J Am Oil Chem Soc
DOI 10.1007/s11746-011-1947-0

ORIGINAL PAPER

Composition Analysis of Free Fatty Acids from *Swertia* Species by a Novel Pre-column Fluorescence Labelling Method Using HPLC-FLD

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Received: 10 April 2011 / Revised: 28 September 2011 / Accepted: 29 September 2011
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Abstract Free fatty acids (FFA) are basic and indispensable components of medicinal plants. Many researches indicate that the efficacy of medicinal plant requires the presence of FFA. In the present study, ultrasonic-assisted extraction of FFA from *Swertia* species was optimized by response surface methodology (RSM), ensuring the highest FFA recoveries. A novel pre-column fluorescence labelling method using 2-(5-benzoacridine)ethyl-*p*-toluenesulfonate (BAETS) as a labelling reagent has been developed for highly sensitive and selective analysis of FFA by HPLC with fluorescent detection (FLD) and online mass spectrometry identification. RSM was also employed to optimize the fluorescence labelling of FFA. HPLC separation of 17 FFA derivatives was carried out on a reversed-phase Hypersil BDS C_8 column (4.6 × 200 mm, 5 μm, Agilent Co.) with a gradient elution. The detection limits and quantifications were as low as 0.60 and 1.22 ng mL^{-1}, respectively. FFA analysis from nine *Swertia* species was performed by the newly developed method, and the FFA composition of these *Swertia* samples was first reported.

Keywords Free fatty acids · HPLC-FLD · Pre-column derivatization · *Swertia* species · Ultrasonic-assisted extraction

Introduction

Swertia species belonging to the Gentianaceae family are well-known for their multiple properties for the treatment of hepatic, choleric, inflammatory, malarial, and diarrheal diseases. Swertiamarin and mangiferin flavonoids, iridoid glycosides, dimeric xanthones, and triterpenoids have all been shown to be the active components [1, 2]. Fatty acids play an important role in the prevention and treatment of coronary artery disease, hypertension, atherosclerosis, diabetes, arthritis, other inflammatory and autoimmune disorders, and cancer [3–6]. More researches indicate that free fatty acids (FFA) are indispensable components for the efficacy of medicinal plants [7–9]. Thus, accurate determination of FFA is not only of vital importance for the safe use of *Swertia* species, but also imperative for quality control.

FFA show little UV absorption and no fluorescence response, and are often present at a low level in plants. Thus, accurate analysis of them using absorptiometry is fairly difficult. The mostly used methods for FA determination are gas chromatography (GC) or GC/MS analysis coupled with diazomethane or silylation [10, 11]. However, the methods based on GC have several limitations in their applications to FFA analysis. For example, the high temperatures used in GC are harmful to the thermally unstable components like unsaturated FA derivatives [12]. Methylation with diazomethane is a hazardous procedure and should be avoided in routine analysis due to the explosiveness, toxicity and carcinogenicity of the reagents. In

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Published online: 28 October 2011

contrast with GC methods, HPLC method coupled with 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 LC [13, 14]. The strategy of pre-column derivatization has been widely used to improve the detection sensitivity and selectivity. Many pre-column labelling reagents have been developed for analysis of the compounds with carboxy groups such as the bromoalkyl reagents, diazomethane reagents and sulfonate reagents [15–18], but these labelling reagents have many disadvantages in their applications such as low detection sensitivity, short detection wavelengths, poor stability, tedious analytical procedures, and so on [19–21]. Recently, we described a new dual-sensitive probe 2-(5-benzoacridine) ethyl-*p*-toluenesulfonate (BAETS) for bile acids and triterpenic acids determination, and analysis using BAETS shows not only excellent fluorescent sensitivity but also high MS ionizable efficiency [22, 23]. In the present study, a novel HPLC method using BAETS as a fluorescent labelling reagent was applied for highly selective and sensitive determination of FFA in medicinal plants. Response surface methodology was utilized to optimize the derivatization reaction between BAETS and FFA. Moreover, quantitative analysis of FFA in the nine *Swertia* species including the flower, stem and root are first reported.

Experimental Section

Chemicals and Standards

All free fatty acid standards were purchased from Sigma Corporation (St Louis, MO, USA). Water was purified by a Milli-Q system (Millipore, Bedford, MA, USA). Acetonitrile of HPLC grade was obtained from Yucheng Chemical Reagent (Shandong Province, China). *N*,*N*-dimethylformamide (DMF), chloroform and potassium carbonate (K_2CO_3) were of analytical grade purchased from Shanghai Chemical Reagent Co. (Shanghai, China). All other reagents used were of analytical grade quality unless otherwise stated.

Samples

Swertia angustifolia (*S. angustifolia*) was collected from the western region of Nepal (elevation 3,200 m), *S. mussotii* from Yushu County, Qinghai province (elevation 4,100 m, China), *S. racemosa* from the southeast region of Tibet (elevation 3,400 m, China), *S. chirayita* from Nagarkot (elevation, 3,300 m, Nepal), *S. nervosa* from the east region of Tibet (elevation 3,300 m, China), *S. franchetiana* from Yushu County, Qinghai province (elevation 4,200 m, China), *S. verticillifolia* from Cuona of the Tibet Autonomous Region (elevation 4,100 m, China), *S. phragmitiphylla* from the southeast region of Tibet (elevation 4,000 m, China), and *S. kingii* from the south region of Tibet (elevation 3,800 m, China). The flower, root, and stem of *Swertia* samples were dehydrated, milled, sized (60 μm) and kept at −20 °C until use.

Equipment

The ultrasonic-assisted extractions of FFA from *Swertia* plants were carried out using an ultrasonic cleaner (SB-5200DTD, 40 kHz, Xinzhi Biotech Co., Ningbo, China). All chromatographic analyses were performed on an Agilent 1100 Series high-performance liquid chromatography/mass spectrometer (HPLC–MSD ion Trap SL, a complete LC–MS/MS), which consists of a vacuum degasser (model G1322A), a quaternary pump (model G1311A), an autosampler (model G1329A), a thermostated column compartment (model G1316A), and a fluorescence detector (model G1321A). The mass spectrometer 1100 Series LC/MSD Trap-SL (ion trap) from Bruker Daltonnik (Bremen, Germany) was equipped with an atmospheric pressure chemical ionization (APCI) source (in positive ion mode). The determination of fluorescence excitation and emission spectra were performed on a 650-10S fluorescence spectrophotometer (Hitachi, Japan). The mobile phase was filtered through a 0.2-μm nylon membrane filter (Alltech, Deerfiled, IL, USA).

Preparation of Standard Solutions

The BAETS reagent solution (1×10^{-2} mol/L) was obtained by dissolving 22.15 mg of DBCETS in 5 mL DMF. BAETS solutions with different concentrations were obtained by diluting the stock solution. A mixed standard stock solution containing 17 FFA was prepared in DMF with the concentration of 1.0×10^{-4} mol/L for each fatty acid. Working standard solutions for calibration curves were prepared by diluting the mixed standard stock solution with DMF.

