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Quantitative Analysis of Fatty Acids from Safflower by Selective and Sensitive Pre-Column Derivatization Method Using HPLC-FLD and Online APCI/MS

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A pre-column derivatization method using 2-(11*H*-benzo[a]carbazol-11-yl) ethyl 4-methylbenzenesulfonate (BCETS) as labeling reagent followed by HPLC with fluorescence detection and on line APCI/MS for the determination and quantitative analysis of fatty acids from safflower has been successfully developed. The fatty acids could be easily and quickly labeled by BCETS at 95 ºC in the presence of K_2CO_3 as catalyst in DMF within 35 min. The derivatives exhibited excellent fluorescence property with excitation and emission wavelengths of 272 and 505 nm, respectively. The method displayed good selectivity, sensitivity, reproducibility and applicability. Qinghai and Xinjiang safflower (QHS and XJS), which were planted in different places in Qinghai province, were analyzed by the established method. The result showed the contents of total fatty acids, saturated fatty acids and unsaturated fatty acids from Qinghai safflower sample were all much higher than that from Xinjiang safflower sample, especially for Qinghai safflower sample planted in Datong county. The main unsaturated fatty acids were C18:1, C18:2 and C18:3 and the dominated saturated fatty acids were C16, C18 and C20, respectively. This work would be helpful for the safety assessment and quality control of the two kinds of safflowers.

Keywords: Fatty acids, Safflower, HPLC-FLD, Pre-column derivatization, APCI/MS.

INTRODUCTION

Safflower, the corolla from *Carthamus tinctorius* L. (Asteraceae) is widely cultivated in China and used as a natural pigment, food additive and cosmetic[1]. It is widely used as traditional medicine for the treatment of inflammatory diseases, arteriosclerosis, hyperlipemia, gynecological disorders and osteoporosis[2]. Previous studies have showed that the bioactive compounds in the dried petals of safflower contained flavonoids[3], safflower yellow A[4], safflomin[5], alkaloids[6], lignans[7], triterpence alcohols[8], *etc*. To the best of our knowledge, no report has been published to analyze the content of fatty acid in the dried petals of safflower. However, there are number of biological effects of fatty acids such as cathartic[9], antioxidative[10], radical-scavenging effect[10], antityrosinase[11], melanine production inhibitory[12], antitumor[13] and fibroblasts growth promoting activities[14]. Recent years, more and more researches have found that fatty acid is indispensable for the efficacy of medicinal plants[15-17].

Since fatty acids show neither natural UV absorption nor fluorescence property, direct LC methods are rarely applied to the analysis of them[18]. So it is a challenging task to analyze the fatty acid mixtures and the most commonly used technique is to transform fatty acids into their less polar methyl ester derivatives (FAMEs) for the analysis by GC-MS[19]. Although this method is well developed and fairly effective, there are some problems associated with volatility of polar compounds, stability of derivatives and their thermal decomposition during analysis[20,21]. Compared with GC, determination of fatty acid derivatives by HPLC holds some unquestionable advantages[22]. Firstly, HPLC allows fatty acid to be converted to a large number of different derivatives and it can overcome some problems such as tailing peaks and low detector sensitivity[23]. Secondly, the HPLC method allows the nonvolatile, thermally labile compounds to be separated with mild operating conditions[23]. Finally, the analyses after HPLC are undamaged, so it is possible to collect the fraction to make further investigations[23].

The fatty acid analysis with HPLC can be achieved by derivatization of the carboxyl moiety with a suitable chromophore or fluorophore. Recently, a fluorescent probe named 2-(11*H*-benzo[a]carbazol-11-yl) ethyl 4-methyl-benzenesulfonate (BCETS) (Fig. 1) was developed and successfully

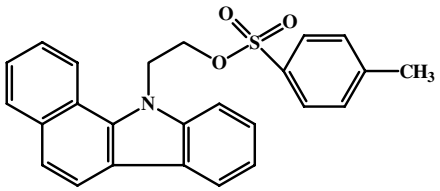

Fig. 1. Chemical structures of BCETS

employed to carboxylic acid analysis[24]. In this study, the reagent was used as pre-column derivatization reagent to determinate fatty acids by HPLC with fluorescent detection and on line atmospheric pressure chemical ionization mass spectrometry and it was successfully applied to analyze the fatty acids in the dried petals of the two kinds of safflowers which planted in different places.

