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Analytical Methods

A developed pre-column derivatization method for the determination of free fatty acids in edible oils by reversed-phase HPLC with fluorescence detection and its application to *Lycium barbarum* seed oil

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ABSTRACT

Free fatty acids (FFAs) in edible oils are one of the most frequently determined quality indices during production, storage, and marketing. In this study, a simple, stable and sensitive method for the simultaneous determination of saturated and unsaturated FFAs from edible oils using 2-(11*H*-benzo[a]carbazol-11-yl)-ethyl-4-methylbenzenesulphonate (BCETS) as labelling reagent with fluorescence detection has been established. FFAs are derivatized by BCETS and separated on a reversed-phase Eclipse XDB-C_8 column with a gradient elution. The results indicated that all FFAs were found to be given an excellent linear response with correlation coefficients of >0.9994. The detection limits at a signal-to-noise ratio of 3 were in the range of 0.22–1.06 ng/mL. When applied to *Lycium barbarum* seed oils, the developed method showed good reproducibility. The effect of extraction methods including supercritical CO_2 and organic solvent extraction on the FFAs composition has also been investigated. Meanwhile, this method exhibits powerful potential for the trace analysis of short- and long-chain free fatty acids from edible oils, foodstuff and other complex samples.

1. Introduction

High quality edible oil is not only healthy but can also increase the delicious flavour to dishes. The quality of edible oil is determined by various factors such as flavour, free fatty acids (FFAs) content, oxidation, hydrolysis and so on (Keurentjes, Doornbusch, & Riet, 1991). FFAs are one of the most frequently determined quality indices during production, storage, and marketing (price dictated by FFA content) (Saad et al., 2007). FFAs in edible oils are undesirable, it results in lower flavour quality and stability of the oil, moreover high levels of FFAs will result in rancidity of the oil (Keurentjes et al., 1991). Technology is not available to avoid the appearance of FFAs in oil. FFAs are usually removed from crude oil by refining in industrial production. However, these refining steps are not 100% efficient (Fadiloglu, Ciftci, & Gogus, 2003), thus, quantitative determination of FFAs is equally important for the quality control, trading and storage of the edible oils. Determination of FFAs in edible oils and fats has been investigated (Canham & Pacey, 1987; Che Man, Moh, & van de Voort, 1999; Ekstrm, 1981; Kwon & Rhee, 1986; Man & Moh, 1998; Mariotti & Mascini, 2001; Nouros, Georgiou, & Polissiou, 1997; Puchades, Suescun, & Maquieira, 1994; Saad et al., 2007; Schooner, Simard, & Pandian, 1991; Tur'yan, Berezin, Kuselman, & Shenhar, 1996; van de Voort, Ismail, Sedman, & Emo, 1994; Zhi, Ríos, & Valcárcel, 1996), but these methods cannot accurately determine each single FFAs and frequently interfere with the sample components. The most used methods for the determination of FFAs are based on liquid–liquid extraction using methyl tert-butyl ether, and then derivatization to their respective methyl-trimethylsilyl-esters, and analysis using Gas Chromatography (GC) or GC coupling with mass spectrometry (MS) analysis (Ai, 1997; Cocito & Delfini, 1994; Kanya, Rao, & Sastry, 2007; Kondyli, Katsiari, Masouras, & Voutsinas, 2002; Poveda & Cabezas, 2006; Refsgaard, Brockhoff, & Jensen, 2000; Tan, Ghazali, Kuntom, Tan, & Ariffin, 2009; Xu, Xu, Zhou, Wang, & Li, 2008). In contrast with GC, use of High Performance Liquid Chromatography (HPLC) allows the FFAs to be directly converted to a large number of different derivatives. Derivatization can overcome some problems, such as tailing peaks and low detector sensitivity, by the formation of less polar compounds, which can be more easily analysed by Liquid Chromatography (LC). Therefore, derivatization

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of these analytes with labelling reagents has been widely adopted because HPLC with UV, especially fluorescence detection, has higher sensitivity. However, Toyo'oka (2002) have reported that many of these reagents have limitations in their applications to samples analysis such as low detection sensitivity, short detection wavelengths, poor stability, tedious analytical procedure, and so on. In this study, 2-(11*H*-benzo[a]carbazol-11-yl)-ethyl-4-methylbenzenesulphonate (BCETS), which is synthesised in our research group, was developed for the simultaneous determination of 30 saturated and unsaturated FFAs from extracted edible oils.

Fruit from *Lycium barbarum* L. (*L. barbarum*) in the family Solanaceae is well known in traditional Chinese herbal medicine and nowadays has been widely used as a popular functional food (Li, Ma, & Liu, 2007). It distributes over China and the southeast of Europe. The pharmacological activities associated with *L. barbarum* include hypoglycaemic, immunomodulation, anti-hypertension, lipotropic, protecting hepatic function, anti-ageing, anti-fatigue, antioxidant and so on (Li, 2007; Yu, Ho, So, Yuen, & Chang, 2006). The seeds of *L. barbarum* contain abundant oil, more than 87% of which are unsaturated fatty acids such as linolenic acid and linoleic acid. To the best of our knowledge, there is no prior report on FFA composition from *L. barbarum* seed oil.