Optimization of FFA Extraction and Derivatization Reaction

A software Design-Expert 7.1.3 Trial (State-Ease, Inc., Minneapolis MN, USA) was dedicated to experimental designs and generate contour plots. The Box-Behnken design (BBD) from response surface methodology was used for optimization of FFA extraction from *Swertia* species and the derivatization reaction between BAETS and FFA. The main parameters affecting FFA extraction were extraction temperature, time and solid–liquid ratio,

and for the derivatization reaction, the main parameters were derivatization time, temperature and amount of BAETS (mole ratio of BAETS to fatty acids). BBD was also employed to evaluate the effects and interactions of the condition variables. The FFA content or the peak area of the labeled fatty acids was taken as the response, *Y*. The model proposed for response (*Y*) was:

$$Y = \beta_0 + \sum_{i=1}^{n} \beta_i X_i + \sum_{i=1}^{n} \beta_{ii} X_i^2 + \sum_{1 \le i \le j}^{n} \beta_{ij} X_i X_j$$

Where *Y* represents the response variable, *n* is the numbers of variables, β_0 is the constant term, β_i, β_{ii} and β_{ij} represents the coefficient of the first order terms, quadratic terms, and interaction terms, respectively.

Preparation of Sample Solutions

The prepared *Swertia* sample (0.5 g) was added to 10 mL chloroform in a volumetric flask. The sample mixture was extracted for 70 min in an ultrasonic bath at 67 °C. After extraction, the sample mixture was filtered through a 0.2 μm membrane filter for the next analysis.

Derivatization

The derivatization was performed following the procedure below: (1) 50 μL standard solution, 100 μL BAETS reagent solution, 60 mg K_2CO_3 and 150 μL DMF were placed in a vial, respectively; (2) the vial was sealed and placed in a water bath at 94 °C for 35 min; (3) the mixture was cooled to room temperature and diluted for HPLC analysis.

HPLC–MS Conditions

HPLC separation of FA derivatives was carried out on a reversed-phase Hypersil BDS C_8 column (4.6 × 200 mm, 5 μm, Agilent Co.) with a gradient elution. The mobile phase A was 30% acetonitrile containing 0.1% ammonium formate and B was 100% acetonitrile. The mobile phase solutions were degassed and filtered through a 0.45-μm filter and pumped at a flow rate of 1 mL/min and the column temperature was set at 35 °C. The injection volume was 10 μL. The fluorescence excitation and emission wavelengths were set at λ_{ex} 272 nm and λ_{em} 505 nm, respectively. The gradient elution program was as follows: 0 min = 50% B, 40 min = 92% B, 45 min = 100% B, and 50 min = 100% B. Before injecting the next sample, the column was equilibrated with the initial mobile phase for 10 min. MS conditions: ion source type, atmospheric pressure chemical ionization (APCI source); nebulizer pressure 60 psi; dry gas temperature, 350 °C; dry gas flow, 5.0 L/min; Vap temperature, 350 °C; capillary voltage, 3,500 V; corona current, 4,000 nA (pos).

Validation of Analytical Method

The method was validated for linearity, limits of detection and quantification (LOD and LOQ), reproducibility, precision (inter-day and intra-day precision) and accuracy. Standard solutions containing 17 FFA were prepared and diluted to appropriate concentrations for construction of the calibration curves. The 17 FFA solutions at 6 different concentrations were injected in triplicate, and the calibration curves were constructed by plotting the peak areas versus the concentrations of each FFA. LOD and LOQ were defined as the compound concentration that produced a signal-to-noise ratio of 3 (S/N = 3) and 10 (S/N = 3), respectively. The method repeatability was evaluated by consecutively injecting a 10-μL standard sample ($n = 6$) and measuring the relative standard deviations (R.S.D.) for peak area and retention time. The inter- and intra-day variability was investigated to evaluate the precision of the proposed method. The accuracy of the method was determined by the % recovery method. The % recovery of the analyte was obtained by spiking a known amount of FFA standards to *Swertia* samples.

Results and Discussion

Optimization of Free Fatty Acid Extraction

The preparation of crude extracts from plants is also a key aspect in the qualitative and quantitative analysis of chemical constituents present in medicinal plants. Before response surface methodology (RSM) optimization, we tried several organic solvents including *n*-hexane, petroleum ether, chloroform and a mixture of chloroform, methanol and water [24]. Results indicated that chloroform gave the highest FFA recovery. The extraction temperature, time and solid–liquid ratio were chosen for further optimization by RSM based on the significant effect on the extraction yield. The three factors and lower, middle and upper design points for RSM in coded and uncoded values are shown in Table 1. In RSM, natural variables are transformed into coded variables that have been defined as dimensionless with a mean zero and the same spread or standard deviation. Multiple regression equations below were generated relating response variable to uncoded levels of the independent variables.

$$Y = 1179 + 148X_1 + 17X_2 - 4.8X_3 - 66X_1^2 - 27X_2^2 - 80X_3^2 - 27.4X_1X_2 + 35.6X_1X_3 - 31.02X_2X_3$$

Table 1 The Box-Behnken design matrix of three test variables in coded and natural units along with the observed responses (total FFA content)

Run	Independent variable			Total FFA content (μg/g)
	X_1 (Temperature, °C)	X_2 (Time, min)	X_3 (Solid–liquid ratio)	
1	30(−1)[a]	40(−1)	22.5(0)	912
2	70(+1)	40(−1)	22.5(0)	1,221
3	30(−1)	90(+1)	22.5(0)	1,009
4	70(+1)	90(+1)	22.5(0)	1,197
5	30(−1)	65(0)	15(−1)	903
6	70(+1)	90(+1)	15(−1)	1,156
7	30(−1)	65(0)	30(+1)	822
8	70(+1)	65(0)	30(+1)	1,238
9	50(0)	40(−1)	15(−1)	1,034
10	50(0)	90(+1)	15(−1)	1,115
11	70(+1)	65(0)	15(−1)	1,164
12	50(0)	90(+1)	30(+1)	1,050
13	50(0)	65(0)	22.5(0)	1,156
14	50(0)	65(0)	22.5(0)	1,213
15	50(0)	65(0)	22.5(0)	1,188
16	50(0)	65(0)	22.5(0)	1,131
17	50(0)	65(0)	22.5(0)	1,213

[a] Natural units (coded)

The results of analysis of variance (ANOVA) showed the regression model was highly significant ($P < 0.001$). The total determination coefficient (R^2) was 0.96, indicating a reasonable fit of the model to the experimental data. The three-dimensional surface plots and two-dimensional contour plots were drawn to illustrate the main and interactive effects of the independent variables on the dependent one (Fig. 1). Figure 1a shows the effect of extraction time and temperature on FFA extraction yield. Both extraction temperature and time have positive effect on FFA content. By increasing the extraction temperature or time, the FFA yield increased and reached a maximum value. Figure 1b depicts the influence of extraction temperature and solid–liquid ratio on extraction yield. The interaction of temperature and solid–liquid ratio have positive effect on the FFA yield. The FFA content increased with the solid–liquid ratio and reached up to a maximum value. Similarly, Fig. 1c clearly presented the combined effect of extraction time and solid–liquid ratio on FFA yields. The optimal conditions given by the model were extraction temperature of 67 °C, extraction time of 70 min and solid–liquid ratio of 20.5. Under the proposed conditions, the FFA content was 1,251 μg/g ($n = 3$), which was in very good agreement with the predicted value. These optimal extraction conditions ensured the highest FFA recoveries within the shortest extraction time.

