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Orthogonal array designs for the optimization of liquid–liquid–liquid microextraction of nonsteroidal anti-inflammatory drugs combined with high-performance liquid chromatography-ultraviolet detection

Jingming Wu, Hian Kee Lee *

Department of Chemistry, National University of Singapore, 3 Science Drive 3, Singapore 117543, Singapore

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Abstract

Orthogonal array designs (OADs) were applied for the first time to optimize liquid–liquid–liquid microextraction (LLLME) conditions for the analysis of three nonsteroidal anti-inflammatory drug residues (2-(4-chlorophenoxy)-2-methylpropionic acid, ketoprofen, and naproxen) in wastewater samples. Six relevant factors were investigated: type of organic solvent, composition of donor phase and acceptor phase, stirring speed, extraction time and salt concentration. In the first stage, mixed-level orthogonal array design, an OA_{16} ($4^1 \times 2^{12}$) matrix was employed to study the effect of six factors, by which the effect of each factor was estimated using individual contributions as response functions. Based on the results of the first stage, 1-octanol was chosen as organic solvent for extraction. The other five factors were selected for further optimization using an OA_{16} (4^5) matrix and a 4 × 4 table to locate more exact levels for each variable. The relative standard deviations for the reproducibility of optimized LLLME varied from 6.2 to 7.1%. The coefficients of determination for calibration curves were higher than 0.9950. The method detection limits for drugs spiked in ultrapure water were in the range of 0.03–0.3 ng/mL. The final optimized conditions were applied to the analysis of drug residues in three wastewater samples in Singapore.
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1. Introduction

Drug residues have become significant contaminants in the aquatic environment in recent years. Nonsteroidal anti-inflammatory drugs (NSAIDs) are amongst the group of pharmaceutical compounds most often used in human health care. The excretion of drugs and their metabolites together with improper waste disposal have led to their presence in wastewater [1–5]. Furthermore, a number of studies have shown that NSAIDs, acidic pharmaceutical compounds are not even eliminated in sewage treatment plants because of their high stability. Thus, they can ultimately reach surface and ground waters [5–7].

Gas chromatography–mass spectrometry (GC–MS) has been successful employed in the analysis of NSAIDs in water samples [1–2,4,6,8,9]. However, derivatization is required which makes analysis tedious. Recently, there have been reports of pharmaceutical residue analysis based on capillary electrophoresis (CE) [7,10,11] because of the rapid analytical time, low running cost and environmental benefit (aqueous-based system, small quantities of reagents, etc.) associated with this technique. However, the high detection limits (ng/mL, even μg/mL) limit the application of CE to real sample analysis. Reversed-phase high-performance liquid chromatography (RP-HPLC) is a popular analytical

* Corresponding author. Tel.: +65 6874 2995; fax: +65 6779 1691.
E-mail address: chemleehk@nus.edu.sg (H.K. Lee).

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method to determine drugs in aqueous samples in recent years [3,5,7,12–18]. In order to detect low levels of the analytes, a preconcentration step is needed in general prior to instrumental determination.

In the past, solid-phase extraction (SPE) has been the most popular sample pretreatment procedure for drug residues [2,5,7]. However, SPE requires a moderate amount of organic solvent and is tedious, unless it is automated, which makes it very expensive. Solid phase microextraction (SPME) based on a partition equilibrium of the analytes in an aqueous sample and a polymer coating on a fused-silica fiber has been successfully used for extracting drug residues [1,4,6]. This simple and solventless extraction technique has proved to be a powerful alternative to traditional extraction techniques. However, SPME fibers are fragile and relatively expensive. They tend to degrade with multiple usage.

Liquid-phase microextraction (LPME) [19–22] is an emerging technique that is based on the use of a small amount of organic solvent to extract analytes from moderate amounts of aqueous matrices. It has been shown to be a viable alternative sample preparation method to conventional extraction techniques. Liquid–liquid–liquid microextraction (LLLME) in which analytes are firstly extracted in an organic phase, and subsequently back-extracted into a second aqueous phase is one type of LPME. In this procedure, a porous-walled polypropylene hollow fiber is used to support the organic phase (held by the wall) and the second aqueous phase (held within the channel of the membrane) [23–25]. After extraction, the acceptor phase is introduced into a HPLC or CE without further treatment.

In this work, orthogonal array experimental designs (OADs) were employed for the first time to optimize LLLME conditions for the extraction of NSAIDs. The theory and methodology of OAD as a chemometric method for the optimization of analytical procedures have been described in detail elsewhere [26–29]. OAD has proved to be a cost-effective optimization strategy that can be used to assign experimental factors in a series of experimental trials. Analysis of variance (ANOVA) is employed for estimating the main significant factors and two-way interaction factors after the OAD procedure has been conducted [26–36]. In the present work, mixed-level OAD procedure with OA_{16} $(4^1 \times 2^{12})$ matrix was applied to study the effect of six factors influencing LLLME efficiency: type of organic solvents, concentrations of donor phase and acceptor phase, stirring speed, extraction time and ionic strength of the sample solution, by which the effect of each factor was estimated using individual contributions as response functions in the first optimization step. Based on the results of this first stage, 1-octanol was chosen as extraction organic solvent. Then, the other five factors were selected for further optimization by using an OA_{16} (4^5) matrix to locate more exact levels for each variable. In addition, the interactions of concentrations of the donor phase and the acceptor phase were also evaluated. The optimized conditions were then applied to the analysis of NSAIDs in wastewater samples.

