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Research Article

Development and application of chemometric-assisted dispersive liquid–liquid microextraction for the determination of suspected fragrance allergens in water samples

A simple and green method based on dispersive liquid–liquid microextraction, mated to chemometrics and followed by mass spectrometric detection for the determination of suspected fragrance allergens in water samples is developed and assessed in this work. Volume of extraction and disperser solvent, pH, ionic strength, extraction time, sample volume, as well as centrifugation time were initially optimized in a fractional factorial design. The obtained significant factors were optimized by using a central composite design and the quadratic model between the dependent and the independent variables was built. The obtained optimal conditions were: aqueous sample of 3.8 mL, 100 μ L chloroform, 1.40 mL acetone, 4 min centrifugation time, natural pH containing 5% (w/v) NaCl, and centrifugation speed 4000 rpm. Method proved to be linear over a wide range of concentration for all analytes with R^2 between 0.9807 and 0.9959. The repeatability and reproducibility of the proposed method, expressed as relative standard deviation, varied between 3–13% and 4–16%, respectively. The limits of detection ranged from 0.007 to 1.0 μ g L⁻¹. The recommended method was applied to water samples including baby bath as well as swimming pool water samples and was compared with a previously reported method.

Keywords: Desirability profile / DLLME / Experimental design / Fragrance allergens / GC-MS
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1 Introduction

Fragrances are a group of chemicals included in almost every product we use in our daily life. Products ranging from perfumes, cosmetics, personal care products, household, and laundry products even baby care ones. According to the International Fragrance Association (IFRA), fragrances are used for its odorous properties, i.e. to give a scent to a product or cover the smell of a malodorous material, or in some cases, for mood benefits and stress or pain relief (IFRA; www.ifraorg.org). Thus, fragrances are being used widely as they give the consumer the idea of well being and good quality product. Fragrance ingredients may be obtained by chemical synthesis from synthetic, fossil, or natural raw materials or by physical operations from natural sources [1]. Some of the active ingredients have been shown to cause skin sensitiv-

ity, rashes, dermatitis, coughing, asthma attacks, migraine, etc. [2–5]. According to the Scientific Committee on Cosmetic Products and Non-Food Products, there are 26 of these ingredients which correspond to the most frequently recognized allergens [1]. Fragrances have been designated by the European Union as requiring labeling on cosmetic and detergent products [6, 7]; their presence must be indicated in the list of ingredients when each individual concentration of the active ingredient exceeds 0.001% in leave-in products and 0.01% in rinse-off products [8].

To date, reports on the presence of fragrances allergens in waters are still scant and only a few studies have dealt with the determination of these analytes in real water samples such as tap water, baby bathwater, recreational place water, public washing place water, and sewage water [9, 10]. Besides, few studies have reported the analysis of fragrances in perfumes, cosmetics, indoor air, and even scented toys [11–21]. The majority of the analytical techniques employed for the determination of this group of substances are mainly based on gas chromatography–mass spectrometry (GC–MS) [9–16]. Up to date, several studies have been developed for the extraction of fragrances allergens, including ultrasound-assisted solvent extraction [11], solid phase dispersion-pressurized liquid extraction [15], ultrasound-assisted emulsification–microextraction (USAEME) [9], solid-phase microextraction

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Abbreviations: CCD, central composite design; DLLME, dispersive liquid–liquid microextraction; EF, enrichment factor; IFRA, International Fragrance Association; USAEME, ultrasound-assisted emulsification–microextraction

[10, 12], matrix solid phase extraction [14], and headspace solid-phase microextraction [16].

DLLME, a microextraction method called dispersive liquid–liquid microextraction based on dispersion of tiny droplets of the extraction liquid within the aqueous solution, was developed by Razaee et al. [22]. DLLME is based on a ternary component solvent system like homogeneous liquid–liquid extraction [23] and cloud point extraction [24]. In this method, the appropriate volume of extraction solvent dissolved in dispersive solvent is rapidly injected into aqueous sample by syringe and a cloudy solution is formed. The fine droplets of the extraction solvent, containing the analyte, are dispersed in the aqueous sample. After centrifugation, the sedimented phase with the enriched analyte is analyzed.

