Chromatographia (2012) 75:571–583
DOI 10.1007/s10337-012-2226-4

ORIGINAL

A Novel Labeling Reagent of 2-(12-Benzo[*b*]acridin-5-(12*H*)-yl)-acetohydrazide for Determination of Saturated and Unsaturated Fatty Acids in Traditional Chinese Herbs by HPLC-APCI-MS

Yinfeng Xie · Guoliang Li · Jinmao You · Xinwei Bai · Chengyan Wang · Lin Zhang · Fuhua Zhao · Xiaoyun Wu · Zhongyin Ji · Zhiwei Sun

Received: 26 March 2011 / Revised: 19 February 2012 / Accepted: 16 March 2012 / Published online: 18 April 2012

Abstract A new fluorescence labeling reagent 2-(12-benzo[*b*]acridin-5(12*H*)-yl)-acetohydrazide (BAAH) has been designed for fatty acids labeling. Eleven fatty acids containing seven saturated and four unsaturated fatty acids were used to evaluate the analytical potential of this reagent. The labeling reaction of BAAH with fatty acids was completed at 85 °C for 60 min using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC·HCl) as the condensing agent. Separation of the derivatized fatty acids was carried out on a reversed-phase Thermo Hypersil Gold C18 column (4.6 mm × 250 mm, 5 μm) in combination with a gradient elution with a good baseline resolution. The fluorescence excitation and emission wavelengths were set at λ_{ex} 280 and λ_{em} 510 nm, respectively. The identification was carried out by the online APCI-MS in positive-ion detection mode. Linear correlation coefficients for all fatty acid derivatives were of >0.9994. Detection limits, at a signal-to-noise ratio of 3:1, were 3.89–12.5 nmol L^{-1} for the labeled fatty acids. The developed method was successfully applied to the accurate determination of fatty acids in five traditional Chinese herbs with satisfactory results.

Keywords HPLC · Pre-column derivatization · 2-(12-Benzo[*b*]acridin-5(12*H*)-yl)-acetohydrazide (BAAH) · Fatty acids · Traditional Chinese herbs

Y. Xie · J. You (✉) · X. Bai · C. Wang · L. Zhang · F. Zhao · X. Wu · Z. Ji
The Key Laboratory of Life-Organic Analysis, College of Chemistry Science, Qufu Normal University, Qufu 273165, Shandong, People's Republic of China
e-mail: jmyou6304@163.com

G. Li · J. You · Z. Sun
Northwest Plateau Institute of Biology, Chinese Academy of Sciences, Xining 810001, Qinghai, People's Republic of China

G. Li · Z. Sun
Graduate University of Chinese Academy of Sciences, Beijing 100049, People's Republic of China

Introduction

Fatty acids are important substances wildly distributed in food, living organisms, and biological fluids. Many kinds of fatty acids play significant physiologically roles at trace levels in the regulation of a variety of physiological and biological functions. For example, saturated fatty acids are involved in energy production, energy storage, lipid transport, the synthesis of phospholipids and sphingolipids needed for membrane synthesis, and the covalent modification of many regulatory proteins [1]. Unsaturated fatty acids, for instance, 2,5,8,11,14,17-docosahexenoic acid (DHA) modulates the carrier-mediated transport of choline, glycine and taurine [2–4], the function of delayed rectifier potassium channels [5], etc. Therefore, there is a widespread interest in isolation and quantitation of these compounds. *Yunnan Caulis Spatholobi, Szechwan Cyathula officinalis Kuan, Tibet Rhodiola crenulata, Qinghai Apocynum Venetum, and Qinghai Plateau Salvia miltiorrhiza Bge* are traditional Chinese herbs, which are commonly used in medical research and production of Chinese patent medicine. As far as we know, there have been few reports about the fatty acid composition in these plants [6–18]. Consequently, quantitative determination of these compounds is equally important for further development and application of these medicinal herbs.

Most fatty acids show neither natural absorption in the visible or ultra-violet (UV) regions nor fluorescence, thus

accurate detection of them at trace levels using absorptiometry is fairly difficult [19]. However, easily detectable fatty acid derivatives by methyl esterification with gas chromatography (GC) or gas chromatography mass spectrometry (GC–MS) have been reported [20, 21]. In contrast with GC, use of high performance liquid chromatography (HPLC) allows the fatty acids to be converted to a large number of different derivatives [22]. 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 analyzed by HPLC. Therefore, derivatization of these analytes with labeling reagents has been widely adopted by HPLC with UV, especially fluorescence detection. The common reagents are as the following: (1) bromomethanes, such as 4-bromomethyl-7-methoxycoumarin(Br-Mmc) [23] and 4-bromomethyl-7-acetoxycoumarin (Br-Mac) [24]; (2) diazomethanes, such as 9-anthryldiazomethane (ADAM) [25, 26] and 1-pyrenyldiazomethane (PDAM) [27]; (3) amines, such as 9-aminophenanthrene (9-AP) [28] and 5-(dimethylamino)-l-naphthalenesulponyl-semipiperazide (dansyl-semipiperazide) [29]; (4) hydrazides, such as 4-(1-methylphenanthro[9,10-*d*]imidazol-2-y1)benzohydrazide (MPIB-hydrazide)[30] and 4-(5,6-dimethoxy-2-benzimidazoyl)-benzohydrazide) (DMBI-hydrazide) [31]; (5) sulfonate ester reagents, such as 2-(2,3-naphthalimino)ethyl trifluoromethanesulphonate (NE-OTF) [32], 2-(2-naphthoxy)-ethyl-2-(piperidino)-ethanesulfonate (NOEPES) [33] and 2-(2,3-anthracene-dicarboximido) ethyl trifluoromethanesulfonate (AE-OTF) [34]. Nevertheless, Toyo'oka [35] has reported that many of these reagents have limitations in their applications such as low detection sensitivity, short detection wavelengths, poor stability, tedious analytical procedure and serious interferences in the biological sample analyses.

In this work, we reported a chemical derivatization strategy based on a condensation reaction for labeling saturated and unsaturated fatty acids using a new reagent of 2-(12-benzo[*b*]acridin-5-(12*H*)-yl)-acetohydrazide (BAAH). The condensation reaction is simple, robust, and routinely performed as pre-column derivatization method for the quantification of fatty acids. The optimal derivatization conditions such as the amount of labeling reagent, condensing agent, reaction temperature, and time were investigated. It was found that BAAH reacted with fatty acids at 85 °C for 60 min using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC·HCl) as the condensing agent to give sensitive fluorescent derivatives. Linearity, detection limits, precision, and recovery were also evaluated. The proposed method applied to free fatty acid analysis from five traditional Chinese medicine extracts to give satisfactory results. The derivatization methods, analysis time, detection limits, and other details of the new reagent were compared with other fatty acid labeling reagents. The result indicated that BAAH was comparable or better than these reagents.

Experimental

Instrumentation

Experiments were performed using a LC-MSD-Trap-SL liquid chromatograph-mass spectrometer (1100 Series LC-MSD Trap, a complete LC–MS–MS instrument). All the HPLC system devices were from the HP 1100 series and consisted of a vacuum degasser (model G1322A), a quaternary pump (model G1311A), an autosampler (model G1329A), a thermostatted column compartment (model G1316A), a fluorescence detector (FLD; model G1321A), and a diode array detector (DAD; model G1315A). The LC system was controlled by HP Chemstation software. The mass spectrometer, from Bruker Daltonik (Bremen, Germany), was equipped with an APCI ion source. The source was operated in positive-ion mode at a nebulizer pressure of 60 psi, a dry gas temperature of 350 °C, and a dry gas flow of 5.0 L min^{-1}. The APCI Vap temperature was 450 °C, the corona current 4,000 nA (pos), and the capillary voltage 3,500 V. The mass spectrometer was controlled by Esquire-LC NT software, version 4.1. Fluorescence excitation and emission spectra were obtained with an F-7000 fluorescence spectrophotometer (Hitachi). Excitation and emission bandpass were both set at 10 nm. Derivatives were separated on a reversed phase Thermo Hypersil Gold C18 column (4.6 mm × 250 mm, 5 μm) by a gradient elution. The mobile phase was filtered through a 0.2-μm nylon membrane filter (Alltech, Deerfield, IL, USA).