2. Experimental

2.1. Standards and reagents

The Accurel Q3/2 polypropylene hollow fiber membrane (600-μm I.D., 200-μm wall thickness, 0.2-μm pore size) was bought from Membrana GmbH (Wuppertal, Germany). Naproxen (NAP) and Ketoprofen (KEP) were provided by Sigma (St. Louis, MO, USA) and 2-(4-chlorophenoxy)-2-methylpropionic acid (CMPA) was purchased from Aldrich (Milwaukee, WI, USA). NaOH and 1-octanol were from Merck (Darmstadt, Germany). Ammonium acetate was purchased from Ajax (Sydney, Australia). HCl and ethyl acetate were obtained from J.T. Baker (Philipsburg, NJ, USA). Toluene, hexane and HPLC-grade methanol were supplied by Fisher (Loughborough, UK). Acetic acid was purchased from Fluka (Buchs, Switzerland). Sodium chloride was obtained from GCE (Chula Vista, CA, USA). Ultrapure water was produced on a Nanopure water-purification system (Barnstead, Dubuque, IA, USA). Individual stock solutions of pure drug standards were dissolved separately in methanol at 1 mg/mL and stored at 4 °C. Working solutions containing the three drugs at different concentrations were prepared by spiking them into ultrapure water every day during the optimization procedure. The concentrations of analytes were 50 ng/mL for the optimization study.

The water samples were collected from a domestic home drain, hospital drain and a local river. Samples were stored at 4 °C after collection. They were filtered through a 0.45-μm membrane filter (Millipore, Billerica, MA, USA) prior to extraction.

2.2. Instrumentation

Analysis was carried out on a Waters (Milford, MA, USA) HPLC system equipped with a UV detector, with detection at a wavelength of 240 nm. The chromatographic system consisted of a Rheodyne (Cotati, CA, USA) 77251 injector equipped with a 20-μL sample loop, a Waters 1525EF binary pump, and a Waters 2487 UV–visible spectrophotometric detector. Data was collected and processed by Empower version 5.0 (Waters) data analysis software.

A column (250 mm × 2 mm I.D.) from Phenomenex (Torrance, CA, USA) packed with BuckySep-RP was used. Methanol–100 mM ammonium acetate (70:30, v/v; pH 5) was used as mobile phase. The flow rate was set at 0.1 mL/min. The column temperature was maintained at 22 °C.

2.3. LLLME procedure

Extractions were carried out according to the following procedure: (1) A 10-mL sample solution was added to the sample vial with a 15 mm × 6 mm magnetic stirring bar; (2) The sample vial was placed on a MR3001K hotplate stirrer (Heidolph, Kelheim, Germany); (3) 5-μL of acceptor phase was withdrawn into a 10-μL microsyringe with a flat needle

Table 1
Assignment of factors and level settings of the experiment runs in the OA_{16} ($4^1 \times 2^{12}$) matrix

Level	Column no.												
	1	2	3	4	5	6	7	8	9	10	11	12	13
	A	*B*	$(A \times B)_1$	$(A \times B)_2$	$(A \times B)_3$	*C*	$(A \times C)_1$	$(A \times C)_2$	$(A \times C)_3$	$B \times C$	*D*	*E*	*F*
1	1-Octanol	0.5				0.5					21	5	0
2	Toluene	0.01				0.01					104	40	200
3	Hexane												
4	Ethyl acetate												

A = Different types of extracting solvent; *B* = HCl concentration (M) (donor phase); *C* = NaOH concentration (M) (acceptor phase); *D* = stirring speed (rad/s); *E* = extraction time (min); *F* = salt concentration (g/L) (ionic strength); $B \times C$ = interactions between HCl concentration and NaOH concentration.

tip (SGE, Sydney, Australia); (4) The syringe needle was then inserted into the clean and dry hollow fiber (2.4-cm length) that was heat-sealed at the other end, and the acceptor solution was introduced into it; (5) The fiber was immersed in organic solvent for 5 s for impregnation; (6) The fiber together with the syringe needle was placed in the donor phase; (7) A piece of aluminum foil (Diamond, Richmond, Virginia, USA) was used to cover the sample vial in order to prevent or reduce evaporation of the organic solvent; (8) After extraction, the hollow fiber and syringe needle was removed from the sample solution, and the extract was withdrawn into the syringe. The hollow fiber was discarded; (9) The extract (5-μL) was injected directly into the HPLC. A fresh hollow fiber was used for each extraction.