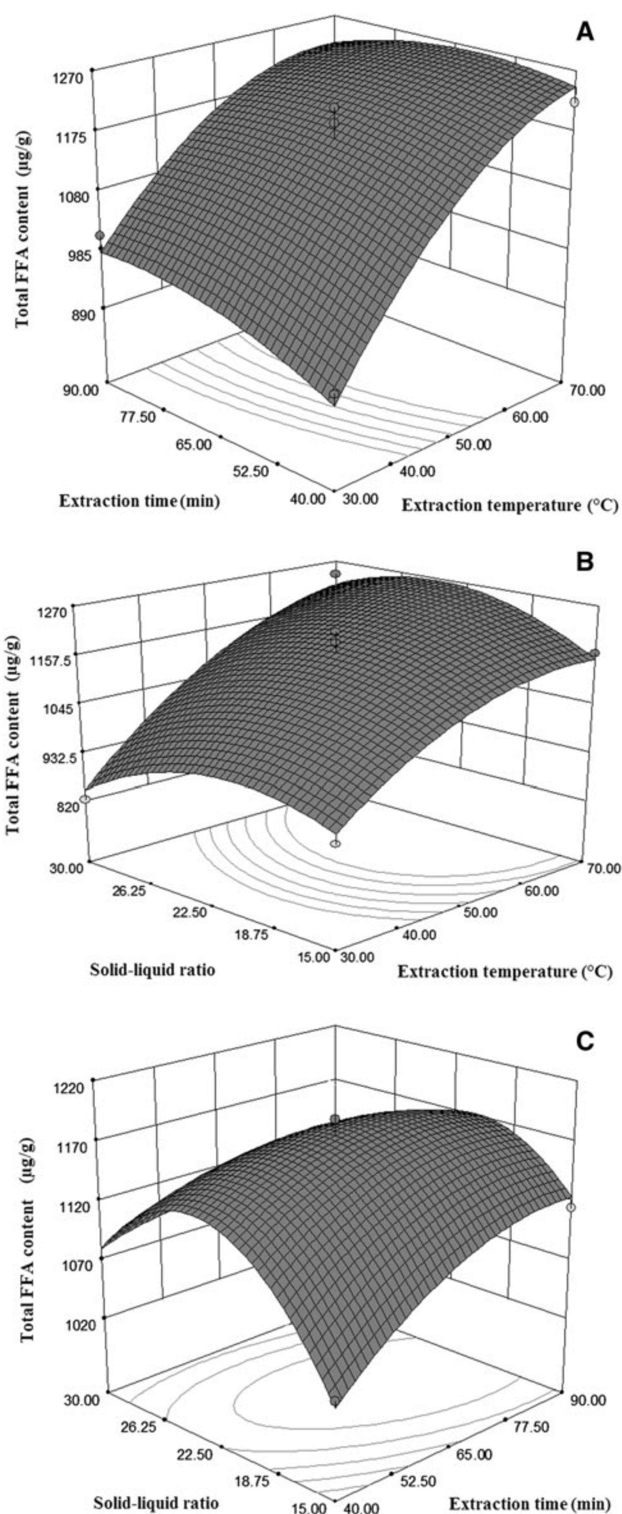

Fig. 1 The 3D response surface and 2D contour plots of total FFA content affected by extraction time (min), extraction temperature (°C) and solid–liquid ratio

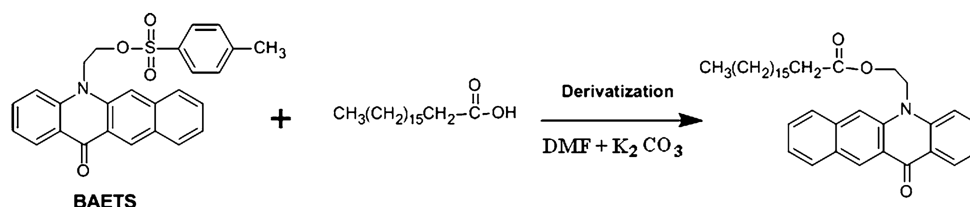

Fig. 2 The representative derivatization scheme of BAETS with C18 fatty acids

Table 2 The Box-Behnken design matrix of three test variables in coded and natural units along with the observed responses (peak area)

Run	Independent variable			Peak area
	X_1 (BAETS ratio)	X_2 (Temperature, °C)	X_3 (Time, min)	
1	8(+1) [a]	80(−1)	30(0)	1,647
2	8(+1)	90(0)	20(−1)	3,678
3	3(−1)	100(+1)	30(0)	2,966
4	5.5(0)	100(+1)	40(+1)	4,325
5	8(+1)	100(+1)	30(0)	3,283
6	3(−1)	80(−1)	30(0)	1,296
7	5.5(0)	90(0)	30(0)	4,304
8	5.5(0)	90(0)	30(0)	4,265
9	5.5(0)	80(−1)	20(−1)	1,843
10	5.5(0)	80(−1)	40(+1)	2,131
11	8(+1)	90(0)	40(+1)	4,291
12	5.5(0)	90(0)	30(0)	4,270
13	5.5(0)	90(0)	30(0)	4,458
14	3(−1)	90(0)	40(+1)	3,658
15	5.5(0)	90(0)	30(0)	4,180
16	5.5(0)	100(+1)	10(−1)	2,940
17	3(−1)	90(0)	20(−1)	2,678

[a] Natural units (coded)

Optimization of the Derivatization Reaction

Derivatization optimization is one key step for the pre-column derivatization strategy, which ensures the sufficient labelling of the analyzed components. The derivatization scheme for BAETS with the representative C18 fatty acids is presented in Fig. 2. Based on a previous study [22], DMF and 60 mg K_2CO_3 were used as the best co-solvent and catalysts, respectively. The main conditions including derivatization temperature, time and amount of BAETS (mole ratio of BAETS to FFA) were chosen for further optimization by employing a three-level, three-variable Box-Behnken design (BBD) from response surface methodology (RSM). Seventeen experiments (Table 2) from BBD were randomly performed. By applying multiple regression analysis methods on the experimental data in Table 2, the predicted model was obtained by a second-order polynomial function, given as follows:

$$Y = 4295 + 287.6X_1 + 824.7X_2 + 408.1X_3 - 615.5X_1^2 - 1381.7X_2^2 - 103.7X_3^2 - 8.64X_1X_2 - 91.4X_1X_3 + 274.1X_2X_3$$