Chemicals

Saturated fatty acids (C10, C12, C14, C16, C18, C20, C22) were purchased from Shanghai Chemical Reagent Co. (Shanghai, China). 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC·HCl) and unsaturated fatty acids 8,11,14-octadecatrienoic acid (C18:3), 2,5,8,11,14,17-docosahexenoic acid (C22:6, DHA), 9,12-octadecadienoic acid (C18:2), and 12-octadecenoic acid (C18:1) were purchased from Sigma (St. Louis, MO, USA). HPLC grade acetonitrile (spectroscopically pure) was purchased from Yucheng Chemical Reagent (Shandong Province, China). *N,N*-Dimethylformamide (DMF), pyridine, chloroform, and formic acid were purchased from Shanghai Chemical Reagent Co. (Shanghai, China). Water was purified on a Milli-Q system (Millipore, Bedford, MA). All other solvents and reagents for synthesis of BAAH were of analytical grade from Jining Chemical Reagent (Jining, Shandong, China).

Synthesis of Labeling Reagent 2-(12-oxobenzo[*b*]acridin-5(12*H*)-yl) acetohydrazide (BAAH)

Synthesis of Benzo[b]acridone

3-Amino-2-naphthoic acid and 3-*N*-phenyl-2-naphthoic acid were prepared by a method as previously described [36]. Benzo[*b*]acridone was prepared by a method as previously described [37].

Synthesis of 2-(12-oxobenzo[b]acridin-5(12H)-yl)acetic acid

KOH (20 g) and DMF (50 mL) were mixed in a 150 mL round-bottom flask and stirred for 20 min at room temperature. Benzo[*b*]acridone (2.5 g) was added and kept stirring for another 30 min. A solution of 5 mL ethyl bromoacetate was added dropwise within 20 min. After stirring at room temperature for 10 h, the contents were transferred into a glass beaker with 500 mL water and 10 g KOH. The mixture was heated continuously at 100 °C for 30 min. The solution was cooled to ambient temperature and then filtrated, the result solution was neutralized to pH 3.0 with 4 mol L^{-1} HCl. The precipitated solid was recovered by filtration, washed with water. The crude product was dried at room temperature for 48 h and recrystallized three times from acetonitrile/pyridine mixed solvent (acetonitrile/pyridine, 1:1, v/v) to afford a golden yellow crystal. Yield 2.4 g (77.67 %). m.p. 248.7–250.8 °C. Found: C 75.18 %, H 4.30 %, N 4.68 %. Calculated: C 75.24 %, H 4.32 %, N 4.62 %. IR (KBr): 3320.98 (*v*O–H), 3023.15 (*v*Ar–H), 1605.94 (Ph-C=O), 1571.62, 1488.63, 1449.10, 1383.14, 1293.15, 757.07, 658.65. LC-APCI-MS: *m/z*: 304.1 $[M + H]^+$ in positive-ion mode; MS–MS: *m/z*: 245.7 (molecular core moiety).

Synthesis of Ethyl-2-(12-oxobenzo[b]acridin-5(12H)-yl)-acetate

2-(12-Oxobenzo[*b*]acridin-5(12*H*)-yl)acetic acid (5 g) and absolute ethanol (150 mL) were mixed in a 250 mL round-bottom flask and stirred for 10 min at room temperature. A solution of 5 mL concentrated sulfuric acid was added dropwise within 10 min. The mixture was heated at 80 °C for 5 h with vigorous stirring. After cooling, the contents were transferred into a glass beaker with 200 mL water. The result solution was neutralized to pH 7.0 with saturated Na_2CO_3 solution. The precipitated solid was recovered by filtration, washed three times with water. The crude product was dried at room temperature for 48 h to obtain a yellow crystal. Yield 4.6 g (84.25 %). m.p. 181.0–183.2 °C. Found: C 76.01 %, H 5.25 %, N 4.22 %. Calculated: C 76.12 %, H 5.17 %, N 4.23 %. IR (KBr): 1736.43 (*v*C=O), 1013.16 (*v*C–O–C), 1454.21 (–CH_3), 1621.35, 1484.39, 1382.34, 1141.44, 1094.16, 863.12, 750.42. LC-APCI-MS: *m/z*: 332.4 $[M + H]^+$ in positive-ion mode; MS–MS: *m/z*: 245.6 (molecular core moiety).

Synthesis of 2-(12-Benzo[b]acridin-5(12H)-yl)-acetohydrazide (BAAH)

Ethyl 2-(12-oxobenzo[*b*]acridin-5(12*H*)-yl)acetate (5 g), 80 % hydrazine hydrate (5 mL), absolute ethanol (100 mL) were mixed in a 250 mL round-bottom flask and reacted at 90 °C for 6 h with vigorous stirring. After cooling, the precipitated solid was recovered by filtration and washed with absolute ethanol. The crude product was dried at room temperature for 48 h and recrystallized from acetonitrile to afford a faint yellow crystal. Yield 3.6 g (75.16 %). m.p. >290 °C. Found: C 71.92 %, H 4.70 %, N 13.27 %. Calculated: C 71.91 %, H 4.76 %, N 13.24 %. IR (KBr): 3459.94 (–NH_2), 1664.06 (*v*C=O), 1516.86, 1157.15 (C–N), 1188.27, 988.10, 834.45, 678.34. LC-APCI-MS: *m/z*: 318.4 $[M + H]^+$ in positive-ion mode; MS–MS: *m/z*: 245.7(molecular core moiety).

High Performance Liquid Chromatography

HPLC separation of fatty acid derivatives was carried out on a reversed-phase Thermo Hypersil Gold C18 column (4.6 mm × 250 mm, 5 μm) by a gradient elution. Eluent A was 30 % of acetonitrile consisting of 0.1 % formic acid; B was 100 % of acetonitrile. Gradient conditions: initial = 80 % A + 20 % B; 20 min = 20 % A + 80 % B; 30 min = 100 % B (kept for 10 min, injection 10 μL). Before injection of the next sample, the column was equilibrated with mobile phase A for 10 min. The flow rate was constant at 1.0 mL min^{-1} and the column temperature was set at 30 °C. The fluorescence excitation and emission wavelengths were set at λ_{ex} 280 and λ_{em} 510 nm, respectively. The detection and identification of derivatives were performed by online post-column fluorescence and APCI-MS in positive ion mode.

Preparation of Standard Solution

The derivatizing reagent solution (1.5×10^{-2} mol L^{-1}) was prepared by dissolving 47.55 mg (BAAH) in 10 mL anhydrous DMF prepared by distilling DMF dried with P_2O_5. Individual stock solutions of fatty acids (1.0×10^{-2} mol L^{-1}) were prepared in acetonitrile/DMF (1:1, v/v) and diluted to the concentration of (5×10^{-4} mol L^{-1}) with the same solvent composition. Standard solution of 0.15 mol L^{-1} coupling reagent was prepared by dissolving 0.2876 g EDC in 10 mL of anhydrous acetonitrile. When not

in use, all reagent solutions were stored at 4 °C in a refrigerator.