2.4. Optimization strategy

In the first optimization stage, six variables were selected for optimization of LLLME. These were: (1) different types of extracting solvent (factor *A*); (2) concentration of donor phase HCl (factor *B*); (3) concentration of acceptor phase NaOH (factor *C*); (4) agitation speed during extraction (factor *D*); (5) duration of extraction (factor *E*); (6) ionic strength of the sample solution (factor *F*). The level setting values of the main variables (*A*, *B*, *C*, *D*, *E* and *F*) used in the mixed-level OAD are shown in Table 1. The OA_{16} ($4^1 \times 2^{12}$) matrix was employed to assign the variables considered because one four-level and five two-level variables had to be considered. According to a previous report [25], the two-variable interactions between HCl concentration (factor *B*) and NaOH concentration (factor *C*) should be taken into account. The assignment of the main-variable and two-variable interactions and their levels has been previously described in detail [29]. The average enrichment factor (defined as the ratio of the equilibrium concentration of each analyte in the acceptor phase and the initial concentration in the donor phase) was calculated from the sum of enrichment factors of three NSAIDs and used as a response function. The results of the OAD experiment were then processed under direct observation analysis [37–39]. Based on the results shown in

Table 2
OA_{16} ($4^1 \times 2^{12}$) matrix with experimental results

	Column no.													Response			
	1	2	3	4	5	6	7	8	9	10	11	12	13	CMPA	KEP	NAP	Sum
1	1	1	1	1	1	1	1	1	1	1	1	1	1	58	28	33	119
2	1	1	1	1	1	2	2	2	2	2	2	2	2	146	174	163	483
3	1	2	2	2	2	1	1	1	1	2	2	2	2	978	686	707	2371
4	1	2	2	2	2	2	2	2	2	1	1	1	1	20	36	30	86
5	2	1	1	2	2	1	1	2	2	1	1	2	2	299	125	184	608
6	2	1	1	2	2	2	2	1	1	2	2	1	1	4	30	53	87
7	2	2	2	1	1	1	1	2	2	2	2	1	1	6	10	26	42
8	2	2	2	1	1	2	2	1	1	1	1	2	2	490	463	558	1511
9	3	1	2	1	2	1	2	1	2	1	2	1	2	7	0.3	0.3	7.6
10	3	1	2	1	2	2	1	2	1	2	1	2	1	3	0.6	1.2	4.8
11	3	2	1	2	1	1	2	1	2	2	1	2	1	7	1	0.1	8.1
12	3	2	1	2	1	2	1	2	1	1	2	1	2	3	0.2	0.1	3.3
13	4	1	2	2	1	1	2	2	1	1	2	2	1	0	0	0	0
14	4	1	2	2	1	2	1	1	2	2	1	1	2	0	0	0	0
15	4	2	1	1	2	1	2	2	1	2	1	1	2	0	0	0	0
16	4	2	1	1	2	2	1	1	2	1	2	2	1	0	0	0	0
r_1	765	164				394				292	292	43	43				
r_2	562	503				272				374	374	623	623				
r_3	6																
r_4	0																
d	765	339				122				82	82	580	580				

Table 3
Assignment of factors and their level values in the OA_{16} (4^5) matrix

Levels	Factors				
	A (C_{NaOH} (M))	*B* (C_{HCl} (M))	*C* (Stirring speed (rad/s))	*D* (Extraction time (min))	*E* (Salt (g/L))
1	1.0	0.005	130	30	150
2	0.5	0.01	104	40	200
3	0.1	0.05	73	50	250
4	0.05	0.1	52	60	300

Table 2, the extraction organic solvent was identified and the other factors were determined to be deserving of further attention.

In the next stage, the optimum levels of three experimental factors were determined according to a four-level OA_{16} (4^5) matrix. These were: stirring speed (factor *C*), extraction time (factor *D*) and ionic strength of solution (salt concentration) (factor *E*). The concentrations of acceptor phase NaOH (factor *A*) and donor phase HCl (factor *B*) were not identified because of their interactions in the initial experiment. More exact levels were selected around the superiority levels obtained from the initial examination. Table 3 illustrates the assignments of the experimental factors (*A*, *B*, *C*, *D*, and *E*) and levels (1–4) for the 16 experimental trials. At this step, interactions among variables were not incorporated in the matrix and focus was placed on the main effects of the five factors. The ANOVA technique was employed where both the purified sum of squares, SS′, and percentage contribution, PC (%), valued for each factor could be calculated.

On the basis of the results above, the optimum values of factors except for concentrations of HCl and NaOH were located, as demonstrated in Tables 4 and 5. In the following step, the interactions between the concentrations of HCl and NaOH were investigated. The experimental design and results are displayed in a 4 × 4 table (Table 6).

