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Development of a New HPLC Method with Precolumn Fluorescent Derivatization for Rapid, Selective and Sensitive Detection of Triterpenic Acids in Fruits

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ABSTRACT: Triterpenic acids are widespread in plants and have multiplicity of biological properties. Unfortunately the method for accurate analysis of these compounds remains poorly investigated. This study proposed a highly sensitive and selective precolumn derivatization method for accurate determination of five triterpenic acids (betulinic acid, betulonic acid, maslinic acid, ursolic acid and oleanolic acid) in fruits using acridone-9-ethyl-*p*-toluenesulfonate (AETS) as fluorescent labeling reagent by HPLC with fluorescence detection (FLD). Response surface methodology was employed to optimize the derivatization reaction, ensuring the sufficient labeling of the analyzed components. The rapid separation of five triterpenic acids could be achieved in as little as 16 min. This developed method offered the exciting detection limits of 1.68−2.04 ng/mL. When applied to several popular fruits in China, it revealed satisfactory applicability and reproducibility. This developed method also exhibits powerful potential for accurate detection of triterpenic acids from other foodstuffs and nature products.

KEYWORDS: triterpenic acids, HPLC-FLD, precolumn fluorescent derivatization, fruits

INTRODUCTION

Consumption of sufficient amounts of fruit and vegetables is recommended as part of a healthy diet, which has been associated with lower incidence and lower mortality rates of many cancers,[1−3] cardio- and cerebrovascular diseases,[4,5] etc. Phytochemicals have been suggested to be responsible for the health benefits of fruits and vegetables.[6] Triterpenic acids as a group of phytochemicals are widespread in plants.[7] They are well-known for their multiple biological effects including hepatoprotective effects, acting at various stages of tumor development to inhibit tumor initiation and promotion,[7−9] cardiovascular, antihyperlipidemic, antioxidant effects,[10] and enhancing the cellular immune system.[11] These attractive biological properties prompt us to develop a novel method for triterpenic acid determination in fruits because accurate analysis of these compounds is imperative for better clarifying the health benefit of fruit consumptions. But it often represents several challenges. For example, triterpenic acids lacking chromophores show very low UV absorption and no fluorescence absorption, thus accurate detection of them using absorptiometry is fairly difficult. There are some matrix interferences because most plant samples contain various triterpenoid compounds with similar structures and polarities. Many triterpenic acids like oleanolic acid and ursolic acid are isomers making the separation more difficult. The reported methods for triterpenic acids analysis are capillary electrophoresis with UV,[12−15] high performance liquid chromatography with UV[16−19] or evaporative light scattering detector (ELSD)[20] or MS/MS[21] and gas chromatography.[22,23] Each of the methods above has its own characteristics, but they show no significant improvement on the detection sensitivity and selectivity. Thus, development of a rapid, selective and sensitive method for triterpenic acid determination in fruits is often required and also valuable.

The precolumn derivatization strategy has been widely used to improve the selectivity and increase the sensitivity in analytical chemistry. In our previous study, we described many labeling reagents containing toluenesulfonate for sensitive and selective determination of carboxylic compounds like free fatty acids[24,25] and bile acids.[26] These labeling reagents can rapidly and sufficiently react with their carboxylic functional group. Acridone-9-ethyl-*p*-toluenesulfonate (AETS) is one of the developed labeling reagents and possesses strong photoluminescence property, ensuring highly sensitive detection.[27] In the present study, we proposed a new precolumn derivatization method using AETS as fluorescent labeling reagent for accurate analysis of triterpenic acids in fruit samples by HPLC with fluorescence detection (FLD). To the best of our knowledge, it is the first trial of exploring the precolumn fluorescent labeling method coupled with HPLC-FLD for rapid, selective and sensitive detection of triterpenic acids in fruits. Five triterpenic acids including betulinic acid, betulonic acid, maslinic acid, ursolic acid and oleanolic acid were chosen as target compounds (scheme in Figure 1), which are some of the most abundant in the plant kingdom.[28] Compared to the reported methods,[12−23] this developed method is capable of offering lower detection limit and higher selectivity, with shorter separation time and minimal sample preparation.

Traditionally, optimization of derivatization in analytical chemistry has been carried out by a one-factor test. Its major disadvantage is that it does not include the interactive effects among the variables studied.[29] Another disadvantage is the