Response surface methodology (RSM) based on statistical design of experiments was introduced by G. E. P. Box and K. B. Wilson in 1951. RSM explores the relationships between variables and one or more response variables and can be effectively used to find a combination of factor levels that produce an optimum response. Nowadays, it has become one of the most widely used optimization techniques applied in several matrices including food, waters, biological samples, cosmetics, etc. [25–30].

The aim of this study is two-fold. First, to examine for the first time (to the best of our knowledge) the analytical utility of DLLME in combination with chemometric tools (RSM and experimental design) and followed by GC/MS for the simultaneous determination of fragrance allergens in water samples. A 2^{7-4} factorial design was planned in order to define the significant experimental variables, and a central composite design (CCD) was then employed for the DLLME extraction of analytes from waters. Second, to assess the applicability of the proposed method with the analysis of real water samples as well as to evaluate its efficiency.

2 Experimental

2.1 Chemicals and solvents

3,7-Dimethyl-2,6-octadien-1-ol, $\geq 96\%$ (geraniol); 2H-1-benzopyran-2-one, 99% (coumarin); 1-methyl-4-prop-1-en-2-yl-cyclohexene 97% ((*R*)-(+)-limonene); 2-methoxy-4-(1-propenyl) phenol, 98% (isoeugenol, *cis/trans*); 2-(phenylmethylene)-heptanal, 95% (amyl cinnamaldehyde); 3-phenyl phenylmethyl ester-2-propenoic acid, 99% (benzyl cinnamate); 3-methyl-4-(2,6,6-trimethyl-2-cyclohexen-1-yl)-3-buten-2-one, $\geq 90\%$ (ionone); 4-(4-hydroxy-4-methylpentyl)cyclohex-3-ene-1-carbaldehyde, $\geq 97\%$ (lyral); 2-(phenylmethylene) octanal, $\geq 95\%$ (hexylcinnamaldehyde); 3-phenyl-2-propenal, $\geq 98\%$ (cinnamaldehyde); and 7-hydroxy-3,7-dimethyloctanal, $\geq 95\%$ (hydroxycitronellal) were purchased from Aldrich (Sigma–Aldrich, Denmark, Germany). 4-methoxybenzene methanol, 98% (anisyl alcohol); 2-methoxy-4-prop-2-enyl phenol, 99% (eugenol); 3,7,11-trimethyldodeca-2,6,10-trien-1-ol, 97% (farnesol, mixture of isomers); 3,7-dimethylocta-2,6-dienal, 95% (citral,

cis/trans); 3,7-dimethyl-1,6-octadien-3-ol, 97% (linalool); benzene methanol, 99% (benzyl alcohol); and 3-phenyl-2-propen-1-ol, 98% (cinnamyl alcohol) were purchased from Merck (Darmstadt, Germany). 3-(4-*tert*-butylphenyl)-2-methylpropanal, $\geq 95\%$ (lilial) and 2-hydroxy-phenylmethyl ester benzoic acid, $\geq 99\%$ (benzyl salicylate) were purchased from Fluka (Denmark, Germany).

Acetone, methanol, ethanol, chloroform, and sodium chloride were provided by Merck (Darmstadt, Germany). Carbon tetrachloride was purchased by Sigma–Aldrich (Denmark, Germany). Individual stock solutions of the 21 selected allergens were prepared by dissolving in acetone each standard (accurately weighed) in volumetric flasks at concentrations of about $1000 \mu\text{g mL}^{-1}$. Working solutions were prepared with further dilution of the stock ones. The solutions were processed individually and in a mixture to optimize the operative conditions.

Water sample (tap water) used for optimization purposes was free from target compounds contamination.

2.2 GC–MS analysis

Analyses were performed using a Trace GC Ultra instrument (Thermo Scientific, Waltham, MA, USA) coupled to an ISQ mass spectrometer controlled by a computer running XCalibur software. Samples of $1 \mu\text{L}$ were injected using an AI/AS 3000 auto sampler (Thermo Scientific). The separation was performed using a DB-5 column, film thickness $0.25 \mu\text{m}$ ($30 \text{ m} \times 0.25 \text{ mm i.d.}$, Thermo Fisher Scientific). Helium was the carrier gas at a flow rate of 1.2 mL min^{-1} . The GC oven temperature program was as follows: initial temperature 45°C for 2 min, 5°C min^{-1} to 100°C , 5°C min^{-1} to 150°C , 5°C min^{-1} to 200°C , (held for 5 min), and 8°C min^{-1} to 225°C , (held for 5 min). The temperatures of the ion source and the interface were set at 280°C . The mass spectrometer was operated in the electron ionization mode at ionization energy of 70 eV, and selected ion monitoring (SIM) mode. Up to three and four ions were selected from the spectrum of each compound to quantify the response under SIM mode. Retention times as well as the identification and quantification ions selected for the target compounds are shown in Table 1. Figure 1 depicts a typical DLLME-GC/MS chromatogram obtained from a spiked (0.025 mg L^{-1}) natural water sample.

