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Simultaneous determination of six triterpenic acids in some Chinese medicinal herbs using ultrasound-assisted dispersive liquid–liquid microextraction and high-performance liquid chromatography with fluorescence detection

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ABSTRACT

A novel analytical method was developed for simultaneous determination of six triterpenic acids using ultrasound-assisted dispersive liquid–liquid microextraction (UA-DLLME) follow by high-performance liquid chromatography (HPLC) with fluorescence detection. Six triterpenic acids (ursolic acid, oleanolic acid, betulinic acid, maslinic acid, betulonic acid and corosolic acid) were extracted by UA-DLLME using chloroform and acetone as the extraction and disperser solvents, respectively. After the extraction and nitrogen flushing, the extracts were rapidly derivatized with 2-(12,13-dihydro-7H-dibenzo[a,g]carbazol-7-yl)ethyl4-methylbenzenesulfonate. The main experimental parameters affecting extraction efficiency and derivatization yield were investigated and optimized by response surface methodology (RSM) combined with Box–Behnken design (BBD). The limits of detection (LODs) and the limits of quantification (LOQs) were in the range of 0.95–1.36 ng mL^{-1} and 3.17–4.55 ng mL^{-1}, respectively. Under the optimum conditions, the method has been successfully applied for the analysis of triterpenic acids in six different traditional Chinese medicinal herbs.

1. Introduction

Triterpenic acids are significant bioactive phytochemicals and ubiquitously present in nature in the form of free acids or aglycones for triterpenoid saponins [1]. Recent researches have demonstrated that triterpenic acids exert a number of multi-target properties, such as anti-inflammatory, antioxidant, antitumor [2], hepatoprotective effects, anti-HIV activity [3], antifungal, antihyperlipidemic [4,5], antiatherogenic, antidiabetic and enhancing the cellular immune system [6], and they are also described as important functional compounds with wide application in cosmetics and healthcare products [7]. Therefore, the development of sensitive and accurate method for rapid determination of triterpenic acids is of great importance and it could help control the quality of related herbs and functional food.

Regarding the determination of triterpenic acids, various methods have been proposed base on capillary electrophoresis (CE) [8,9], HPLC [10,11], gas chromatography (GC) [12,13], thin-layer chromatography (TLC) [7,14] and NMR [15,16]. Among these cited techniques, HPLC is frequently used for the separation and quantification of triterpenic acids. However, because of the lack of suitable chromophoric or fluorescent moieties in triterpenic acid molecules, employing post-column derivatization has been widely accepted to enhance the selectivity and sensitivity [17]. In this study, a new novel pre-column fluorescence labeling reagent 2-(12,13-dihydro-7H-dibenzo[a,g]carbazol-7-yl)ethyl4-methylbenzenesulfonate (DDCETS) has been synthesized and successfully applied to detect triterpenic acids. Compared with traditional derivatization reagents for labeling carboxyl moiety, such as 6-Oxy-(acetyl piperazine) fluorescein [18] and 9-anthryldiazomethane [19], DDCETS has much bigger conjugate structure, which could offer stronger photoluminescence property

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and improve the detection sensitivity. In addition, its derivatization procedure has the advantages of mild reaction conditions, simple procedure and short reaction min. To the best of our knowledge, DDCETS was first employed as the pre-column derivatization reagent for the determination of six triterpenic acids in real samples.

However, there are still many challenges remaining in the course of analyzing triterpenic acids. The target compounds in natural products are usually found at trace levels. Moreover, the complex matrix in samples not only interferes with accurate measurement of the analytes, but also contaminates the chromatographic column and shortens column lifetime, so a sample preparation prior to chromatographic analysis is apparently necessary and significant. Currently the most commonly used techniques for sample preparation were liquid–liquid extraction (LLE) [20] and solid phase extraction (SPE) [21,22], but various inevitable defects have also been found in their application. As a classical pretreatment method, LLE suffers from disadvantages such as being time-consuming and the consumption for large amounts of samples and toxic organic solvents [23]. Although SPE takes much less organic solvent and time than LLE, it is relatively expensive and still needs several complex steps prior to instrumental analysis. In addition, it may result in analytes losses and contamination [24]. Recently, dispersive liquid–liquid microextraction (DLLME) is a novel and emerging microextraction technique, which was proposed by Assadi and coworkers previously [25]. In comparison with the conventional extraction methods, DLLME could be a wiser choice because of its many distinct advantages such as high enrichment ability, simple operation, low organic solvent consumption, high recovery and low cost [26].

The aim of this work was to establish a sensitive and selective UA-DLLME/HPLC-FLD (ultrasound-assisted dispersive liquid–liquid microextraction/high-performance liquid chromatography with fluorescence detection) method for simultaneous determination of six triterpenic acids. The main experimental parameters that influence the derivatization yield and UA-DLLME efficiency were investigated and optimized by Box–Behnken design (BBD) of response surface methodology (RSM). Under the optimal conditions, the proposed method has been successfully applied to the analysis of six triterpenic acids in six traditional Chinese medicinal herbs, which possessed good sensitivity, satisfactory recoveries as well as repeatability.

2. Materials and methods

2.1. Chemicals

2-(12,13-Dihydro-7H-dibenzo[a,g]carbazol-7-yl)ethyl4-methylbenzenesulfonate (DDCETS) was synthesized in our laboratory. Ursolic acid (UA) and oleanolic acid (OA) were obtained from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Maslinic acid (MA), betulinic acid (BIA), corosolic acid (CA) and betulonic acid (BOA) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Acetonitrile (HPLC grade), ethanol, chloroform and acetone were purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). Other reagents were of analytical grade from Jining Chemical Reagent (Jining, Shandong Province, China). Water was purified on a Milli-Q system (Millipore, Bedford, MA, USA).

2.2. Instrumentation

An Agilent 1100 Series HPLC/mass spectrometry system was used for all analysis. The mass spectrometer (MSD Trap SL, model G2445D) from Bruker Daltonik (Bremen, Germany) was equipped with an atmospheric pressure chemical ionization (APCI) source (model G1947A). Ion source conditions: APCI in positive ion detection mode; nebulizer pressure 60 psi; dry gas temperature, 350 °C; dry gas flow, 5 L/min. APCI Vap temperature 350 °C; corona current 4000 nA; capillary voltage 3500 V. An ultrasonic cleaner (KQ3200E, Kunshan Ultrasonic Instrument, Jiangsu, China) set at 40 kHz (equivalent to the wavelength of 37.5 mm) was used to emulsify the solutions. A XH-100A microwave-oven (Xianghu Science and Technology Development Co., Ltd, Beijing, China) was used for microwave assisted extraction, which equipped with a monitor of temperature, microprocessor programmer software and a microwave power of 1000 W.

2.3. Chromatographic parameters

Chromatographic separation was performed on a Hypersil C18 (4.6 mm × 200 mm, 5 μm) column with a gradient elution. The mobile phase was water containing 5% acetonitrile (A) and 100% acetonitrile (B) with a flow rate of 1 mL min^{-1}, and the column temperature was set at 30 °C. The elution program was as follows: 0–5 min, 65–90% B; 5–15 min, 90–92% B; 15–35 min, 92–92.5% B; 35–38 min, 92.5–100% B. The injection volume was 10 μL for each analysis and the detection wavelength was set at $\lambda_{ex}/\lambda_{em}$ = 292/402 nm.