EXPERIMENTAL

The HPLC analysis was performed using an Agilent 1100 series HPLC system, equipped with a vacuum degasser (model G1332), an autosampler (model G1329A), a quaternary pump (model G1311A), a thermostated column compartment (model G1316A) and the FLD (model G1321A) adjusted at wavelengths of 272 and 505 nm for excitation and emission. Chromatographic separation was achieved on a Hypersil BDS C8 column (200 mm × 4.6 mm, 5 μm, Dalian Elite Analytical Instruments Co., Ltd., China). Solvent A was 5 % acetonitrile in water and B was acetonitrile. The flow rate was constant at 1.0 mL min^{-1} and the column temperature was kept at 30 ºC. The injection volume was 10 μL. The column was equilibrated with the initial mobile phase for 5 min before the next injection. The gradient condition of mobile phase was as follows: 65-83 % B from 0-35 min; 83-88 % B from 35-50 min; 88-100 % B from 50-55 min and then held for 5 min.

All fatty acids used as standards were purchased from Sigma Reagent Co. (USA). HPLC grade acetonitrile was purchased from Yuwang Company, China. Dimethylformamide (DMF), potassium carbonate (K_2CO_3) 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. BCETS was obtained from professor You with purity higher than 99 %.

Samples: The seed of Qinghai safflower was purchased from Qinghai Datong county and the seed of Xinjiang safflower was purchased from Xinjiang Tacheng county. Then these two kinds of seeds were planted in Qinghai Datong, Minhe and Duoba county of Qinghai province, respectively. The petals of safflower were collected, dried naturally and smashed for the next step.

Preparations of standard solutions and samples: Each of the low concentrations was obtained by diluting the stock solution with acetonitrile and the labeling reagent was prepared by dissolving 22.15 mg BCETS with 10 mL DMF. Individual standard of fatty acid was prepared by dissolving the fatty acid in HPLC grade acetonitrile to a concentration of 1×10^{-2} mol L^{-1}. Standards of 20 mixed fatty acids were prepared by diluting the corresponding individual stock solution with acetonitrile and then mixing them up. When not in use, all reagent solutions were stored at 4 ºC in a refrigerator.

The sample solution was prepared as following: 0.1 g petal powder of safflowers was added to 5 mL chloroform/methanol mixture (1:1, v/v) in a 10 mL graduated test tube with stopper and then extracted with ultrasonication for 1 h. The purpose of the application of chloroform/methanol mixture was to make sufficient exaction[25]. After the extraction, the supernatant was collected.

Derivatization of fatty acids: 150 μL standard fatty acid mixtures, 200 μL BCETS and 100 μL DMF were added orderly to a 2 mL vial containing 15 mg K_2CO_3. The vial was sealed and allowed to react in a water bath at 90 ºC for 35 min. 100 μL supernatant obtained above was removed to dry under a stream of nitrogen gas in a 2 mL vial and then 15 mg K_2CO_3, 200 μL BCETS and 100 μL DMF were added orderly into the vial. The vial was sealed and allowed to react in a water bath at 90 ºC for 35 min. After the reaction was completed, the mixture was cooled to room temperature. The derivatization solution was syringe filtered using a 0.22 mm nylon filter and injected directly into the chromatograph column. The injected volume was 10 μL. The derivatization procedure of fatty acids with BCETS was shown in Fig. 2.

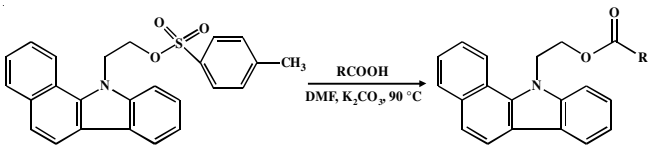

Fig. 2. Scheme of derivatization reaction of BCETS with fatty acids

MS conditions: The mass spectrometer 1100 series LC-MSD Trap-SL (ion trap) from Bruker Daltonik (Bremen, Germany) was equipped with an ESI source. Ion source conditions: ESI in positive ion mode, nebulizer pressure 241.3 kPa, dry gas temperature 350 ºC, dry gas flow 9.0 L min^{-1} and capillary voltage-3500 V.