Extraction of Fatty Acids from Five Traditional Chinese Herbs

The freshly obtained five traditional Chinese medicines were washed with water and dried at room temperature, respectively. *Yunnan Caulis Spatholobi* (3.1620 g), *Szechwan Cyathula officinalis Kuan* (0.6754 g), *Tibet Rhodiola crenulata* (1.6849 g), *Qinghai Apocynum Venetum* (0.4095 g), and Qinghai *Plateau Salvia miltiorrhiza Bge* (0.7838 g) were separately triturated and then taken in five 50-mL volumetric flasks, respectively (each containing 20 mL chloroform). The contents were sonicated for 30 min and allowed to stand for 24 h and then filtered. The residues in each flask were again washed by chloroform (5 mL). The combined chloroform layers were saturated with pyridine and evaporated to dryness under a stream of nitrogen, and the residues were re-dissolved in DMF and stored at 4 °C until derivatization analysis [38, 39].

Derivatization Procedure

To a solution containing 20 μL (5×10^{-4} mol L^{-1} each) of a standard fatty acids mixture in a vial, 125 μL (1.5×10^{-2} mol $L^{-1)}$ derivatization reagent and 50 μL (0.15 mol L^{-1}) EDC were added. The vial was sealed and heated at 85 °C for 60 min in a thermostatic water-bath and then left to cool at room temperature. A 200 μL acetonitrile was added to dilute the derivatization solution. The diluted solution (10 μL) was injected directly into the LC system. The derivatization procedure is shown in Fig. 1.

Results and Discussion

Stability of BAAH and its Derivatives

The stability of BAAH and its derivatives were investigated. When a solution of BAAH in DMF was stored at 4 °C in darkness for 2 weeks, derivatization yields for fatty acids were no different from those obtained with freshly prepared BAAH solution. When the labeled fatty acid derivatives were stored at 4 °C in darkness for 48 h, careful examination of the reaction mixture by HPLC indicated no obvious degradation had occurred. It indicated that BAAH and its derivatives were stable enough for further HPLC analysis.

Spectral Properties of BAAH and its Derivatives

Ultraviolet Absorption

Acridone derivatives are among the most studied and important classes of photochromic molecules. They usually have extremely interesting photochromic properties. The ultraviolet absorption of 2-(12-benzo[*b*]acridin-5(12*H*)-yl) acetohydrazide (BAAH) was investigated in acetonitrile solution. The absorption wavelength of BAAH was obtained with the scanning range of 200–400 nm. The maximum absorption wavelength (λ) and molar absorption coefficients (ε, L mol^{-1} cm^{-1}) were 272 nm (1.317×10^5) and 294 nm (4.99×10^4), respectively.

Fluorescence Excitation and Emission

The excitation and emission spectra of BAAH and its derivatives were collected by using the scanning mode of the fluorescence detector. Maximum fluorescence responses of BAAH and its derivatives were achieved at the excitation wavelength 280 nm and the emission wavelength 510 nm. Therefore, $\lambda_{ex}/\lambda_{em}$ (280/510 nm) was chosen for all subsequent experiments.

Optimization of Derivatization Conditions

The derivatization conditions, such as the amount of labeling reagent BAAH, condensing agent EDC, reaction temperature and time, were investigated with decoic acid (C10), dodecanoic acid (C12), 12-octadecenoic acid (C18:1), octadecanoic acid (C18), and eicosoic acid (C20), chosen to represent fatty acid derivatives. The total volume was kept constant when optimizing. The results were shown in Fig. 2.

The concentration of BAAH used in our study was 1.5×10^{-2} mol L^{-1}. The amount of BAAH added was 1 to 20-fold molar reagent excess to total molar fatty acids. The fluorescence intensity of BAAH-derivatives increased with increasing amounts of reagent and constant fluorescence

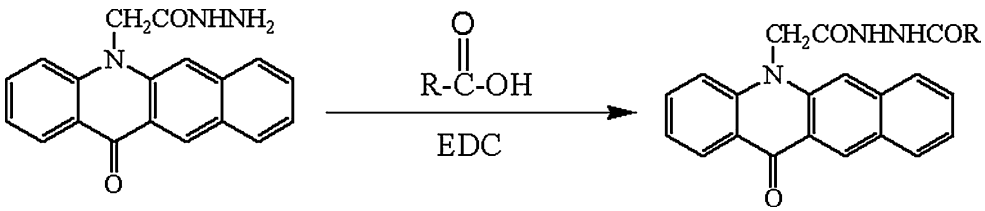

Fig. 1 Derivatization scheme of 2-(12-benzo[*b*]acridin-5(12*H*)-yl)-acetohydrazide (BAAH) with fatty acids

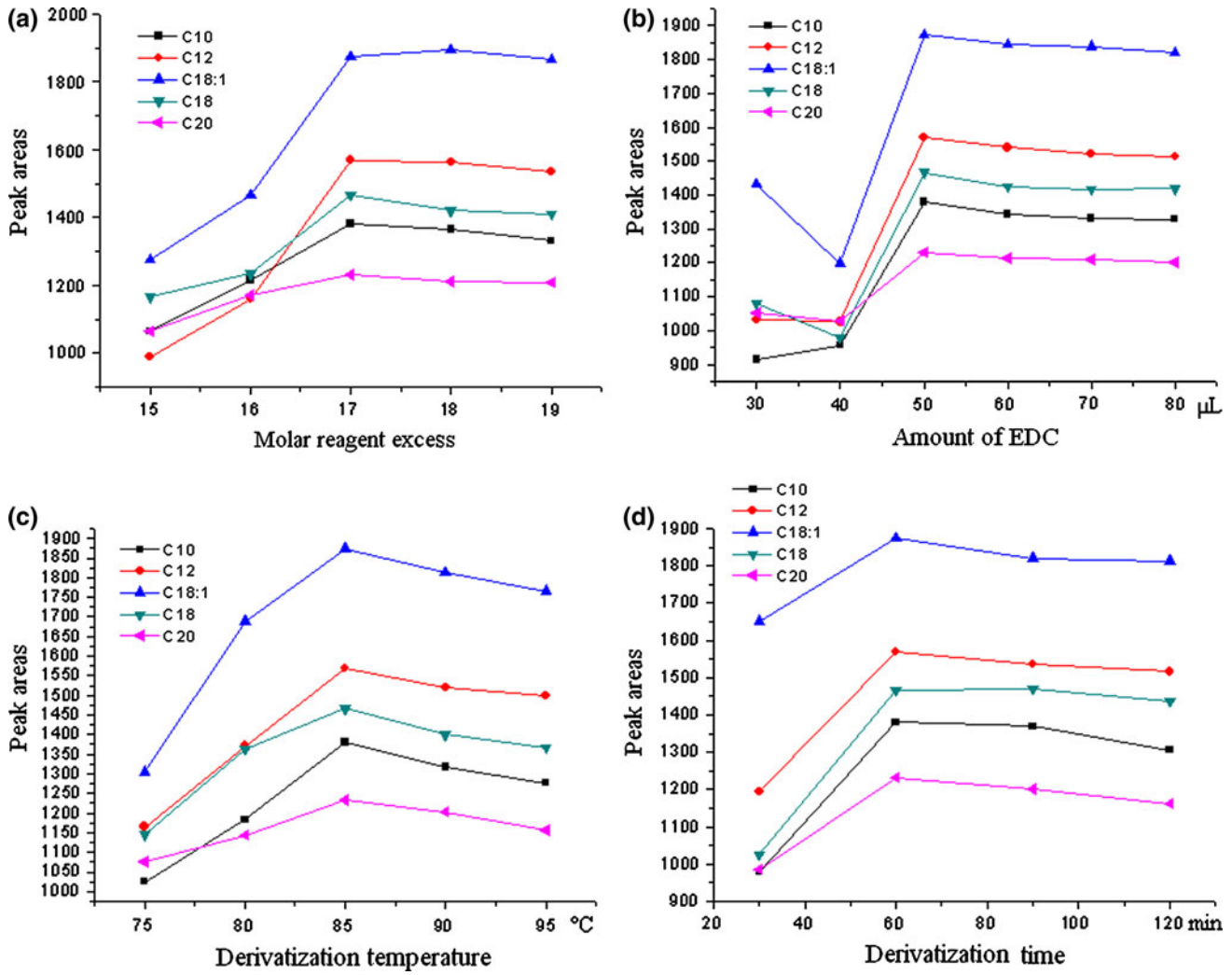

Fig. 2 Effect of **a** molar reagent excess; **b** amount of EDC; **c** derivatization temperature; **d** derivatization time on the peak areas of fatty acid derivatives. (*filled square*) C10, decoic acid; (*filled circle*) C12, dodecanoic acid; (*filled triangle*) C18:1, 12-octadecenoic acid; (*filled downward triangle*) C18, octadecanoic acid; (*filled leftward triangle*) C20, eicosoic acid

intensity was achieved by addition of 17-fold molar excess of reagent; further increasing the amount had no significant effect on yields. With less than a 17-fold molar excess of reagent, derivatization of the fatty acids was incomplete and obviously resulted in low detection responses (see Fig. 2a). To an unknown concentration of sample, complete derivatization was guaranteed by using a large excess of BAAH until constant peak intensity for detector responses.