2.4. Synthesis of derivatization reagent (DDCETS)

2.4.1. Syntheses of 12, 13-dihydro-7H-dibenzo [a,g] carbazole

According to the previously reported method [27], DDCETS was synthesized and shown in Fig. S1. Concentrated hydrochloric acid (90 mL), water (250 mL) and naphthylhydrazine (19 g) were well mixed and rapidly heated to reflux with stirring. Next, 3.4-dihydro-1(2H)-naphthalenone (1 mL) was added dropwise within 1 h. After refluxing for 1 h, the mixture was cooled. Then the precipitated solid was recovered by filtration, washed with water and 75% ethanol, and dried at room temperature for 48 h. The crude product was recrystallized three times to afford a rufous crystal, yield 80%. m.p. 193.7–194.2 °C. Found (%): C 89.12%, H 5.63%, N 5.28%; calculated (%): C 89.22%, H 5.57%, N 5.20%. IR (KBr): 3403.56 (N–H), 3048.03, 2952.89, 2882.32, 1503.86 (Ar), 1392.13 (C–N), 802.21, 765.83; MS: m/z $(M+H)^+$: 270.1.

2.4.2. Synthesis of 2-(12, 13-dihydro-7H-dibenzo [a,g]carbazol-7-yl) ethanol

12,13-Dihydro-7H-dibenzo[a,g] carbazole (25 g), KOH (20 g), and 2-butanone (80 mL) were well dissolved at 40 °C in a 500-mL round-bottomed flask and rapidly cooled to 0 °C with ice-water by vigorous stirring. A freezing mixture of oxane (7.5 g) in 50 mL 2-butanone solution was added dropwise within 1 h. After keeping the temperature constant for 2 h with stirring, the solution was heated to 55 °C for 2 h and concentrated by rotary evaporation. After cooling, the residue was transferred into 200 mL ice-water with vigorous stirring for 0.5 h. The precipitated solid was recovered by filtration, washed with water and 75% ethanol and dried at room temperature for 48 h. The crude product was recrystallized three times to afford white crystals, yield 78%. m.p. 131.4–132.2 °C. Found (%): C 84.28%, H 6.12%, N 4.50%; calculated (%): C 84.35%, H 6.07%, N 4.47%. IR (KBr): 3368.54 (O–H), 3050.03 (Ar), 1137.21 (C–O), 816.51, 725.83; MS: m/z $(M+H)^+$: 313.5.

2.4.3. Synthesis of 2-(12,13-dihydro-7H-dibenzo[a,g]carbazol-7-yl)ethyl 4-methylbenzenesulfonate (DDCETS)

A mixture of 2-(12,13-dihydro-7H-dibenzo[a,g]carbazol-7-yl) ethanol (5 g) in 50 mL of pyridine solution was added dropwise

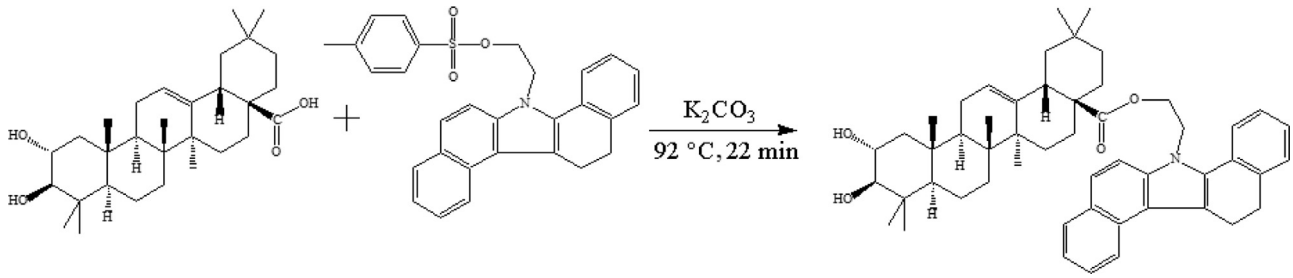

Fig. 1. The representative derivatization scheme of DDCETS with maslinic acid.

within 30 min with vigorous stirring, to a solution of 3.7 g p-toluenesulfonyl chloride in 30 mL pyridine (0 °C) in a 100-mL of round-bottomed flask. After stirring at 0 °C for 4 h, the contents were left to stand at ambient temperature for another 4 h with vigorous stirring. The solution was then concentrated by rotary evaporation. The crude product was recrystallized twice from methanol to give white crystals, 6.2 g (71.7%), m.p. 182.8–183.3 °C. Found (%): C 74.43%, H 5.42%, N 2.92%, S 6.79%; calculated (%): C 74.49%, H 5.39%, N 3.01%, S 6.84%. IR (KBr): 3048.53 (Ar); 2980.12 (CH_3), 1175.58 (C–O), 802.21, 765.83; MS: m/z $(M+H)^+$: 468.2.

2.5. Preparation of standard solutions

The DDCETS solution (4.67 mg mL^{-1}) was prepared by dissolving 46.7 mg of DDCETS in 10 mL of acetonitrile. The low concentration solution was obtained by diluting the prepared DDCETS solution with acetonitrile. Individual stock solutions (2.0 mg mL^{-1}) of triterpenic acids were prepared by dissolving 20.0 mg triterpene acid standard in 10 mL of acetonitrile, respectively. The mixed standard solutions for HPLC analysis were prepared by diluting the stock solutions with acetonitrile. Then all standard solutions were stored at 4 °C until use.

2.6. Plant materials and sample preparation

Swertia racemosa, *Corydalis impatiens*, *Meconopsis henrici*, *Dracocephalum tanguticum* Maxim, *Comastoma pulmonarium* and *Punica granatum* were purchased from Qinghai Province and identified by Professor Chang-Fan Zhou from Northwest Plateau Institute of Biology, Chinese Academy of Sciences. These herbs were dried at 40 °C for 24 h in an electrical furnace, then milled and kept at 4 °C until use.

According to several reported studies, the microwave assisted extraction of triterpenic acids was performed with minor revision [28]. For each material, dried samples (1 g) were individually placed in a three-necked flask and then extracted thrice with 15 mL 80% aqueous ethanol for 20 min at 500 W. Next, the extracts were collected, made up to 50 mL with water and then stored at 4 °C.

2.7. UA-DLLME and derivatization procedures

1 mL of extract (or 30 μL mixed standard solution) was added to a 10 mL conical test tube and then diluted to 8 mL with water. Next, the mixture of acetone and chloroform (1012 μL of acetone and 89 μL of chloroform) was rapidly injected into the conical test tube using a glass syringe. The tube was immersed immediately in an ultrasonic water bath for 1 min. After centrifugation at 5000 rpm for 5 min (800B centrifuge, Shanghai Anting Instruments Inc., China), the organic phase containing the targeted analytes was deposited at the bottom of the vial, transferred to a vial with a glass syringe and then dried under nitrogen. Next, 125 μL N,N-dimethylformamide (DMF), 150 μL DDCETS solution, 225 μL ACN and 5 mg K_2CO_3 catalyst were placed together in above vial. The vial was shaken for 10 s, sealed and then allowed to stand for 22 min at 92 °C. After the reaction, the mixture was cooled to room temperature and then directly injected in HPLC for analysis. The derivatization scheme for the representative maslinic acid was shown in Fig. 1.

