# Study on Determination of Pentacyclic Triterpenoids in *Chaenomeles* by HPLC–ELSD

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## Abstract

**A new direct high-performance liquid chromatography analytical method using evaporative light scattering detection (ELSD) for the simultaneous determination of the major pentacyclic triterpenoids in *Chaenomeles* (Chinese medicinal herb) has been developed. The simultaneous separation of the seven pentacyclic triterpenoids was achieved on a Zorbax Stable Bound (4.6 mm × 100 mm i.d., 1.8 µm) $C_{18}$ column by gradient elution with acetonitrile–water as the mobile phase and detected with ELSD. This method provides good reproducibility and sensitivity for the quantification of seven major pentacyclic triterpenoids, namely erythodiol, betulin, acetyl ursolic acid, ursolic acid, oleanolic acid, betulinic acid, and pomolic acid, respectively. The relative standard deviation (RSD) of overall intra-day variations was less than 2.2%, and the RSD of inter-day variations was less than 2.8%. The standard recoveries (three different concentrations of markers: 0.1, 0.5, and 2.0 mg) ranged from 96–104%. The results demonstrate that this method is simple, sensitive, selective, and suitable for the quality control of this commonly used Chinese medicinal herb.**

## Introduction

The Chinese folk drug, Mugua, is the dried fruits of the *Chaenomeles* plant. It has been one of the most important drugs in traditional Chinese medicine (TCM) for thousands of years and can be used for the treatment of asthma, the common cold, sore throat, tuberculosis, mastitis, and hepatitis by the local people. There are five different *Chaenomeles* varieties in P.R. China: *Chaenomeles cathayensis, Chaenmoeles lagenaria, Chaenomeles thibetica, Chaenomeles sieneis,* and *Chaenomeles japonica* (1–3).

Various chemical and pharmacological studies have demonstrated that the major biologically active ingredients present in this TCM herb are pentacyclic triterpenoids, flavonoids, and organic acids (1–5). Therefore, both quality and quantity controls of the major active pentacyclic triterpenoids in this herb have always been an important issue to ensure its effective and safe clinical usefulness (1,4–7).

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Most of the pentacyclic triterpenoids in Mugua (Figure 1) are nonchromophoric, which make the use of direct UV detection without pre-or post-column derivatization impossible. Therefore, only a few methods have been previously reported to analyze the major active pentacyclic triterpenoids. One reported high-performance liquid chromatography (HPLC)–UV method could only determine ursolic acid and oleanolic acid at a very low wavelength of 205 nm (8). Our research has previously developed by HPLC–UV methods with pre-column derivatization. However, the pre-column derivatization methods require time-consuming sample preparation and a complication of derivatizing reaction (9).

Recently, publications on the use of HPLC coupled with evaporative light scattering detection (ELSD) have markedly increased, and the published results demonstrated that ELSD is an excellent detection method for the analysis of nonchromophoric compounds (10–14). Because the response of ELSD depends on the size, shape, and number of eluate particles rather than the structure and/or chromophore of analytes, ELSD should also be a suitable detection method for HPLC analysis of pentacyclic triterpenoids in Mugua. Therefore, in this paper, a simple and sensitive direct HPLC analytical method using ELSD for the simultaneous determination of the major biologically active pentacyclic triterpenoids in Mugua was developed. This