The aims of the present work are: (1) to develop a simple, sensitive method for the simultaneous determination of saturated and unsaturated FFAs from the extracted edible oils using 2-(11*H*-benzo-[a]carbazol-11-yl)-ethyl-4-methylbenzenesulphonate (BCETS) as labelling reagent by HPLC with fluorescence detection; (2) to evaluate the effect of different extracting methods on the FFA composition from extracted *L. barbarum* seed oils.

2. Material and methods

2.1. Instrumentation

The HPLC system was Agilent HP 1100 series (Waldbronn, Germany) with a fluorescence detector (FLD) (model G1321A). The mass spectrometer 1100 Series LC/MSD Trap-SL (ion trap) from Bruker Daltonik (Bremen, Germany) was equipped with an atmospheric pressure chemical ionisation (APCI) source. Derivatives were separated on a reversed-phase Eclipse XDB-C_8 column (150× 4.6 mm, 5 μm, Agilent) by a gradient elution. Fluorescence excitation and emission spectra were obtained at a F7000 fluorescence spectrophotometer (Hitachi, Japan).

2.2. Plant material

The matured fruits of *L. barbarum* were collected from Qinghai province (China) in October 2006. *L. barbarum* seeds were dried under a stream of nitrogen and ground by a rotary mill and then sieved (0.5 mm diameter) for supercritical CO_2 or organic solvent extraction.

2.3. Chemicals and materials

All fatty acids used as standards were of chromatographic grade and purchased from Sigma Reagent Co. (USA). Spectroscopically pure acetonitrile (ACN) was purchased from Yuwang Company, China. *N*,*N*-dimethylformamide (DMF), potassium carbonate (K_2CO_3), pyridine, and chloroform were of analytical grade obtained from Shanghai Chemical Reagent Co. (Shanghai, China). Water was purified on a Milli-Q system (Millipore, Bedford, MA, USA). All other reagents used were also of analytical grade unless otherwise stated.

2.4. Synthesis of 1,2-benzo-carbazole-9-ethyl-p-toluenesulfonate (BCETS)

In brief, 1,2-benzo-3,4-dihydrocarbazole was synthesised according to the method previously described by You et al. (2004). The synthesis procedure is presented in Fig. 1. BCETS are white crystals. m.p.155.8–156.5 °C, found (%): C 72.32, H 4.98, N 3.35, O 11.56, S 7.79; calculated (%): C 71.94, H 5.52, N 3.36, O 11.51, S 7.67. IR (KBr): 3053.20(Ph–N–CH_2–); 2998.66, 2953.25, 2937.57, 2898.95, 2836.57(Ph); 1594.93(Ph); 1468.75, 1368.70(C–H), 1360.36 (–C–SO_2–); 1175.50, 1095.42 (Ph–S–), 974.15, 745.16, 769.8; MS: *m/z* $[M+H]^+$: 416. The fluorescence excitation and emission wavelengths of BCETS in acetonitrile or methanol were λ_{ex} 279 nm and λ_{em} 380 nm, respectively.

2.5. Supercritical CO_2 oil extraction from L. barbarum seed

Supercritical CO_2 fluid (SFE) extraction measurements were conducted in a semi-bath flow extraction apparatus (Hua'an Supercritical Fluid Extraction Corp., Nantong, China). The schematic flow diagram was described in detail as previously described by Koga, Iwai, Hata, Yamamoto, and Arai (1996). The extraction conditions were as follows: extraction pressure, 30 MP; extraction temperature, 45 °C; extraction time, 90 min. Extractions in the presence and absence of modifier (ethanol 10%, v/v) were carried out for the purpose of comparison.

2.6. Organic solvent extraction

The prepared seed samples (25 g) were macerated with 300 mL solvent (*n*-hexane or petroleum ether) at 45 °C for 4 h. The extraction process was repeated three times. The resulting extracts were combined; the solvent was removed under reduce pressure (40 °C); the residue was weighed and analysed.

2.7. Preparation of standard solutions

BCETS solution (2.0×10^{-3} mol L^{-1}) was prepared by dissolving 8.32 mg BCETS in 10 mL anhydrous acetonitrile obtained by distiling HPLC grade acetonitrile dried with P_2O_5. Individual stock solutions of the fatty acids (1.0×10^{-3} mol L^{-1}) were prepared in ACN/DMF (1:1, v/v) and diluted to the concentration of 5.0×10^{-5} mol L^{-1} with the same solvent composition. When not in use, all reagent solutions were stored at 4 °C in a refrigerator.

2.8. Derivatization design and statistical analysis

The derivatization is of great importance for the sufficient labelling of the analysed components. In traditional methods, derivatization parameters are optimised by one-factor-at-a-time experiments. Procedure is troublesome and time-consuming as well as ignoring the interaction effect of parameters. Compared to the classical methods, response surface methodology (RSM) is more efficient, requires fewer data and provides interaction effects on the response besides factor effects.