2.8. Experimental design and data analysis

In the present study, a three-variable and three-level BBD was applied to optimize UA-DLLME and derivatization conditions. The input variables and levels used for the optimization of UA-DLLME were: X_1, pH (5, 7 and 9), X_2, extraction solvent volume (70, 100 and 130 μL) and X_3, disperser solvent volume (600, 950 and 1300 μL), and the variables and levels used for derivatization optimization were: X_1, derivatization temperature (80, 90 and 100 °C), X_2, derivatization time (10, 25 and 40 min) and X_3, the molar ratio of DDCETS to the triterpenic acids (3, 7 and 11). The response value (Y) was peak area. The experimental designs for UA-DLLME and derivatization were given in Tables S1 and S2, respectively. The experimental data obtained from the design approach were analyzed by the Design Expert software (Version 7.1.6, Stat-Ease Inc., Minneapolis, MN, USA) and were fitted to a second-order polynomial model, which can be described as the following equation:

$$Y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{23} x_2 x_3 + \beta_{11} x_1^2 + \beta_{22} x_2^2 + \beta_{33} x_3^2$$

where x_1, x_2 and x_3 are the independent variables; β_0 is the constant term; β_1, β_2 and β_3 are the regression coefficients of the first order terms, β_{12}, β_{13} and β_{23} are that of quadratic terms and β_{11}, β_{22} and β_{33} are that of interaction terms; and Y is the response.

3. Results and discussion

3.1. Optimization of UA-DLLME parameters

In order to obtain the highest extractive yield, those parameters influencing UA-DLLME procedure need to be evaluated in detail by designing a series of experiments.

3.1.1. Selection solvent pair for UA-DLLME

In UA-DLLME process, extraction and disperser solvents were critical elements influencing extraction efficiency, so it is essential to select the best solvent pair. The experiments for optimizing solvent pair were performed with 1 mL of dispersive solvent and 100 μL of extraction solvent. Based on the characteristics of extraction and disperser solvents, chloroform, carbon tetrachloride, dichloromethane, bromoethane and 1,2-dichloroethane were used as extraction solvents (Fig. 2a), and acetonitrile, acetone, ethanol and methanol were selected as dispersive solvents in study (Fig. 2b). The results showed that maximum of DLLME efficiencies were obtained, when extraction solvent was chloroform and dispersive solvent was acetone. Thus, chloroform and acetone were

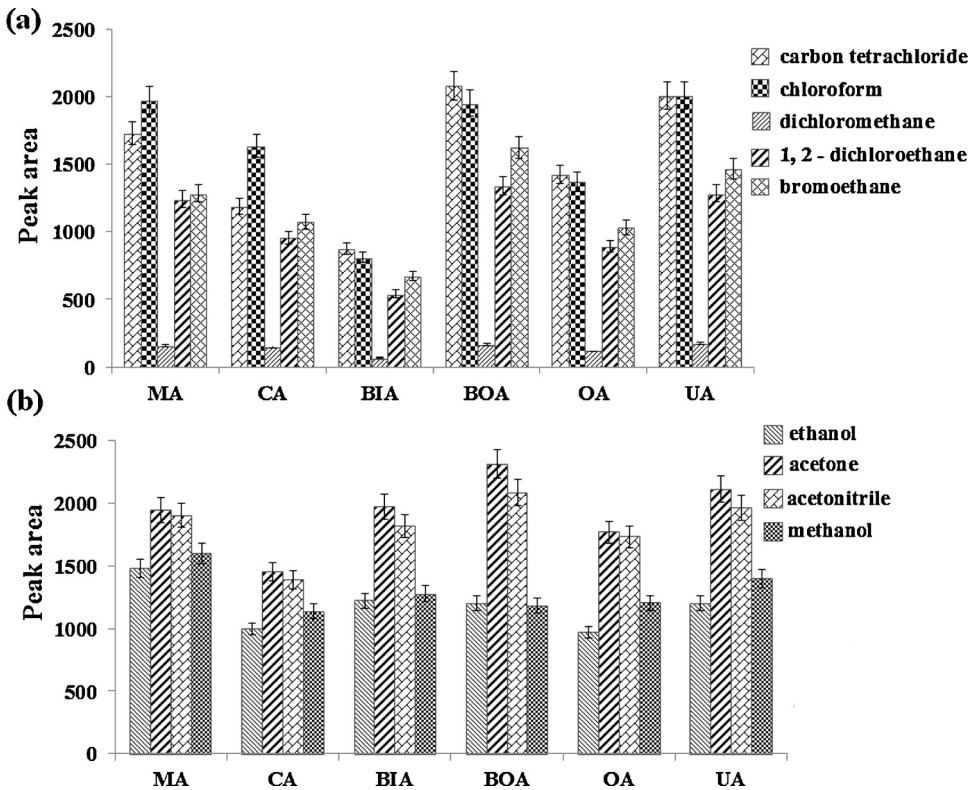

Fig. 2. Effect of different extraction solvents on the extraction efficiency (a); effect of different disperser solvents on the extraction efficiency (b) Abbreviations: maslinic acid (MA), betulinic acid (BIA), corosolic acid (CA), betulonic acid (BOA), ursolic acid (UA) and oleanolic acid (OA).

employed as the optimal extraction and disperser solvents for further studies, respectively.

3.1.2. *Effect of the ionic strength and ultrasonic time*

The effect of ultrasonic time for UA-DLLME was investigated in the range of 0 – 4 min. The analysis results showed the extraction efficiency reached the maximum value when the ultrasonic time was set at 1 min (Fig. 3a). Thus, 1 min was selected as ultrasonic time in this work.

The ionic strength was evaluated by adding sodium chloride into aqueous phase in the range of 0–5% (w/v). The best extraction efficiency was obtained when no sodium chloride was added (Fig. 3b). Thus, extraction procedure in this study was not necessary to add sodium chloride.

3.1.3. *UA-DLLME optimization using response surface methodology*

In order to obtain the optimal extraction conditions, a BBD was employed to optimize the significant variables (pH, extraction solvent volume and disperser solvent volume) and further investigated the existing interaction among these variables. The examined levels and experimental results were listed in Table S1. The analysis of variance (ANOVA) was used to assess the significance of each factor and interaction terms. The ANOVA showed that F value of 460.51 was significant at p-value < 0.0001 level and the lack of fit was no significant, indicating that the second-order polynomial model was very adequate to approximate the functional relationship between response features and experimental design variables. The determination coefficient (R^2) was 0.9983 and the adjusted R^2 was 0.9961, indicating the model has a good relationship with the experimental data. Based on experimental data, a second-order polynomial model was constructed by BBD, which can be described as follows:

$$Y = 1680.40 - 365.88X_1 + 117.13X_2 + 79.25X_3 - 94.75X_1X_2 - 84.00X_1X_3 + 36.50X_2X_3 - 977.58X_1^2 - 263.58X_2^2 - 254.82X_3^2$$

where Y is the predicted response value, and X_1, X_2 and X_3 are the coded values of the pH, extraction solvent volume and disperser solvent volume mentioned. In the optimum design process, three-dimensional surface and contour plots were seen as a visual process of the predicted model equation to study the interactive effect between the two factors selected on the response (Fig. 4a–c). Fig. 4a showed the combined effect of the pH and extraction solvent volume and both of extraction solvent volume and pH have positive effect on the response. By increasing the pH in the range of 5–7, the response value rapidly increased, but it signally decreased from 7 to 9. The response value increased with increasing extraction solvent volume, and reached a maximum value, follows by a slightly declined with its further increased. Fig. 4b described the interactive effect of the pH and dispersion solvent volume on the response value. With increasing of disperser solvent volume, the response value increased firstly, reached the highest value and then decreased slightly. Fig. 4c depicted the combined effect of disperser solvent volume and extraction solvent volume at the fixed value of pH. According to the overall results of the optimization study, the optimal conditions were chosen as follows: pH, 7; extraction solvent volume, 89 μL and disperser solvent volume, 1012 μL.