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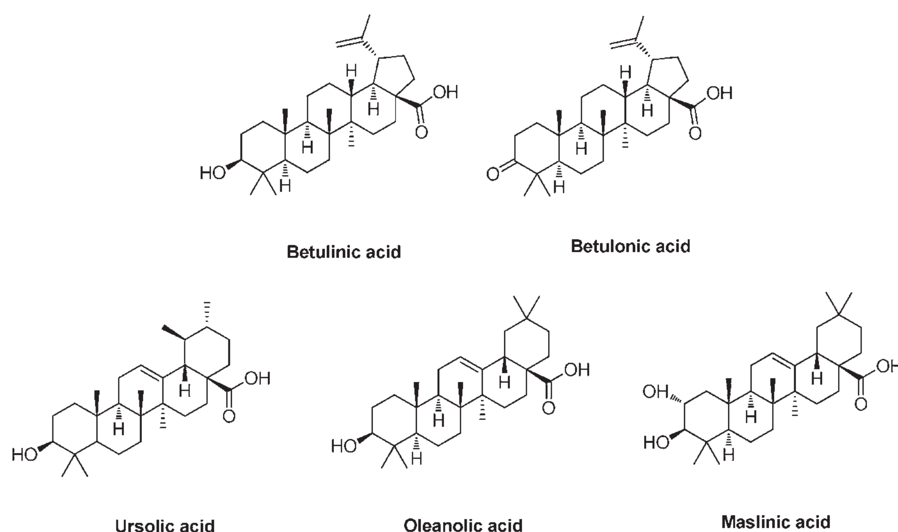

Figure 1. The chemical structures for betulinic acid, betulonic acid, maslinic acid, ursolic acid and oleanolic acid.

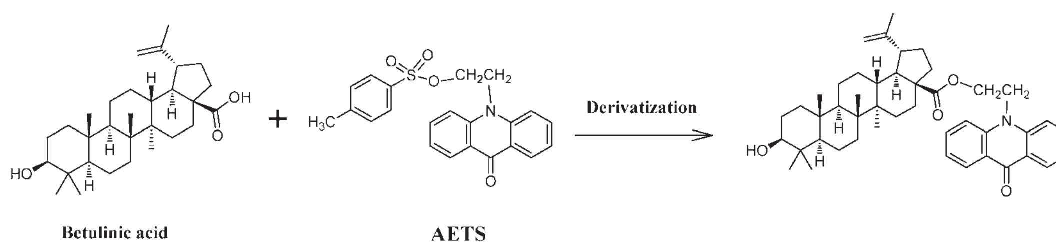

Figure 2. The representative derivatization scheme for AETS with betulinic acid.

increase in the number of experiments, which leads to an increase of time, reagents and materials consumption.[30] Response surface methodology (RSM) as a multivariate statistic technique can overcome this problem.[30] In the present study, Box−Behnken design from RSM was used to optimize the main parameters affecting the derivatization yield, ensuing the sufficient labeling of the analyzed components.

Triterpenic acids in some fruits have been determined like *Chaenomeles sinensis*,[31] apple peels,[32] fruits of *Ziziphus* species,[20] etc.; for most fruits the triterpenic acid analysis remains poorly investigated. Here by employing the developed method we investigated the content of triterpenic acid from several popular fruits in China containing *Punica granatum*, *Crataegus pinnatifida*, *Ziziphus montana*, *Citrus limon*, *Citrus reticulate*, and *Actinidia chinensis*.

■ MATERIALS AND METHODS

Solvents and Chemicals. Oleanolic acid and ursolic acid were obtained from national institute for the control of pharmaceutical and biological products (China). Betulinic acid, maslinic acid and betulonic acid were purchased from Sigma (St. Louis, MO, USA). HPLC grade acetonitrile was from Yucheng Chemical Reagent (Shandong Province, China). *N,N*-Dimethylformamide (DMF), potassium carbonate (K_2CO_3) and ethanol were of analytical grade obtained from Shanghai Chemical Reagent Co. (Shanghai, China). Acridone-9-ethyl-*p*-toluenesulfonate (AETS) was synthesized in our laboratory.[27]

Plant Material. *Punica granatum*, *Crataegus pinnatifida*, *Ziziphus montana*, *Citrus limon*, *Citrus reticulate* and *Actinidia chinensis* were purchased from a marketplace in Jining (Shandong province, China) and authenticated by Prof. Chang-Fan Zhou from Northwest Plateau Institute of Biology, Chinese Academy of Sciences. The sarcocarp, peel and seed of *Punica granatum,* the sarcocarp and seed of *Crataegus pinnatifida* and *Ziziphus montana,* the sarcocarp and peel of *Citrus limon* and *Citrus reticulate* and the whole fruit of *Actinidia chinensis* were prepared and dried at 45 °C under a stream of nitrogen, then milled and stored at 4 °C until analysis.

Preparation of Standard Solutions. An accurately weighed amount (5 mg) of each of betulinic acid, oleanolic acid, ursolic acid, maslinic acid and betulonic acid was transferred into a 10 mL volumetric flask and dissolved in methanol to produce the stock solutions of 0.5 mg mL^{-1}. The stock solution was stored at 4 °C. The working standard solutions with a concentration range of 0.05−6.5 μg mL^{-1} were obtained by diluting the standard solution. The AETS solution (5.0×10^{-2} mol L^{-1}) was prepared by dissolving 0.0983 g of AETS in 5 mL of *N,N*-dimethylformamide (DMF). A low concentration of labeling reagent (5.0×10^{-3} mol L^{-1}) was obtained by diluting the prepared AETS solution with DMF.