Three factors including the amount of BCETS (mol (BCETS)/mol (total fatty acids)), reaction temperature and reaction time, were chosen based on single-factor designs for further optimisation by employing a three-level, three-variable Box–Behnken design (BBD) from RSM. Lauric acid ($C_{12}H_{24}O_2$) and docosanoic acid ($C_{22}H_{44}O_2$) were used as standards of the tested compounds. The derivatization scheme of BCETS with fatty acids is shown in Fig. 1A. The coded and uncoded independent variables used in the RSM design and their respective levels were listed in Table 1. A total of 17 experiments were designed (Table 1). The average peak area of fatty acids (lauric acid and docosanoic acid) was taken

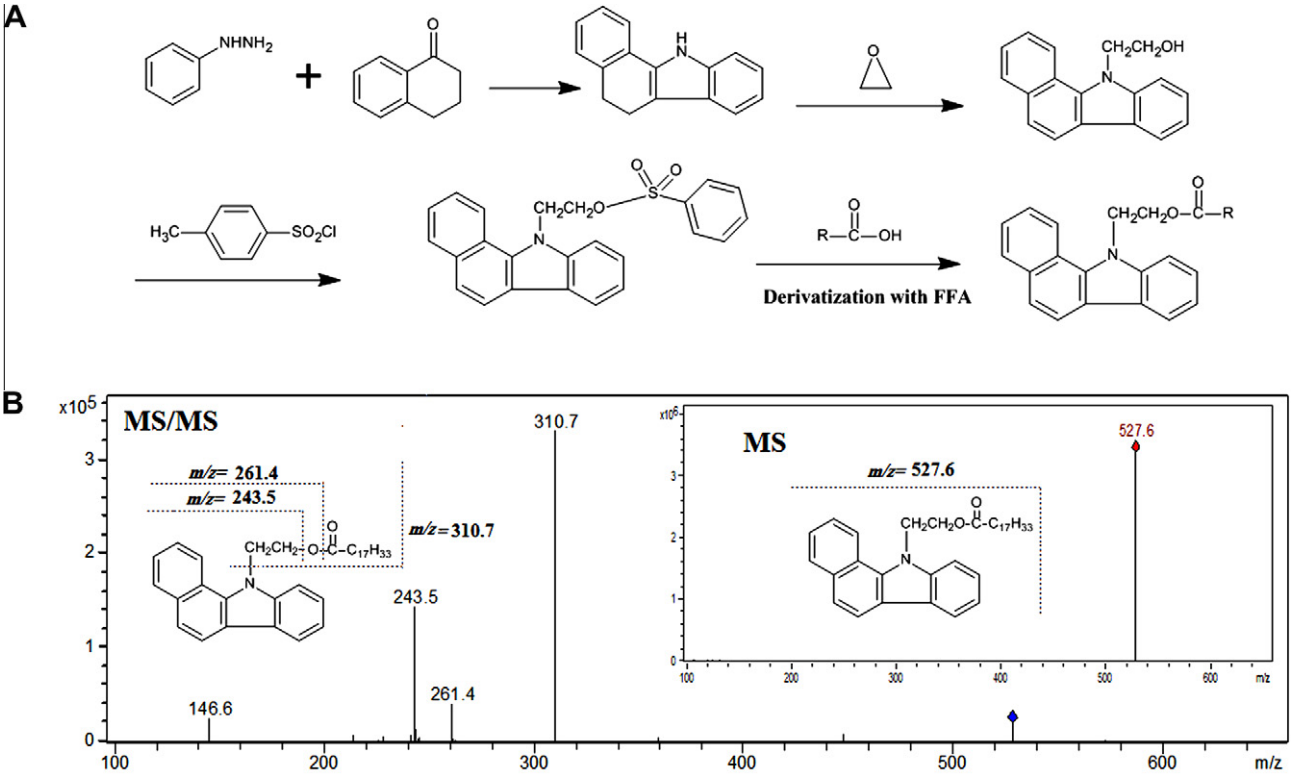

Fig. 1. Synthesis scheme of BCETS reagent and derivatization with fatty acids (A); the cleavage mode of the typical BCETS-C_{18} fatty acid derivative (MS and MS/MS) (B).

Table 1
Experimental data for the peak area obtained from Box–Behnken design (n = 3).

Run	Independent variable			Y (peak area)
	X_1 (amount of BCETS)	X_2 (temperature, °C)	X_3 (time, min)	
1	4(−1)	80(0)	40(+1)	850
2	12(+1)	80(0)	10(−1)	800
3	4(−1)	80(0)	10(−1)	650
4	4(−1)	100(+1)	25(0)	700
5	12(+1)	100(+1)	25(0)	750
6	12(+1)	60(−1)	25(0)	380
7	8(0)	80(0)	25(0)	1100
8	8(0)	80(0)	25(0)	1050
9	8(0)	60(−1)	10(−1)	450
10	8(0)	100(+1)	40(+1)	900
11	8(0)	60(−1)	40(+1)	500
12	8(0)	80(0)	25(0)	1080
13	8(0)	80(0)	25(0)	1060
14	8(0)	100(+1)	10(−1)	700
15	8(0)	80(0)	25(0)	1120
16	12(+1)	80(0)	40(+1)	1000
17	4(−1)	60(−1)	25(0)	300

as the response, Y, which reflects the detector response. Regression analysis was performed, based on the experimental data, and was fitted into an empirical second-order polynomial model as shown below in the following equation.