2.3 DLLME procedure

A total of 3.8 mL of aqueous sample solution with ionic strength of 5% (w/v) NaCl, containing $0.025 \mu\text{g mL}^{-1}$ of target analytes was placed in a 10.0 mL screw cap test tube with conical bottom. A mixture of 1.40 mL acetone (disperser solvent) containing $100 \mu\text{L}$ chloroform (extraction solvent) were rapidly injected into the sample solution with a 5.00 mL glass syringe and the mixture was gently shaken (extraction time: 1 min). A cloudy solution was formed and the analytes

Table 1. Retention times and selected ions for the analysis of the target compounds

a/a	Compound	<i>t_R</i> (min)	LogKow	Solubility (mg L ⁻¹)	Quantifier and qualifier (<i>m/z</i>)
1	Limonene	11.86	4.57	13.8	68, 93 , 121
2	Benzyl alcohol	12.29	1.08	40 000	77, 79, 108
3	Linalool	14.02	1.54	1589	71, 80, 93 , 121
4	Citral	18.20	1.52	590	67 , 82/ 69 , 84
		19.04			
5	Geraniol	18.45	3.28	531	69 , 93, 111
6	Cinnamaldehyde	19.50	2.22	1420	77, 103, 131
7	Hydroxy-citronellal	19.63	1.54	23 800	59 , 71, 81
8	Anisyl alcohol	19.70	1.10	2070	77, 109, 138
9	Cinnamyl alcohol	20.35	1.93	1800	77, 92, 134
10	Eugenol	21.42	2.20	<1000	103, 131, 164
11	Ionone	23.15	4.41	16	93, 121, 136
12	Isoeugenol	23.98	2.45	984	103, 131, 164
13	Coumarin	24.04	1.39	2500	89, 118 , 146
14	Lilial	25.88	4.07	33	131, 147, 189
15	Lylal	29.20	2.53	185–1045	71, 93, 136
16	Amyl cinnamyl alcohol	29.51	4.80	8.5	91, 115, 133
17	Farnesol	30.03	5.31	267	69 , 81, 93
18	Hexylcinnamaldehyde	30.89	4.82	2.8	115, 129 , 216
19	Benzyl benzoate	31.57	3.97	19.8	105 , 194, 212, 91
20	Benzyl salicylate	33.78	4.31	<1000	65, 91 , 228
21	Benzyl cinnamate	40.25	3.65	9	91, 131 , 192

in the water sample were extracted into fine droplets [31]. The mixture was then centrifuged for 4 min at 4000 rpm. The sedimented phase was withdrawn with a 100 μ L syringe and was finally injected into GC/MS system (1 μ L).

2.4 Experimental design

An experimental 2^{7-4} design was employed in the present study to screen the important variables that statistically affected allergens extraction, namely: volume of extraction and dispersive solvent, pH, ionic strength, extraction time, cen-

trifuging time, as well as sample volume were considered during the multivariable approach. Each variable was examined at two levels: -1 for the low level and $+1$ for the high level. The fractional factorial design in total included eight experiments plus three central points in order to estimate the experimental error [32].

Next, a 2^3 CCD with sixstar points placed at a distance α from the central point was performed in order to determine the optimal conditions of the extraction process using DLLME procedure. The value of α was 1.682 to establish the rotatability condition [33]. Seventeen experiments including three central points were carried out in random order.