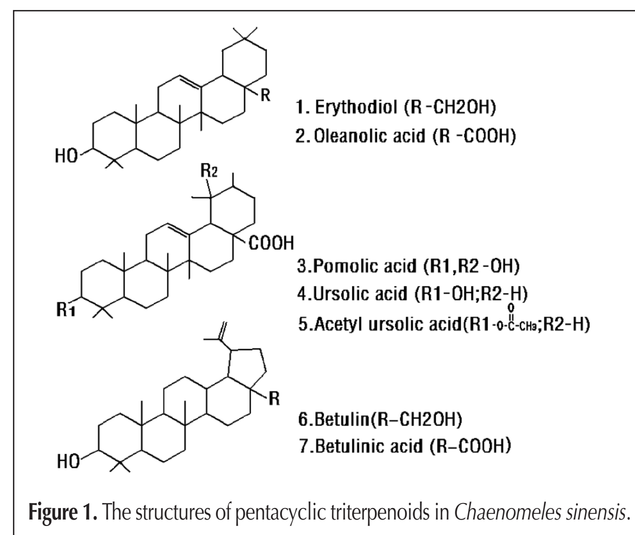


**Figure 1.** The structures of pentacyclic triterpenoids in *Chaenomeles sinensis*.

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has been applied to the analysis of triterpenoids in samples of different *Chaenomeles* varieties, different areas, different parts, and different growing stages with good results.

## Experimental

### Isolation of the triterpenoids

The erythodiol, betulin, acetyl ursolic acid, ursolic acid, oleanolic acid, betulinic acid, and pomolic acid were isolated from *Chaenomeles sieneis* in our laboratories, according to the literature (6,7). To separate the triterpenoids, the plant material of *Chaenomeles sieneis* (2.0 kg, Dali Prefecture, Yunnan Province, China) was exhaustively extracted with acetone (Shanghai Experiment Reagent Co., Shanghai, China) at room temperature three times. The solvent was evaporated in a vacuum, and the crude extract (150 g) was obtained. This crude extract was chromatographed on a silica gel column (10 × 120 cm, 200–300 mesh, Qingdao Marine Chemical, Inc., Qingdao, China) eluting with chloroform–acetone (Shanghai Experiment Reagent Co., Shanghai, China) (1:0, 9:1, 8:2, 2:1, 1:1, and 0:1) to afford fractions of I–VI. The fraction I and II (22.5 g) was repeatedly chromatographed with a semipreparative HPLC Agilent 1100 equipped with a Zorbax SB-$C_{18}$ column (9.4 × 250 mm, 5 μm, Agilent Technologies Inc., Santa Clara, CA) using 80% methanol (Fisher Scientific Inc., Madison, WI) as the mobile phase at a flow rate of 3.0 mL/min. The retention times are pomolic acid (6.24 min), betulinic acid (8.02 min), oleanolic acid, (11.12 min), ursolic acid (12.8 min), acetyl ursolic acid (18.4 min), betulin (26.6 min), and erythodiol (29.6 min). To make further purification, the seven triterpenoids were repeatedly chromatographed with a Sephadex LH-20 column (1.5 × 150 cm, Beijing pharmacia Inc., Beijing, China) using methanol (Fisher Scientific Inc, Madison, WI) as the mobile phase. The pomolic acid (48.5 mg), betulinic acid (63.8 mg), oleanolic acid (240 mg), ursolic acid (160 mg), acetyl ursolic acid (35.2 mg), betulin (43.6 mg), and erythodiol (26.4 mg) were obtained. Their identities were confirmed by comparing the data of IR, $^{1}$H- and $^{13}$C-nuclear magnetic resonance (NMR), and MS analyses with literature (6,7). The purities were identified at ≥ 98%.