RESULTS AND DISCUSSION

Chromatographic separation and mass spectrometry identification: The BCETS-fatty acid derivatives were successfully separated with the method mentioned above (Fig. 3) and the chromatographic peaks were simultaneously identied by online post-column mass spectrometry in positive mode. All molecular ions $[M + H]^+$ of 20 fatty acids derivatives were listed in Table-1. Fig. 4 showed the MS, MS/MS and cleavage mode of the typical C18:1 derivative. As expected, the BCETS-fatty acid derivative exhibited intense molecular ion peaks. C18:1 derivative produced an intense molecular ion peak at *m/z* 309 and specic fragment ions at *m/z* 216.8, *m/z* 243.8 and *m/z* 261.4. The characteristic fragment ion at *m/z* 216.8 was from the cleavage of N-C bond of the N-linked side chain. The specific fragment ion at *m/z* 243.8 was generated from the cleavage of C-OCO bond. The peculiar fragmention at *m/z* 261.4 was from the cleavage of O-CO bond of the BCETS-fatty acid derivative. Despite the amount of other endogenous acidic compounds present in samples that were co-extracted and then derivatized with the target fatty acids, a clear composition of fatty acids from samples could be doubly identified by chromatographic retention time and on-line MS identification for the highly specific parent mass-to-charge ratio.

TABLE-1
MS DATA, LINEAR REGRESSION EQUATIONS, CORRELATION COEFFICIENTS, LODs, LOQs, REPRODUCIBILITY AND ACCURACY

Fatty acid	MS data	Y = AX + B	r^2	LOD (ng mL^{-1})	LOQ (ng mL^{-1})	Repeatability RSD (%, n = 6)		Accuracy (n = 3)	
						Retention time	Peak area	Recovery	RSD (%)
C10	416.3	Y = 37.82X – 1.07	0.9998	1.965	4.878	0.05	1.60	92.8	1.8
C11	430.1	Y = 34.49X + 1.38	0.9999	2.014	5.681	0.03	2.05	96.4	1.4
C12	444.3	Y = 36.88X.33	0.9997	1.732	4.174	0.02	1.65	101.4	2.3
C13	458.3	Y = 33.31X + 7.15	0.9999	2.287	6.715	0.06	1.95	97.1	1.4
C18:3	521.9	Y = 34.38X + 1.63	0.9999	1.624	3.741	0.02	1.25	99.6	1.6
C14	472.3	Y = 25.73X + 6.19	0.9998	1.857	4.594	0.01	1.40	95.7	2.1
C16:1	497.8	Y = 35.88X + 9.05	0.9999	1.769	4.278	0.03	1.75	93.1	1.8
C18:2	523.9	Y = 25.18X + 4.52	0.9999	1.672	3.931	0.03	1.30	100.1	1.2
C15	486.3	Y = 23.70X – 0.13	0.9998	1.499	3.422	0.04	1.25	93.7	1.5
C16	500.2	Y = 32.05X + 2.22	0.9997	1.584	3.758	0.02	1.60	92.3	2.1
C18:1	525.8	Y = 22.25X + 5.09	0.9996	1.367	3.284	0.03	1.20	102.4	1.7
C17	514.4	Y = 24.60X – 1.54	0.9999	1.472	3.531	0.06	2.01	98.1	2.0
C18	528.3	Y = 11.95X – 2.16	0.9996	1.459	3.497	0.05	1.25	100.6	1.8
C20:1	553.9	Y = 13.65X + 4.02	0.9993	1.631	3.867	0.07	1.55	96.6	1.6
C19	542.4	Y = 19.04X + 5.25	0.9995	1.458	3.552	0.02	1.10	93.2	1.5
C20	556.3	Y = 4.22X + 1.47	0.9997	1.475	3.691	0.04	1.40	104.5	2.4
C21	570.3	Y = 17.69X – 4.22	0.9999	1.534	3.855	0.03	1.15	99.1	2.3
C22	584.1	Y = 10.90X – 0.68	0.9998	1.697	1.067	0.04	2.16	98.6	1.9
C23	598.4	Y = 7.20X – 4.46	0.9995	2.078	5.068	0.07	1.22	95.4	1.3
C24	612.3	Y = 14.69X – 4.22	0.9997	2.156	6.047	0.05	1.67	102.5	2.3

Note: X: Injeced amount (pmol), Y: Peak area.