3.2. *Optimization of pre-column derivatization conditions*

In order to obtain the maximum derivatization yield, a series of experiments was designed to optimize the important derivatization parameters. The effects of co-solvent and catalysts on the derivatization yield were evaluated before starting the response surface optimization process. DMF, dimethylsulfoxide (DMSO), ethyl acetate, dichloromethane, chloroform and acetone were selected as co-solvents and carefully optimized. By comparison,

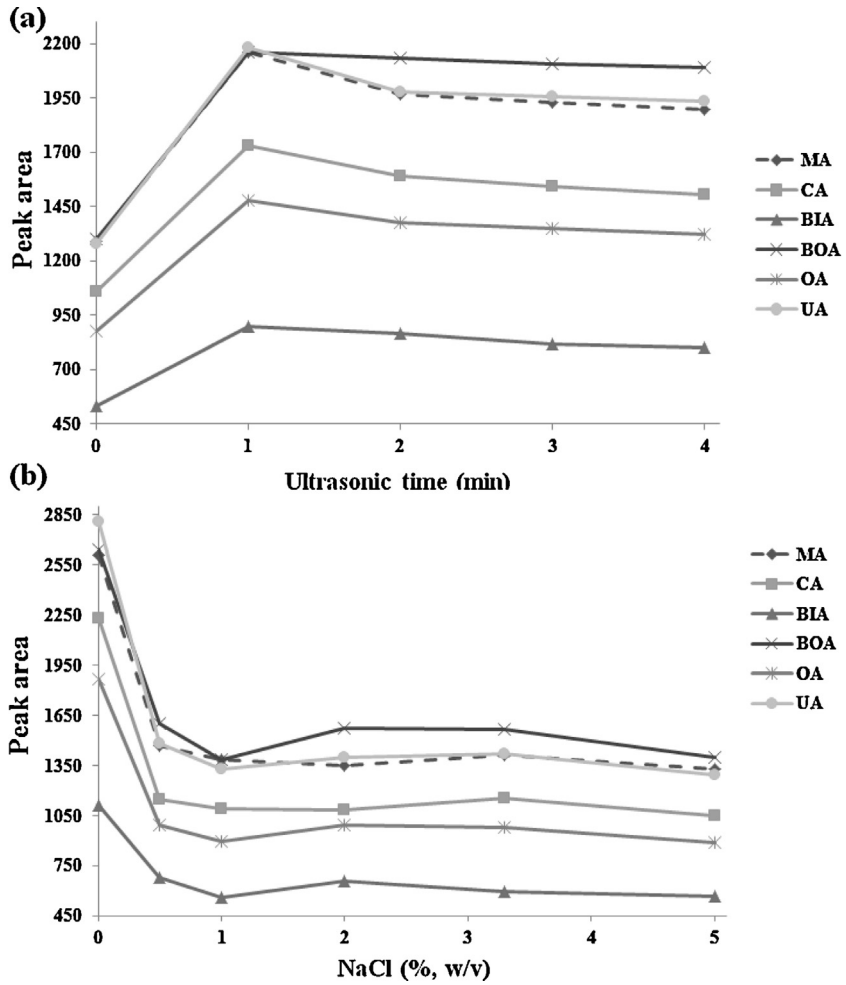

Fig. 3. Effect of ultrasonic time on the extraction efficiency (a); effect of ionic strength on the extraction efficiency (b) Abbreviations: maslinic acid (MA), betulinic acid (BIA), corosolic acid (CA), betulonic acid (BOA), ursolic acid (UA) and oleanolic acid (OA).

the maximum derivatization yield was obtained when DMF was the co-solvent. For the basic catalyst, $(CH_3)_4NCO_3$, K_2CO_3, Na_2CO_3, 2-methylpyridine, $K_2C_2O_4$ and pyridine were investigated. The obtained results showed K_2CO_3 was more suitable than others. In addition, the addition amount of K_2CO_3 was evaluated from 0 to 20 mg. The labeling yield reached the maximum when the amount of K_2CO_3 was 5 mg.

In the following step, a three-level BBD was applied to optimize the main variables affecting the efficiency of derivatization, which were reaction temperature, reaction time and the molar ratio of DDCETS to triterpenic acids. The experimental runs and results were shown in Table S2. The ANOVA results showed the model was significant with p-value <0.0001 and the lack of fit was insignificant. The R^2 was 0.9992 and the adjusted R^2 was 0.9982. The results indicated the quadratic model could effectively describe the experiment response. The quadratic model was shown as below:

$$Y = 1252.20 + 203.00X_1 + 213.50X_2 + 211.75X_3 - 41.25X_1X_2 - 50.75X_1X_3 - 58.25X_2X_3 - 290.98X_1^2 - 261.48X_2^2 - 190.97X_3^2$$

where Y was the predicted response, X_1, X_2 and X_3 were reaction temperature, reaction time and the molar ratio of DDCETS to triterpenic acids, respectively. By three-dimensional graphs, the interactive effects between different variables were visually illustrated in Fig. 4d–f. Fig. 4d depicted the combined effect of reaction temperature and reaction time on the response. Fig. 4e described the combined effect of reaction temperature and the molar ratio of DDCETS to triterpenic acids at the fixed value of reaction time. Fig. 4f showed the combined effect of reaction time and the molar ratio of DDCETS to triterpenic acids at the fixed value of reaction temperature. Based on the analysis results, the optimum experimental conditions were obtained as follows: reaction temperature, 92 °C; time, 22 min and the molar ratio of DDCETS to triterpenic acids, 7.5.

3.3. HPLC separation and mass spectrometry identification

In order to obtain a set of suitable separation conditions, several chromatographic parameters were investigated in detail. In this study, Hypersil C18 (4.6 mm × 200 mm, 5 μm) column, ZORBAX SB-C18 (4.6 mm × 150 mm, 5 μm) column, Eclipse XDB-C8 (4.6 mm × 150 mm, 5 μm) column, Hypersil BDS C8 (4.6 mm × 200 mm, 5 μm) column, Akasil-C18 column (4.6 mm × 250 mm, 5 mm) and Spherisorb C18 (4.6 mm × 200 mm, 5 μm) column were evaluated and compared. The analysis results revealed that good separation could obtain on Hypersil C18 (4.6 mm × 200 mm, 5 μm) column. Due to the hydrophobic character of the triterpenic acids derivatives, methanol-water and acetonitrile-water were investigated and compared as mobile phases. The flow rate was set at 1 mL min^{-1} and injection volume 10 μL. The results suggested that a better separation and peak shape was obtained, when acetonitrile-water was selected as mobile