The experimental data were analyzed by analysis of variance (ANOVA). The low probability 'P' value of <0.001 indicated a high significance of the regression model. The coefficient of determination (R^2) was 0.98, indicting a reasonable fit of the model to the experimental data. The three-dimensional surface plots and two-dimensional contour plots in Fig. 3 were the graphical representations of regression function. They explained the type of interactions between two tested variables, and the relationships between responses and experiment level. Figure 3a describes the combined effect of molar ratio of BAETS to free fatty acids and derivatization temperature on the derivatization yields. Both temperature and amount of BAETS have positive effects on the derivatization reaction. As shown in Fig. 3a, the peak area increased with the temperature and reached a maximum value, and a further increase in the derivatization temperature had a slight effect on the peak area. The derivatization yield increased rapidly with the amount of BAETS and reached a maximum value, and the further increase of BAETS

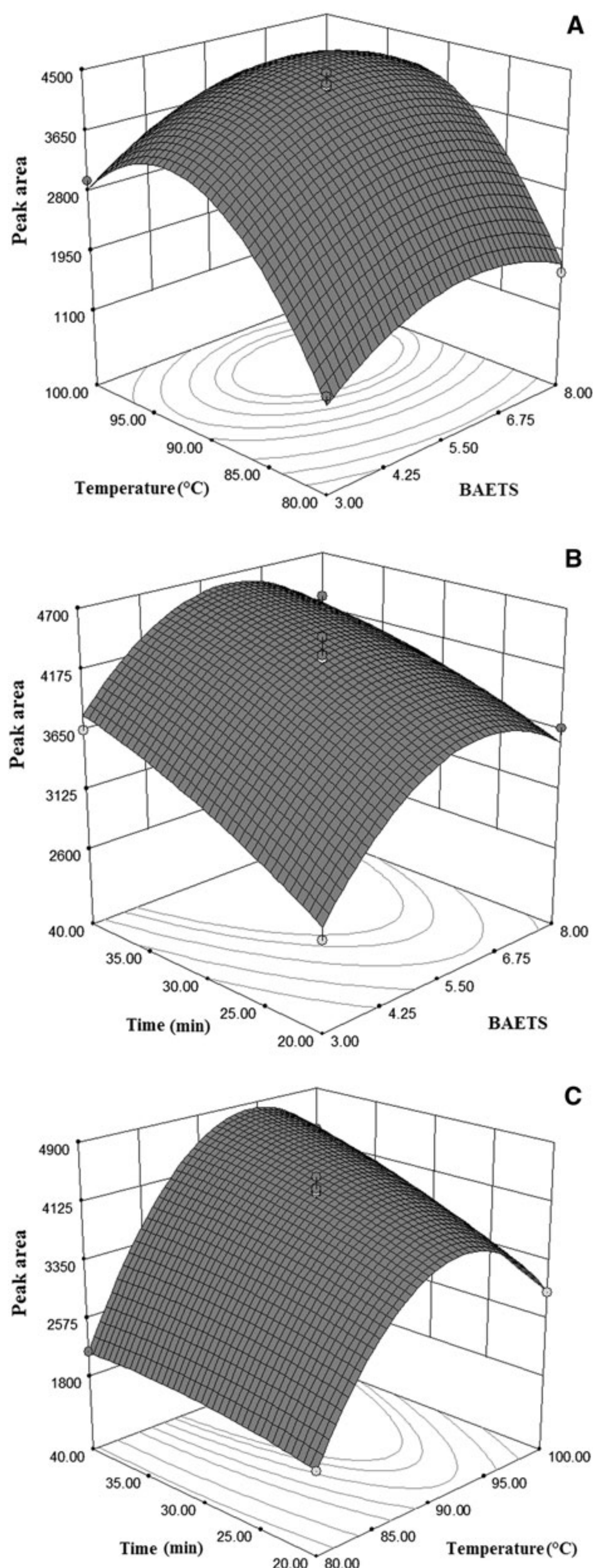

Fig. 3 The 3D response surface and 2D contour plots of derivatization yield (expressed in terms of peak area) affected by derivatization temperature (°C), derivatization time (min) and molar ratio of BAETS to FFA

resulted in a small decrease in the derivatization yield. Similarly, Fig. 3b vividly shows the effect of derivatization time and amount of BAETS on the response, and Fig. 3c presents the effect of derivatization time and temperature on the response.

The prediction optimization values were calculated using the second-order polynomial equation. The optimal conditions obtained by calculating the regression equation were as follows: molar ratio of BAETS to FFA = 4.6; derivatization temperature = 94.4 °C; and derivatization time = 35.2 min. To test validity of response surface analysis method, the derivatization was carried out under the optimal condition and peak area was 4,380 ($n = 3$), which was close to the theoretical predicted value of 4,466, indicating that the experimental design model may better reflect the derivatization parameters.

HPLC Separation Optimization and Mass Spectrometry Identification

The first step in developing a HPLC method is to select an analytical column. Various analytical columns including Hypersil C_{18} (200 mm × 4.6 mm, 5 μm), Hypersil BDS C_8 (200 mm × 4.6 mm, 5 μm), Hypersil BDS C_{18} (200 mm × 4.6 mm, 5 μm), and Spherisorb C_{18} (200 mm × 4.6 mm, 5 μm) were investigated, and results showed that a Hypersil BDS C_8 (200 mm × 4.6 mm, 5 μm) gave the best resolution compared to other columns. Retention factor, tailing factor and pH of the mobile phase were evaluated for different proportions of acetonitrile-containing aqueous solvents. Eluent A (30% acetonitrile containing 0.1% ammonium formate) and Eluent B (100% acetonitrile) offered good resolution, peak shape and better retention times of 17 FFA derivatives. The best flow rate of mobile phase and column temperature were found to be 1.0 ml/min and 35 °C, respectively. The representative chromatography for 17 FFA standards is shown in Fig. 4a.

Chromatographic peaks were simultaneously identified by retention time and online post-column mass spectrometry in positive mode. The mass spectrometry data for all of derivatized FFA are shown in Table 3. The mass spectrometry data indicated that FFA derivatives exhibited intense quasi-molecular ion peak of $[M + H]^+$. The MS, MS/MS and cleavage mode for the typical C18 derivative are shown in Fig. 5. As expected, C18 derivative produced an intense molecular ion peak at *m/z* 556.4, and the specific fragment ions at *m/z* 290.5, *m/z* 272.5, *m/z* 311.4 and 246.5. Although other endogenous acidic compounds present in samples were presumably co-extracted and derivatized by BAETS, no interference was observed due to the highly specific parent mass-to-charge ratio and the characteristic product ions.