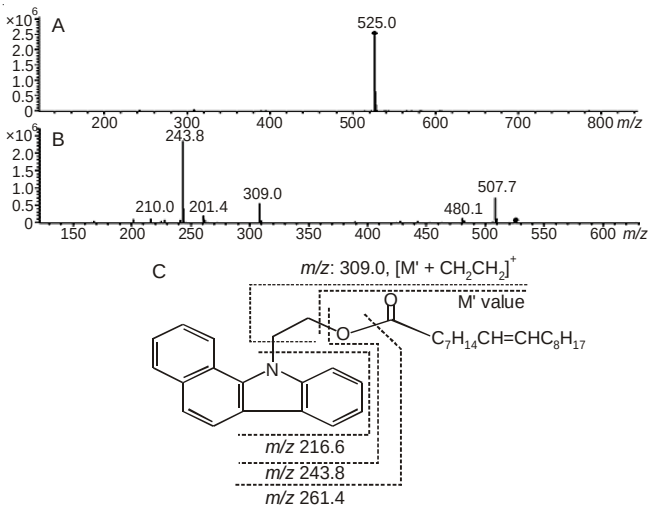

Fig. 3. Mass spectra of oleic acid (C18:1) derivative and its cleavage mode

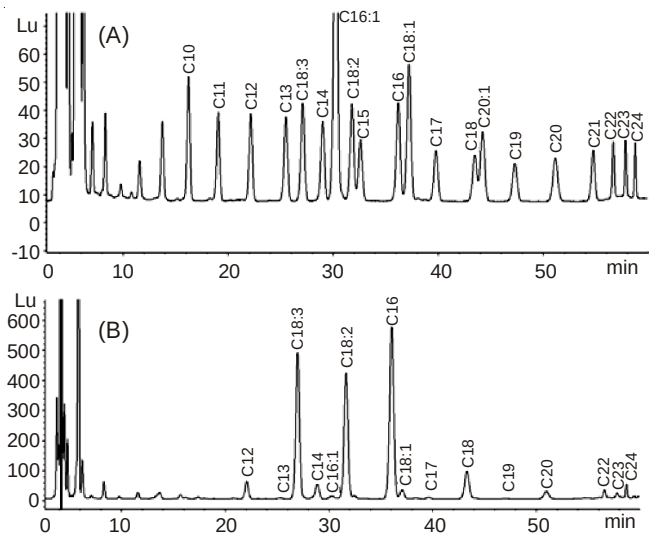

Fig. 4. A representative chromatogram of fatty acid standard solution (A) and a chromatogram of fatty acids in safflower (B) peak labels: C10 (decoic acid); C11 (undecanoic acid); C12 (dodecanoic acid); C13 (tridecanoic acid); C18:3 (linolenic acid); C14(tetradecanoic acid); C16:1 (palmitoleic acid); C18:2 (linoleic acid); C15 (pentadecanoic acid); C16 (hexadecanoic acid); C18:1 (oleic acid); C17 (heptadecanoic acid); C18 (octadecanoic acid); C20:1 (8-eicosenoic acid); C19 (nonadecanoic acid); C20 (eicosoic acid); C21 (heneicosoic acid); C22 (docosanoic acid); C23 (tricosanoic acid); C24 (lignoceric acid)

Method valuation: Linearity, limit of detections and limit of quantifications (LODs and LOQs), precision and accuracy of this method were evaluated (Table-1). The standard curve was established by sample injection with six concentrations according to the regression of the peak area *versus* concentration of each fatty acid standard. All the correlation coefficients of fatty acids were found to be greater than 0.9995, which implied that all fatty acid standards had excellent linear responses. Limit of detection were defined as the compound concentration that produced a signal-to-noise ratio of 3 (S/N = 3), which ranged from 0.562-1.587 ng mL^{-1}. Limit of quantification were defined as the compound concentration that produced a signal-to-noise ratio of 10 (S/N = 10), which was in the range of 1.463-6.068 ng mL^{-1} (Table-1). The precisions of the method were carried out by six sample injection and the RSD value of the retention time and peak area were 0.01-0.07 and 1.10-2.16 %, respectively. The recoveries of this method were measured by analyzing samples spiked with 1.0, 5.0 and 10.0 μg g^{-1} fatty acids. The recoveries were calculated based on the formula of (measured value-endogenous value)/added value × 100 %. All analysis procedures were carried out in triplicate. The results showed that the recoveries of all fatty acids were in the range 92.8-104.5 % and the values of RSD ranged from 1.3-2.4 %. It is indicated that the method has preferable practicability.