Owing to its excellent dehydration property, EDC is one of the most used condensing agents which are used for the condensation of hydrazide reagent with fatty acids in many studies [30, 31, 40, 41]. It was observed that if condensing agent was insufficient to obtain maximal yield, addition of more condensing agent could reproducibly increase the yield to the maximum. With 0.15 mol L^{-1} EDC, the derivatization yield was evaluated by addition of different volume of EDC solution in the range of 30–80 μL (see Fig. 2b). The detector responses increased with increasing amounts of EDC, and constant peak intensity was achieved by addition of 50 μL EDC solution; further increasing EDC showed slightly low fluorescent responses. Therefore, amount of EDC (50 μL) was selected as the optimal derivatization.

Temperature has a significant effect on reaction time and yields. It was found that when temperature was raised beyond 85 °C, the position of equilibrium reduced the reaction proportion of BAAH with fatty acids because of forming some by-products; while below 85 °C the reaction rate was decreased and led to longer derivatization time. Therefore, derivatization temperature selected in this experiment was set at 85 °C (see Fig. 2c). With derivatization temperature at 85 °C, the peak heights for all fatty acid derivatives reached almost maximum after heating for 60 min (see Fig. 2d).

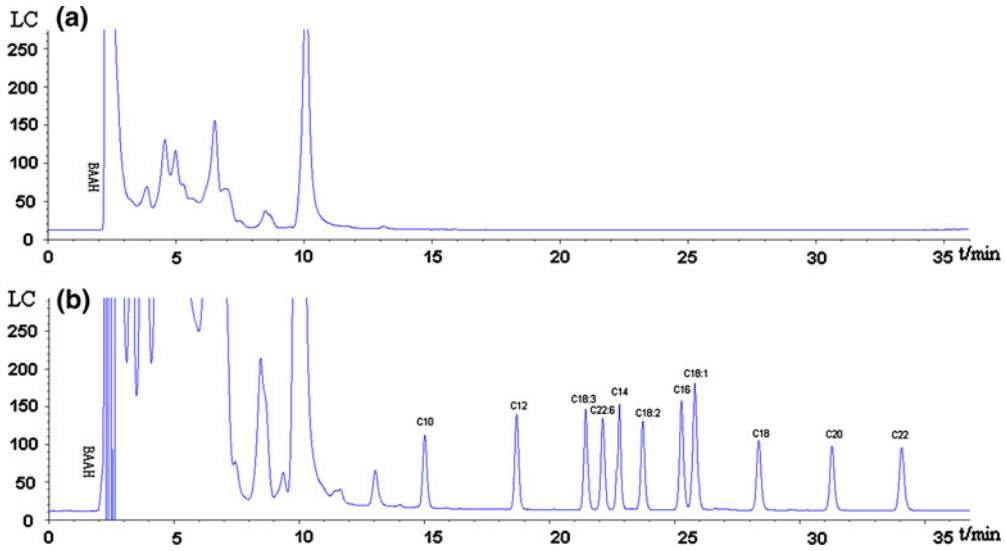

Fig. 3 Chromatographic conditions: column temperature at 30 °C; excitation wavelength λ_{ex} 280 nm, emission wavelength λ_{em} 510 nm; a reversed-phase Thermo Hypersil Gold C18 column (4.6 mm × 250 mm, 5 μm); flow rate = 1.0 mL min^{-1}. **a** Chromatogram of a blank (10 pmol, 10 μL injection). **b** Chromatogram of a mixture of fatty acid standards (80 pmol, 10 μL injection). *Peaks*: *C10*, decoic acid; *C12*, dodecanoic acid; *C18:3*, 8,11,14-octadecatrienoic acid; *C22:6*, 2,5,8,11,14,17-docosahexenoic acid; *C14*, tetradecanoic acid; *C18:2*, 9,12-octadecadienoic acid; *C16*, hexadecanoic acid; *C18:1*, 12-octadecenoic acid; *C18*, octadecanoic acid; *C20*, eicosoic acid; *C22*, docosanoic acid

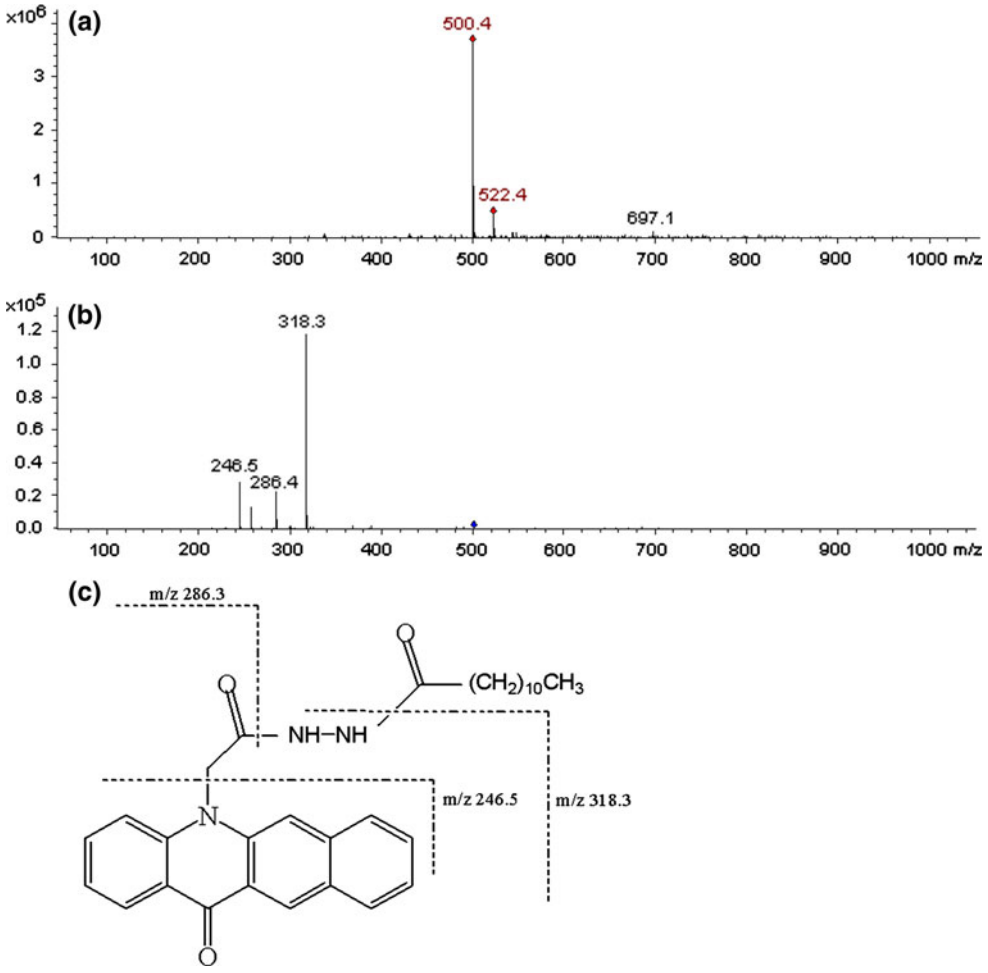

Fig. 4 The profiles of molecular ion chromatogram and scanning of the isolated representative n-C12 acid derivative (BAAH-C12). **a** Typical LC–MS profile of n-C12 acid derivative (BAAH-C12) from full scanning range from 100 to 1,000 amu with APCI in positive-ion detection mode. **b** Typical APCI-MS–MS profile of n-C12 acid derivative (BAAH-C12) from full scanning range from 100 to 1,000 amu with APCI in positive-ion detection mode. **c** The MS–MS cleavage mode of BAAH-C12 derivative

