determination of partition coefficients

Partition coefficients between the acceptor phase and the organic phase $K_{a/org}$ were determined by LLE of 1 ml of the analytes of interest present at 100 μg/ml in 10 m*M* HCl (simulated LPME acceptor phase) with 1 ml of dihexyl ether (simulated LPME organic phase) in a 4 ml sample vial. LLE was accomplished for 15 min with vibration at 1500 rpm, and subsequently the aqueous phase was analyzed by CE to establish the amount of analyte remaining in this phase. $K_{a/org}$ was calculated according to the following equation:

$$K_{a/org} = \frac{C_{eq,aq}}{C_{eq,org}} = \frac{n_{eq,aq}V_{aq}}{n_{eq,org}V_{org}} = \frac{n_{eq,aq}V_{aq}}{(n_i - n_{eq,aq})V_{org}} \quad (17)$$

where $C_{eq,aq}$ and $C_{eq,org}$ were the concentrations of analytes in the aqueous and organic phases respectively, $n_{eq,aq}$ and $n_{eq,org}$ the amounts of analyte at equilibrium in the aqueous and organic phases respectively, V_{aq} and V_{org} the volumes of the aqueous and organic phases respectively, and n_i the amount of analyte originally present in the aqueous phase.

Partition coefficients between the organic phase and the sample (donor phase) $K_{org/d}$ were determined by LLE of 2 ml of the analytes of interest present at 1 μg/ml in 100 m*M* NaOH (simulated LPME donor phase) with 1 ml of dihexyl ether (simulated LPME organic phase) in a 4 ml sample vial. Also in this case, LLE was accomplished for 15 min with vibration at 1500 rpm. Subsequently, 1 ml of the aqueous phase was collected and exposed to LPME according to the procedure described above, and the LPME extract was analyzed by CE to establish the amount of analyte remaining in the original NaOH phase. For calibration, the results were compared with LPME and CE of the analytes of interest present at 50 ng/ml in pure water. $K_{org/d}$ was calculated according to the following equation:

$$K_{org/d} = \frac{C_{eq,org}}{C_{eq,aq}} = \frac{n_{eq,org}V_{org}}{n_{eq,aq}V_{aq}} = \frac{(n_i - n_{eq,aq})V_{org}}{n_{eq,aq}V_{aq}} \quad (18)$$

where the symbols are similar to those used in Eq. (17).

3.9. Determination of immobilized dihexyl ether

The mass of dihexyl ether immobilized within the pores of the fibre was determined by exact weighing of the fibre before and after immobilization. The volume of the immobilized solvent was calculated after adjusting for solvent density.

4. Results and discussion

4.1. Practical considerations

In order to enable comparison between the different extraction methods, all the extractions performed in the present work was based on 1.0 ml of human plasma or urine samples, and the extraction conditions for both LPME and conventional LLE were selected to match typical procedures reported in recent publications. Thus, for two-phase LLE, this included pH adjustment of the biological sample and subsequent dilution with water to a total volume of 2.0 ml. The diluted biological samples were extracted with 5 ml of *tert.*-butyl methyl ether, subsequently the solvent was evaporated to dryness and the extracts were reconstituted in 75 μl of *tert.*-butyl methyl ether. Both the volumes selected for the extraction and for the reconstitution were in accordance with recent procedures published in the literature for robust and reliable drug analysis in biological samples [25–39]. *tert.*-Butyl methyl ether was selected as the extracting solvent because it has similar properties as dihexyl ether used as the preferred LPME solvent and because the volatile nature of the former solvent enabled rapid evaporation. In three phase LLE, 1.0 ml biological sample was pH adjusted and diluted to 2.0 ml (with water), extracted with 5 ml *tert.*-butyl methyl ether, and back extracted with 150 μl of 10 m*M* HCl. Again, the conditions matched typical procedures for back extraction LLE of drugs in biofluids published recently [25–39]. For all LPME experiments, the conditions were as published in recent reports [12–16].