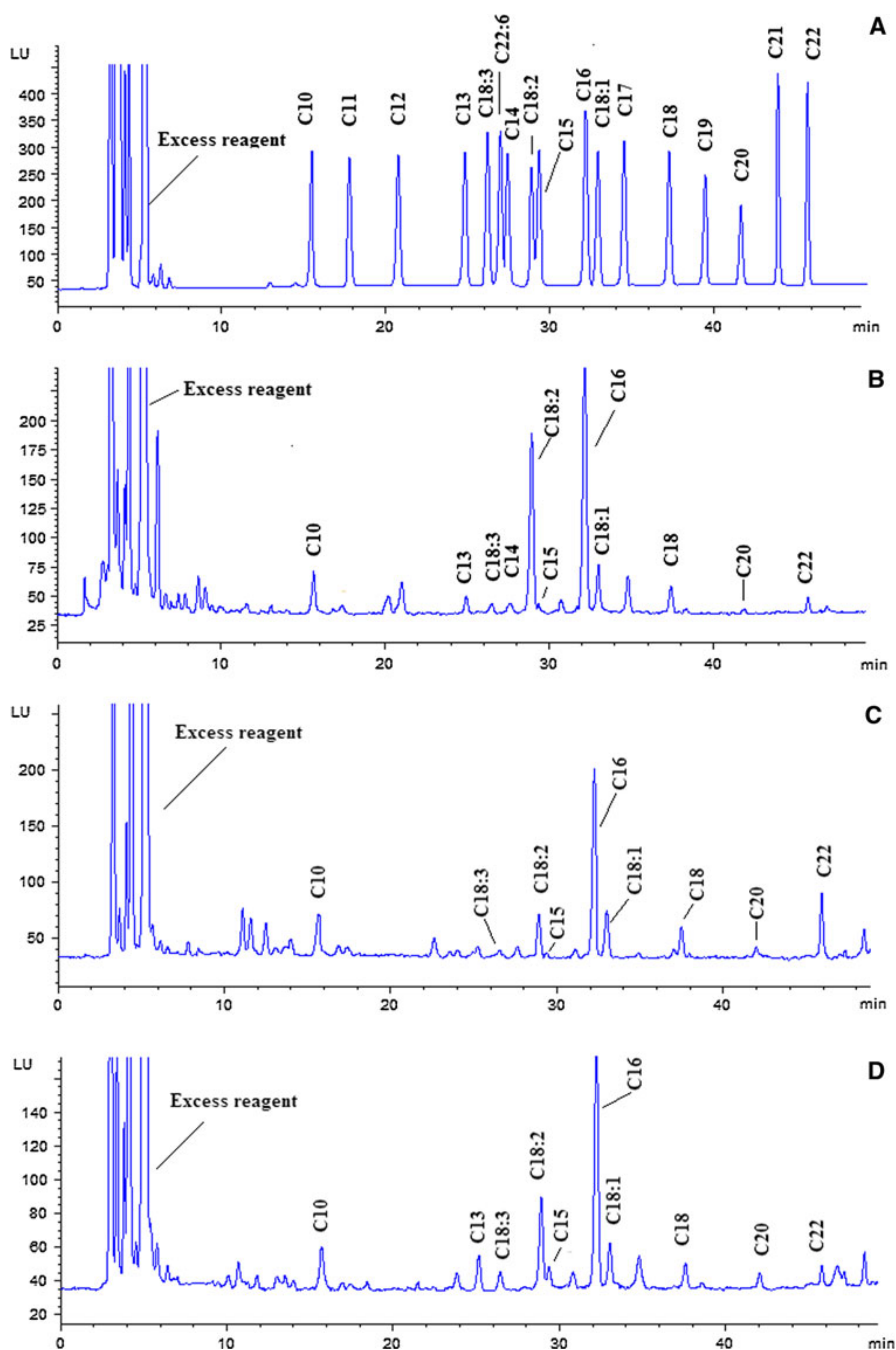

Fig. 4 The representative chromatograms for 17 FFA standards (**a**), and the stem, root and flower of *Swertia nervosa* (**b**–**d**) HPLC conditions: FA derivatives separation was carried out on a reversed-phase Hypersil BDS C_8 column (4.6 × 200 mm, 5 μm); the mobile phase A was 30% acetonitrile containing 0.1% ammonium formate and B was 100% acetonitrile; the fluorescence excitation and emission wavelengths were set at λ_{ex} 272 nm and λ_{em} 505 nm, respectively

Method Validation

The results for the method validation are shown in Table 3. The linearity of an analytical method is its ability, within a definite range, to obtain results directly proportional to the concentrations of the analyte in the sample. The correlation coefficients were found to be >0.9994, indicating excellent linearity. As presented in Table 3, the proposed method offered the low LOD of 0.60–1.11 ng mL^{-1} and LOQ of 1.22–3.41 ng mL^{-1}, which are significantly lower than the common methods based on GC [25, 26]. The high detection sensitivity would enable us to accurately analyze the

Table 3 Linear regression equations, correlation coefficients, LOD, LOQ, reproducibility of retention time and peak area, and accuracy

FAA	MS data[a]	$Y = AX + B$[b]	r	LOD[c] (ng/mL)	LOQ[d] (ng/mL)	Repeatability R.S.D (%) (n = 6)		Accuracy (n = 3)	
						Retention time	Retention time	Recovery	R.S.D (%)
C10	443.9	$Y = 44.60X + 12.26$	0.9999	0.60	1.55	0.04	1.60	92.8	2.7
C11	457.9	$Y = 39.71X + 7.81$	0.9997	1.00	2.41	0.02	2.05	96.4	1.8
C12	471.9	$Y = 43.43X + 16.20$	0.9998	0.77	1.80	0.01	1.65	101.4	2.5
C13	486.0	$Y = 44.10X + 15.24$	0.9995	0.74	1.90	0.01	1.95	97.1	2.4
C18:3	552.2	$Y = 61.86X + 24.06$	0.9997	0.48	1.22	0.03	1.25	99.6	1.8
C22:6	600.4	$Y = 37.62X + 6.54$	0.9999	1.11	2.77	0.04	1.40	95.7	1.1
C14	500.0	$Y = 34.53X + 5.40$	0.9998	0.97	3.03	0.03	1.75	93.1	1.5
C18:2	554.3	$Y = 48.73X + 35.83$	0.9996	0.89	2.60	0.03	1.30	100.1	1.7
C15	514.0	$Y = 29.33X + 8.31$	0.9997	1.05	3.18	0.06	1.25	93.7	1.4
C16	528.4	$Y = 54.03X + 41.47$	0.9995	0.63	2.18	0.01	1.60	92.3	2.4
C18:1	555.5	$Y = 90.23X + 18.75$	0.9994	0.69	2.06	0.02	1.20	102.4	1.6
C17	542.1	$Y = 46.29X + 9.22$	0.9996	1.07	3.23	0.02	2.01	98.1	1.0
C18	556.4	$Y = 41.84X + 9.06$	0.9999	0.95	3.20	0.04	1.25	100.6	1.4
C19	570.1	$Y = 47.78X + 9.09$	0.9999	1.06	3.41	0.05	1.55	96.6	2.3
C20	584.0	$Y = 33.57X + 1.69$	0.9998	0.91	3.05	0.02	1.10	93.2	2.5
C21	598.1	$Y = 59.64X + 15.82$	0.9997	0.87	2.87	0.04	1.40	104.5	1.7
C22	613.5	$Y = 113.89X + 25.54$	0.9998	0.78	2.49	0.03	1.15	99.1	1.3