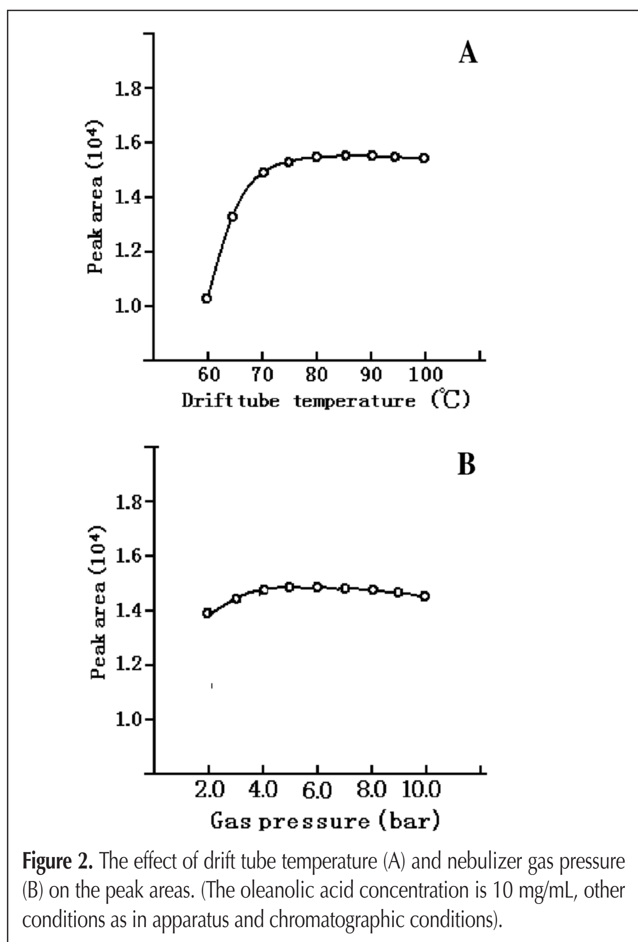


**Figure 2.** The effect of drift tube temperature (A) and nebulizer gas pressure (B) on the peak areas. (The oleanolic acid concentration is 10 mg/mL, other conditions as in apparatus and chromatographic conditions).

### Plant material

The samples analyzed are dried fruits of *Rosaceae*, *Chaenomeles*. The samples of different *Chaenomeles* varieties (*Chaenomeles cathayensis*, *Chaenmoeles lagenaria*, *Chaenomeles thibetica*, *Chaenomeles sieneis*, *Chaenomeles japonica*, collected in Dali Prefecture, Yunnan Province, P.R. China) were provided by Professor Rong He, Yunnan Academy of Forest Science, China. The *Chaenomeles sieneis* samples of the main producing areas in China (Yuxi Prefecture, Yunnan Province; Dali Prefecture, Yunnan Province; Chuxiong Prefecture, Yunnan Province; Zhaotong Prefecture, Yunnan Province; Leshan Prefecture, Sichuan Province) were purchased in local TCM shops in Kunming, China. The different parts and different growing stages of *Chaenomeles sieneis* samples (Dali Prefecture, Yunnan Province, China) were provided by Professor Guang-Ming Liu (Dai Medical College, China). All of the samples were identified by Professor Xi-Wen Li