Table 1 MS and MS–MS data for fatty acid derivatives

Fatty acids	Molecular weight	Molecular weight of derivatives	$[M + H]^+$	MS–MS data
C10	172	471	472.4	246.5, 258.5, 286.5, 318.4
C12	200	499	500.4, 522.4 (+ Na^+)	246.4, 286.5, 318.3
C18:3	278	577	578.3	246.3, 286.5, 318.2, 560.3
C22:6	328	627	628.3, 650.3 (+ Na^+)	246.5, 286.5, 318.3, 610.4
C14	228	527	528.2, 550.3 (+ Na^+)	246.5, 286.3, 318.3
C18:2	280	579	580.2	246.4, 286.4, 318.3, 562.2
C16	256	555	556.4	246.4, 286.3, 318.4
C18:1	282	581	582.1	246.5,286.5, 318.4, 564.1
C18	284	583	584.2	246.5, 286.4, 318.3
C20	312	611	612.4	246.4, 286.3, 318.3
C22	340	639	640.1	246.5, 286.2, 318.1

Table 2 Linear regression equations, correlation coefficients, detection limits, repeatability for peak area and retention time

Fatty acids	Regression equation	Correlation coefficients	Detection limit/nmol L^{-1}	RSD (%, $n = 6$)	
				Retention time	Peak area
C10	$Y = 14.31X + 4.89$	0.9998	6.33	0.07	0.41
C12	$Y = 17.68X + 5.28$	0.9995	6.25	0.07	0.35
C18:3	$Y = 20.98X + 9.26$	0.9999	4.38	0.05	0.65
C22:6	$Y = 16.58X + 8.58$	0.9996	5.83	0.06	0.34
C14	$Y = 22.81X + 10.82$	0.9995	5.25	0.07	0.91
C18:2	$Y = 17.03X + 6.12$	0.9999	6.18	0.05	0.67
C16	$Y = 20.76X + 11.51$	0.9998	4.86	0.04	0.28
C18:1	$Y = 37.15X + 23.83$	0.9998	3.89	0.04	1.23
C18	$Y = 14.90X + 5.20$	0.9998	9.45	0.18	1.03
C20	$Y = 13.64X + 1.39$	0.9998	7.10	0.29	2.01
C22	$Y = 15.40X + 10.49$	0.9994	12.5	0.35	2.12

X injected amount (pmol), *Y* peak area

Taken together, 1.5×10^{-2} mol L^{-1} BAAH (17-fold molar excess), 0.15 mol L^{-1} EDC (50 μL) and heating at 85 °C for 60 min were chosen as the optimal reaction conditions.

HPLC Separation for Derivatized Fatty Acids

The separation of fatty acid derivatives was evaluated by using different columns such as Hypersil BDS-C18, Hypersil Gold C18 column, and Eclipse XDB-C8. The results indicated that C18:3 and C22:6 could not obtain a complete baseline resolution with BDS-C18 and XDB-C8 columns. Therefore, a reversed-phase Thermo Hypersil Gold C18 column (4.6 mm × 250 mm, 5 μm) was selected in conjunction with a gradient elution. The proper concentration of polar solvent and water is essential for perfect separation of all studied fatty acids. Several gradient programs were investigated to ensure satisfactory HPLC separation within the shortest time. With methanol as the mobile phase, low resolution and long elution time (45 min) were observed. Acetonitrile was used as mobile phase to give the shortest retention time and the sharpest peak shape. The compositions of two mobile phases were as follows: (A) 30 % acetonitrile containing 0.1 % formic acid and (B) 100 % acetonitrile. The gradient conditions were carried out as described in the experimental section. The gradient elution from (A) to (B) within 35 min was used to give the best separation. Under these conditions, all fatty acid derivatives were separated within 35 min. Chromatogram of a blank (Fig. 3a). Chromatogram of a mixture of fatty acid standards (Fig. 3b). Separation of all the fatty acids reflects the elution behavior of these compounds on Thermo Hypersil Gold C18 column as a function of double bonds and chain length. The longer the chain of fatty acids or the fewer the double bonds of fatty acids, the stronger is the hydrophobicity of fatty acids. When the hydrophobicity is stronger, the retention of fatty acids increased due to the strong interaction between the

Table 3 The addition recovery in *Yunnan Caulis Spatholobi* ($n = 3$)

Fatty acids	Added (ng mL^{-1})	Found (ng mL^{-1})	RSD (%, $n = 3$)	Recovery (%)
C10	40	38.51	1.84	92.23
	80	75.01	2.42	87.60
	120	112.5	2.15	87.47
C12	40	37.04	3.40	86.53
	80	74.87	2.67	87.01
	120	114.5	2.73	89.26
C18:3	40	36.25	3.47	85.64
	80	76.44	2.86	90.53
	120	120.4	2.41	100.1
C22:6	40	38.73	2.69	93.42
	80	75.96	2.24	89.13
	120	117.6	1.52	95.55
C14	40	36.07	4.25	85.47
	80	73.24	3.64	86.14
	120	118.0	3.28	96.29
C18:2	40	41.16	2.65	100.2
	80	77.43	2.50	91.49
	120	116.0	2.03	92.10
C16	40	37.95	3.74	88.43
	80	76.65	4.33	89.75
	120	115.0	2.64	90.12
C18:1	40	38.62	1.78	92.31
	80	76.16	2.45	90.24
	120	116.5	2.53	92.25
C18	40	41.57	4.37	103.4
	80	78.42	3.72	95.67
	120	124.8	4.28	98.7
C20	40	37.68	2.44	90.21
	80	75.12	2.63	87.74
	120	113.5	3.08	88.84
C22	40	40.76	4.04	100.1
	80	80.01	3.21	100.0
	120	121.8	2.59	100.8

Table 4 The addition recovery in *Szechwan Cyathula officinalis Kuan* ($n = 3$)

Fatty acids	Added (ng mL^{-1})	Found (ng mL^{-1})	RSD (%, $n = 3$)	Recovery (%)
C10	40	41.54	2.36	94.67
	80	78.93	2.02	89.25
	120	127.6	2.82	95.11
C12	40	37.28	2.77	85.43
	80	77.62	3.01	85.24
	120	116.4	2.23	87.80
C18:3	40	37.50	1.92	88.74
	80	81.29	3.84	93.26
	120	123.5	4.01	102.3
C22:6	40	35.48	1.65	86.31
	80	80.23	3.97	94.02
	120	122.5	2.68	95.44
C14	40	41.09	1.57	94.91
	80	78.00	1.88	92.28
	120	120.7	2.18	93.42
C18:2	40	35.61	2.05	86.83
	80	77.43	2.93	91.54
	120	120.4	1.78	94.37
C16	40	35.95	2.43	86.75
	80	82.13	3.51	100.2
	120	117.6	4.12	92.42
C18:1	40	37.69	1.85	89.23
	80	77.40	2.44	91.76
	120	121.7	4.22	93.10
C18	40	39.02	3.25	88.41
	80	76.54	2.63	90.67
	120	122.8	1.75	102.1
C20	40	35.64	4.09	85.73
	80	80.12	3.41	92.52
	120	114.7	2.83	87.90
C22	40	39.08	2.55	88.74
	80	76.58	3.11	88.69
	120	120.5	2.71	91.58

stationary phase and the analyte. Therefore, the shorter chain fatty acids are eluted earlier than the longer ones, and furthermore, fatty acids containing more double bonds elute earlier than the fewer ones, which are also displayed in the chromatogram (Fig. 3b). As can be seen from Fig. 3b, all saturated and unsaturated fatty acid derivatives were separated with a good baseline resolution.