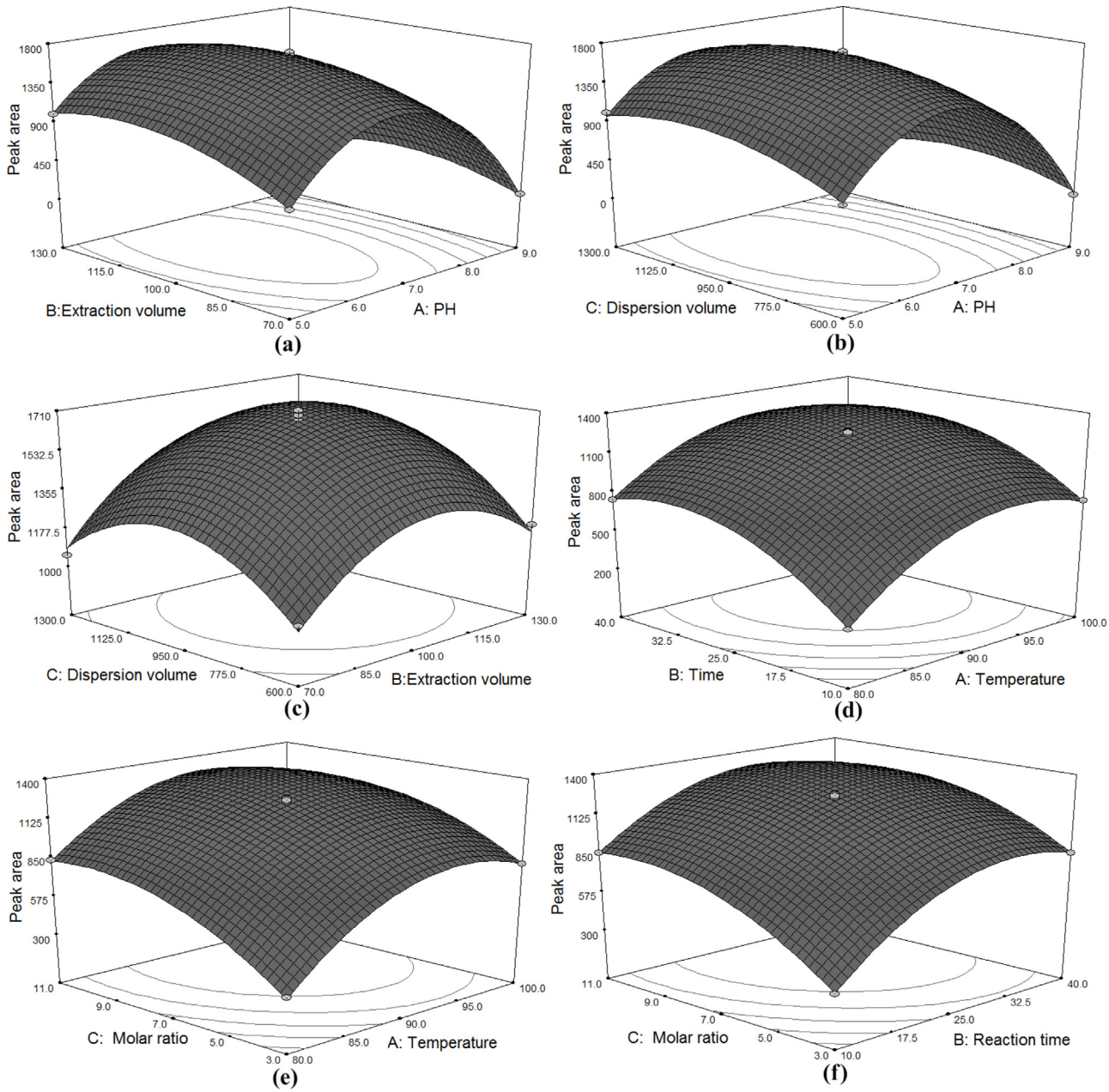

Fig. 4. 3D surface plot showing the significant interaction effects of the UA-DLLME parameters (a–c): (a) extraction solvent volume versus pH; (b) disperser solvent volume versus pH and (c) disperser solvent volume versus extraction solvent volume; response surfaces for triterpenic acids derivatization using the BBD obtained by plotting (e–f): (e) reaction time versus reaction temperature; (f) molar ratio versus reaction temperature and (g) molar ratio versus reaction time.

phase. Finally, the optimized chromatographic conditions were as follows: analytical column, Hypersil C18 (4.6 mm × 200 mm, 5 μm) column; flow-rate, 1 mL min^{-1}; volume injected, 10 μL; temperature, 30 °C and mobile phase, acetonitrile–water. The typical chromatograms of the blank sample, standard analytes and real samples were presented in Fig. 5a–d.

In the present study, the chromatogram peak of analyte was doubly confirmed by comparing its retention time and online mass spectrometry identification. Fig. 6 showed the MS and MS/MS spectrum of representative maslinic acid derivative. Obviously, maslinic acid derivative has the protonated molecule ($[M+H]^+$) peak at m/z 768.18 with fragments at m/z 295.92, 499.24 and 544.25, which were characteristics to maslinic acid derivative. Thus, the characteristic molecular ion and its fragments could be helpful for identifying related triterpenic acid derivative.

3.4. Validation and comparison of the proposed method

Under the optimal conditions, the proposed method was validated by linearity, LODs, LOQs, reproducibility, precision and recovery. Linearity was calculated by preparing eight different concentrations of each triterpenic acid and summarized in Table 1. The linear range of triterpenic acid ranged from 4 to 2000 ng mL^{-1}. Good linearity was observed with correlation coefficients in the range of 0.9989–0.9998. LODs calculated according to a signal-to-noise ratio of 3 ranged from 0.95 to 1.36 ng mL^{-1} and LOQs calculated according to a signal-to-noise ratio of 10 ranged from 3.17 to 4.55 ng mL^{-1}. A 10 μL standard sample was analyzed six times in order to investigate the repeatability by measuring the relative standard deviations (RSD) for peak area and retention time. The RSDs for the retention time and peak area were less than 0.07 and 2.3, respectively. In