Preparation of Sample Solutions. Triterpenic acid extractions were made with modification of an earlier described procedure.[19] Aliquots of 0.5 g of the powdered materials were extracted by 10 mL of ethanol (two times, 30 min each) in an ultrasonic bath at room temperature. The extracts were combined and filtered through analytical filter paper. The extracts were dried under vacuum and then redissolved in 10 mL of ethanol for analysis.

Derivatization Optimization. Box−Behnken designs from response surface methodology (RSM) with three variables were employed to optimize the derivatization reaction and determine the response pattern, and then to establish a model. The derivatization scheme for the representative betulinic acid is presented in Figure 2. The three design variables were the molar ratio of AETS to triterpenic acids (X_1, AETS), derivatization temperature (X_2, °C) and derivatization time (X_3, min). A

Table 1. The Box−Behnken Design Matrix of Three Test Variables in Coded and Natural Units along with Observed Responses (Peak Area)[a]

	independent variable			
run	X_1[b] (AETS)	X_2 (temp, °C)	X_3 (time, min)	response (peak area[c])
1	4(−1)	90(0)	35(+1)	2032
2	6.5(0)	100(+1)	35(+1)	2128
3	6.5(0)	80(−1)	35(+1)	1184
4	6.5(0)	90(0)	27.5(0)	2477
5	6.5(0)	90(0)	27.5(0)	2523
6	6.5(0)	80(−1)	20(−1)	1024
7	9(+1)	90(0)	20(−1)	2043
8	4(−1)	90(0)	20(−1)	1488
9	9(+1)	80(−1)	27.5(0)	915
10	6.5(0)	90(0)	27.5(0)	2640
11	6.5(0)	90(0)	27.5(0)	2646
12	9(+1)	90(0)	35(+1)	2384
13	4(−1)	80(−1)	27.5(0)	720
14	6.5(0)	90(0)	27.5(0)	2528
15	6.5(0)	100(+1)	20(−1)	1634
16	4(−1)	100(+1)	27.5(0)	1648
17	9(+1)	100(+1)	27.5(0)	1824

[a] The 17 runs from the Box−Behnken design were given by the software Design-Expert 7.1.3 Trial. [b] X_1: Molar ratio of AETS to triterpenic acid. [c] Peak area of the tested compounds (betulonic acid).

total of 17 runs were designed, and the coded and natural/uncoded independent variables used in the RSM design are shown in Table 1. The experimental data were statistically analyzed by the software Design-Expert 7.1.3 Trial (e.g., ANOVA, determination of the estimated effects and interaction) and were fitted to a second-order polynomial model as follows:

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

in which n is the number of variables, β_0 is the constant term, and β_i, β_{ii} and β_{ij} represent the coefficients of the first order terms, quadratic terms and interaction terms, respectively. The design expert software was also used to generate response surfaces plots.

Chromatographic Instrumentation and Conditions. HPLC separation, MS identification and sample analysis were performed on an Agilent 1100 series high-performance liquid chromatography/mass spectrometry system (quaternary pump, degasser, and autosampler) with a diode array and fluorescence detector. The mass spectrometer 1100 series LC-MSD Trap-SL (ion trap) from Bruker Daltonik (Bremen, Germany) was equipped with an electrospray ionization (ESI) source. Ion source conditions: ESI in positive ion mode, nebulizer pressure 241.3 kPa, dry gas temperature 350 °C, dry gas flow 9.0 L/min and capillary voltage-3500 V.

HPLC separation of the triterpenic acid derivatives was carried out on a Hypersil BDS C8 column (200 mm × 4.6 mm, 5 μm, Yilite Co Dalian, China) combining with a gradient elution. Mobile phase A and B were acetonitrile/H_2O (30:70; v/v) and 100% acetonitrile, respectively. The gradient elution program was as follows: 0 min = 60% B, 20 min = 80% B. Before injecting the next sample, the column was equilibrated with the initial mobile phase for 10 min. The flow rate was constant at 1.0 mL/min and the column temperature was set to 32 °C. The fluorescence excitation and emission wavelengths were set to λ_{ex} = 404 and λ_{em} = 440 nm, respectively.