Identification with APCI-MS at Positive-ion Detection Mode

The ionization and fragmentation of the isolated BAAH-fatty acid derivatives were studied by mass spectrometry with atmospheric pressure chemical ionization detection in positive-ion detection mode. As expected, the BAAH-fatty acid derivative produced an intense molecular ion peak at *m/z* $[M + H]^+$. Some derivatives such as C14-derivative and C22:6-derivative, also showed weak molecular ion peaks at *m/z* $[M + Na]^+$, however, the intensity of $[M + Na]^+$ peak was fairly low relative to that of molecular ion peak ($[M + H]^+/[M + Na]^+ = 4.75 \sim 6.33$). With MS/MS analysis of fatty acid derivatives, the collision-induced dissociation spectra of *m/z* $[M + H]^+$ produced the fragment ions at *m/z* 246, 286, and 318. In most cases, the collision-induced dissociation spectra of *m/z* $[M + H]^+$ for the unsaturated fatty acid derivatives

Table 5 The addition recovery in *Tibet Rhodiola crenulata* ($n = 3$)

Fatty acids	Added (ng mL^{-1})	Found (ng mL^{-1})	RSD (%, $n = 3$)	Recovery (%)
C10	40	37.69	2.54	90.17
	80	80.41	2.03	94.25
	120	117.4	2.21	86.86
C12	40	37.29	2.57	89.32
	80	78.44	1.63	92.43
	120	120.8	1.87	93.26
C18:3	40	35.38	3.03	86.52
	80	73.82	2.57	88.79
	120	117.9	4.38	92.83
C22:6	40	41.45	3.53	96.51
	80	79.89	1.94	93.13
	120	123.6	2.81	96.08
C14	40	39.46	2.03	92.06
	80	77.41	4.04	91.95
	120	117.3	2.85	94.33
C18:2	40	43.59	3.64	100.6
	80	81.43	2.60	99.83
	120	122.2	2.88	98.76
C16	40	37.58	4.09	92.73
	80	78.03	3.92	91.58
	120	117.9	2.14	92.49
C18:1	40	39.40	2.37	94.01
	80	78.56	2.68	90.08
	120	117.4	3.64	88.15
C18	40	41.83	3.88	102.5
	80	82.76	2.73	97.61
	120	123.2	3.45	101.8
C20	40	36.55	4.78	87.70
	80	77.11	4.63	87.35
	120	118.2	3.48	89.66
C22	40	40.16	1.53	97.62
	80	81.33	2.42	96.54
	120	122.6	2.79	100.1

Table 6 The addition recovery in *Qinghai Apocynum Venetum* ($n = 3$)

Fatty acids	Added (ng mL^{-1})	Found (ng mL^{-1})	RSD (%, $n = 3$)	Recovery (%)
C10	40	38.20	1.64	91.38
	80	76.94	1.82	90.57
	120	119.8	1.71	89.86
C12	40	39.43	2.83	87.45
	80	81.62	2.09	86.84
	120	119.4	2.60	87.63
C18:3	40	37.92	2.87	87.49
	80	75.88	3.16	92.56
	120	121.1	2.58	95.78
C22:6	40	40.56	2.79	96.73
	80	79.36	1.54	92.55
	120	122.7	2.92	94.98
C14	40	39.37	1.95	94.33
	80	78.48	2.60	90.27
	120	117.9	1.92	91.84
C18:2	40	41.23	2.84	97.62
	80	81.67	4.52	95.34
	120	122.3	3.59	95.30
C16	40	37.56	3.04	89.51
	80	78.82	3.87	90.66
	120	116.5	1.49	88.73
C18:1	40	40.21	4.34	95.75
	80	79.89	3.87	93.69
	120	121.7	2.73	93.52
C18	40	38.66	2.78	92.20
	80	75.32	1.71	91.74
	120	120.5	3.67	94.39
C20	40	36.58	4.05	87.13
	80	77.00	3.45	88.36
	120	118.3	3.43	90.17
C22	40	42.86	2.88	101.2
	80	81.05	3.57	98.04
	120	122.8	3.09	97.60

produced a specific fragment ions by losing H_2O molecules, giving the ion at *m/z* $[MH\text{-}H_2O]^+$, which was a specific fragment ion for the identification of unsaturated fatty acid derivatives. There was no detectable signal from the blank water sample using this transition. Although other endogenous acidic compounds present in natural environmental samples were presumably co-extracted and derivatized by BAAH reagent, no interference was observed due to the highly specific parent mass-to-charge ratio and the characteristic product ions in the *m/z* 246, 286, and 318. To reduce the disturbance to minimum from other unknown components presented in sample, the gradient elution with HPLC for the separation and determination of derivatized fatty acids was an efficient method. The cleavage mode for a representative BAAH-C12 derivative is shown in Fig. 4 (a–c). All molecular ions $[M + H]^+$ and corresponding specific fragment ions for fatty acid derivatives are shown in Table 1.

Repeatability, Precision, Linearity, Detection Limits and Recovery

The method repeatability was investigated by measuring the relative standard deviations (RSD) for peak area and retention time. A 10-μL standard sample was injected into the chromatograph by means of an automatic sampler

Table 7 The addition recovery in *Qinghai Plateau Salvia miltiorrhiza Bge* ($n = 3$)

Fatty acids	Added (ng mL^{-1})	Found (ng mL^{-1})	RSD (%, $n = 3$)	Recovery (%)
C10	40	38.78	2.36	91.24
	80	77.18	1.49	90.25
	120	115.3	2.66	89.50
C12	40	40.84	2.83	96.37
	80	79.52	1.96	94.80
	120	121.5	4.03	93.69
C18:3	40	38.63	2.58	89.04
	80	78.77	2.92	90.11
	120	120.3	3.59	93.86
C22:6	40	37.54	2.78	92.27
	80	76.03	3.82	92.01
	120	119.8	2.52	91.95
C14	40	36.79	2.63	86.62
	80	75.54	1.97	86.50
	120	118.5	1.88	91.14
C18:2	40	38.26	2.59	100.1
	80	80.52	3.47	95.40
	120	122.76	2.93	98.05
C16	40	36.67	1.76	86.95
	80	76.58	1.39	86.74
	120	118.9	2.54	91.42
C18:1	40	37.32	2.35	90.10
	80	78.46	2.53	91.87
	120	118.2	2.19	90.95
C18	40	40.61	3.47	97.40
	80	77.49	2.92	95.08
	120	122.5	3.22	96.84
C20	40	38.98	4.74	94.99
	80	76.52	1.86	87.05
	120	118.4	2.98	91.76
C22	40	41.56	2.47	100.3
	80	82.10	2.81	98.52
	120	123.9	2.79	101.4

($n = 6$, injected amount 50 pmol). The relative standard deviations (RSDs) of the retention times and peak areas varied from 0.04 to 0.35 % and from 0.28 to 2.12 %, respectively. The RSDs of the retention times and peak areas are listed in Table 2. Method precision was also determined by measuring intra-day and inter-day variability of retention time and peak area for each of the tested fatty acids ($n = 6$). The precision was calculated as the RSD for six successive injections of each of the tested fatty acids at a concentration of 5 μmol L^{-1}. The results showed that the intra-day precision was <0.42 % for the retention time and 2.46 % for the peak areas, and the inter-day