4.2. Two-phase LPME

In Table 1, extraction recoveries and analyte enrichment were calculated by Eqs. (6) and (7). Since the total volume of organic solvent was 45 μl while the volume available for analysis was only 25 μl, the maximum practical extraction recovery was 55.6% with the membrane based LPME concept. This was summarized in the first recovery column of Table 1. In the second column, total recoveries were calculated based on a 45 μl total volume of organic phase. These values may be representative for the different microdrop approaches of LPME where the total volume of organic phase may be available for the final analysis. As illustrated by the calculations, $K_{org/d}$ values as high as 500–1000 were required to obtain almost quantitative extraction in two-phase LPME, whereas the extraction recovery was significantly reduced for analytes with lower $K_{org/d}$.

Table 1
Calculated recovery and enrichment in two-phase LPME and LLE at different $K_{org/d}$-values

$K_{org/d}$	Two-phase LPME[a]			Two-phase LLE[b]	
	Recovery[c] (%)	Total recovery[d] (%)	Enrichment	Recovery (%)	Enrichment
1	0.6	1.1	1.0	71.4	19.0
5	3.0	5.4	4.7	92.5	24.8
10	5.6	10.1	9.0	96.2	25.6
50	20.0	36.0	32.0	99.2	26.4
100	29.4	52.9	47.0	99.6	26.6
500	47.2	85.0	75.5	99.9	26.6
1000	51.0	91.8	81.6	100.0	26.6
5000	54.6	98.3	87.4	100.0	26.6
10 000	55.1	99.2	88.1	100.0	26.6

[a] $V_a = 45$ μl and $V_d = 4$ ml.
[b] $V_a = 5$ ml (evaporation and reconstitution in 75 μl) and $V_d = 2$ ml.
[c] 25 of 45 μl acceptor phase available for analysis.
[d] 45 μl acceptor phase available for analysis.

Nevertheless, because of the small volume of the acceptor phase, two-phase LPME provided very high analyte enrichments from the 4 ml sample volumes even for analytes with $K_{org/d}$ values in the range 50–100.

Extraction recovery and enrichment were also calculated for conventional two-phase LLE. Because of the large volume of solvent used during extraction, LLE provided high recoveries even for compounds with relatively low $K_{org/d}$ values. However, analyte enrichments were relatively low because volumes in the range 75 to 250 μl has to be used in the final step for reliable and quantitative reconstitution. In comparison, two-phase LLE provided moderate enrichment and high recovery for compounds in a broad $K_{org/d}$ range, whereas two-phase LPME provided relatively high recovery and high enrichment for compounds with high $K_{org/d}$ values. The latter type of compounds are typically moderately or highly hydrophobic compounds containing acidic or basic groups, or neutral compounds of similar hydrophobicity.

From an practical point of view, several important conclusions may be derived from the theoretical discussion above; the application range of two-phase LLE is broader than for two-phase LPME, but for compounds amenable to two-phase LPME, this technique may provide substantially higher analyte enrichment and selectivity. The latter effect is caused by the discriminative nature of two-phase LPME; hydrophilic compounds present in the sample are not extracted or poorly extracted into the organic acceptor phase. The major conclusions concerning two-phase LPME and LLE were illustrated in Figs. 2 and 3, where the two basic drugs methadone and promethazine present at the 2.5 μg/ml level were extracted from human urine and plasma by both techniques and subsequently analyzed by GC–FID. For both drugs, the peak height was substantially higher with two-phase LPME as compared to LLE, supporting that higher enrichments are obtained with the former technique. In addition, several matrix components were observed in the chromatograms obtained following LLE, whereas the traces obtained from LPME were almost free of interferences detected by GC–FID. A third aspect, which was not covered by the calculations; whereas LLE required time consuming and cumbersome evaporation of solvent, the LPME extract was directly transferred to the GC system and analyzed without further handling.

4.3. Three-phase LPME

Calculations of extraction recovery and enrichment were accomplished for three-phase LPME as demonstrated in Table 2 utilizing the Eqs. (15) and (16). In the case of three-phase LPME, the total 25 μl volume of acceptor phase was available for the final analysis and consequently, Eq. (15) was utilized