3. Results and discussion

3.1. Initial experiments using mixed-level OA_{16} ($4^1 \times 2^{12}$) matrix

The corresponding enrichment factors used as responses for each experimental trial were calculated and are tabulated in Table 2 after accomplishing 16 experimental trials predesigned, according to the OA_{16} ($4^1 \times 2^{12}$) matrix. The average of responses (r_1, r_2, r_3 and r_4) for each factor at different levels were also calculated and are given in Table 2. Direct observation analysis was statistically employed to estimate the importance of a given factor and their interactions. The mean value difference (*d*) between two levels of each factor except for extraction organic solvent was used for evaluating the importance of the factors. For a factor with four levels (extraction organic solvent), the mean value difference is the range between the maximum and the minimum values. The mean value difference (*d*) is related to the factors involved as well as the level settings [33]. From Table 2, it

Table 4
Assignment of factors and levels of the optimization experiments using an OA_{16} (4^5) matrix along with the enrichment factors

Trial no.	Factor					Enrichment factors			
	A	*B*	*C*	*D*	*E*	1	2	3	Sum
1	1	1	1	1	1	1095	1208	977	3280
2	1	2	2	2	2	2238	2349	1781	6368
3	1	3	3	3	3	2568	2033	2122	6723
4	1	4	4	4	4	2012	1723	2075	5810
5	2	1	2	3	4	2985	3110	2710	8805
6	2	2	1	4	3	3720	3119	3738	10577
7	2	3	4	1	2	1996	1519	1702	5217
8	2	4	3	2	1	2416	2121	2455	6992
9	3	1	3	4	2	4637	4855	4885	14377
10	3	2	4	3	1	3883	4605	4243	12731
11	3	3	1	2	4	3485	3478	2797	9760
12	3	4	2	1	3	1737	2148	1858	5743
13	4	1	4	2	3	1961	1866	1913	5740
14	4	2	3	1	4	1844	1962	1903	5709
15	4	3	2	4	1	3804	3188	3783	10775
16	4	4	1	3	2	2470	2395	2479	7344
r_1	5545	8051	7740	4987	8445				
r_2	7898	8846	7923	7215	8327				
r_3	10653	8119	8450	8901	7196				
r_4	7392	6472	7375	10385	7521				

Table 5
An ANOVA table for experimental responses in the OA_{16} (4^5) matrix

Source	SS	d.f.	MS	F[a]	SS′	PC (%)
NaOH concentration (A)	1.785×10^7	3	5.95×10^6	88.94***	1.765×10^7	37.13
HCl concentration (B)	0.401×10^7	3	1.37×10^6	20.48***	0.381×10^7	8.01
Stirring speed (C)	0.08×10^7	3	0.27×10^6	4.04	0.0599×10^7	1.26
Extraction time (D)	2.152×10^7	3	7.17×10^6	107.17***	2.132×10^7	44.85
Salt concentration (E)	0.122×10^7	3	0.41×10^6	6.13	0.102×10^7	2.15
Error	0.214×10^7	32	6.69×10^4		0.314×10^7	6.60
Total	4.754×10^7	47			4.754×10^7	100.00

SS = sum of squares; d.f. = degrees of freedom; MS = mean squares; SS′ = purified sum of squares; PC = percentage contribution.
[a] Critical value is 6.96 (*** $P < 0.001$) and 2.27 ($P < 0.1$).

is obvious that the most significant factor was the type of organic solvent. The next most significant factors were the extraction time and salt concentration. The concentration of the donor phase (HCl) and the concentration of the acceptor phase (NaOH) also have important influence on LLLME efficiency. Agitation speed has no significant influence on extraction compared with the other factors. It seems plausible that there are interactions between the concentrations of the donor phase and the acceptor phase. The interaction experiments were designed and the results are discussed below.

Superiority (level at which the best experiment result is obtained) and inferiority (level at which the worst experiment result is obtained) levels of the six factors were evaluated by comparing the mean effect of these factors at different levels. Table 2 gives the mean effect of these factors and possible two-variable interaction at different levels. It is clear that 1-octanol as extraction organic solvent will give the best extraction result. It is indicated that a low concentration of HCl (0.01 M), a high concentration NaOH (0.5 M), and high stirring speed (104 rad/s) will increase extraction efficiency. In addition to this, a longer extraction time (40 min) and the addition of salt (sodium chloride: 200 g/L) (ionic strength) will also improve the extraction.

The type of organic solvent immobilized in the pores of the hollow fiber in LLLME is very important in order to reach satisfactory analyte enrichment factors [3,19,25]. In general, the organic solvent selected should be compatible with the fiber so as to be able to fill the pores on the wall of the fiber effectively, and to represent a suitable medium for extraction. The nonmiscibility with water should also be considered. Additionally, the higher solubility of analytes in the acceptor phase than in the organic solvent is critical as well as the higher solubility of analytes in the organic solvent than in the donor phase [3,19]. Otherwise, the analytes cannot be extracted into the acceptor phase from the donor phase. Based on this consideration, 1-octanol, toluene, hexane and ethyl acetate were studied for their effect on extraction. It is clear that both hexane and ethyl acetate provided poor extraction (Table 2). Almost no analytes were extracted when ethyl acetate was employed. Toluene showed better extraction results compared with hexane and ethyl acetate. As can be seen, 1-octanol demonstrated the highest enrichment factors among the four organic solvents. The possible reason is its greater affinity for the acidic NSAIDs resulting from its relatively higher polarity and hydrogen-bonding ability. Thus, 1-octanol was chosen as the extraction organic solvent in subsequent experiments.