Table 8 Comparison of between BAAH and other reported reagents for fatty acids

Reagents	Analytes	Derivatization procedure	Seperation method	Wavelength	Analysis time (min)	DL (S/N = 3, nmol L^{-1})	Applications	References
BAAH	Fatty acids (C10–C22)	17-fold molar excess, 0.15 mol L^{-1} EDC (50 μL), 85 °C, 60 min	HPLC	280/510	35	3.89–12.5 (38.89–125 fmol/10 μL)	Traditional Chinese herbs	This work
DAABD-AE	Fatty acids (C4–C20)	8 mmol L^{-1}EDC, 20 mmol L^{-1} DMAP, 60 °C, 30 min	HPLC	430/570	40	11–66 (55–330 fmol/5 μL)	Rat plasma	42
MePZBD-AE	Fatty acids (C4–C20)	32 mmol L^{-1}EDC, 80 mmol L^{-1} DMAP, 60 °C, 30 min	HPLC	430/570	40	9–90 (45–450 fmol/5 μL)	Rat plasma	43
ABD-PZ	Fatty acids (C12–22)	5 μL DEPC, room temperature, 6 h	HPLC	440/580	60	5–25 (10–50 fmol/2 μL)	–	44
MDC	Fatty acids (C12–C18)	12 mmol L^{-1} MDC (50 μL), 2 μL DEPC, room temperature, 25 min	HPLC	340/518	50	Below 20 (below 100 fmol/1 μL)	Rabbit plasma	45
4-AF	Fatty acids (C2–C10)	DCC, room temp., multi-step phase transfer and extraction	CE		9	3–150	Atmospheric air sample	46

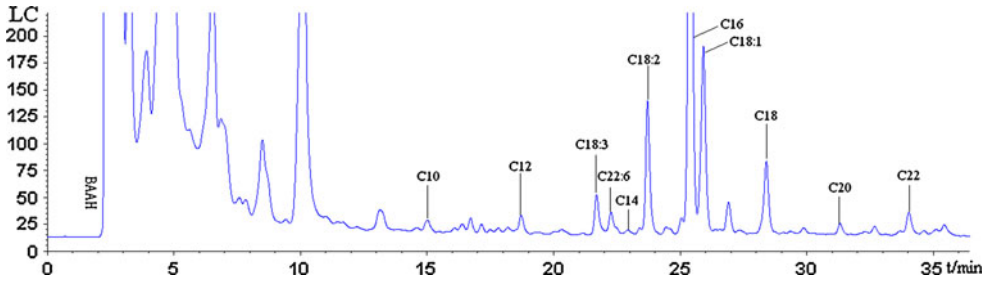

Fig. 5 Chromatogram for the separation of fatty acids from *Yunnan Caulis Spatholobi* derivatized with BAAH. Column temperature is set at 30 °C; excitation wavelength: λ_{ex} 280 nm, emission wavelength: λ_{em} 510 nm; Thermo Hypersil Gold C18 column (4.6 mm × 250 mm, 5 μm); flow rate = 1.0 mL min^{-1}; *peaks* as Fig. 3b

precision was <0.64 % for the retention time and 3.85 % for the peak areas.

Based on the optimum derivatization conditions, linearity of the procedure was evaluated in the range of 0.625–80.0 pmol by fluorescence detection. All of the fatty acids were found to give the excellent correlation coefficients of >0.9994. The detection limits for each derivatized fatty acids (at a signal-to-noise ratio = 3:1) were from 3.89 to 12.5 nmol L^{-1} (38.89–125 fmol/10 μL injection). The linear regression equations, correlation coefficients and detection limits for all fatty acid derivatives are listed in Table 2.

The recoveries of 11 fatty acids were evaluated by the addition of a known amount of standard (see Tables 3, 4, 5, 6, 7) into all samples. The extraction and derivatization methods were the same as described in the experimental section, and the analysis was carried out by three duplicates. The results indicated that the method was sufficiently accurate for the simultaneous determination of the above analytes.

Comparison between BAAH and Other Reported Reagents for Fatty Acids

To comment on the attributes of the proposed method, the comparison of derivatization methods, analysis time, detection limits and other details obtained with BAAH and other fatty acid labeling reagents were shown in Table 8 [42–46].

Compared with other reagents, the detection limit obtained with BAAH is comparable or better than those reagents. Despite the comparably high temperature and long time involved in derivatization process, it is free of using expensive or virulent activators, such as 4-(dimethylamino)pyridine (DMAP), diethyl phosphorocyanidate (DEPC), etc. In addition, the derivatization condition of the developed method is simpler in contrast with that using other fluoresce in-based fatty acid-reactive reagent, such as 4-AF [46]. 4-AF was used in CE, but compared with BAAH, its derivatization with fatty acids is tedious and time-consuming owing to multi-step phase transfers and extractions. What's more, the analysis time using BAAH is much shorter than most of regents. Although 4-AF exhibits shorter time than BAAH, its detection limit is too high.

Analysis of Samples

The chromatogram for the analysis of free fatty acids from the extracted *Yunnan Caulis Spatholobi* was shown as representative of traditional Chinese herbs in Fig. 5. Chromatographic peaks were identified by retention time and simultaneously confirmed by on-line APCI-MS in positive-ion detection mode. As expected, the saturated and unsaturated fatty acids were simultaneously separated with a good baseline resolution. The compositional data for the saturated and unsaturated free fatty acids are shown in Table 9. It was found that five samples all contain seven saturated and four unsaturated fatty acids, and their contents might be dependent upon the type of samples.

Conclusions

The objective of this study was to develop a sensitive method using BAAH as labeling reagent for simultaneous analysis of saturated and unsaturated fatty acids. Fluorescence detection was used for quantitative analysis in this method and MS determination was used for qualitative analysis. The proposed method offers a number of advantages: (1) Derivatization of fatty acids used BAAH is relatively simple and can also improve the detection selectivity; (2) Derivatives are sensitive to fluorescence and could be simultaneously obtained an online sensitive APCI-MS identification; (3) Maximal yields close to 100 % are observed with a 17-fold molar reagent excess; (4) Low background interference was observed due to high emission wavelength. Detection limits are in the femtomole range. The HPLC separation for the derivatized fatty acids shows good repeatability. The established method

Table 9 Contents of free fatty acids from five traditional Chinese herbs

Fatty acids	*Yunnan Caulis Spatholobi*		*Szechwan Cyathula officinalis Kuan*		*Tibet Rhodiola crenulata*		*Qinghai Apocynum Venetum*		*Qinghai Plateau Salvia miltiorrhiza Bge*	
	Contents ($\mu g\ g^{-1}$)	RSD (%, $n = 6$)	Contents ($\mu g\ g^{-1}$)	RSD (%, $n = 6$)	Contents ($\mu g\ g^{-1}$)	RSD (%, $n = 6$)	Contents ($\mu g\ g^{-1}$)	RSD (%, $n = 6$)	Contents ($\mu g\ g^{-1}$)	RSD (%, $n = 6$)
C10	2.37	2.89	0.39	3.17	0.23	1.31	75.36	3.24	8.77	2.55
C12	3.10	2.34	4.23	1.75	1.84	3.45	5.74	2.69	7.26	1.74
C18:3	8.53	3.70	31.50	3.56	34.00	2.73	284.82	2.56	100.91	1.46
C22:6	4.56	3.25	7.95	2.96	3.74	2.68	14.68	4.51	7.47	2.67
C14	0.81	1.91	11.21	4.01	0.64	4.36	15.20	1.73	6.87	3.05
C18:2	27.07	2.67	233.16	1.78	96.65	4.12	124.79	3.51	131.87	2.63
C16	154.18	3.02	127.88	2.50	46.58	3.37	581.52	2.07	109.94	3.78
C18:1	21.07	1.76	25.68	2.41	14.67	2.80	29.54	4.38	35.50	4.11
C18	24.98	2.44	11.57	3.18	6.64	1.43	88.00	2.28	38.93	2.34
C20	3.66	3.53	3.28	2.52	9.67	2.25	25.17	2.84	7.25	3.51
C22	7.75	4.53	12.52	3.44	35.18	3.23	20.94	2.32	6.83	2.96

can also be applied to the determination of various drugs and plants containing fatty acids. A possible disadvantage of the proposed method is that the reagent BAAH can only be used in the pre-column derivatization.