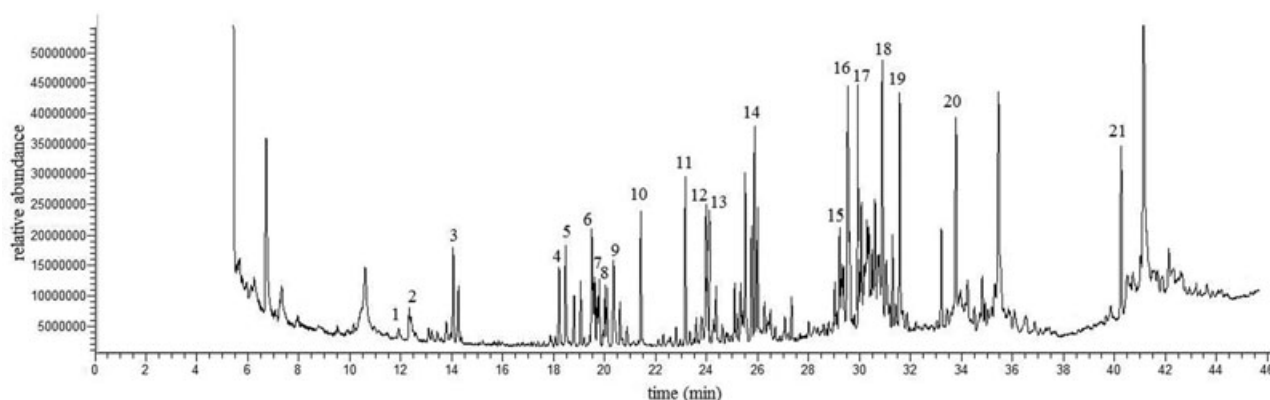
**Figure 1.** Total ion chromatogram obtained for a water sample spiked with the target analytes at 0.025 mg L⁻¹ after concentration by DLLME.

Table 2. Experimental conditions and responses obtained with the 2³ central composite design

Factors	Levels			Star point	
	Low (−1)	Central (0)	High (+1)	−a	+a
(X ₁) Volume of acetone (mL)	1.10	1.25	1.4	0.998	1.502
(X ₂) Volume of CHCl ₃ (μL)	84	90	96	79.92	100.08
(X ₃) Volume of sample (mL)	3.70	4	4.30	3.50	4.5
Run	X ₃	X ₁	X ₂	Average extraction efficiency (R%)	
1	−1	−1	−1	62	
2	−1	−1	1	61	
3	−1	1	−1	73	
4	−1	1	1	64	
5	1	−1	−1	61	
6	1	−1	1	69	
7	1	1	−1	53	
8	1	1	1	72	
9	−1.682	0	0	48	
10	+1.682	0	0	76	
11	0	−1.682	0	70	
12	0	+1.682	0	67	
13	0	0	−1.682	72	
14	0	0	+1.682	62	
15	0	0	0	62	
16	0	0	0	57	
17	0	0	0	64	

The conditions set in each experiment are listed in Table 2. Moreover, the profile for predicted values and desirability option was used for the optimization of the extraction process.

In all cases, the measured response was selected as the mean extraction yield of all analytes (average extraction efficiency, R%) in order to reach the so-called global optimum of the DLLME method since being a multiresidue method. STATISTICA 7.0 (StatSoft Inc., Tulsa, OK, USA) statistical package was used to generate the experimental matrix and to evaluate the results.

2.5 Calculation of enrichment factor (EF) and recovery

EF is defined as the ratio between the analyte concentration in the sedimented phase (C_{sed}) and the initial concentration

of the analytes (C_0) in the aqueous sample ($EF = C_{\text{sed}}/C_0$). The extraction recovery (R%) was defined as the percentage of the total analyte which was extracted in the sedimented phase [V_{sed}]: $(R\%) = [(C_{\text{sed}} \times V_{\text{sed}}) \times 100]/(C_0 \times V_{\text{aq}}) = (EF \times V_{\text{sed}})/V_{\text{aq}}$ (V_{aq} is the volume of the aqueous sample).

3 Results and discussion

Special care was taken in order to avoid the risk of contamination, resulting in false of positive results. Laboratory glassware were washed with ultra pure water, acetone, and dried at 100°C, while screw cap test tubes were disposable. Moreover, experiments were carried out in an isolated room with clean air without any fragrance odor. Blank runs and direct injections of solvents were made to confirm the absence of suspected allergens in the chromatographic system.

3.1 Screening design

DLLME efficiency was examined for the target analytes using several combinations of dispersive (acetone, methanol, and ethanol) and extraction solvents (carbon tetrachloride and chloroform). Results obtained have shown that acetone as dispersive solvent and chloroform as extraction solvent displayed the highest extraction efficiencies of the target analytes.