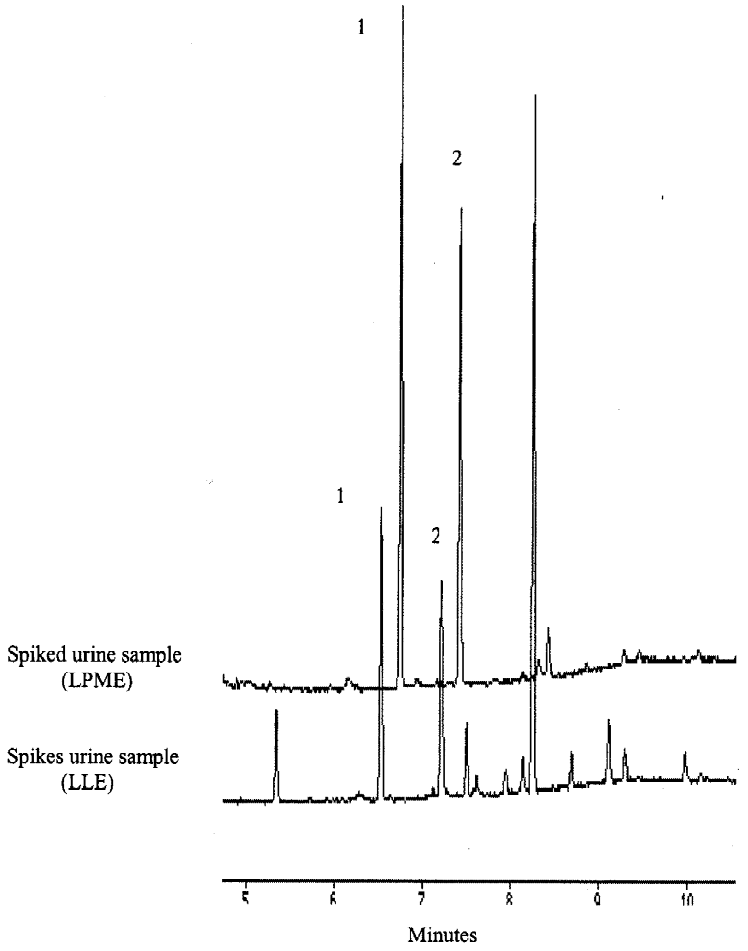

Fig. 2. Two-phase LPME (upper chromatogram) and two-phase LLE (lower chromatogram) combined with GC–FID of 2.5 μg/ml of methadone (peak 1) and promethazine (peak 2) in humane urine.

directly for the calculation of recoveries which theoretically may range up to 100%. As illustrated in Table 2, relatively high $K_{a/d}$ values were required in order to obtain high extraction recoveries in three-phase LPME. For a 80 to 90% recovery, $K_{a/d}$ values above 500–1000 were required. With three-phase LPME, the extraction recoveries were dependent on both $K_{org/d}$ and $K_{a/org}$ of which the product was $K_{a/d}$; in general a $K_{a/org}$-value above 10 was required to effectively transfer the analytes to the acceptor phase resulting in a high recovery. In cases where the $K_{a/org}$-value was below 10, substantial amounts of the analyte remained in the organic phase resulting in reduced extraction recovery. In addition to recoveries, analyte enrichments were calculated in Table 2. For analytes with $K_{a/d}$ values above 100, very high enrichments were obtained from the 4 ml samples. Analytes with high $K_{a/d}$ values are typically moderately or highly hydrophobic compounds containing acidic or basic functionalities; neutral compounds are not or very poorly extracted into the acceptor phase in three-phase LPME.

Two- and three-phase LPME were comparable by the fact that both techniques provided high recovery only for compounds with high overall partition coefficients ($K_{a/d}$ and $K_{org/d}$ respectively), whereas extraction was inefficient or suppressed for compounds with lower partition coefficients. With three-