The concentrations of the donor phase and the acceptor phase are of great importance in LLLME. The pH value of the donor phase (HCl) should be lower than the pK_a's of

Table 6
The effect of concentrations of the donor phase and the acceptor phase on LLLME enrichment factors

Concentration of NaOH	Compound	Concentration of HCl			
		B_1 (0.005 M)	B_2 (0.01 M)	B_3 (0.05 M)	B_4 (0.10 M)
A_1 (1.00 M)	CMPA	1143	1152	1381	1090
	KEP	1026	1007	1183	1036
	NAP	1008	970	1084	916
A_2 (0.50 M)	CMPA	1258	1244	1428	1219
	KEP	1240	1192	1428	1115
	NAP	1221	1159	1423	1108
A_3 (0.10 M)	CMPA	1576	1649	1501	1545
	KEP	1681	1857	1643	1607
	NAP	1748	1904	1724	1604
A_4 (0.05 M)	CMPA	1583	1520	1162	1189
	KEP	1792	1738	1248	814
	NAP	1891	1763	1226	602

the acidic analytes so that analytes are completely deionized and therefore exist as neutral molecules. In experiments with 0.01 M HCl, the pH was lower than the pK_a's of CMPA (3.10), KEP (4.45), and NAP (4.15) and relatively good extraction efficiency was achieved. However, when a lower pH was employed (HCl at 0.5 M), relatively poor extraction was observed. The reason for this is unclear at this juncture. It is possible that ionized species were formed as the target acidic drugs accepted an extra proton at low pH, thus reducing the distribution ratios (referring to the ratios of the concentration of all species of each analyte in organic solvent and those in the donor phase), although the highly acidic donor phase would increase the extraction efficiency of 1-octanol. For analytes, this ionizing effect of a highly acidic donor phase may exceed that of higher extraction capability of 1-octanol, therefore possibly decreasing the extraction efficiency. Relatively high enrichment factors were reached with a high concentration of NaOH (0.5 M) compared with a low concentration (0.01 M). Based on the above discussion, 0.01 M HCl and 0.5 M NaOH were selected as reference for the discrete level assignments in the further optimization procedure.

Agitation speed plays an important pole in LLLME. To improve the extraction efficiency, agitation permits the continuous exposure of extraction solvent to fresh aqueous sample [40]. As seen from Table 2, higher enrichment factors were obtained when high stirring speed (104 rad/s) was used compared to a lower speed (21 rad/s).

Extraction time is another important factor for consideration. In general, the amount of analytes extracted increased significantly with increasing exposure time. It was also true in this work. As shown in Table 2, the average sum of enrichment factors for three target drugs was 623 with an extraction time of 40 min and 43 with an extraction time of 5 min. Subsequent experiments were carried out with several extraction times around 40 min to investigate their effects on extraction efficiency.

As reported before [40], salt added to the donor phase in LPME improved extraction efficiency in most cases. It was also true in the present work. The average sum of enrichment factors for three analytes was 643 when sodium chloride concentration (ionic strength) was 200 g/L (Table 2). When no salt was added, an enrichment factor of only 43 was obtained. On the basis of the observations above, sodium chloride concentration close to 200 g/L were selected for level settings in the subsequent optimization approach.

3.2. Experiments using OA_{16} (4^5)

The results of the experiments designed using OA_{16} (4^5) matrix are shown in Tables 4 and 5. In these experiments, 1-octanol was used as extraction solvent. The effects of five important factors (concentration of HCl, concentration of NaOH, stirring speed, salt concentration added and the duration of extraction) on response functions were studied in more detail using a four-level design. Assignment of five factors and their level values in the OA_{16} (4^5) matrix are depicted in Table 3.

ANOVA was used to assess the OAD results. The results of the sums of squares (SS) for different variables were calculated and are shown in Table 5, according to the methods given [26–29,37]. Sixteen experimental trials were repeated three times. The error estimation of the experiments was calculated and used in ANOVA since no dummy columns (in which no actual factor is assigned) were assigned in OA_{16} (4^5) matrix. The SS of error is obtained by subtracting all the SS of the items from the total SS [37].

From the ANOVA results in Table 5, it can be seen that factor *A* (NaOH concentration), factor *B* (HCl concentration) and factor *D* (extraction time) are statistically significant at $P < 0.001$ while both factors *C* (stirring speed) and *E* (salt concentration or ionic strength) are significant at $P < 0.1$. Furthermore, from the percentage contribution (Table 5), it can be deduced that, the most important factor contributing to the extraction efficiency is factor *D* (extraction time, 44.85%), followed by factor *A* (NaOH concentration, 37.13%) and lastly, factor *B* (HCl concentration, 8.01%).