[a] Mass spectrometry

[b] X Injected amount (pmol) Y Peak area

[c] Limit of detection, S/N = 3, per 10 μL injection volume

[d] Limit of quantification S/N = 10, per 10 μL injection volume

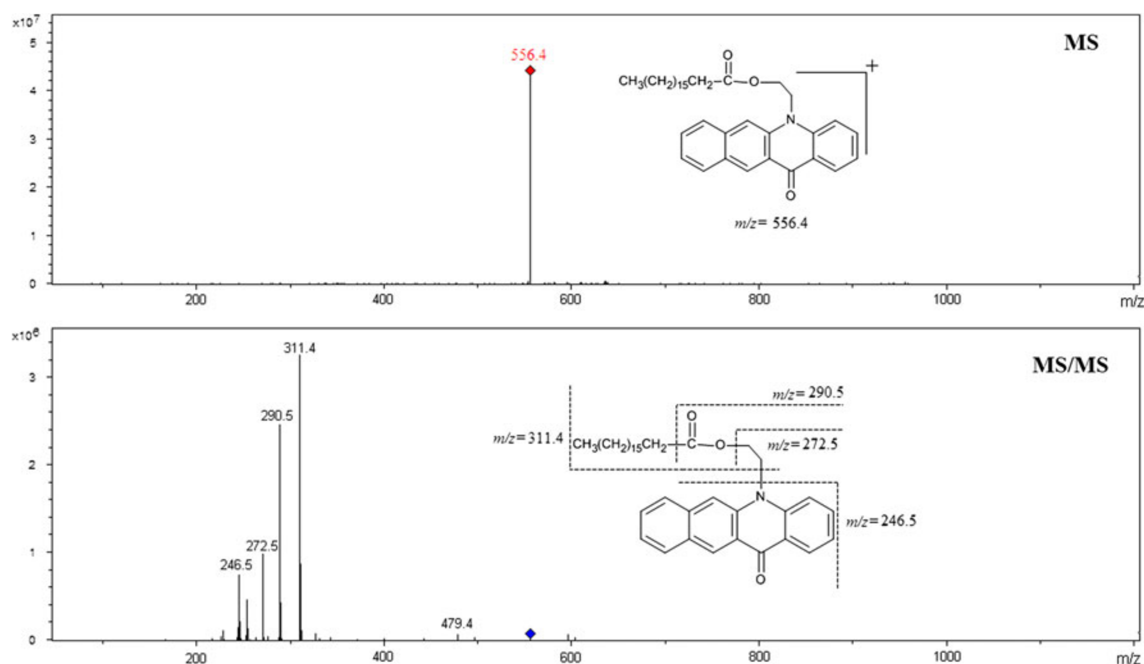

Fig. 5 MS spectra of the representative C18 fatty acid derivative and the cleavage mode of protonated molecular ion

FFA in medicinal plants where usually very low content of FFA is expected. The reproducibility of retention time and peak area were obtained with R.S.D. values lower than 0.05 and 2.05%, respectively. The experimental accuracies were found to be in the range of 92.3–104.5%, which indicated the excellent accuracy of the proposed method.

Table 4 The content of free fatty acids in nine *Swertia* species [Mean value ± S.D. (standard deviation)]