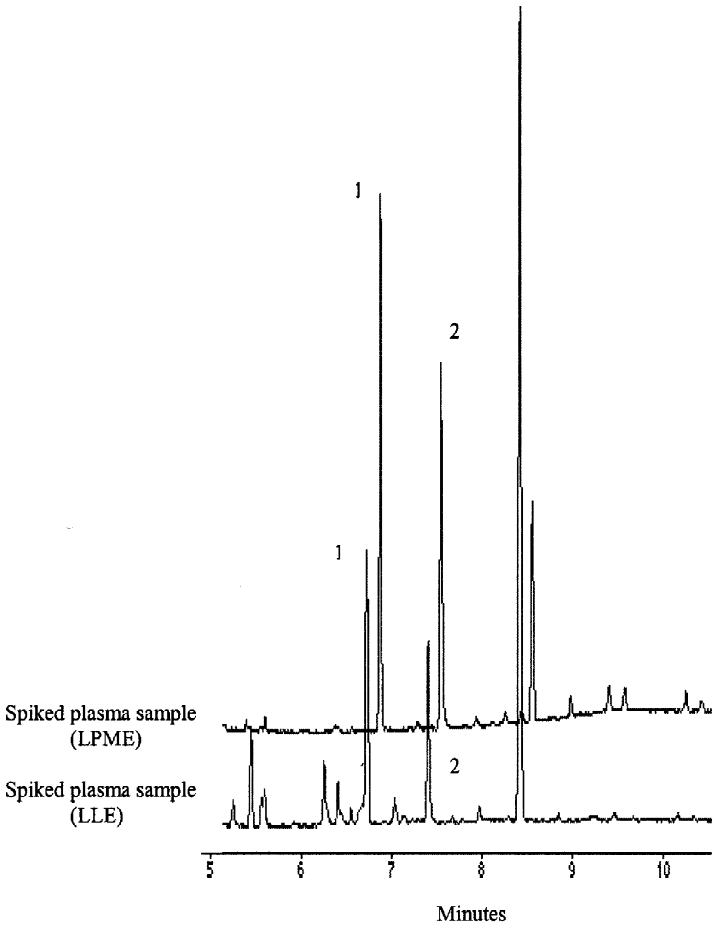

Fig. 3. Two-phase LPME (upper chromatogram) and two-phase LLE (lower chromatogram) combined with GC–FID of 2.5 μg/ml of methadone (peak 1) and promethazine (peak 2) in humane plasma.

phase LPME, enrichments were higher than with hollow fibre based LPME performed in the two-phase mode because the volume of the acceptor phase was smaller in the former technique. In addition, three-phase LPME provided higher selectivity because neutral compounds were not extracted with this technique.

Compared with two-phase LLE (see Table 1 and 2), also three-phase LPME was applicable to a more limited group of compounds with relatively high $K_{a/d}$ values, but three-phase LPME provided substantially higher enrichments and higher selectivity. In addition to two-phase LLE, LLE is frequently accomplished with back extraction into a new aqueous phase. Calculations of recovery and enrichment for this type of three-phase LLE were included in Table 2, where extraction was simulated from 2 ml of aqueous sample and into 5 ml of organic solvent in the first step. In the second step, back extraction was accomplished into 150 μl of a new aqueous phase. Because of the large volume of organic solvent used in the first step, the analytes were effectively extracted into the organic phase. However, because of the volume difference between the organic solvent and the receiving aqueous phase, high extraction recoveries were only obtained in cases where the partition coefficient between the receiving phase and the organic phase ($K_{a/org}$) was high; in all other cases substantial amounts of the analyte remained within the organic solvent. As seen from Table 2, three-phase LPME generally provided much higher recoveries and enrichments than three-phase LLE. For

Table 2
Calculated recovery and enrichment in three-phase LPME and LLE at different $K_{a/d}$-values

$K_{a/d}$	$K_{org/d}$	$K_{a/org}$	Three-phase LPME[a]		Three-phase LLE[b]	
			Recovery (%)	Enrichment	Recovery (%)	Enrichment
1	1	1	0.6	1.0	1.1	0.3
5	1	5	3.0	4.8	5.1	1.4
	5	1	3.0	4.8	1.7	0.4
10	1	10	5.9	9.4	9.7	2.6
	5	2	5.7	9.1	2.7	0.7
	10	1	5.6	9.0	1.4	0.4
50	1	50	23.7	37.9	34.9	9.3
	5	10	23.4	37.4	6.7	1.8
	10	5	22.9	36.6	6.7	1.8
	50	1	20.0	32.0	1.5	0.4
100	1	100	38.3	61.3	51.7	13.9
	5	20	37.9	60.6	21.7	5.9
	10	10	37.3	59.7	12.6	3.5
	50	2	33.3	53.3	2.9	0.8
	100	1	29.4	47.0	1.5	0.4
500	1	500	75.7	121.1	84.3	22.4
	5	100	75.3	120.5	58.1	15.5
	10	50	74.9	119.8	41.9	11.2
	50	10	71.4	114.2	13.0	3.5
	100	5	67.6	108.2	7.0	1.9
	500	1	47.2	75.5	1.5	0.4
1000	1	1000	86.1	137.8	91.5	24.5
	5	200	85.9	137.4	73.5	19.7
	10	100	85.6	137.0	59.1	15.7
	50	20	83.3	133.3	22.9	6.1
	100	10	80.6	129.0	13.0	3.5
	500	2	64.1	102.6	2.9	0.8
	1000	1	51.0	81.6	1.5	0.4