Analysis of variance (ANOVA) was performed to examine whether the studied experimental factors, were significant in the performance of the proposed method. In the studied experimental domain, ionic strength, sample volume, dispersive volume, and extractive volume, were statistically significant at 95% confidence level, while centrifugation time, extraction time, and pH proved to be nonsignificant. Positive signs indicate that increasing these variables will result in an increase in the analytical signal. According to the obtained results, ionic strength was kept fixed at 5% (w/v) NaCl while sample volume, dispersive volume, and extractive volume were evaluated in the CCD for further assessment. Centrifugation time, extraction time, and pH were eliminated for further studies.

3.2 CCD

Following the results of the previous design, the next step was to optimize the analytical method for the remaining three factors by the employment of a CCD (Table 2).

The main effects, interaction effects, as well as quadratic effects were evaluated through ANOVA. Moreover, lack-of-fit (LOF; i.e. the variation of the data around the fitted model) was also checked and was shown to be not significant relative to the pure error, indicating good response to the model. The model regression coefficient (R^2) of 0.9559 is in reasonable agreement with the experimental results, indicating 95.59% of the variability can be revealed by the model and is left with 4.41% residual variability. As well, the adjusted determination coefficient (adjusted $R^2 = 0.8992$) is also very high to

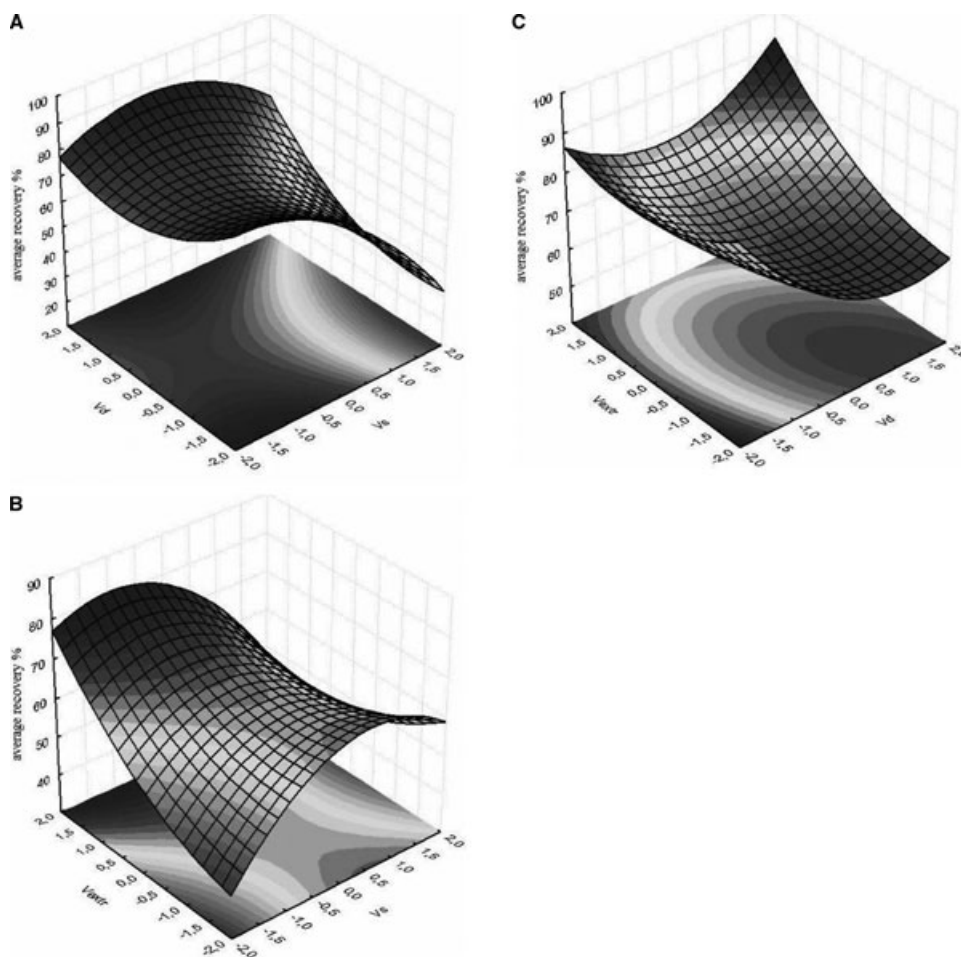


Figure 2. Response surfaces for the 2^3 central composite design: (A) volume of sample (V_s) – volume of dispersive solvent (V_d), (B) volume of extractive solvent (V_{ext}) – volume of sample (V_s), (C) volume of extractive solvent (V_{ext}) – volume of dispersive solvent (V_d). Response value: average extraction efficiency of all analytes ($R\%$).

advocate for a high significance of the model. Both values ensured a satisfactory adjustment of the polynomial model to the experimental data.