Swertia species	Organs	Free fatty acid content (μg/g)												UFFA[a] (μg/g)	TFFA[b] (μg/g)
		C10	C13	C18:3	C14	C18:2	C15	C16	C18:1	C18	C20	C21	C22		
Swertia racemosa	Stem	20.3 ± 0.8	6.5 ± 0.4	29.2 ± 0.4	11.3 ± 0.4	105.3 ± 1.6	7.6 ± 0.1	254.9 ± 3.8	10.8 ± 0.5	23.1 ± 1.2	11.7 ± 0.4	1.4 ± 0.1	11.5 ± 0.8	145.3 ± 2.7	638.8 ± 5.1
	Root	38.1 ± 1.9	30.5 ± 0.9	44.5 ± 0.5	31.7 ± 0.3	209.1 ± 3.2	11.3 ± 0.3	361.3 ± 4.1	50.0 ± 1.8	135.4 ± 3.4	77.8 ± 0.5	6.1 ± 0.7	31.5 ± 1.3	304.0 ± 5.3	1332 ± 17.0
	Flower	59.3 ± 2.3	42.2 ± 1.3	149.2 ± 3.8	5.3 ± 0.2	194.1 ± 2.6	3.1 ± 0.0	223.4 ± 2.9	41.4 ± 1.3	26.1 ± 1.3	25.1 ± 1.0	–[d]	63.6 ± 3.4	384.8 ± 4.9	1218 ± 15.3
Swertia angustifolia	Stem	122.0 ± 3.7	9.0 ± 0.6	20.2 ± 0.7	10.7 ± 0.8	60.7 ± 0.5	6.6 ± 0.1	127.7 ± 2.0	20.0 ± 0.6	20.9 ± 0.9	11.1 ± 0.7	–[d]	9.4 ± 0.6	101.0 ± 3.3	519.8 ± 9.7
	Root	11.1 ± 0.8	+[c]	11.2 ± 0.4	4.7 ± 0.7	122.3 ± 2.7	2.7 ± 0.4	167.1 ± 2.2	16.8 ± 0.8	21.3 ± 1.4	8.3 ± 0.4	–[d]	13.4 ± 1.4	150.4 ± 6.1	529.5 ± 7.8
	Flower	53.5 ± 0.6	44.7 ± 2.6	12.5 ± 0.7	–[d]	38.6 ± 0.4	4.4 ± 0.5	223.7 ± 1.9	37.0 ± 1.7	27.2 ± 0.5	31.2 ± 0.2	–[d]	13.8 ± 0.5	88.1 ± 2.6	575.1 ± 8.5
Swertia chirayita	Stem	34.8 ± 1.1	3.36 ± 0.1	11.0 ± 0.4	9.6 ± 0.8	9.3 ± 0.7	2.7 ± 0.1	280.4 ± 3.3	29.5 ± 0.4	110.1 ± 3.1	43.6 ± 3.3	7.9 ± 0.2	18.1 ± 0.7	49.8 ± 3.2	610.2 ± 10.3
	Root	32.9 ± 2.0	+[c]	+[c]	63.5 ± 2.5	12.0 ± 0.8	5.9 ± 0.2	344.4 ± 2.9	40.9 ± 0.5	52.3 ± 1.5	31.2 ± 1.2	–[d]	24.0 ± 1.2	53.0 ± 1.5	660.4 ± 9.5
	Flower	6.2 ± 0.1	18.6 ± 0.8	+[c]	16.4 ± 0.8	9.1 ± 0.2	2.7 ± 0.1	377.4 ± 4.2	55.1 ± 1.3	89.7 ± 3.3	130.6 ± 4.2	9.7 ± 0.8	44.2 ± 2.5	64.3 ± 2.5	824.3 ± 9.8
Swertia verticillifolia	Stem	37.1 ± 0.4	+[c]	40.9 ± 0.4	9.0 ± 0.4	157.1 ± 2.0	5.3 ± 0.2	218.3 ± 2.6	14.2 ± 1.9	34.8 ± 1.6	10.9 ± 1.3	–[d]	19.0 ± 2.3	212.3 ± 4.1	759.2 ± 12.6
	Root	52.6 ± 1.7	+[c]	15.9 ± 0.2	19.8 ± 0.7	301.6 ± 4.5	2.3 ± 0.6	380.1 ± 4.0	42.6 ± 1.4	49.4 ± 1.5	2.0 ± 0.1	–[d]	8.8 ± 0.4	360.1 ± 4.6	1235 ± 15.9
	Flower	57.6 ± 0.6	6.9 ± 0.1	7.2 ± 0.2	3.8 ± 0.1	70.5 ± 0.8	9.7 ± 0.5	274.4 ± 2.5	44.7 ± 0.8	53.6 ± 2.4	83.8 ± 2.7	–[d]	45.9 ± 3.6	122.3 ± 3.9	782.7 ± 12.3
Swertia nervosa	Stem	39.4 ± 0.5	13.2 ± 0.8	27.9 ± 0.3	6.4 ± 0.2	95.6 ± 2.7	20.5 ± 0.5	245.4 ± 3.4	23.5 ± 1.2	29.2 ± 0.3	14.1 ± 1.2	–[d]	7.9 ± 0.2	147.0 ± 4.0	670.3 ± 11.0
	Root	33.9 ± 1.4	+[c]	31.0 ± 0.4	–[d]	256.0 ± 3.1	16.6 ± 0.3	229.3 ± 2.6	19.3 ± 0.7	20.5 ± 0.8	16.7 ± 2.4	–[d]	13.7 ± 0.9	306.2 ± 3.6	943.6 ± 7.8
	Flower	34.7 ± 1.9	25.8 ± 1.9	29.1 ± 0.3	–[d]	120.1 ± 1.6	6.6 ± 0.1	176.4 ± 2.0	31.1 ± 1.3	13.3 ± 0.9	23.6 ± 0.9	–[d]	33.8 ± 0.3	180.4 ± 2.1	675.1 ± 6.6
Swertia phragmitiphylla	Stem	12.9 ± 0.6	+[c]	40.8 ± 0.5	17.6 ± 0.8	108.7 ± 2.8	10.9 ± 0.2	131.4 ± 2.9	35.8 ± 1.1	33.4 ± 2.4	29.9 ± 1.3	–[d]	23.3 ± 1.2	185.3 ± 2.5	630.2 ± 11.1
	Root	1.4 ± 0.2	+[c]	27.4 ± 0.9	12.7 ± 0.5	152.6 ± 3.0	6.6 ± 0.5	77.6 ± 1.5	93.0 ± 1.6	53.0 ± 3.6	75.4 ± 1.7	–[d]	11.3 ± 0.3	273.0 ± 4.1	784.2 ± 10.2
	Flower	+[c]	+[c]	150.4 ± 3.1	25.9 ± 0.9	91.4 ± 1.5	14.7 ± 0.2	162.0 ± 2.0	138.4 ± 1.2	101.4 ± 4.5	85.8 ± 2.6	–[d]	22.9 ± 0.5	380.2 ± 5.9	1173 ± 14.7
Swertia kingii	Stem	39.5 ± 1.5	+[c]	51.3 ± 0.4	4.6 ± 0.2	170.8 ± 2.7	6.8 ± 0.1	412.9 ± 6.6	47.8 ± 1.5	59.0 ± 0.6	34.5 ± 2.2	–[d]	17.5 ± 1.2	269.9 ± 4.6	1114 ± 13.6
	Root	17.7 ± 0.7	+[c]	29.7 ± 0.8	11.8 ± 0.6	165.7 ± 1.9	13.2 ± 1.1	210.0 ± 3.0	23.5 ± 0.5	41.8 ± 2.3	14.7 ± 1.0	–[d]	4.1 ± 0.1	218.8 ± 3.1	751.1 ± 6.4
	Flower	21.4 ± 0.3	+[c]	13.4 ± 0.3	9.2 ± 0.4	49.2 ± 1.7	5.9 ± 0.3	108.2 ± 1.4	21.0 ± 0.7	22.8 ± 1.0	16.5 ± 0.8	–[d]	24.2 ± 0.8	83.5 ± 2.6	375.5 ± 10.1
Swertia mussotii	Stem	29.4 ± 0.2	+[c]	24.0 ± 0.4	9.0 ± 0.6	60.6 ± 0.9	14.1 ± 0.7	352.2 ± 4.6	27.7 ± 0.3	40.2 ± 2.3	21.2 ± 2.1	–[d]	20.7 ± 0.4	112.2 ± 2.5	711.6 ± 8.8
	Root	82.6 ± 2.4	+[c]	43.2 ± 1.4	4.2 ± 0.2	393.4 ± 5.1	8.0 ± 0.4	424.4 ± 5.1	56.2 ± 0.4	55.5 ± 1.7	46.6 ± 1.2	–[d]	28.1 ± 0.3	492.7 ± 6.3	1635 ± 20.6
	Flower	68.4 ± 0.8	18.6 ± 0.2	42.0 ± 1.9	10.1 ± 0.4	351.6 ± 4.2	2.4 ± 0.3	379.3 ± 2.1	117.2 ± 2.0	49.7 ± 3.4	118.8 ± 2.8	–[d]	61.0 ± 4.8	510.8 ± 7.6	1732 ± 21.7
Swertia franchetiana	Stem	28.1 ± 1.2	6.5 ± 0.1	90.3 ± 1.0	+[c]	324.5 ± 4.1	3.7 ± 0.2	269.5 ± 3.2	25.9 ± 1.4	16.6 ± 1.1	6.8 ± 0.6	–[d]	12.3 ± 2.2	440.7 ± 8.9	1225 ± 17.2
	Root	53.5 ± 1.8	3.9 ± 0.0	60.8 ± 0.7	+[c]	294.9 ± 3.7	3.2 ± 0.8	277.3 ± 4.5	22.6 ± 1.6	25.3 ± 3.2	16.9 ± 1.2	–[d]	2.1 ± 0.2	378.2 ± 4.5	1138 ± 19.9
	Flower	27.1 ± 0.9	43.6 ± 0.5	235.4 ± 2.9	+[c]	105.4 ± 2.3	2.2 ± 0.0	208.4 ± 3.2	18.9 ± 0.3	8.8 ± 0.6	16.3 ± 0.8	–[d]	16.5 ± 2.3	359.7 ± 4.8	1046 ± 15.4

[a] Total contents of unsaturated fatty acids (μg/g)

[b] Total contents (μg/g)

[c] Below the limit of quantification

[d] Below the limit of detection

The intra- and inter-day precision (expressed in terms of % R.S.D.) were found to be in the range of 1.1–4.3 and 3.5–5.6%, respectively, which demonstrated the good precision of the established method.