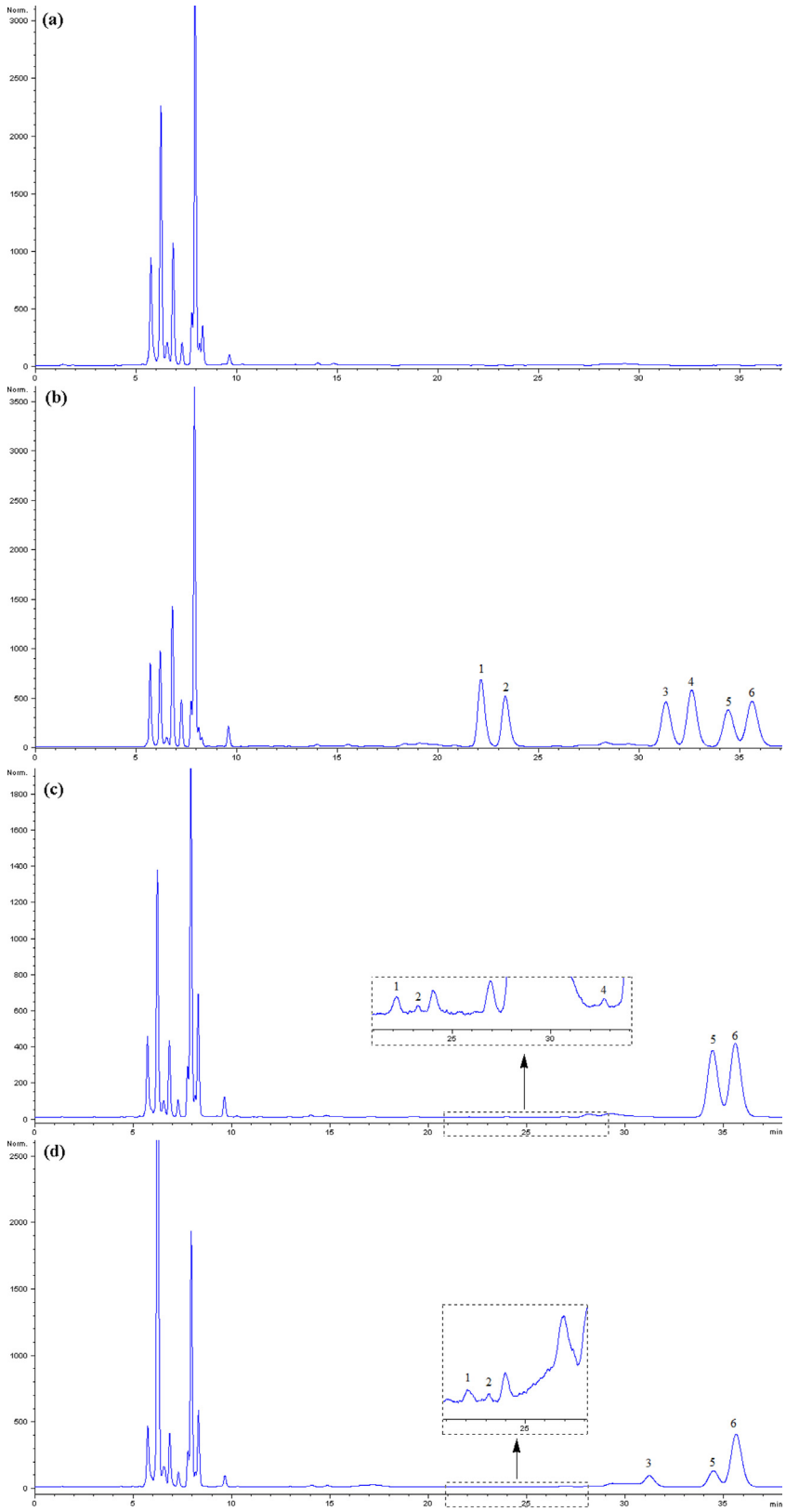

Fig. 5. The representative chromatograms for blank (a), standards (b), *Meconopsis henrici* (c) and *Dracocephalum tanguticum* Maxim (d). Chromatographic conditions: column temperature at 30 °C; excitation wavelength: λ_{ex} 292 nm, emission wavelength: λ_{em} 402 nm; Hypersil C18 (4.6 mm × 200 mm, 5 μm) column; flow rate = 1 mL min^{-1}; peak labels: 1 (maslinic acid), 2 (corosolic acid), 3 (betulinic acid), 4 (betulonic acid), 5 (oleanolic acid) and 6 (ursolic acid).

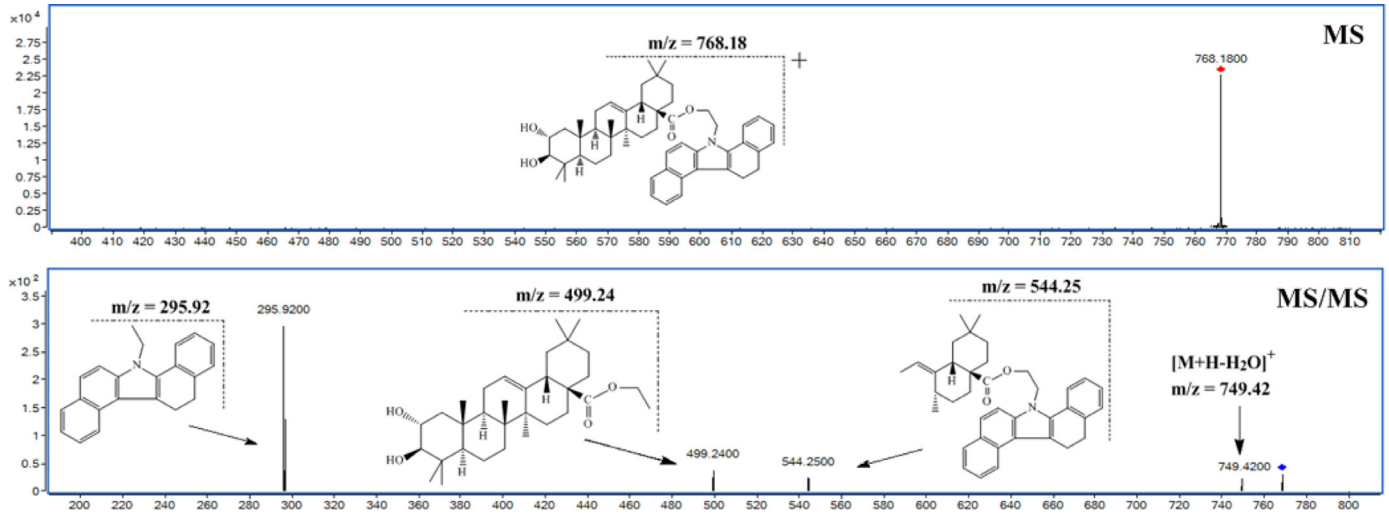

Fig. 6. MS spectra of representative maslinic acid and the cleavage mode of protonated molecular ion.

Table 1
Linear regression equation, correlation coefficients, LODs, LOQs, reproducibility of retention time and peak area, intra- and interday precisions.

Peak	Analyte	Regression equation[a]	r	LOD[b] (ng mL^{-1})	LOQ[c] (ng mL^{-1})	Repeatability (RSD%, n = 6)		Precision (RSD %, n = 6)	
						Retention time	Peak area	Intraday	Interday
1	Maslinic acid	$y = 17.976x - 14.313$	0.9992	1.12	3.74	0.03	1.6	2.9	4.6
2	Corosolic acid	$y = 14.526x - 20.806$	0.9989	1.34	4.48	0.07	2.3	2.1	3.8
3	Betulinic acid	$y = 15.707x - 27.834$	0.9996	1.30	4.34	0.01	1.2	2.6	4.1
4	Betulonic acid	$y = 20.023x - 76.839$	0.9997	0.95	3.17	0.06	2.1	3.3	3.9
5	Oleanolic acid	$y = 13.964x - 36.961$	0.9994	1.36	4.55	0.06	1.9	2.8	5.2
6	Ursolic acid	$y = 18.670x - 67.865$	0.9998	1.08	3.61	0.05	1.2	1.9	3.5

[a] y = peak area; x = theoretical concentration of triterpenic acids.
[b] Signal/noise ratio = 3:1.
[c] Signal/noise ratio = 10:1.

addition, the precision of the proposed method was an important parameter and evaluated in terms of intraday and interday variability. The intraday and interday precision (RSD) ranged from 1.9 to 3.3% and from 3.5 to 5.2%, respectively. The accuracy of the method was determined by spiking three different levels of the mixed standards into real samples and analyzing the percentage recoveries. The percentage recoveries were calculated by comparing the concentration obtained from the spiked samples with actual added concentration and reported in Table 2. The percentage recovery ranged from 92.5 to 106.5%.

A comparison of the present method with other reported methods for determination of triterpene acids was summarized in Table 3. The LODs achieved by the proposed method were in the range of 0.95–1.36 ng mL^{-1}, which were significantly lower than those reported HPLC-UV [21,29] or ELSD [30] methods (Table 3). Although the LODs were comparable to those reported HPLC-FLD methods [6,31], DDCETS has stronger photoluminescence property and shorter derivatization time. In addition, UA-DLLME technique was firstly used to pretreat triterpene acids extracts before chromatographic analysis, and it did not require expensive instrumentation and complex operation, consumed much less toxic organic solvent and exhibited excellent extraction capacity. All these results indicated that the developed method was sensitive, rapid, reliable and suitable for the determination of triterpene acids in real samples.