[a] $V_a = 25$ μl, $V_{org} = 20$ μl and $V_d = 4$ ml.
[b] $V_a = 150$ μl, $V_{org} = 5$ ml and $V_d = 2$ ml.

both techniques, $K_{a/d}$-values above 500 were required for almost quantitative extraction. Thus, both techniques are applicable only for moderately or highly hydrophobic analytes with basic or acidic groups. Three-phase LLE was very sensitive towards the magnitude of $K_{a/org}$, whereas this was not the case of three-phase LPME. This aspect further limit the applicability of three-phase LLE. From Table 2 it may also be concluded that both techniques provided a high selectivity since more hydrophilic compounds remained in the sample solution. In three-phase LLE, this was principally caused by the small volume of the receiving phase relative to the organic phase, while three-phase LPME discriminated hydrophilic compounds principally because of the limited volume of the organic phase.

The aspects of three-phase LPME and three-phase LLE were verified by Figs. 4 and 5, where the three basic drugs methadone, promethazine, and haloperidol present at the 100 ng/ml level were extracted from human urine and plasma by both techniques and subsequently analyzed by CE–UV. For all the drugs, the peak height was substantially higher with three-phase LPME as compared to LLE. This was in agreement with the three-phase calculations of Table 2; LPME provided superior enrichment as compared with LLE. With both extraction techniques, only a few other peaks emerged in the electropherograms supporting the high selectivity. A third aspect, which was not covered by the calculations; whereas LLE required extraction in two steps, the LPME was accomplished in a single operation.

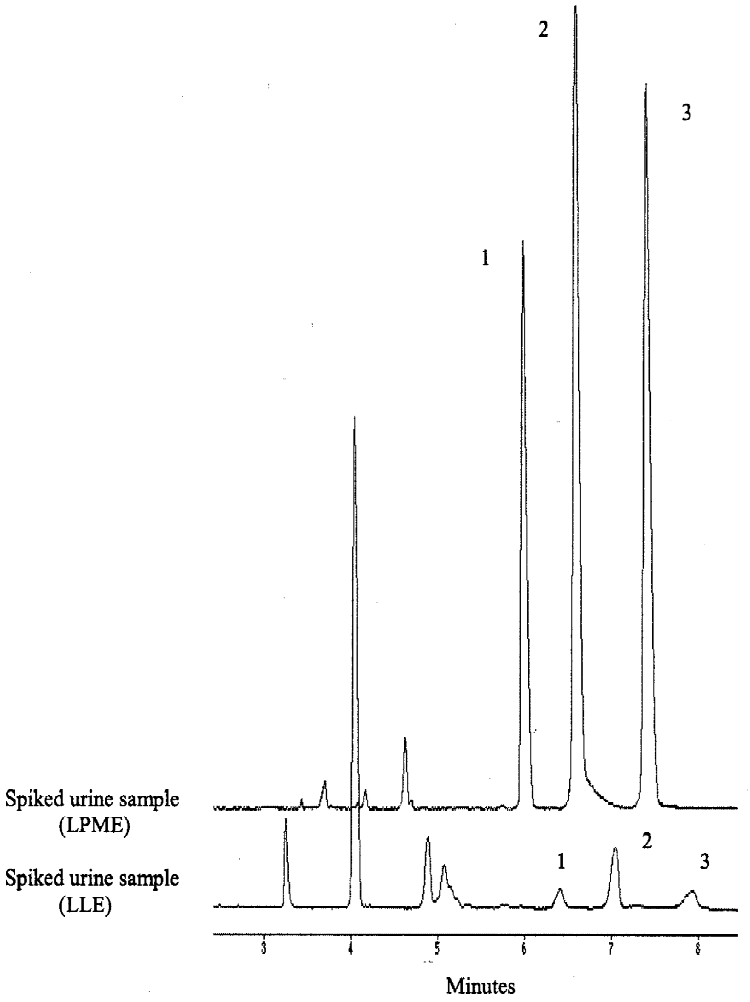

Fig. 4. Three-phase LPME (upper electropherogram) and three-phase LLE (lower electropherogram) combined with CE–UV of 100 ng/ml of promethazine (peak 1), methadone (peak 2) and haloperidol (peak 3) in humane urine.