Data analysis using the STATISTICA software at 95% of confidence level permitted to obtain a semiempirical expression (Eq. (1) in terms of significant coded factors):

$$\begin{aligned}
 Y = & 63.48(\pm 0.64) - 3.92(\pm 0.30) V_s - 3.80(\pm 0.33) V_s^2 \\
 & + 3.06(\pm 0.33) V_d^2 + 1.26(\pm 0.30) V_{ext} + 1.66(\pm 0.33) V_{ext}^2 \\
 & + 3.07(\pm 0.39) V_s V_d - 2.93(\pm 0.39) V_s V_{ext} \\
 & + 2.22(\pm 0.39) V_d V_{ext}
 \end{aligned} \quad (1)$$

where V_s is sample volume, V_d is dispersive volume, and V_{ext} is volume of extractive solvent.

Positive coefficients indicate that extraction efficiency is favored in the presence of high values of the respective variables within the range studied, while negative coefficients indicate that the reaction is favored in the presence of low values. Positive quadratic coefficients of $V_s V_d$ variables indicate a synergistic effect, while negative coefficients of $V_s V_{ext}$, an antagonistic effect between the variables.

The overall interaction effects are displayed in Fig. 2; a 3-D representation of the polynomial (Eq. (1) obtained from the experimental data), depicting the surface plots of extraction yield versus the significant variables.

As shown in Fig. 2A, the extraction efficiency of DLLME method depends on the volumes of acetone (dispersive solvent, V_d) and the aqueous sample (V_s), respectively. Lower extraction yields ($R\%$) were observed at low levels of acetone and sample volume >4 mL. At such conditions, even if chloroform, was used at highest volume (100 μ L), the extraction solvent could not be dispersed efficiently and therefore, cloudy solution was not formed completely. At moderate volumes of aqueous solutions and higher volumes of acetone, extraction of target analytes is favored.

Considering the interaction between extraction volume (chloroform) and sample volume (Fig. 2B) at fixed acetone amount (1.4 mL), one can see that when low amounts of chloroform and sample are introduced, extraction efficiency is diminished. Recovery percentage rate enhancement at moderate sample volumes and high extractive solvent is likely due to the optimized formation of cloudy solution which allows the efficient extraction of the target analytes. Figure 2C depicts that at fixed moderate amounts of sample (3.8 mL), the extraction efficiency of the proposed method is maximized

Table 3. Recoveries, r^2 , LODs, repeatability, and reproducibility of water sample

Compound	r^2 ^{a)}	Repeatability ^{b)} (intra-day) (RSD%)	Reproducibility ^{c)} (inter-day) (RSD%)	LOD ^{d)} (ng mL ⁻¹)	Recovery % ^{e)} (RSD%)
Limonene	0.9823	13	11	0.250	34.7
Benzyl alcohol	0.9888	5	4	0.250	29.0
Linalool	0.9895	12	4	0.050	89.9
Citral	0.9814	10	16	0.060	106
	0.9916				
Geraniol	0.9807	13	7	0.300	92.3
Cinnamal- dehyde	0.9880	11	16	0.010	64.5
Hydroxy- citronellal	0.9894	7	5	0.050	49.3
Anisyl alcohol	0.9954	11	11	1.000	31.3
Cinnamyl alcohol	0.9931	6	13	0.150	52.0
Eugenol	0.9928	12	5	0.007	89.0
Ionone	0.9905	3	5	0.010	88.2
Isoeugenol	0.9959	9	16	0.250	69.6
Coumarin	0.9908	13	5	0.050	49.1
Lilial	0.9956	4	4	0.010	78.3
Lylal	0.9858	10	10	0.050	50.4
Amyl cinnamyl alcohol	0.9890	6	8	0.250	79.7
Farnesol	0.9899	12	13	0.100	91.3
Hexylcinnam- aldehyde	0.9841	9	10	0.015	112.2
Benzyl benzoate	0.9907	5	6	0.025	90.8
Benzyl salicylate	0.9827	8	10	0.025	91.8
Benzyl cinnamate	0.9809	10	12	0.025	99.2

a) Coefficient of determination.