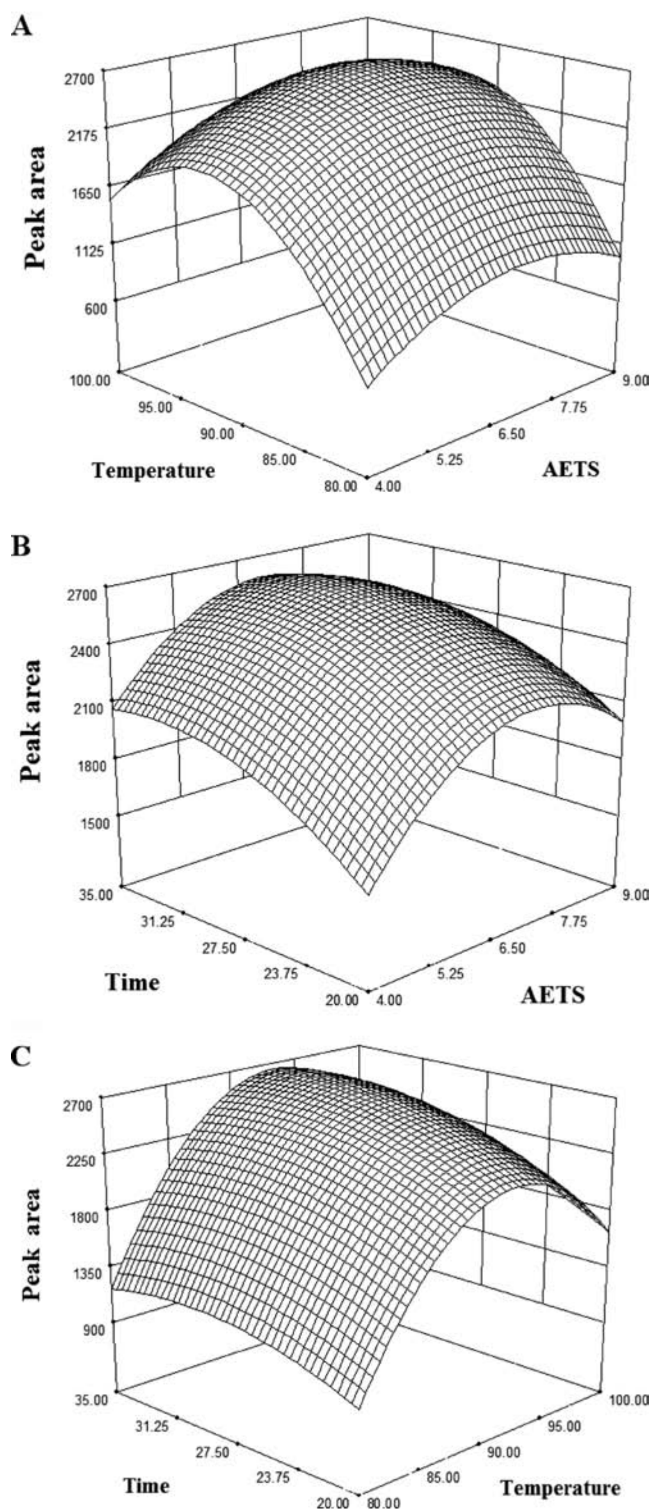

Figure 3. The 3D response surface of the derivatization yield (expressed in terms of peak area) affected by the varying derivatization temperature and molar ratio of AETS to triterpenic acids (A), derivatization time and the molar ratio of AETS to triterpenic acids (B), and the varying derivatization time and temperature (C).

The prepared sample solution (10 μL) was directly injected into the HPLC−MS system for analysis.

Method Validation. The analytical method was validated for linearity, limit of detection (LOD), limit of quantification (LOQ),