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3$$

where Y represents the response variable, β_0 is a constant, β_i, β_{ii} and β_{ij} are the linear, quadratic and interactive coefficients, respectively.

A software Design-Expert 7.1.3 (State-Ease, Inc., Minneapolis MN, USA) was used to obtain the coefficients of the quadratic polynomial model. The quality of the fitted model was expressed by the determined coefficient (R^2), and its statistical significance was checked by an F-test.

2.9. HPLC–MS conditions

HPLC separation of fatty acid derivatives was carried out on a reversed-phase Eclipse XDB-C_8 column (150 × 4.6 mm, 5 μm, Agilent) with a gradient elution. Eluent A was water, B was a mixed solvent of ACN/DMF (1:1.v/v), and C was acetonitrile (100%). The flow rate was constant at 1.0 mL min^{-1} and the column temperature was set at 30 °C. The injection volume was 10 μL. The fluorescence excitation and emission wavelengths were set at λ_{ex} 279 nm and λ_{em} 380 nm, respectively. The gradient elution programme was as follows: 45–10% (A), 50–80% (B) from 0 to 30 min; 10–3% (A), 80–87% (B) from 30 to 40 min; 3–2% (A), 87–88% (B) from 40 to 50 min; and 2–0% (A), 88–85%(B) from 50 to 70 min.

FFA derivatives separated by HPLC were identified by online mass spectra equipped with an APCI source. The APCI probe was heated to 400 °C to ensure complete vaporisation of the column effluent. Other mass spectra conditions were as follows: APCI source in positive-ion detection mode; nebulizer pressure 60 psi; dry gas temperature, 350 °C; dry gas flow, 5.0 L/min. APCI Vap temperature 350 °C; corona current (nA) 4000 (pos); capillary voltage 3500 V.

3. Results and discussion

3.1. Fluorescence spectra of BCETS–FFA derivatives

Maximum excitation and emission wavelengths which are set in HPLC fluorescence detector are important for the detection sensitivity of FFA derivatives. To determine the fluorescence spectra of FFA derivatives, each of FFA derivatives was prepared by individual derivatization and purification on a solid-phase extraction column

(ODS C18, Dalian Elite Analytic Instruments Co. Ltd., Dalian, China), and scanned on the 650-10S fluorescence spectrophotometer. Results indicated the maximum excitation and emission wavelengths for BCETS–FFA derivatives were 279 and 380 nm, respectively.

3.2. Optimisation of derivatization parameters

DMF and K_2CO_3 were chosen as the reaction co-solvents and basic catalyst based on previous experiments (You, Zhao, Suo, Li, &