Since the two-variable interaction between NaOH concentration and HCl concentration was in all likelihood significant in the direct observation analysis for the initial experiments, the choice of the optimum conditions for these two factors could be determined based on the results in the interaction experiments designed. It is shown that the other three factors (stirring speed, salt concentration added and the duration of extraction) had a different influence on the extraction efficiency. The extraction efficiency improved continuously when the extraction time was increased from 30 to 60 min. This is in good agreement with previous work [25,40]. Extraction efficiency also improved considerably when the stirring speed increased from 52 to 73 rad/s, but continuously decreased with further increase in the stirring speed from 73 to 130 rad/s. This latter observation may be explained in the following way. With extraction at 104 rad/s or faster stirring speed, excessive air bubbles were generated which in turn could interfere with extraction. With sodium chloride concentration increased from 150 to 250 g/L, the extraction efficiency decreased continuously. It subsequently increased with the salt concentration up to 300 g/L. Based on the discussion above, the optimized condition for factor *C* (stirring speed) was 73 rad/s since factor *C* was independent on the other factors. The optimum extraction time and sodium chloride concentration were 60 min and 150 g/L, respectively.

3.3. Experiment for interactions between HCl and NaOH

Based on the direct observation analysis in the initial experiments, the choice of the optimum concentrations of HCl and NaOH must depend on their interactions. The other extraction conditions optimized were: 1-octanol used as extraction organic solvent, 60 min as extraction time, 150 g/L of added sodium chloride at a stirring speed of 73 rad/s. The

Table 7
Performance of LLLME

Compound	Enrichment factor	RSD% ($n = 6$)	Linear range (ng/mL)	Coefficient of determination (r^2)	LOD (ng/mL)	Recovery (%)	
						5 ng/mL[a]	10 ng/mL[a]
CMPA	1649	6.2	1.0–500	0.9990	0.3	84.9	78.8
KEP	1857	6.3	0.2–500	0.9986	0.07	79.7	82.1
NAP	1904	7.1	0.2–500	0.9959	0.03	89.7	80.2

LLLME conditions: 0.01 M HCl as donor phase; 0.1 M NaOH as acceptor phase; extraction time: 60 min; extraction stirring speed: 73 rad/s; salt concentration: 150 g/L.
[a] The final concentration of each analyte after spiking in ultrapure water.

experimental design and experiment results are presented in Table 6. The levels of two factors in this interaction investigation are the same as those in the OA_{16} (4^5) matrix. It is obvious that the combination of A_3 (0.1 M NaOH) and B_2 (0.01 M HCl) would provide the maximum enrichment factors for all three analytes. The possible reason is that the pH value of the donor phase HCl (0.01 M) is more than 1 unit lower than the pK_a's of all of analytes (CMPA, 3.10; KEP, 4.45; NAP, 4.15), which decrease the partition coefficients of the acidic analytes in the donor phase. However, greater acidity of the donor phase (HCl concentration $\geq$ 0.05 M) reduces the distribution ratios of analytes between in the organic solvent, 1-octanol and in the donor phase because of the formation of ionized species (see above). For the acceptor phase, a high concentration of NaOH at 0.5 M is too basic for the BuckySep-RP column. Thus, 0.1 M NaOH and 0.01 M HCl were selected as the acceptor phase and the donor phase, respectively.

3.4. The optimized LLLME conditions

Under optimized conditions, the performance of this method was investigated and the results are shown in Table 7.