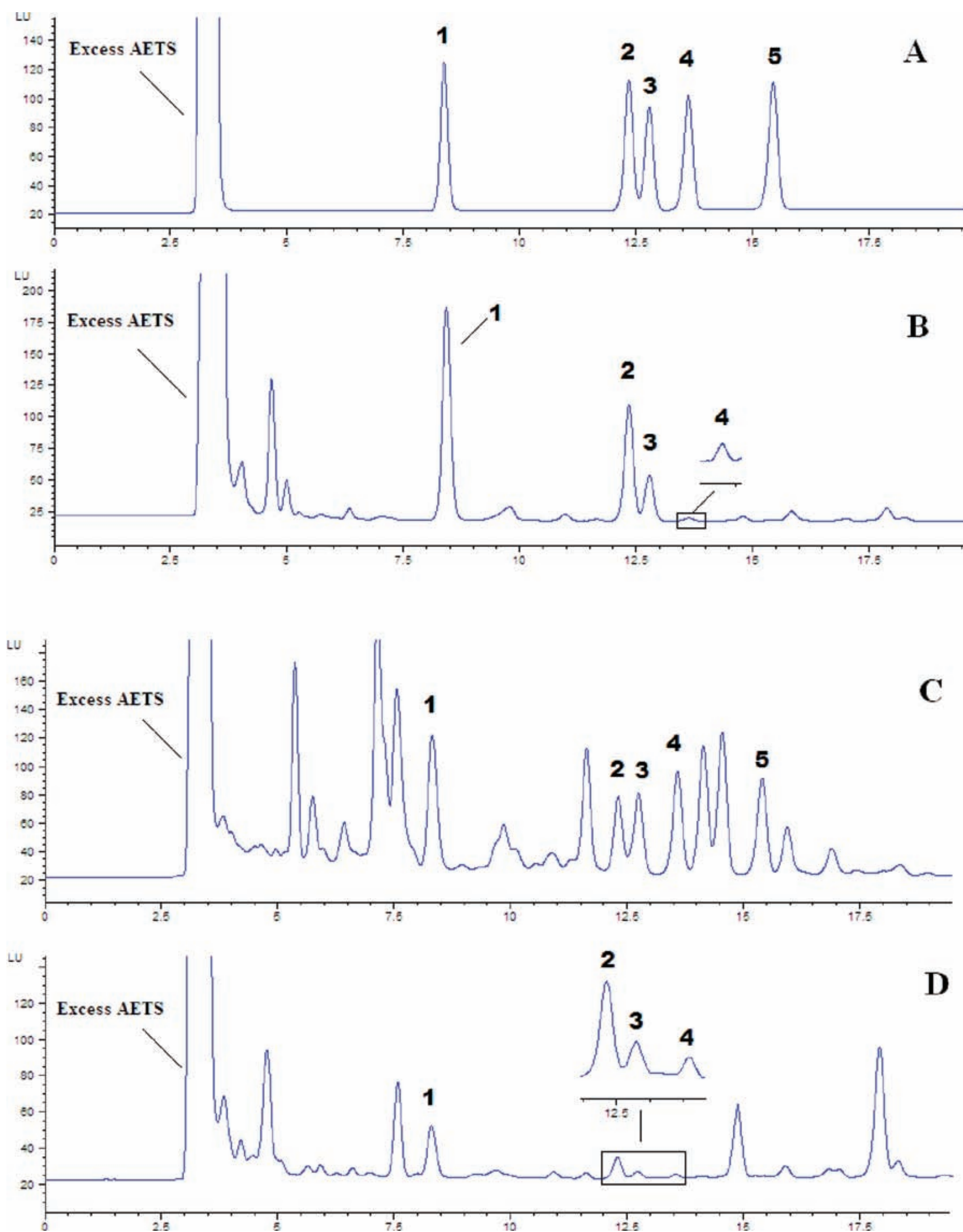

Figure 4. The typical chromatograms for triterpenic acid standards (A), peel of *Punica granatum* (B), sarcocarp of *Ziziphus montana* (C) and *Actinidia chinensis* (D). Peak labels: maslinic acid (1), ursolic acid (2), oleanolic acid (3), betulinic acid (4) and betulonic acid (5).

accuracy and precision following the International Conference on Harmonization (ICH) guideline[33] and some studies on the development of HPLC method.[19,20,34]Linearity was measured at seven concentration levels, and calibration curves were constructed by plotting peak area versus concentration in the range of 0.05−6.5 μg mL^{-1} for each triterpenic acid. The limit of detection (LOD) was defined as the compound concentration that produced a signal-to-noise ratio of 3 (S/N = 3). The limit of quantification (LOQ) was evaluated as the concentration equal to 10 times the signal-to-noise ratio (S/N = 10). The method repeatability was investigated by injecting 10 μL of standard sample (n = 6, injected amount 0.5 ng) and measuring the relative standard deviations (RSD) for peak area and retention time. The accuracies were calculated as follows: accuracy (%) = $100(a - b)/c$, where a was the measured concentration obtained from the extracted *Crataegus pinnatifida* samples which were spiked with triterpenic acid standards; b was the concentration of triterpenic acid in the matrix and c