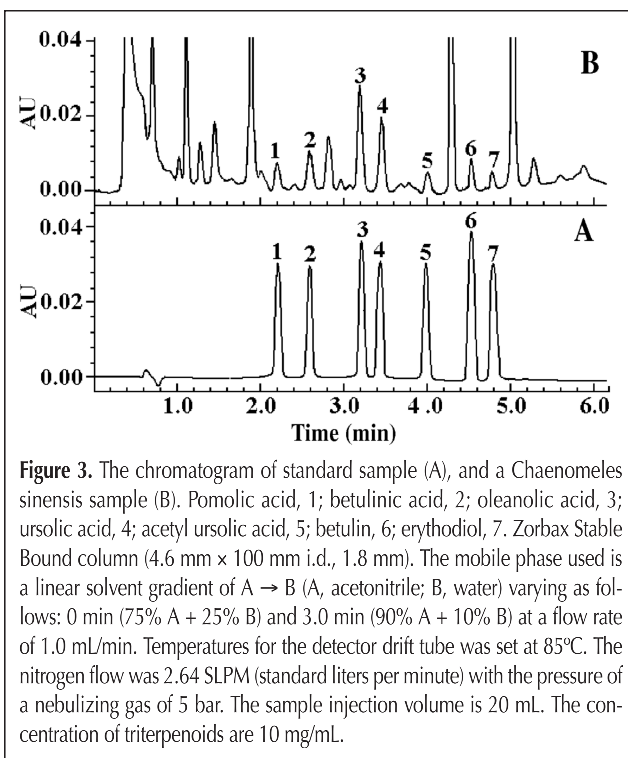


**Figure 3.** The chromatogram of standard sample (A), and a Chaenomeles sinensis sample (B). Pomolic acid, 1; betulinic acid, 2; oleanolic acid, 3; ursolic acid, 4; acetyl ursolic acid, 5; betulin, 6; erythodiol, 7. Zorbax Stable Bound column (4.6 mm × 100 mm i.d., 1.8 mm). The mobile phase used is a linear solvent gradient of A → B (A, acetonitrile; B, water) varying as follows: 0 min (75% A + 25% B) and 3.0 min (90% A + 10% B) at a flow rate of 1.0 mL/min. Temperatures for the detector drift tube was set at 85°C. The nitrogen flow was 2.64 SLPM (standard liters per minute) with the pressure of a nebulizing gas of 5 bar. The sample injection volume is 20 mL. The concentration of triterpenoids are 10 mg/mL.

(Kunming Institue of Botany, Chinese Academy of Sciences). The voucher specimens were deposited at the State Key Laboratory of Phytochemistry and Plant Resources in West China (Kunming Institute of Botany, Chinese Academy of Sciences, China). For each sample, at least 0.5 kg of herbal samples were dried at room temperature for constant weight and pulverized to 200 mesh.

### Chemicals, apparatus, and chromatographic conditions

The HPLC analysis was performed on a Waters 2695 Alliance separation system with an ELSD 2000 and a Nitrox nitrogen generator (Waters Corporation, Milford, MA).

A Zorbax Stable Bound column (4.6 mm × 100 mm i.d., 1.8 μm, Agilent Technologies Inc., Santa Clara, CA) was utilized.

HPLC-grade acetonitrile (mobile phase), methanol and chloroform (for sample preparation) were provided by Fisher Scientific Inc. (Madison, WI). The ultrapure water used was obtained from a Milli-Q50 SP Water system (Millipore Inc, Bedford, MA). The mobile phase used is a linear solvent gradient of A → B (A, acetonitrile; B, water) varying as follows: 0 min (75% A + 25% B) and 3.0 min (90% A + 10% B) at a flow rate of 1.0 mL/min. The temperature for the detector drift tube was set at 85°C. The nitrogen (Kunming Cylinder Charging Plants, Kunming, China) flow was 2.64 SLPM (standard liters per minute) with the pressure of a nebulizing gas of 5 bar. The sample injection volume is 20 μL.

### Preparation of sample

0.25 g of dried powders sample was extracted with 50 mL of methanol and chloroform (1:9) by reflux at 60°C for 1 h. The extracts were cooled and diluted to the 50 mL with chloroform. Then, 1.0 mL of the solution was concentrated to dryness under reduced pressure. The residue was dissolved in 1.0 ml of methanol. This solution was filtered through a 0.45-μm syringe filter and ready for HPLC analysis.

### Preparation of standard solution

To prepare standard solutions, an accurately weighed amount of erythodiol, betulin, acetyl ursolic acid, ursolic acid, oleanolic acid, betulinic acid, and pomolic acid were dissolved in methanol for HPLC. Five concentrations were chosen with the range 0.8–120 μg/mL respectively. Calibration graphs were plotted subsequently for linear regression analysis of the peak area with concentrations.

## Results and Discussion

### Optimal ELSD condition

The important parameters that need to be controlled for the optimization of ELSD response are the flow rate of nebulizer gas (pressure) and drift tube temperature (15). Under the fixed chromatographic conditions, two parameters were evaluated by the injection of oleanolic acid, which are the testing triterpenoid for optimizing ELSD conditions at different detector temperatures from 60°C to 100°C and the pressure from 2.0 bar to 10 bar. In this study, the results (Figure 2) show that the drift tube temperature of 85°C and gas pressure of 5.0 bar (flow was 2.64) give a maximum and constant peak area valves. This drift tube temperature and gas pressure were selected for detecting the analytes. These optimized parameters allow for complete solvent evaporation and produce negligible baseline noise.