Sample Analysis

The newly established analytical method was subsequently applied to simultaneous determination of 17 FFA in nine *Swertia* species including root, stem and flower (27 samples). All samples were analyzed using the optimized derivatization conditions and HPLC conditions. Each sample was analyzed in triplicate to determine the mean content (μg/g) and experiment results are presented in Table 4. Figure 4b–d shows the representative chromatograms for FFA in the stem, root and flower from *Swertia nervosa*. The FFA content variances in nine *Swertia* species were significant. *Swertia angustifolia* and *Swertia mussotii* represented the samples with the highest and lowest FFA content. The total FFA contents in stem, root and flower were ranged from 519.8 to 1,225 μg/g, from 529.5 to 1,635 μg/g, and from 575.1 to 1,732 μg/g, respectively. The most abundant FFA in *Swertia* plant was C16 with a content ranging from 77.8 to 424.4 μg/g, and the next FFA was C18:2 with a content range of 60.8–393.4 μg/g. The main unsaturated FFA in order of content were C18:2, C18:3 and C18:1.

Conclusion

This work reported a novel HPLC-FLD method coupled with pre-column derivatization using BAETS as a labelling reagent for FFA determination in medicinal plants. RSM was employed to optimize the FFA extraction and derivatization reaction ensuring the highest FFA recoveries within the shortest extraction time and the sufficient labelling of the analyzed components. The developed method here was capable of providing higher detection sensitivity and selectivity than the common methods based on GC. The FFA composition analysis in nine *Swertia* species was performed using the established method.

Acknowledgments This work was supported by the 100 Talents Program of The Chinese Academy of Sciences (No. 328) and the CAS Special Grant for Postgraduate Research, Innovation and Practice.

References

1. Brahmachari G, Mondal S, Gangopadhyay A (2004) *Swertia* (Gentianaceae): chemical and pharmacological aspects. Chem Biodivers 1:1627–1651
2. Cao Y, Wang Y, Ye J (2005) Differentiation of Swertia Mussotii Franch from Artemisiae Capillaris Herba by capillary electrophoresis with electrochemical detection. J Pharm Biomed Anal 39:60–65
3. Wang Z, Wang L, Li T (2006) Rapid analysis of the essential oils from dried *Illicium verum* Hook. f. and *Zingiber officinale* Rosc. by improved solvent-free microwave extraction with three types of microwave-absorption medium. Anal Bioanal Chem 386:1863–1868
4. Simopoulos A (1999) Essential fatty acids in health and chronic disease. Am J Clin Nutr 70:560S
5. von Schacky C, Harris W (2007) Cardiovascular benefits of omega-3 fatty acids. Cardiovas Res 73:310
6. Beydoun M, Kaufman J, Satia J, Rosamond W, Folsom A (2007) Plasma n-3 fatty acids and the risk of cognitive decline in older adults: the Atherosclerosis Risk in Communities Study. Am J Clin Nutr 85:1103
7. Bent S, Ko R (2004) Commonly used herbal medicines in the United States: a review* 1. Am J Med 116:478–485
8. Shidoji Y, Ogawa H (2004) Natural occurrence of cancer-preventive geranylgeranoic acid in medicinal herbs. J Lipid Res 45:1092
9. Wheat J, Currie G (2008) Herbal medicine for cancer patients: an evidence based review. Int J Alter Med 5:1–20
10. Kanya T, Rao L, Sastry M (2007) Characterization of wax esters, free fatty alcohols and free fatty acids of crude wax from sunflower seed oil refineries. Food Chem 101:1552–1557
11. Tan C, Ghazali H, Kuntom A, Tan C, Ariffin A (2009) Extraction and physicochemical properties of low free fatty acid crude palm oil. Food Chem 113:645–650
12. Bauza T, Blaise A, Daumas F, Cabanis J (1995) Determination of biogenic amines and their precursor amino acids in wines of the Vallée du Rhône by high-performance liquid chromatography with precolumn derivatization and fluorimetric detection. J Chromatogr A 707:373–379
13. Li G, You J, Suo Y et al (2011) 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 in *L. barbarum* seed oil. Food Chem 125(4):1365–1372
14. Ruiz-Rodriguez A, Reglero G, Ibaez E (2010) Recent trends in the advanced analysis of bioactive fatty acids. J Pharm Biomed Anal 51:305–326
15. Zinellu A, Sotgia S, Bastianina S et al (2009) Taurine determination by capillary electrophoresis with laser-induced fluorescence detection: from clinical field to quality food applications. Amino acids 36:35–41
16. Gatti R, Gioia M, Leoni A, Andreani A (2010) 2, 5-Dimethyl-1*H*-pyrrole-3,4-dicarbaldehyde as a precolumn derivatization reagent for HPLC/UV detection of amino acids. J Pharm Biomed Anal 53:207–211
17. Nimura N, Kinoshita T, Yoshida T, Uetake A, Nakai C (1988) 1-Pyrenyldiazomethane as a fluorescent labelling reagent for liquid chromatographic determination of carboxylic acids. Anal Chem 60:2067–2070
18. Su Y, Lin Y, Cheng F, Jen J (2010) In-capillary derivatization and stacking electrophoretic analysis of γ-aminobutyric acid and alanine in tea samples to redeem the detection after dilution to decrease matrix interference. J Agric Food Chem 58:120–126
19. Toyo'oka T (2002) Fluorescent tagging of physiologically important carboxylic acids, including fatty acids, for their detection in liquid chromatography. Anal Chim Acta 465:111–130
20. You J, Zhang W, Zhang Y (2001) Simple derivatization method for sensitive determination of fatty acids with fluorescence detection by high-performance liquid chromatography using 9-(2-hydroxyethyl)-carbazole as derivatization reagent. Anal Chim Acta 436:163–172

21. Lin Y, Su Y, Jen J (2007) Capillary electrophoretic analysis of γ-aminobutyric acid and alanine in tea with in-capillary derivatization and fluorescence detection. J Agric Food Chem 55:2103–2108
22. You J, Fu Y, Sun Z, Suo Y (2010) 2-(5-Benzoacridine)ethyl-*p*-toluenesulfonate as sensitive reagent for the determination of bile acids by HPLC with fluorescence detection and online atmospheric chemical ionization-mass spectrometric identification. Anal Bioanal Chem 396:2657–2666
23. Li G, Zhang X, You J et al (2011) Highly sensitive and selective pre-column derivatization HPLC approach for rapid determination of triterpenes oleanolic and ursolic acids and application to Swertia species: optimization of triterpenic acids extraction and pre-column derivatization using response surface methodology. Anal Chim Acta 688:208–218
24. Bligh EG, Dyer WJ (1959) A rapid method of total lipid extraction and purification. Can J Physiol Pharmacol 37:911–917
25. Kangani CO, Kelley DE, DeLany JP (2008) New method for GC/FID and GC-C-IRMS analysis of plasma free fatty acid concentration and isotopic enrichment. J Chromatogr B 873:95–101
26. Yang F, Feng K, Zhao J, Li S (2009) Analysis of sterols and fatty acids in natural and cultured *Cordyceps* by one-step derivatization followed with gas chromatography–mass spectrometry. J Pharm Biomed Anal 49:1172–1178