3.5. Analysis of real samples

To evaluate the practical applicability, the proposed method was applied for the determination of six triterpene acids from several traditional Chinese herbs including *S. racemosa*, *C. impatiens*, *M. henrici*, *D. tanguticum* Maxim, *C. pulmonarium* and *P. granatum* peel. The analytical results of these herbs were given in Table 4. The typical chromatograms of *M. henrici* and *D. tanguticum* Maxim are illustrated in Fig. 5c and d. From Table 4, it can be found that the varieties and contents of the triterpene acids in six herbs were

Table 2
Recovery studies of the proposed method at three concentrations levels.

Analyte	Concentration 1			Concentration 2			Concentration 3		
	Added (μg mL^{-1})	Found (μg mL^{-1})	Recovery (%)	Added (μg mL^{-1})	Found (μg mL^{-1})	Recovery (%)	Added (μg mL^{-1})	Found (μg mL^{-1})	Recovery (%)
Maslinic acid	0.4	0.38	95	0.8	0.77	96.3	2.0	2.11	105.5
Corosolic acid	0.5	0.53	106	1.0	0.98	98	2.5	2.39	95.6
Betulinic acid	0.5	0.47	94	1.0	0.96	96	2.5	2.46	98.4
Betulonic acid	0.3	0.29	96.7	0.6	0.62	103.3	1.5	1.54	102.7
Oleanolic acid	0.4	0.37	92.5	0.8	0.79	98.8	2.0	2.13	106.5
Ursolic acid	0.4	0.41	102.5	0.8	0.75	93.8	2.0	1.90	95

Table 3
Comparison of performance of the proposed method with that of other analytical methods.

Methods	Extraction method	Derivatization Reagent and reaction conditions	Detection	Sample type	Repeatability	LOD (ng mL^{-1})	Reference
HPLC	SPE-SI-PANI[a]	NO	UV at 200 nm	Plant materials	–	110–150	[21]
HPLC	SLE[b]	NO	UV at 208 nm	Fomes officinalis	<4.68	390–830	[29]
HPLC	SLE	NO	ELSD	Fruits	<2.7	3200–7030	[30]
HPLC	SLE	CPMS,[c] 30 min, 90 °C	FLD $\lambda_{ex}/\lambda_{em}$ = 293/360 nm	Fruits	–	0.5–1.0	[31]
HPLC	SLE	AETS,[d] 28 min, 92 °C	FLD $\lambda_{ex}/\lambda_{em}$ = 404/440 nm	Fruits	<1.52	1.68–2.04	[6]
HPLC	SLE-DLLME	DDCETS, 22 min, 92 °C	FLD $\lambda_{ex}/\lambda_{em}$ = 292/402 nm	Chinese medicinal herbs	< 2.3	0.95–1.36	This work

Abbreviations: [a]SPE-SI-PANI, solid phase extraction with silica gel coated with a film of polyaniline; [b]SLE, solid–liquid extraction; [c]CPMS, 1-(9H-carbazol-9-yl) propan-2-yl-methanesulfonate; [d]AETS, acridone-9-ethyl-p-toluenesulfonate.

Table 4
Triterpenic Acid Content in herbs Samples.

Samples	Maslinic acid (mg kg^{-1})	Corosolic acid (mg kg^{-1})	Betulinic acid (mg kg^{-1})	Betulonic acid (mg kg^{-1})	Oleanolic acid (mg kg^{-1})	Ursolic acid (mg kg^{-1})
Comastoma pulmonarium	2.6 ± 13.5[a]	1.9 ± 0.6	ND[b]	3.5 ± 9.3	722.9 ± 2.8	631.5 ± 5.1
Meconopsis henrici	3.3 ± 9.7	2.1 ± 11.8	ND	5.2 ± 16.5	229.7 ± 27.3	154.4 ± 0.4
Swertia racemosa	2.5 ± 2.1	1.6 ± 7.1	ND	3.5 ± 3.3	963.5 ± 11.6	71.6 ± 0.7
Corydalis impatiens	13.5 ± 16.4	8.1 ± 10.1	ND	11.7 ± 0.5	783.3 ± 5.9	128.8 ± 8.2
Dracocephalum tanguticum Maxim	3.2 ± 21.2	2.0 ± 4.5	169.26 ± 0.8	ND	252.9 ± 4.7	643.1 ± 13.7
Punica granatum peel	8.4 ± 5.9	16.3 ± 10.7	4.4 ± 7.5	ND	28.2 ± 1.6	44.9 ± 7.8

[a] Data are expressed as mean value ± S.D.
[b] Not detected.

significantly different. *C. pulmonarium* has the highest amount of triterpene acids, followed by *D. tanguticum* Maxim, *S. racemosa*, *C. impatiens*, *M. henrici* and *P. granatum* peel. Ursolic and oleanolic acids were the two major constituents in six herbs, and maslinic acid, betulonic acid and corosolic acid were minor constituents. In addition, betulinic acid was only detected in *D. tanguticum* Maxim and *P. granatum* peel. These experimental data may be valuable to control the herbs quality and better understand these herbs. These data further suggested the proposed method can be an effective analytical method for the determination of triterpene acids in real samples.

4. Conclusions

In the present study, a novel method was developed and applied to pretreat and detect six triterpene acids. The main variables affecting derivatization yield and UA-DLLME efficiency were optimized by response surface methodology based on Box–Behnken design. Under the best experimental conditions, the proposed method could offer favorable extraction efficiency, high derivatization yield, good sensitivity and low detection limit. Moreover, the method was successfully applied to detect triterpene acids in several Chinese tradition medicinal herbs. The results further indicated that the proposed method was sufficiently competent to determine triterpene acids in real samples, and it had demonstrated great potential to analyze triterpenic acids in other medical herbs.

Conflict of interest

Hongliang Wu declares that he has no conflict of interest. Guoliang Li declares that he has no conflict of interest. Shucheng Liu declares that he has no conflict of interest. Guang Chen declares that he has no conflict of interest. Di Liu declares that she has no conflict of interest. Na Hu declares that she has no conflict of interest. Yourui Suo declares that he has no conflict of interest. Jinmao You declares that he has no conflict of interest. This article does not contain any studies with human or animal subjects.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jpba.2014.10.031.

References

[1] R.T.S. Frighetto, R.M. Welendorf, E.N. Nigro, N. Frighetto, A.C. Siani, Isolation of ursolic acid from apple peels by high speed counter-current chromatography, Food Chem. 106 (2008) 767–771.
[2] F. Gbaguidi, G. Accrombessi, M. Moudachirou, J. Quetin-Leclercq, HPLC quantification of two isomeric triterpenic acids isolated from *Mitracarpus scaber* and antimicrobial activity on *Dermatophilus congolensis*, J. Pharm. Biomed. Anal. 39 (2005) 990–995.
[3] L. Novotny, M.E. Abdel-Hamid, H. Hamza, I. Masterova, D. Grancai, Development of LC–MS method for determination of ursolic acid: application to the analysis of ursolic acid in *Staphylea holocarpa* Hemsl, J. Pharm. Biomed. Anal. 31 (2003) 961–968.
[4] A. Gupta, N.R. Sheth, S. Pandey, D.R. Shah, J.S. Yadav, Determination of ursolic acid in fractionated leaf extracts of *Ocimum gratissimum* Linn and in developed herbal hepatoprotective tablet by HPTLC, Phcog. J. 5 (2013) 156–162.
[5] N.P. Joshi, V.V. Vaidya1, S.V. Patankar, M.B. Kenkare, A rapid bioanalytical method for simultaneous determination of coroslic acid and asiatic acid in human plasma by soild phase extraction and direct injection into liquid chromatrography mass spectrometry, Int. J. Bioassays 02 (12) (2013) 1585–1591.
[6] G. Li, J. You, C. Song, L. Xia, J. Zheng, Y. Suo, Development of a new HPLC method with precolumn fluorescent derivatization for rapid, selective and sensitive detection of triterpenic acids in fruits, J. Agric. Food Chem. 59 (2011) 2972–2979.