Acknowledgments This work was supported by 100 Talents Programme of The Chinese Academy of Sciences (No. 328).

References

1. Spector AA (1999) Lipids 34:S1–S3. doi:10.1007/BF02562220
2. Yorek MA, Strom DK, Spector AA (1984) J Neurochem 42: 254–261. doi:10.1111/j.1471-4159.1984.tb09726.x
3. Hyman BT, Spector AA (1982) J Neurochem 38:650–656. doi: 10.1111/j.1471-4159.1982.tb08680.x
4. Yorek MA, Hyman BT, Spector AA (1983) J Neurochem 40:70–78. doi::10.1111/j.1471-4159.1983.tb12654.x
5. Poling JS, Karanian JW, Jr NS, Vicini S (1995) Mol Pharm 47:381–390
6. Ha ES, Lee EO, Yoon TJ, Kim JH, Park JO, Lim NC, Jung SK, Yoon BS, Kim SH (2004) Biol Pharm Bull 27:1348–1352
7. Liu YT, Wang F, Wang GX, Han J, Wang Y, Wang YH (2010) Parasitol Res 106:1233–1239. doi:10.1007/s00436-010-1799-9
8. Chen XM, Tian GY (2003) Carbohydr Res 338:1235–1241. doi: 10.1016/S0008-6215(03)00073-9
9. Liu J, Xu J, Zhao XJ, Gao WY, Zhang SZ, Guo YQ (2010) Chin Chem Lett 21:70–72. doi:10.1016/j.cclet.2009.08.006
10. Zhou R, Li BG, Zhang GL (2005) J Integr Plant Biol 47:368–374. doi:10.1111/j.1744-7909.2005.00048.x
11. Zhou R, Li BG, Zhang GL (2005) J Asian Prod Res 7:245–252. doi:10.1080/10286020410001721159
12. Lei Y, Gao H, Tsering T, Shi S, Zhong Y (2006) Genet Mol Biol 29:339–344
13. Han X, Zhang TY, Wei Y, Cao XL, Ito Y (2002) J Chromatogr A 971:237–241. doi:10.1016/S0021-9673(02)01041-5
14. Zhang J, Chen XH, Wang P, Huo L, Shen ZD, Guo XR, Cheng WM, Bi KS (2008) Chromatographia 67:695–700. doi: 10.1365/s10337-008-0585-70009-5893/08/05
15. Butterweck V, Nishibe S, Sasaki T, Uchida M (2001) Biol Pharm Bull 24:848–851. doi:10.1248/bpb.24.848
16. Cao YH, Zhang X, Fang YZ, Ye JN (2001) Mikrochim Acta 137:57–62. doi:10.1007/s006040170028
17. Butterweck V, Simbrey K, Seo S, Sasaki T, Nishibe S (2003) Pharmacol Biochem Behav 75:557–564. doi:10.1016/S0091-3057(03)00118-7
18. Hao GP, Shi RJ, Wang JM, Qi B (2009) Afr J Biotechnol 8:2027–2035
19. Ingalls ST, Minkler PE, Hoppel CL (1984) J Chromatogr A 299:365–376. doi:10.1016/S0021-9673(01)97852-5
20. Brondz I, Olsen I, Haapasalo M, Winkelhoff AJV (1991) J Gen Microbiol 137:1445–1452. doi:10.1099/00221287-137-6-1445
21. Mukwaya GM, Welch DF (1989) J Clin Microbiol 27:2640–2646
22. Christie WW (1987) HPLC and lipids, 2nd edn. Pergamon Press, Oxford
23. Dünges W (1977) Anal Chem 49:442–445
24. Tsuchiya H, Hayashi T, Naruse H, Takagi N (1982) J Chromatogr A 234:121–130. doi:10.1016/S0021-9673(00)81786-0
25. Yoshida T, Uetake A, Yamaguchi H, Nimura N, Kinoshita T (1988) Anal Biochem 173:70–74. doi:10.1016/0003-2697(88)90161-3
26. Yamauchi Y, Tomita T, Senda M, Hirai A, Terano T, Tamura Y, Yoshida S (1986) J Chromatogr A 357:199–205. doi: 10.1016/S0021-9673(01)95821-2

27. Nimura N, Kinoshita T (1988) Anal Chem 60:2067–2070
28. Ikeda M, Shimada K, Sakaguchi T (1984) J Chromatogr B 305:261–270. doi:10.1016/S0378-4347(00)83341-1
29. Yanagisawa I, Yamane M, Urayama T (1985) J Chromatogr B 345:229–240. doi:10.1016/0378-4347(85)80160-2
30. Iwata T, Hirose T, Nakamura M, Yamaguchi M (1994) Analyst 119:1747–1751. doi:10.1039/AN9941901747
31. Iwata T, Nakamura M, Yamaguchi M (1992) Anal Sci 8:889–892
32. Yasaka Y, Tanaka M, Shono T, Tetsumi T, Katakawa JI (1990) J Chromatogr A 508:133–140. doi:10.1016/S0021-9673(00)91246-9
33. Lu CY, Wu HL, Chen SH, Kou HS (2000) Chromatographia 51:315–321. doi:10.1007/BF02490609
34. Akasaka K, Ohrui H, Meguro H (1993) Analyst 118:765–768. doi:10.1039/AN9931800765,Paper
35. Toyo'oka T (2002) Anal Chim Acta 465:111–130
36. You JM, Fu YY, Sun ZW, Suo YR (2010) Anal Bioanal Chem 396:2657–2666. doi:10.1007/s00216-010-3467-4
37. Shirley D, Earl Tatum W (1960) J Org Chem 25:2238–2240. doi:10.1021/jo01082a616
38. Bai XW, Song CH, You JM, Sun ZW, Fu YY, Li GL (2010) Chromatographia 71:1125–1129. doi:10.1365/s10337-010-1552-7
39. Zhao XN, Suo YR, Ding XC (2006) Chin J Chromatogr 24:456–461
40. Miwa H, Yamamoto M, Nishida T, Nunoi K, Kikuchi M (1987) J Chromatogr B 416:237–245. doi:10.1016/0378-4347(87)80507-8
41. Miwa H, Hiyama C, Yamamoto M (1985) J Chromatogr A 321:165–174. doi:10.1016/S0021-9673(01)90433-9
42. Du XL, Zhang HS, Guo XF, Deng YH, Wang H (2007) J Chromatogr A 1169:77–85. doi:10.1016/j.chroma.2007.08.038
43. Tsukamoto Y, Santa T, Saimaru H, Imai K, Funatsu T (2005) Biomed Chromatogr 19:802–808. doi:10.1002/bmc.523
44. Toyo'oka T, Ishibashi M, Takeda Y (1991) Analyst 116:609–613. doi:10.1039/AN9911600609
45. Lee YM, Nakamura H, Nakajima T (1989) Anal Sci 5:681–685
46. Kibler M, Bächmann K (1999) J Chromatogr A 836:325–331. doi:10.1016/S0021-9673(99)00012-6