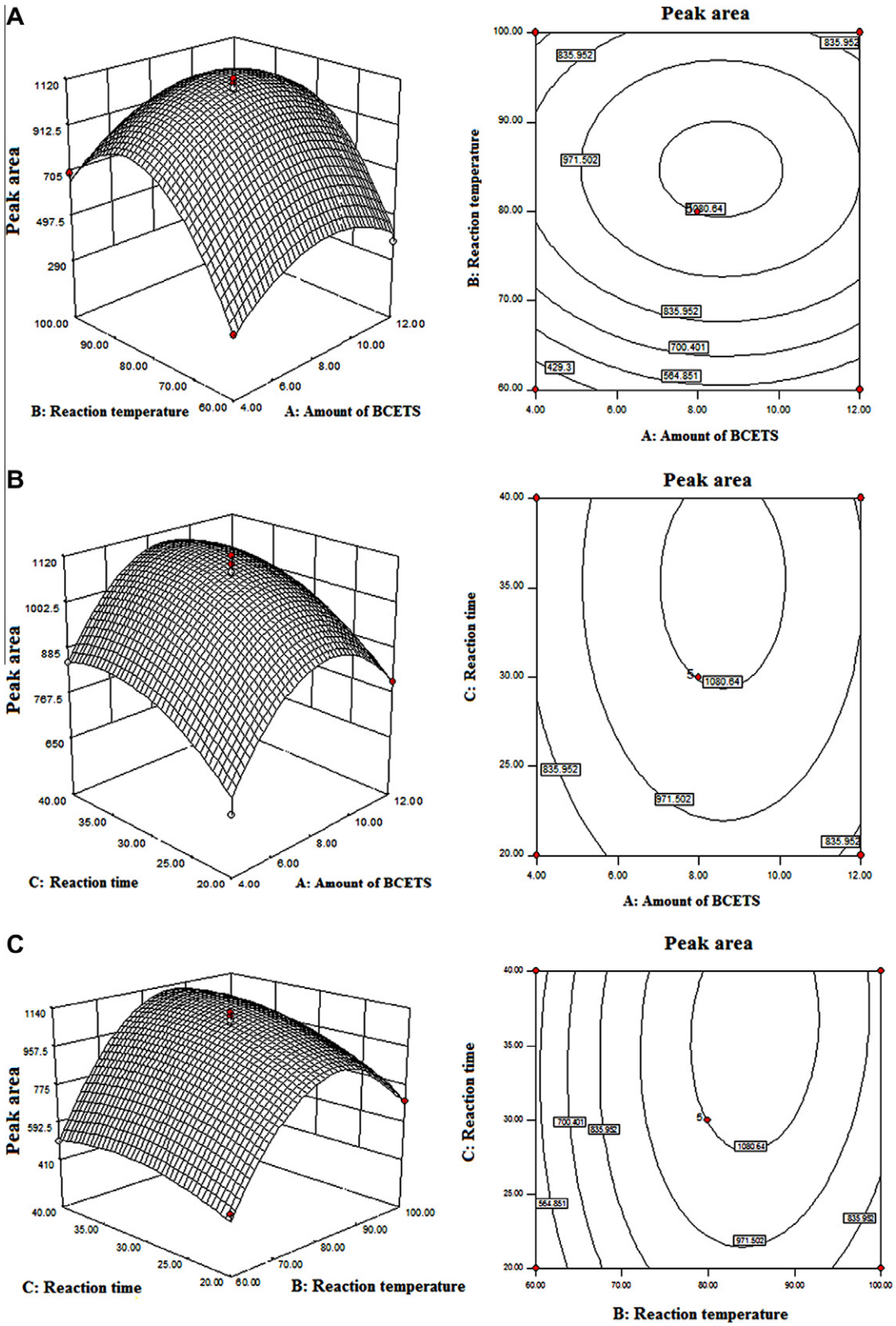

Fig. 2. The 3D response surface and 2D contour plots of detection responses (peak area) affected by the amount of BCETS (mol (BCETS)/mol (total fatty acids)), reaction temperature and reaction time.

Chen, 2007). Table 1 shows the experimental data. The independent and dependent variables were analysed to obtain a regression equation that could predict the response within the given range. The predicted second-order polynomial model was:

$$Y = 1102.39 + 53.75X_1 + 202.50X_2 - 20.08X_3 - 181X_1^2 - 368.50X_2^2 - 76.00X_3^2 - 7.50X_1X_2 + 37.50X_2X_3$$

The analysis of variance (ANOVA) for the experimental results of BBD indicated that all the linear parameters and quadratic parameters were significant at the level of $p < 0.05$ or $p < 0.01$, but all the interaction parameters were not significant. The value of R^2 (0.99) revealed that the experimental data were in good agreement with the predicted values of peak area. *F*-value for the lack of fit was insignificant ($p > 0.05$), meaning that this model was sufficiently accurate for predicting the relevant responses. Coefficient of variation (C.V.%) of less than 5.15% indicated that the model was reproducible.

The 3D response surface and 2D contour plots in Fig. 2 provide a method to visualise the relationship between responses and experimental levels of each variable and the type of interactions between two test variables. Fig. 2A depicts the effect of reaction temperature and amount of BCETS on the peak area. With a given amount of BCETS, the peak area increased rapidly with increasing reaction temperature and reached a maximum value, followed by a decline with its further increase. The interaction between reaction time and amount of BCETS is shown in Fig. 2B. With a definite reaction time, the peak area increased rapidly with the increase of the added amount of BCETS and reached the highest value, and then no obvious variation for peak areas with the further increase amount of BCETS were observed. Fig. 2C shows the interaction between reaction temperature and reaction time. The interaction between them displayed a negative effect on the peak area. Effect of reaction time on the peak area was not important to the case of reaction temperature.

The optimal conditions obtained using the model were as follows: added amount of BCETS, 8.8-fold molar excess to total molar fatty acids; reaction temperature, 84 °C; reaction time, 28 min. Under these conditions, the model gave predicted values of *Y* (peak area) being 1121. To test validity of response surface analysis method, the derivatization was carried out under the optimal condition and peak area was 1174 ($n = 3$). The value was close to the