[7] M. Wójciak-Kosior, Separation and determination of closely related triterpenic acids by high performance thin-layer chromatography after iodine derivatization, J. Pharm. Biomed. Anal. 45 (2007) 337–340.
[8] H. Cheung, Q. Zhang, Enhanced analysis of triterpenes, flavonoids and phenolic compounds in *Prunella vulgaris* L. by capillary zone electrophoresis with the addition of running buffer modifiers, J. Chromatogr. A 1213 (2008) 231–238.
[9] P. Yang, Y. Li, X. Liu, S. Jiang, Determination of free isomeric oleanolic acid and ursolic acid in *Pterocephalus hookeri* by capillary zone electrophoresis, J. Pharm. Biomed. Anal. 43 (2007) 1331–1334.
[10] H. Wang, Z. Wang, W. Guo, Comparative determination of ursolic acid and oleanolic acid of *Macrocarpium officinalis* (Sieb. et Zucc.) Nakai by RP-HPLC, Ind. Crop. Prod. 28 (2008) 328–332.
[11] M.K. Lee, Y.M. Ahn, K.R. Lee, J.H. Jung, O.S. Jung, J. Hong, Development of a validated liquid chromatographic method for the quality control of *Prunellae* Spica: determination of triterpenic acids, Anal. Chim. Acta 633 (2009) 271–277.
[12] G. Janicsák, K. Veres, A. Zoltán Kakasy, I. Máthé, Study of the oleanolic and ursolic acid contents of some species of the Lamiaceae, Biochem. Syst. Ecol. 34 (2006) 392–396.
[13] G. Janicsák, k. Veres, M. Kállai, I. Máthe, Gas chromatographic method for routine determination of oleanolic and ursolic acids in medicinal plants, Chromatographia 58 (2003) 295–299.
[14] A. Banerjee, R.T. Sane, K. Mangaonkar, S. Shailajan, A. Deshpande, G. Gundi, Quantitation of oleanolic acid in *Oldenlandia corymbosa* L. whole-plant powder by high-performance thin-layer chromatography, J. Planar Chromatogr. 19 (2006) 68–72.
[15] N. Banno, T. Akihisa, H. Tokuda, K. Yasukawa, H. Higashihara, M. Ukiya, K. Watanabe, Y. Kimura, J. Hasegawa, H. Nishino, Triterpene acids from the leaves of Perilla frutescens and their anti-inflammatory and antitumor-promoting effect, Biosci. Biotechnol. Biochem. 68 (2004) 85–90.
[16] S. Werner, S. Nebojsa, W. Robert, S. Robert, K. Olaf, Complete assignments of 1H and 13C NMR resonances of oleanolic acid, 18α-oleanolic acid, ursolic acid and their 11-oxo derivatives, Magn. Reson. Chem. 41 (2003) 636–638.
[17] S. Morales-Muñoz, M.D.L. de Castro, Dynamic ultrasound-assisted extraction of colistin from feeds with on-line pre-column derivatization and liquid chromatography-fluorimetric detection, J. Chromatogr. A 1066 (2005) 1–7.
[18] X.L. Du, H.S. Zhang, X.F. Guo, Y.H. Deng, H. Wang, 6-Oxy-(acetyl piperazine) fluorescein as a new fluorescent labeling reagent for free fatty acids in serum using high-performance liquid chromatography, J. Chromatogr. A 1169 (2007) 77–85.
[19] T. Yoshida, A. Uetake, H. Yamaguchi, N. Nimura, T. Kinoshita, New preparation method for 9-anthryldiazomethane (ADAM) as a fluorescent labeling reagent for fatty acids and derivatives, Anal. Biochem. 173 (1988) 70–74.
[20] M. Song, T.J. Hang, Y. Wang, L. Jiang, X.L. Wu, Z. Zhang, J. Shen, Y. Zhang, Determination of oleanolic acid in human plasma and study of its pharmacokinetics in Chinese healthy male volunteers by HPLC tandem mass spectrometry, J. Pharm. Biomed. Anal. 40 (2006) 190–196.
[21] I. Sowa, M. Wojciak-Kosior, K. Rokicka, R. Kocjan, G. Szymczak, Application of solid phase extraction with the use of silica modified with polyaniline film for pretreatment of samples from plant material before HPLC determination of triterpenic acids, Talanta 122 (2014) 51–57.
[22] B. Claude, P. Morin, M. Lafosse, A.S. Belmont, K. Haupt, Selective solid-phase extraction of a triterpene acid from a plant extract by molecularly imprinted polymer, Talanta 75 (2008) 344–350.
[23] M. Moradi, Y. Yamini, S. Seidi, M. Ghambarian, A. Esrafili, Ultrasound-assisted emulsification microextraction using low density solvent for analysis of toxic nitrophenols in natural waters, Int. J. Environ. Anal. Chem. 93 (2013) 199–212.
[24] G. Vas, K. Vekey, Solid-phase microextraction: a powerful sample preparation tool prior to mass spectrometric analysis, J. Mass Spectrom. 39 (2004) 233–254.
[25] M. Rezaee, Y. Assadi, M.R. Milani Hosseini, E. Aghaee, F. Ahmadi, S. Berijani, Determination of organic compounds in water using dispersive liquid–liquid microextraction, J. Chromatogr. A 1116 (2006) 1–9.
[26] Y. Han, X. Jia, X. Liu, T. Duan, H. Chen, DLLME combined with GC–MS for the determination of methylparaben, ethylparaben, propylparaben and butylparaben in beverage samples, Chromatographia 72 (2010) 351–355.
[27] J. You, Y. Shi, Y. Ming, Z. Yu, Y. Yi, J. Liu, Development of a sensitive reagent, 1,2-benzo-3,4-dihydrocarbazole-9-ethyl-p-toluenesulfonate, for determination of bile acids in serum by HPLC with fluorescence detection, and identification by mass spectrometry with an APCI source, Chromatographia 60 (2004) 527–535.
[28] E. Xia, B. Wang, X. Xu, L. Zhu, Y. Song, H. Li, Microwave-assisted extraction of oleanolic acid and ursolic acid from *Ligustrum lucidum* Ait, Int. J. Mol. Sci. 12 (2011) 5319–5329.
[29] E.N. Li, J.G. Luo, L.Y. Kong, Qualitative and quantitative determination of seven triterpene acids in *Eriobotrya japonica* Lindl. by high-performance liquid chromatography with photodiode array detection and mass spectrometry, Phytochem. Anal. 20 (2009) 338–343.
[30] S. Guo, J.A. Duan, Y.P. Tang, N.Y. Yang, D.W. Qian, S.L. Su, E.X. Shang, Characterization of triterpenic acids in fruits of *ziziphus* species by HPLC-ELSD-MS, J. Agric. Food Chem. 58 (2010) 6285–6289.
[31] S. Zhang, Y. Sun, Z. Sun, X. Wang, J. You, Y. Suo, Determination of triterpenic acids in fruits by a novel high performance liquid chromatography method with high sensitivity and specificity, Food Chem. 146 (2014) 264–269.