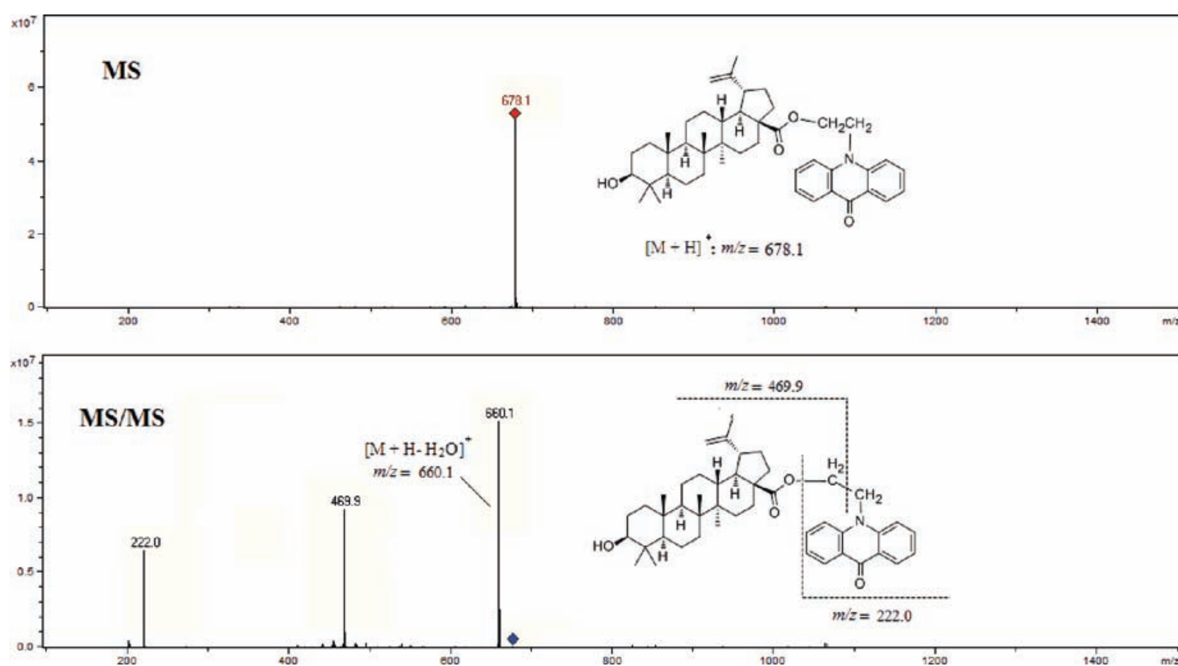

Figure 5. MS spectra of the representative betulinic acid derivative and the fragmentation pattern of protonated molecular ion.

Table 2. Linear Regression Equation, Correlation Coefficients, Limit of Detection and Quantification, Reproducibility of Retention Time and Peak Area

					reproducibility (RSD, %, n = 6)	
triterpenic acids	regression equation[a]	r	LOD[b] (ng/mL)	LOQ[c] (ng/mL)	retention time	peak area
maslinic acid	$Y = 39.01X - 12.39$	0.9998	1.80	5.62	0.04	1.41
ursolic acid	$Y = 36.59X - 2.71$	0.9999	2.04	6.34	0.03	1.35
oleanolic acid	$Y = 28.80X - 4.22$	0.9999	1.77	5.37	0.01	1.16
betulinic acid	$Y = 35.69X - 9.33$	0.9999	1.91	5.85	0.02	1.44
betulonic acid	$Y = 39.30X - 1.48$	0.9999	1.68	4.92	0.01	1.52

[a] Y, peak area; X, injected amount of each triterpenic acid (ng), 10 μL injection volume. [b] Signal-to-noise ratio = 3. [c] Signal-to-noise ratio = 10.

was the added known concentration to the matrix. The inter- and intraday precisions were estimated by analyzing six replicates containing the spiked samples at three different concentrations (40, 120, and 360 ng mL^{-1}).

RESULTS AND DISCUSSION

Optimization of Derivatization Procedure. The optimization of precolumn derivatization as a key step is of great importance for the sufficient labeling of the analyzed components. The cosolvents for derivatization including dichloromethane, *N*,*N*-dimethylformamide (DMF), ethyl acetate, acetone, dimethyl sulfoxide (DMSO) and chloroform were investigated. DMF generated the most intense fluorescence responses and was chosen as the cosolvent. Moreover, DMF used as the derivatization cosolvent can also avoid the problem of precipitation of hydrophobic derivatives. The basic catalysts including pyridine, 2-methylpyridine, K_2CO_3, Na_2CO_3, $K_2C_2O_4$ and $(CH_3)_4NCO_3$ were evaluated for the derivatization. The added K_2CO_3 amount of 70 mg was found to be the best basic catalyst and offered the highest detection responses.

According to Box−Behnken designs from RSM, a total of 17 runs were designed to optimize the main factors affecting derivatization yields including molar ratio of AETS to triterpenic acids, derivatization temperature and time. Betulonic acid was used as the tested compound, and the experimental results are shown in Table 1.

The analysis of variance (ANOVA) for the response surface quadratic model showed that the model, linear parameters except for amount of AETS and all quadratic parameters were significant at the level of $p < 0.05$, but all the interaction parameters were not significant. The value of R^2 was calculated to be 0.98, revealing that the experimental data were in good agreement with the predicted values of peak area. F-value of 3.03 for the lack of fit was insignificant ($p > 0.05$), indicating that the model was sufficiently accurate for predicting the relevant responses. Coefficient of variation (CV %) of less than 5.6 indicated that the model was reproducible. The final estimative response model equation (based on the actual value) was given as follows:

$$Y = 2563 + 160X_1 + 424X_2 + 191X_3 - 395X_1^2 - 890X_2^2 - 180X_3^2 - 5X_1X_2 - 51X_1X_3 + 84X_2X_3 \quad (2)$$

Three-dimensional response surfaces (Figure 3) were plotted on the basis of the model equation, to investigate the interaction