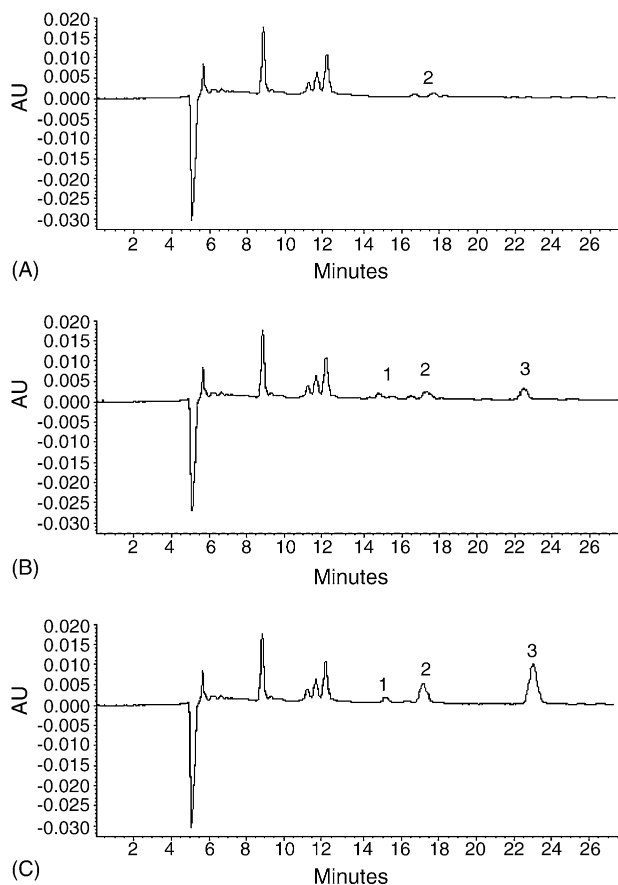

Fig. 1. HPLC-UV chromatograms of domestic wastewater extracted by the optimized LLLME method. (A) Blank domestic wastewater sample, (B) domestic wastewater sample spiked with 1 ng/mL of each analyte, (C) domestic wastewater sample spiked with 5 ng/mL of each analyte. Peaks: 1 = CMPA, 2 = KEP, 3 = NAP. HPLC conditions as in the text.

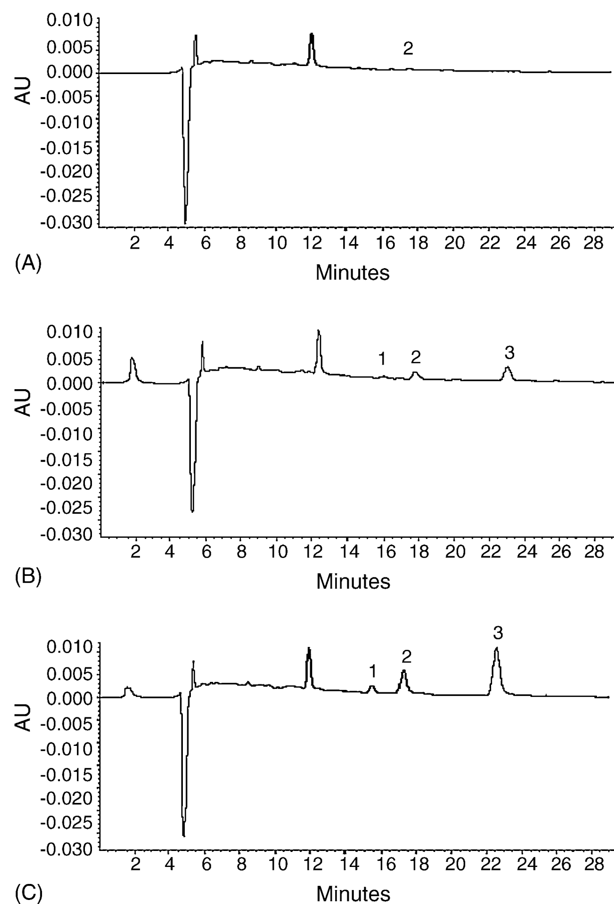

Fig. 2. HPLC-UV chromatograms of river water extracted by the optimized LLLME method. (A) Blank river water sample, (B) river water sample spiked with 1 ng/mL of each analyte, (C) river water sample spiked with 5 ng/mL of each analyte. Peaks: 1 = CMPA, 2 = KEP, 3 = NAP. HPLC conditions as in the text.

Table 8
Summary of results of analysis of NSAIDs in spiked water samples

Compound	Domestic wastewater				River water				Hospital drain water			
	1 ng/mL		5 ng/mL		1 ng/mL		5 ng/mL		1 ng/mL		5 ng/mL	
	Recovery[a] (%)	RSD% ($n=3$)	Recovery[a] (%)	RSD% ($n=3$)	Recovery[a] (%)	RSD% ($n=3$)	Recovery[a] (%)	RSD% ($n=3$)	Recovery[a] (%)	RSD% ($n=3$)	Recovery[a] (%)	RSD% ($n=3$)
CMPA	97.1	5.1	85.1	6.1	99.2	6.3	97.1	0.1	NC[b]	9.3	NC[b]	6.9
KEP	NC[b]	2.5	NC[b]	5.5	NC[b]	4.8	NC[b]	2.1	94.1	11.4	94.8	5.8
NAP	95.3	1.2	86.3	4.9	99.5	6.4	92.4	2.3	104.0	7.2	89.9	6.4

[a] $n=3$.
[b] Not considered since they were detected in water samples.

The maximum enrichment factor can reach as high as 1904. Good linearity of response was observed in the range of 0.2–500 ng/mL, and the coefficients of determination for calibration curves, r^2, were higher than 0.9959. The limits of detection (LODs) calculated at a signal-to-noise (S/N) ratio of 3 (HPLC-UV detection), ranged between 0.03 and 0.3 ng/mL. The relative standard deviations (RSDs) were 6.2% (CMPA), 6.3% (KEP) and 7.1% (NAP) respectively based on the peak areas for six replicates. The recoveries for ultrapure water sample spiked at 5 ng/mL of each analyte, were 84.9% (CMPA), 79.7% (KEP) and 89.7% (NAP). Recoveries were 78.8% (CMPA), 82.1% (KEP) and 80.2% (NAP) when analytes were spiked at 10 ng/mL in ultrapure water.