b) Repeatability was investigated at concentration: 0.025 mg L⁻¹ of each analyte.

c) Reproducibility was investigated at concentration: 0.025 mg L⁻¹ of each analyte.

d) Limits of detection (LOD), for a S/N $\geq$ 3.

e) Recovery of each analyte in the optimum condition.

at high values of both extractive (chloroform) and dispersive solvents (acetone).

The desirability function for the extraction efficiency% was defined by assigning a desirability value of 0.0 to minimum $R\%$ (48.03%), 1.0 for the maximum (76.22%), and 0.5 for middle (62.12%). In order to achieve the highest desirability score (desirability 1), software optimized 76.28% average recovery of target analytes with calculating the optimized model variables of 100.0 μ L CHCl₃, 1.4 mL acetone, and 3.8 mL sample volume.

For validation purposes, duplicate assenting experiments were conducted using the optimized parameters. The results are closely correlated with the data obtained from desirability optimization analysis using CCD, indicating that CCD with desirability function could be effectively used to optimize the extraction recovery of the target analytes.

3.3 Analytical performance and analysis of natural waters

In order to evaluate the analytical performance of the proposed method, limit of detection (LOD), repeatability (intra-day), reproducibility (inter-day), and linear range were investigated according to the optimum conditions. The LODs (Table 3) for each compound according to the signal-noise ratio ($s/n \geq 3$) ranged from 0.007 to 1 ng mL⁻¹. Method linear range was tested at the range of 3.5–50 ng mL⁻¹ with squared correlation coefficients (r^2) ranging from 0.9807 to 0.9959. Intra- and inter-day precision was carried out by extracting a spiked water sample at 0.025 μ g mL⁻¹. The repeatability and reproducibility of the proposed method, expressed as relative standard deviation, varied between 3–13% and 4–16%, respectively. Recoveries were satisfactory for the

most analytes with the exception of limonene (34.7), benzyl alcohol (29.0), hydroxy citronellal (49.3), anisyl alcohol (31.3), and coumarin (49.1). This behavior was expected for these allergens due to their low logK_{ow} partition coefficient. The only exception was limonene that although it displays a high logK_{ow}, its extraction efficiency was low, even with ultra pure water which is in agreement with the findings of Becerril-Bravo et al. [9] when USAEME was employed. Analytical characteristics of USAEME were comparable with the proposed DLLME method with LODs ranging between 0.006 and 1.15 ng mL⁻¹, while recovery values varied in the range of 75–110%.

Finally, the proposed method was applied to the analysis of water samples, including baby bathwater, as well as swimming pool water. Target analytes were determined at concentrations ranging from 0.118 to 724.1 µg L⁻¹. Highest concentrations were observed at baby bathwater and swimming pool water from recreational places recognized as an important source of exposure since these chemicals are present in a wide variety of cosmetics and sun creams.

The presence of the target analytes in recent studies and in the present study, at several real samples including tap water, baby bathwater, recreational place water, public washing place water, and sewage water demonstrates the ubiquity of these cosmetic ingredients that may be accumulated in the aquatic environment through direct (swimming, bathing) and indirect sources (wastewater-treatment plants). The accuracy of the proposed DLLME method was assessed by comparing the results with those obtained by USAEME [9] at selected water samples. Application of paired *t*-test at 95% confidence level showed that no significant difference was observed between the two methods.

4 Conclusions

In this study, DLLME method coupled to GC–MS has been evaluated for the determination of suspected fragrances allergens in real water samples for the first time (to the best of our knowledge). The extracting organic phase was separated from the aqueous phase, and the analytes were finally determined with GC–MS. With the aid of RSM and experimental design, acetone, chloroform, and volume sample were the most significant factors affecting the proposed method. This simple and rapid extraction method provides good repeatability and reproducibility range, high extraction efficiency, and short time compared to other methods with the requirement of low volume of organic solvents. DLLME can be successfully applied for routine analysis for the determination of the target analytes in aqueous samples.

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The authors have declared no conflict of interest.

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