Table 3. Intra- and Interday Precision and Accuracy of Five Triterpenic Acids

compds	spiked concentration (ng mL^{-1})	intraday (n = 6) measd concn (ng mL^{-1})	intraday precision (%)	intraday accuracy (%)	interday (n = 6) measd concn (ng mL^{-1})	interday precision (%)	interday accuracy (%)
maslinic acid	40	39.28	2.23	98.2	38.49	4.83	96.2
	120	114.72	3.12	95.6	112.43	3.43	93.7
	360	347.04	3.63	96.4	343.21	3.95	95.3
ursolic acid	40	41.36	1.81	103.4	40.53	4.31	101.3
	120	113.64	2.08	94.7	114.65	4.94	95.5
	360	342.36	2.70	95.1	352.63	5.01	98.0
oleanolic acid	40	40.56	2.40	101.4	40.10	3.56	100.2
	120	116.40	3.56	97.0	111.74	4.10	93.1
	360	356.04	1.75	98.9	349.00	2.47	96.9
betulinic acid	40	38.88	3.24	97.2	37.32	4.92	93.3
	120	120.72	3.43	100.6	118.31	3.64	98.6
	360	368.28	3.99	102.3	360.91	4.03	100.3
betulonic acid	40	39.04	2.97	97.6	38.26	5.30	95.7
	120	117.60	2.29	98.0	123.48	2.55	102.9
	360	361.44	2.97	100.4	343.51	4.36	95.4

among the variables and to determine the optimum conditions of each factor for maximum derivatization yield. Figure 3A is the response surface showing the effect of derivatization temperature and molar ratio of AETS to triterpenic acids on the response (peak area) at a fixed derivatization time of 27.5 min. Figure 3B intuitively presents the variations of peak areas with derivatization time and the molar ratio of AETS to triterpenic acids at a constant derivatization temperature of 90 °C. Figure 3C described the effects of different derivatization time and temperature at a fixed AETS-to-triterpenic acid molar ratio of 6.5:1.

The optimal values of the selected variables were obtained by solving the regression equation (eq 2) using Design-Expert software. The optimal derivatization conditions estimated by the model equation were as follows: molar ratio of AETS to triterpenic acid = 7, derivatization temperature = 92 °C and derivatization time = 28 min. In order to verify the prediction of the model, the optimal reaction conditions were applied to three independent replicates for derivatization. The average peak area was 2620, a figure well within the estimated value of the model equation. This demonstrated that response surface methodology with appropriate experimental design can be effectively applied to the optimization of the derivatization reaction. Finally, we obtained the optimal derivatization procedure: To a 2 mL vial, 30 μL (mixed triterpenic acid solution) or 100 μL (extracted solution), 70 mg of K_2CO_3, 90 μL of DMF, and 180 μL of AETS solution were successively added. The solution was placed in a water bath at 92 °C with shaking at 5 min intervals for 28 min. After derivatization, the mixture was diluted by 800 μL of acetonitrile for analysis.

Compared to the traditional methods for derivatization optimization based on single factor experiments,[35,36] response surface methodology as an efficient tool exhibited several advantages including lessening laborious time and reagent consumption and providing interaction effects on the response besides factor effects, which is also an added benefit of this study.

HPLC Separation and MS Identification. To obtain the best separation of the triterpenic acid derivatives in the shortest time, the main variables with influence on the chromatographic separation, including different analytical columns, composition of the mobile phase, flow-rate and column temperature, were optimized, respectively. A series of analytical columns containing Hypersil BDS C8 (200 mm × 4.6 mm, 5 μm), Hypersil C18 (200 mm × 4.6 mm, 5 μm), Spherisorb C18 (200 mm × 4.6 mm, 5 μm) and Hypersil BDS C18 (200 mm × 4.6 mm, 5 μm) were investigated, and results indicated Hypersil BDS C8 (200 mm × 4.6 mm, 5 μm) could result in good resolution. The best mobile phases were found to be eluent A acetonitrile/H_2O (30:70; v/v) and eluent B 100% acetonitrile. The optimum flow rate and column temperature were 1 mL min^{-1} and 32 °C, respectively. The representative chromatogram for standard solutions under the proposed conditions is shown in Figure 4A. It is noteworthy to mention that the complete separation of the five triterpenic acid derivatives could be achieved in as little as 16 min, which was shorter than that of the reported methods based on CE,[13] GC[23] or LC.[17,19] Furthermore, our method was proved to be more facile in terms of analytical column, mobile phase and elution program used in LC separation.

The chromatographic peaks were simultaneously identified by retention time and online MS with ESI in positive-ion detection mode. As expected, AETS-triterpenic acid derivatives produced an intense molecular ion peak at m/z $[M + H]^+$. The typical MS and MS/MS spectra for the AETS-labeled betulinic acid derivative are presented in Figure 5. The betulinic acid derivative produced an intense molecular ion peak at m/z 678.1, and the specific fragment ions at m/z 222.0, m/z 469.9, and m/z 660.1. Other endogenous acidic compounds may be present in real samples and were presumably coextracted, and then derivatized by AETS reagent. But the highly intense molecular ions and the characteristic fragment ions monitored by online mass spectrometry indicated no matrix interference from other compounds.