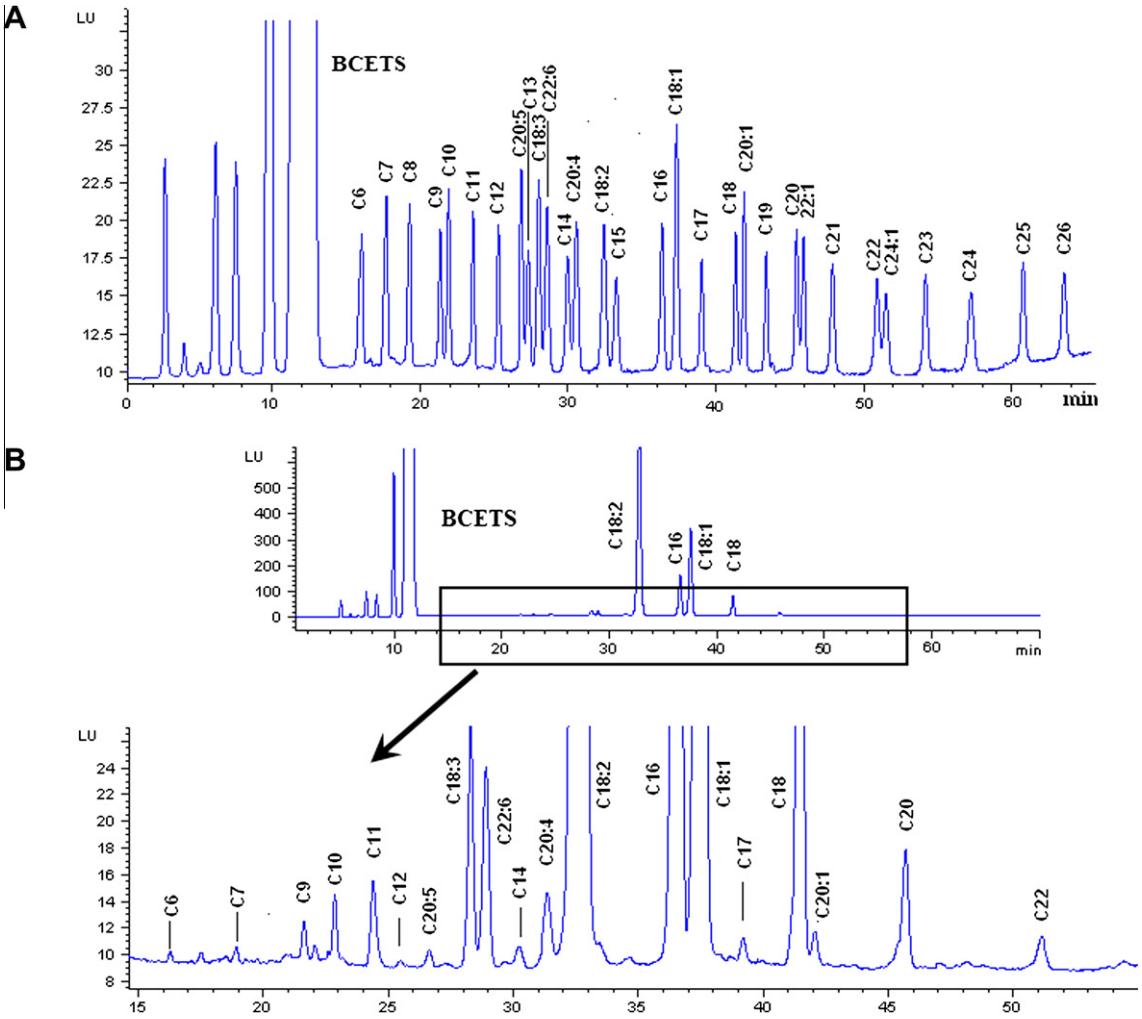

Fig. 3. Chromatogram of fatty acid standard derivative (A) and FFA derivative in extracted oil (B). Chromatographic conditions: Column temperature at 30 °C; excitation wavelength λ_{ex} 279 nm, emission wavelength λ_{em} 380 nm; Eclipse XDB-C_8 column (4.6 × 150 mm, 5 μm); flowrate = 1.0 mL min^{-1}; Peak labels: C6 (hexanoic acid); C7 (heptoic acid); C8 (caprylic acid); C9 (pelargonic acid); C10 (decoic acid); C11 (undecanoic acid); C12 (dodecanoic acid); C13 (tridecanoic acid); C20:5 (5,8,11,14,17-eicosapentaenoic acid,); C18:3 (8,11,14-octadecatrienoic acid); C22:6 (2,5,8,11,14,17- docosahexaenoic acid); C14 (myristic acid); C20:4 (6,9,12,15-arachidonic acid); C18:2 (9,12-octadecadienoic acid); C15 (pentadecanoic acid); C16 (hexadecanoic acid);C18:1 (12-octadecenoic acid); C17 (heptadecanoic acid); C18 (octadecanoic acid); C20:1 (11-eicosenoic acid); C19 (nonadecanoic acid); C20 (arachidic acid); C22:1 (12-docosenoic acid); C21 (heneicosoic acid); C22 (docosanoic acid); C24:1 (20-tetracosenoic acid); C23 (tricosanoic acid); C24 (tetracosanoic acid); C25 (pentacosanoic acid); C26 (hexacosanoic acid).

theoretical predicted value, indicating that the experimental design model may better reflect the derivatization parameters.

Finally, we got the optimum derivatization procedure: (1) to a solution containing 20 μL of a standard fatty acid mixture (or extracted sample) in a vial, 130 μL reagent solution, 10 mg K_2CO_3 and 200 μL DMF was added, respectively; (2) the vial was sealed and allowed to place in water bath at 84 °C with shaking at 5 min intervals for 28 min; (3) the mixture was cooled down to room temperature and diluted with 200 μL DMF for HPLC analysis.

3.3. Chromatographic separation and mass spectrometry analysis

An Eclipse XDB-C_8 column was selected in conjunction with a gradient elution, and several programs were investigated to ensure satisfactory HPLC separation. To achieve optimal separation, DMF was added in mobile phase B, which could raise solubility of fatty acid derivatives to obtain fast separation with sharp peaks. With the chromatographic conditions described above, a satisfactory resolution for 30 fatty acid derivatives was obtained within 70 min with the shortest retention-time and the sharpest peaks (see Fig. 3A).