TABLE-2
CONTENT OF FATTY ACIDS IN SAFFLOWER (μg g^{-1})

Fatty acid		Qinghai safflower sample			Xinjiang safflower sample		
		Datong	Minhe	Duoba	Datong	Minhe	Duoba
Saturated fatty acids	C10	nd	nd	nd	Nd	nd	nd
	C11	nd	nd	nd	Nd	nd	nd
	C12	277.01	201.90	320.46	305.77	245.45	150.61
	C13	29.52	18.23	22.60	13.03	23.90	30.25
	C14	515.86	330.84	282.85	203.89	303.55	322.30
	C15	nd	nd	nd	Nd	nd	nd
	C16	3833.08	3424.96	3563.10	2011.94	2389.85	1875.72
	C17	56.94	47.17	45.84	24.98	34.81	36.71
	C18	2209.33	1930.33	1692.39	978.58	1246.45	1376.00
	C19	39.31	27.32	30.72	18.69	19.08	32.32
	C20	2816.90	1762.11	2092.13	1387.51	1532.67	2455.12
	C21	nd	nd	nd	Nd	nd	nd
	C22	533.80	375.04	369.12	230.47	254.43	459.15
	C23	430.00	411.85	311.72	251.15	261.04	405.21
	C24	471.44	370.61	381.79	252.05	275.92	363.71
	Total content	11213.20	8900.38	9112.73	5678.06	6587.16	7507.09
Unsaturated fatty acids	C16:1	57.66	44.38	37.59	8.30	17.34	42.21
	C18:1	271.98	201.37	237.81	140.07	188.42	341.02
	C18:2	3704.73	3352.26	376.18	2459.13	2689.97	1748.49
	C18:3	3343.61	2782.91	3561.44	1985.00	2340.27	1109.50
	C20:1	nd	nd	nd	Nd	nd	nd
	Total content	7377.98	6380.92	4213.01	4592.50	5236.00	3241.22
Total content		18591.17	15281.30	13325.74	10270.56	11823.16	10748.31

Note: nd: No detection or below the LOQ.

Fatty acid analysis in the petals of safflower: The method mentioned above was successfully employed to analyze the fatty acids in the petals of safflower. The separation chromatogram of standard fatty acids and safflower were showed in Fig. 4A,B. The composition and content of fatty acids in the petals of safflower were listed in Table-2. Table-2 showed that the content of total fatty acids in Qinghai safflower sample planted in Datong, Minhe and Duoba was 18.59, 15.28 and 13.33 mg g^{-1}, respectively and the content of unsaturated fatty acids was 7.38, 6.38 and 4.21 mg g^{-1}, respectively. The content of total fatty acids in Xinjiang safflower sample planted in Datong, Minhe and Duoba was 10.27, 11.82 and 10.75 mg g^{-1} and the content of unsaturated fatty acids was 4.59, 5.24 and 3.24 mg g^{-1}, respectively. The result indicated that the content of total fatty acids in Qinghai safflower samples was all higher than that of Xinjiang safflower samples. The highest content of total fatty acids and unsaturated fatty acids in Qinghai safflower sample were all planted in Datong county and the second highest content of these were planted in Minhe county. The lowest content of total fatty acids existed in the Xinjiang safflower planted in Datong county, wheras the lowest content of unsaturated fatty acids was the Xinjiang safflower sample planted in Duoba county.

Conclusion

The HPLC-FLD-APCI/MS method based on pre-column derivatization developed in this work proved to be reproducible (excellent sensitivity, linearity and precision) and accurate. This proposed method was successfully applied to analyze the content and composition of fatty acids in Qinghai safflower and Xinjiang safflower samples planted in different places in Qinghai province. The result showed that the composition of fatty acids in the safflower samples from various origins were about the same. Among the saturated fatty acids, the content of palmitic acid (C16) was the highest in all safflower samples from various places with the amount of 3.83 mg g^{-1} (Qinghai safflower sample planted in Datong county) and among the unsaturated fatty acids, the content of linoleic acid (C18:2) was the highest with the amount of 3.70 mg g^{-1} (Qinghai safflower sample planted in Datong county). The result also indicated that the content of total fatty acids, unsaturated fatty acids and saturated fatty acids in safflower samples from various places were significantly different. The content of total fatty acids in Qinghai safflower sample was all higher than Xinjiang safflower sample. The highest content of total fatty acids and unsaturated fatty acids in Qinghai safflower sample were all planted in Datong county and the second highest content of these were planted in Minhe county. The lowest content of total fatty acids existed in the Xinjiang safflower sample planted in Datong county, wheras the lowest content of unsaturated fatty acids was the Xinjiang safflower sample planted in Duoba county. So, the Qinghai safflower was selected to be planted in Datong county in Qinghai province in order to have a good production of fatty acids in safflower. This work also would be helpful for the safety assessment and quality control of safflower.

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