HPLC Method Validation. The linear regression equation, correlation coefficients, limit of detection and quantification, and reproducibility of retention time and peak area are presented in Table 2. The correlation coefficients were found to be >0.9998, indicating excellent linearity. The reproducibility of retention time and peak area were lower than 0.04% and 1.52%, respectively. The proposed method offered the exciting LOD of 1.68−2.04 ng/mL, which are significantly lower than the reported

Table 4. Triterpenic Acid Content in Fruit Samples

fruits	test samples	triterpenic acids (μg/g, n = 3)					total content (μg/g)
		maslinic acid	ursolic acid	oleanolic acid	betulinic acid	betulonic acid	
Punica granatum	sarcocarp	10.76 ± 0.24	1.09 ± 0.06	nd[a]	nd	nd	11.85 ± 0.27
	peel	106.77 ± 1.10	58.88 ± 0.83	26.96 ± 0.93	2.27 ± 0.20	nd	194.87 ± 3.13
	seed	15.45 ± 0.31	0.78 ± 0.01	1.12 ± 0.09	1.06 ± 0.05	133.45 ± 2.61	151.86 ± 3.03
Crataegus pinnatifida	sarcocarp	24.18 ± 0.37	930.75 ± 6.43	172.81 ± 1.27	25.48 ± 0.62	2.83 ± 0.40	1156.05 ± 9.23
	seed	9.57 ± 0.17	2.98 ± 0.29	10.19 ± 0.52	7.80 ± 0.14	2.68 ± 0.26	33.22 ± 1.29
Ziziphus montana	sarcocarp	57.18 ± 0.44	25.48 ± 0.71	39.33 ± 0.68	46.88 ± 0.70	41.70 ± 1.17	210.57 ± 3.90
	seed	9.61 ± 0.18	nd	17.26 ± 0.52	15.08 ± 0.33	0.58 ± 0.05	42.53 ± 1.16
Citrus limon	sarcocarp	3.67 ± 0.07	0.91 ± 0.03	nd	nd	nd	4.58 ± 0.16
	peel	7.83 ± 0.25	3.27 ± 0.23	0.62 ± 0.01	nd	nd	11.73 ± 0.55
Actinidia chinensis	whole fruit	17.27 ± 0.32	7.67 ± 0.40	3.07 ± 0.11	0.95 ± 0.08	nd	28.97 ± 0.84
Citrus reticulata	sarcocarp	1.18 ± 0.09	nd	nd	nd	nd	1.18 ± 0.09
	peel	17.70 ± 0.48	0.62 ± 0.01	1.05 ± 0.04	14.52 ± 0.17	23.05 ± 0.77	56.94 ± 1.40

[a] Not detected.

methods.[12−23] For example, Lee et al.[19] described a HPLC-DAD method for determination of triterpenic acids in *Prunellae Spica* that gave the detection limits of 1160 and 1500 ng/mL for ursolic acid and oleanolic acid, respectively. Guo et al.[20] reported an approach for characterization of triterpenic acids in fruits of *Ziziphus* species by HPLC-ELSD−MS. This approach gave detection limits of 2050−6800 ng/mL.

The experimental precision and accuracy are given in Table 3. The intra- and interday accuracies were found to be in the range of 94.7−103.4% and 93.1−102.9%, respectively, indicating the excellent accuracy. The intra- and interday precisions (expressed in terms of % RSD) ranged from 1.75 to 3.99% and from 2.47 to 5.30%, respectively, which demonstrated the good precision of the proposed method.

Determination of Triterpenic Acids in Fruit Samples. The proposed method was applied to analyze triterpenic acids from several popular fruits in China including *Punica granatum*, *Crataegus pinnatifida*, *Ziziphus montana*, *Citrus limon*, *Citrus reticulate* and *Actinidia chinensis.* The contents of five triterpenic acids in these fruits are expressed in μg/g dry matter and summarized in Table 4. The typical chromatograms for peel of *Punica granatum,* sarcocarp of *Ziziphus montana* and *Actinidia chinensis* are given in Figure 4 (B−D). In all test samples *Crataegus pinnatifida* represented the fruit with the highest amount of triterpenic acid, followed by *Ziziphus montana, Punica granatum, Citrus reticulate, Actinidia chinensis* and *Citrus limon.* Results also indicated the content variations in different tissues were significant. For example, in *Punica granatum*, peel possessed the highest values of total triterpenic acids (194.87 ± 3.13 μg/g); while seed and sarcocarp had the content of 151.86 ± 3.03 and 11.85 ± 0.27 μg/g, respectively. In sarcocarp of *Crataegus pinnatifida,* total content of the five target compounds could reach up to 1156.05 ± 9.23 μg/g, yet in the seed the content was 33.22 ± 1.29 μg/g. These data should be valuable for the further understanding and development of these fruits. Meanwhile, the method reported here also exhibits powerful potential for accurate determination of triterpenic acids from other foodstuffs and nature products.

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