The ionisation and fragmentation of BCETS-fatty acid derivatives were investigated by atmospheric chemical ionisation–mass spectrometry (APCI/MS) in positive-ion detection mode. As expected, the BCETS-fatty acid derivative produced an intense molecular ion peak at m/z $[M + H]^+$. The collision-induced dissociation spectra (MS/MS) of molecular ions (MS, $[M + H]^+$ ion) produced intense and stable fragment ions at m/z 243.5, 261.4 and 310.7 (Fig. 1), which was specific fragment ions for DBCEC-labelled AA derivatives. In most cases, the collision-induced dissociation spectra of m/z $[M + H]^+$ for the unsaturated fatty acid derivatives produced an specific fragment ions by losing H_2O molecules, giving the ion at m/z $[MH–H_2O]^+$ which was a specific fragment ion for the identification of unsaturated fatty acid derivatives. With APCI in positive-ion detection mode, intense ion current signals for fatty acid derivatives should be attributed to the introduction of the weakly basic nitrogen atoms in the corresponding BCETS molecular core structure, resulting in high ionisation efficiency. The corresponding cleavage mode and MS/MS analysis for the representative BCETS-C_{18} derivative is shown in Fig. 1B. MS and MS/MS data for all the fatty acid derivatives are shown in Table 2.

3.4. Linearity, detection limits, precision and recovery

The linearity was established over a 1000-fold concentration range for fatty acids with analysis of serial standards in the concentration range of 25 pmol–25 fmol. All of the fatty acids were found to give linear responses over this range with correlation coefficients of >0.9994 (see Table 2). The detection limits at a signal-to-noise ratio (S/N) of 3 were in the range of 0.22–1.06 ng/mL (Table 2). The relative standard deviations (RSDs) of the peak areas and retention times for BCETS derivatives are shown in Table 2. The experimental recoveries were in the range of 87.8–106.4%.

3.5. FFA analysis in L. barbarum seed oil

The proposed method was applied to the determination of FFAs in *L. barbarum* seed oil (LBSO). The representative chromatograms of the standard solutions and the extracted seed oil by supercritical CO_2 extraction (SFE) with ethanol as modifier are shown in Fig. 3B. As expected, the saturated and unsaturated fatty acids could be simultaneously separated with a good baseline resolution. The compositional data for FFAs are shown in Table 3. LBSO were found to be rich in unsaturated FFAs, which accounted for about 81%, and the high to low concentrations were as follows: linoleic acid (C18:2), oleic acid (C18:1), docosahexaenoic acid (C22:6), linolenic acid (C18:3), eicosenoic acid (C20:1) and eicosapentaenoic acid (C20:5). The main saturated FFAs were hexadecanoic acid (C16), octadecanoic acid (C18), arachidic acid (C20) and so on.

Table 2
MS data of BCETS-fatty acid derivatives, correlation coefficients, detection limits, and repeatability of peak area and retention time ($n = 6$).

Fatty acids	$[M + H]^+$	Specific MS/MS data	Correlation coefficient	Detection limit (ng/mL)	Retention time RSD (%)	Peak area RSD (%)
C6	360.2	261.4, 133.2	0.9996	0.28	0.18	0.71
C7	374.1	261.4, 157.6	0.9995	0.22	0.15	0.60
C8	388.3	261.4, 171.3	0.9998	0.47	0.11	0.60
C9	402.3	261.4, 185.4	0.9997	0.32	0.09	0.49
C10	416.3	261.4, 199.5	0.9995	0.34	0.07	0.43
C11	430.1	261.4, 213.4	0.9994	0.38	0.08	0.51
C12	444.3	261.4, 227.3	0.9996	0.38	0.06	0.50
C20:5	546.1	261.4, 328.6, 528.2	0.9997	0.49	0.06	0.40
C13	458.3	261.4, 231.2	0.9994	0.74	0.06	0.22
C18:3	521.9	261.4, 304.9, 503.9	0.9996	0.44	0.06	0.26
C22:6	572.1	261.4, 354.9, 553.8	0.9998	0.53	0.06	0.30
C14	472.3	261.4, 255.6	0.9994	0.48	0.04	0.30
C20:4	547.9	217.0, 330.9, 529.9	0.9999	0.46	0.07	0.36
C18:2	523.9	261.4, 306.7, 516.0	0.9996	0.40	0.04	0.3
C15	486.3	261.4, 269.6	0.9994	0.29	0.04	0.20
C16	500.2	261.4, 283.5	0.9994	0.28	0.04	0.16
C18:1	525.4	243.5261.4, 310.7	0.9994	0.35	0.03	0.14
C17	514.4	261.4, 297.1	0.9998	0.33	0.03	0.19
C18	527.6	261.4, 311.3	0.9999	0.34	0.02	0.18
C20:1	553.9	261.4, 337.4, 536.0	0.9995	0.48	0.04	0.22
C19	542.4	261.4, 325.1	0.9996	0.32	0.02	0.19
C20	556.3	261.4, 339.4	0.9997	0.46	0.05	0.42
C22:1	582.6	261.4, 365.6, 564.3	0.9998	0.64	0.09	0.51
C21	570.3	261.4, 353.2	0.9995	0.52	0.07	0.84
C22	584.1	261.4, 367.5	0.9997	0.56	0.10	1.22
C24:1	610.5	261.4, 393.3, 592.1	0.9994	0.75	0.18	1.14
C23	598.4	261.4, 371.2	0.9994	1.00	0.12	1.40
C24	612.3	261.4, 395.4	0.9999	0.91	0.19	2.00
C25	626.5	261.4, 409.1	0.9995	0.98	0.24	2.41
C26	640.3	261.4, 423.0	0.9998	1.06	0.23	1.32