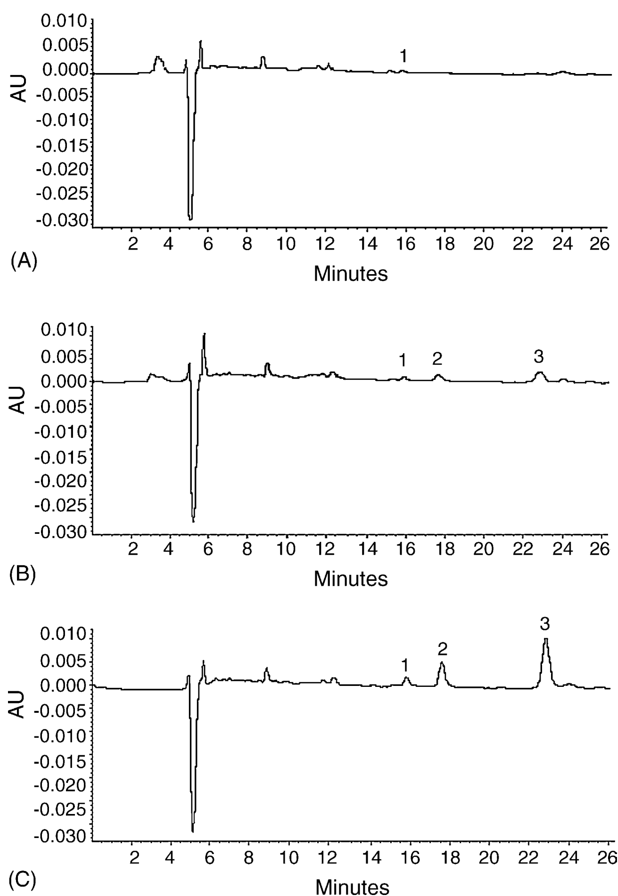

Fig. 3. HPLC-UV chromatograms of hospital drain water extracted by the optimized LLLME method. (A) Blank hospital drain water sample, (B) hospital drain water sample spiked with 1 ng/mL of each analyte, (C) hospital drain water sample spiked with 5 ng/mL of each analyte. Peaks: 1 = CMPA, 2 = KEP, 3 = NAP. HPLC conditions as in the text.

3.5. Application to real wastewater samples

Domestic wastewater, drain water from a hospital and river water were extracted using the optimized LLLME technique developed and the extracts were analyzed by HPLC-UV. In the domestic wasterwater sample, KEP was detected at a concentration of 0.452 ng/mL (Fig. 1A) and its presence was confirmed by spiking the three drugs into the sample and reanalyzing it (Fig. 1B and C). 0.290 ng/mL KEP was also found in the river sample (Fig. 2A). Fig. 2B and C show the chromatograms of the spiked river water sample after LLLME. In the hospital drain water, CMPA was detected and determined to be at a level of 1.13 ng/mL (Fig. 3A). Fig. 3B and C depict the chromatograms of this sample spiked with the three drugs and then reanalyzed. Several unidentified peaks were present in all of water samples, but these did not interfere with the analysis.

To assess matrix effects, all the water samples were spiked with the drug standards at various concentrations. As employed in the literature [41,42], the relative recoveries (defined as the ratio of HPLC peak areas of the respective spiked water sample extracts to spiked ultrapure water extracts) were calculated to evaluate matrix effects. Results of relative recoveries and RSDs of three water samples fortified at 1.0 ng/mL and 5.0 ng/mL in triplicate are shown in Table 8. The data demonstrate that the relative recoveries were in the range between 85 and 105% for all NSAIDs. These results show that the matrix had little effect on LLLME.

4. Conclusions

For the first time, orthogonal array design (OAD) was efficiently employed to optimize LLLME conditions for analyzing nonsteroidal anti-inflammatory drug (NSAID) residues in wastewater samples. The use of OAD not only led to considerable time saving, but also enabled the consideration of interactions among extraction conditions which was not pos-

sible in a univariate approach. An OA_{16} ($4^1 \times 2^{12}$) matrix was used to study the effects of six factors. The effect of each factor was estimated using individual contributions as response functions in the first stage. The extraction organic solvent selected was 1-octanol. Then an OA_{16} (4^5) matrix and a 4×4 table were applied for further optimization and the more exact levels of other five factors were located. Up to 1904-fold enrichment factor could be achieved. The reproducibility of the optimized LLLME method varied from 6.2 to 7.1%. The linearity range of analytes were from 0.2 to 500 ng/mL with r^2 higher than 0.9959. Recoveries of analytes from spiked ultrapure water samples at low ng/mL level ranged from 78 to 90%. Recoveries of analytes from spiked real water samples were similar. This study demonstrated that OAD is an effective approach for optimizing LLLME conditions, suitable for the extraction and subsequent determination by HPLC of NSAIDs in the sub- to low ng/mL range in real water samples.

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