4.4. Determination of partition coefficients and verification of experimental results

Based on the models discussed above, actual partition coefficients were determined according to the procedure described under experimental, the experimental values were used to calculate theoretical recoveries, and these were compared with experimental values obtained by three-phase LPME. As illustrated in Table 3, the $K_{a/d}$ values were high for all the three basic drugs and the individual partition coefficients ($K_{org/d}$ and $K_{a/org}$) promoted efficient simultaneous extraction from the sample through the organic phase and further into the acceptor phase. Based on the individual partition coefficients, theoretical recoveries were calculated and these were in acceptable agreement with the values determined experimentally by three-phase LPME of the three drugs dissolved at the 100 ng/ml level in pure water. Based on Eqs. (2)–(7), it was found that more than 99.97% of the analytes not extracted into the acceptor phase (1.4 to 7.5% in total) was trapped within the organic phase after equilibrium, whereas the amount of analyte remaining in the donor phase after LPME was insignificant. The procedure for estimating the individual partition coefficients were rela-

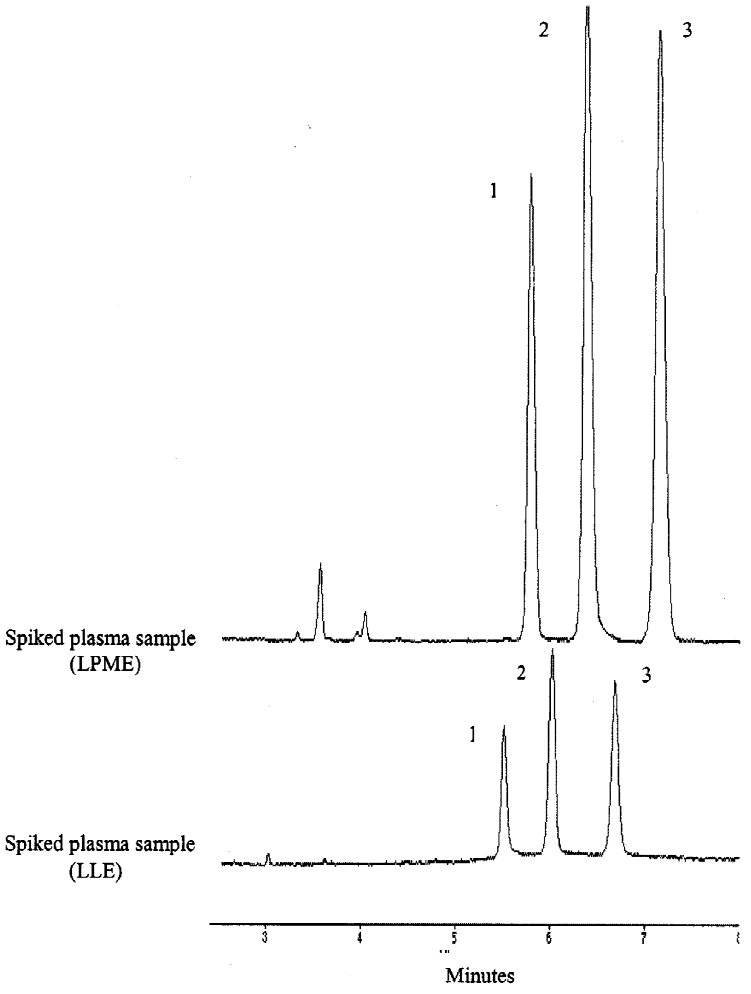

Fig. 5. Three-phase LPME (upper electropherogram) and three-phase LLE (lower electropherogram) combined with CE–UV of 100 ng/ml of promethazine (peak 1), methadone (peak 2) and haloperidol (peak 3) in humane plasma.