Table 3
Content of FFAs from *L. barbarum* seed oil with different extraction methods (data are average values of three runs).

Free fatty acids	SFE without ethanol μg/mL (%)[a]	SFE with ethanol μg/mL (%)[a]	Petroleum ether μg/mL (%)[a]	*n*-hexane μg/mL (%)[a]
C6	3.8 (0.01)	7.2 (0.02)	3.8 (0.02)	4.4 (0.02)
C7	1.6 (0.01)	12.6 (0.03)	0.4 (0.00)	1.1 (0.01)
C9	25.6 (0.09)	38.2 (0.10)	18.8 (0.09)	16.5 (0.08)
C10	16.5 (0.06)	11.3 (0.03)	9 (0.05)	12 (0.06)
C11	65.3 (0.22)	80.5 (0.20)	23 (0.12)	16 (0.08)
C12	19.5 (0.07)	8.7 (0.02)	22 (0.11)	14 (0.07)
C20:5	24.5 (0.08)	12.6 (0.03)	6.6 (0.03)	0.2 (0.00)
C18:3	350.3 (1.19)	518.9 (1.31)	259.5 (1.31)	272.2 (1.31)
C22:6	378.1 (1.28)	445.4 (1.12)	207.4 (1.04)	205.3 (0.99)
C14	30.6 (0.10)	46.5 (0.12)	23.6 (0.12)	25.1 (0.12)
C20:4	69.6 (0.24)	90.8 (0.23)	58.4 (0.29)	84.6 (0.41)
C18:2	17921.4 (60.64)	24558.8 (61.78)	12439.2 (62.66)	12580.5 (60.77)
C16	3034.2 (10.27)	4120.8 (10.37)	2027.3 (10.21)	2273.1 (10.98)
C18:1	4932.5 (16.69)	6571.4 (16.53)	3158.9 (15.91)	3347.4 (16.17)
C17	42.9 (0.15)	58.2 (0.15)	35 (0.18)	36.8 (0.18)
C18	2034.3 (6.88)	2582.3 (6.50)	1159.9 (5.84)	1354.1 (6.54)
C20:1	38.6 (0.13)	48.6 (0.12)	43.3 (0.22)	28.3 (0.14)
C20	428.7 (1.45)	322.5 (0.81)	242.4 (1.22)	291.5 (1.41)
C22	137.5 (0.47)	214.9 (0.54)	114.1 (0.57)	151.3 (0.73)
Saturated FFA	5840.5 (19.76)	7503.7 (18.88)	3679.3 (18.53)	4182.8 (20.21)
Unsaturated FFA	23715.0 (80.24)	32246.5 (81.12)	16173.3 (81.47)	16518.5 (79.79)
Total FFA	29555.5 (100)	39750.2 (100)	19852.6 (100)	20701.3 (100)

[a] Absolute content (μg/mL, μg (free fatty acid)/mL(oil)), relative percent (%, ratio of the content of a FFA with that of all FFAs).

As observed in Table 3, LBSO extracted by different methods gave different amount of FFAs. SFE extracted more FFAs than organic solvents (*n*-hexane or mineral). SFE with ethanol yielded the highest content of FFAs. FFA content from high to low orders were: SFE with ethanol > SFE without ethanol > *n*-hexane > petroleum ether. SFE without ethanol yielded FFAs at the concentration level of 29555.5 μg/mL. When ethanol was added, the FFAs could be achieved at the concentration level of 39750.2 μg/mL. This is probably due to the fact that modifier (ethanol) can significantly counterbalance the hydrogen bonds and ionic forces between the membrane-associated lipids and proteins allowing the lipids to be available for extraction by the supercritical CO_2 (Koga et al., 1996; Sánchez-Vicente, Cabaas, Renuncio, & Pando, 2009). The result indicated that ethanol as modifier for SFE could cause a great increase in FFA content.

4. Conclusion

In this study, simultaneous determination of saturated and unsaturated fatty acids from extracted edible oil using BCETS as labelling reagent with fluorescence detection coupled with on-line MS identification has been successfully obtained. The established method shows good correlation, and high sensitivity. This method exhibits powerful potential for the trace analysis of short- and long-chain FFAs from edible oils, foodstuff and other complex samples. Through comparative analysis of different extracting methods, the results indicated that different extraction methods resulted in obvious difference in FFAs content. SFE extraction with ethanol as modifier gave the highest yields of FFAs.

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