Table 3
Experimental partition coefficients, theoretical recovery, and experimental recovery of methadone, promethazine, and haloperidol for three-phase LPME

Compound	Partition coefficients Experimental			Recovery (%)	
				Theoretical[a]	Experimental[a]
	$K_{org/d}$	$K_{a/org}$	$K_{a/d}$		
Promethazine	27	81	2187 (RSD=4.8%)*	92.5	86.8 (RSD=6.7%)*
Methadone	141	82	11 562 (RSD=6.8%)*	98.6	92.5 (RSD=5.9%)*
Haloperidol	35	75	2625 (RSD=8.3%)*	96.3	97.2 (RSD=7.3%)*

* Three replicates.
[a] V_a=25 μl, V_{org}=20 μl and V_d=4 ml.

tively simple and may be used in future studies of LPME.

4.5. Practical considerations in LPME optimization

Basically, two-phase LPME may be utilized in combination with GC because the organic acceptor phase may be directly compatible with GC systems, whereas three-phase LPME is preferred in combination with HPLC and CE because the acceptor phases are aqueous. In addition to these considerations, the nature of the analytes have to be considered prior to LPME. For successful two-phase LPME, high $K_{org/d}$ are required. As discussed above, compounds with high $K_{org/d}$ are relatively hydrophobic acidic or basic structures or neutral components of high hydrophobicity. Acidic or basic compounds with relatively low $K_{org/d}$ may be successfully extracted in three-phase LPME provided that their $K_{a/org}$ value is high resulting in a high overall partition coefficient ($K_{a/d}$). Thus, for chargeable compounds, the applicability range may be expanded changing from two- to three-phase LPME.

For method optimization, selection of the organic phase is of high importance both in two-phase LPME and three-phase LPME. In two-phase LPME, the solvent should be selected in order to maximize the $K_{org/d}$ partition coefficient; thus a very good solvent for the analyte immiscible with water should be selected. For three-phase LPME in contrast, the organic solvent should be selected to ensure both high $K_{org/d}$ and $K_{a/org}$ values; especially the latter is of high importance in order to avoid analyte trapping in the organic phase and consequently reduced analyte recovery.

In both LPME and LLE, inaccuracies may occur owing to variations in the measured volumes of extracting solvent or acceptor phase. In LPME based on the hollow fibre formate, an additional source of variation may be the immobilization of organic solvent within the pores of the hollow fibre. Since the length and the wall thickness may vary slightly from fibre to fibre, fluctuations in the volume of organic solvent may be experienced. In Table 4, the impact on analyte enrichment was calculated both for two- and three-phase LPME simulating 25% variations in the volume of immobilized organic solvent. In the two-phase system, substantial variations in analyte enrichment was observed as the volume of organic solvent immobilized in the pores decreased from 20 to 15 μl. This effect was most significant for analytes with high $K_{org/d}$ values. In the three-phase system in contrast, a similar reduction of the volume of the organic phase resulted in almost no change in the analyte enrichment. Thus, three-phase LPME is relatively insensitive to small variations in the fibre thickness and length. Nevertheless, in both two- and three-phase LPME, addition of an internal standard

Table 4
Sensitivity towards fluctuations in the volume of immobilized organic solvent

Two-phase LPME			Three-phase LPME				
$K_{org/d}$	V_{org}	Enrichment	$K_{a/d}$	$K_{org/d}$	$K_{a/org}$	V_{org}	Enrichment
100	15	**50**	100	5	20	15	**61**
100	20	**47**	100	5	20	20	**61**
			100	20	5	15	**61**
			100	20	5	20	**61**
500	15	**83**	500	5	100	15	**121**
	20	**75**	500	500	100	20	**120**
			500	100	5	15	**111**
			500	100	5	20	**108**
1000	15	**91**	1000	10	100	15	**138**
	20	**82**	1000	1000	100	20	**137**
			1000	100	10	15	**131**
			1000	100	10	20	**129**

is recommended to compensate for several other experimental parameters which may vary from sample to sample.

5. Conclusion

The present work has clarified fundamental differences between LPME and LLE. Although the principles are equal, the large phase-ratio differences of LPME results in several aspects of high importance for successful implementation of the technique. Basically, the range of compounds amenable to the technique is limited as compared with traditional LLE. Because of the small volumes of organic solvent used in both two- and three-phase LPME, the techniques are basically limited to moderately or highly hydrophobic analytes. For these compounds, LPME provides higher analyte enrichment and superior selectivity as compared to LLE. With the inherent advantages and limitations of LPME in mind, different formates will hopefully be developed into attractive alternatives for sample preparation within the near future.

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