**Table I. Regression Equation, Coefficient, and LOD**

| Components | Regression Equation C (mg/mL) | Linearity Range (mg/mL) | Coefficient | σ % ($n$ = 7) | LOD (μg/mL) | RSD% ($n$ = 9) |
|---|---|---|---|---|---|---|
| Pomolic acid | A = 1.28 × 103C + 121 | 1.2–120 | $r$ = 0.9995 | 0.46 | 0.4 | 1.2 |
| Betulinic acid | A = 1.32 × 103C – 82 | 1.0–100 | $r$ = 0.9992 | 0.68 | 0.3 | 1.1 |
| Oleanolic acid | A = 1.56 × 103C + 106 | 1.4–120 | $r$ = 0.9994 | 0.48 | 0.4 | 0.82 |
| Ursolic acid | A = 1.22 × 103C – 1147 | 1.5–110 | $r$ = 0.9992 | 0.71 | 0.4 | 0.73 |
| Acetyl ursolic acid | A = 1.26 × 103C + 94 | 1.2–90 | $r$ = 0.9992 | 0.65 | 0.4 | 0.76 |
| Betulin | A = 1.48 × 103C – 76 | 1.0–140 | $r$ = 0.9993 | 0.57 | 0.3 | 0.91 |
| Erythodiol | A = 1.33 × 103C –114 | 1.5–150 | $r$ = 0.9993 | 0.62 | 0.4 | 1.1 |

**Table II. Determination Results (%) of the Triterpenoids in Different *Chaenomeles* Varieties**

| Components | Samples of Different Chaenomeles Varieties (%) | | | | | RSD% ($n$ = 5) | Recovery% ($n$ = 5) |
|---|---|---|---|---|---|---|---|
| | *Chaenomeles cathayensis* | *Chaenmoeles lagenaria* | *Chaenmoeles lagenaria* | *Chaenomeles sieneis* | *Chaenomeles japonica* | | |
| Pomolic acid | 0.148 | 0.102 | 0.208 | 0.243 | 0.173 | 2.2 | 97 |
| Betulinic acid | 0.211 | 0.308 | 0.273 | 0.328 | 0.187 | 2.5 | 101 |
| Oleanolic acid | 0.523 | 0.407 | 0.434 | 1.12 | 0.718 | 2.6 | 106 |
| Ursolic acid | 0.334 | 0.208 | 0.348 | 0.570 | 0.485 | 1.6 | 98 |
| Acetyl ursolic acid | 0.103 | 0.111 | 0.101 | 0.284 | 0.144 | 2.0 | 102 |
| Betulin | 0.148 | 0.117 | 0.107 | 0.211 | 0.121 | 2.2 | 101 |
| Erythodiol | 0.130 | 0.088 | 0.108 | 0.195 | 0.094 | 2.3 | 103 |

### Optimal of chromatographic separation

Optimal chromatographic conditions were obtained after testing different mobile phase systems with two reversed-phase columns ($C_8$ and $C_{18}$). In the case of the $C_8$ column, the two major titerpenoids, betulin and erythodiol, could not be resolved as a baseline separation, although all other analytes were separated. However, all analytes were resolved well with a baseline separation using the $C_{18}$ column. Furthermore, among the various mobile phases examined, the mobile phase used is a linear solvent gradient of A → B (A, acetonitrile; B, water). The best separation was found to be at 0 min (75% A + 25% B) and 3.0 min (90% A + 10% B) at a flow rate of 1.0 mL/min was found to be the best separation. Therefore, acetonitrile–water gradient elution was selected as the mobile phase in this experience. To shorten the chromatographic separation time, a Zorbax Stable Bound rapid analysis column (4.6 mm × 100 mm i.d., 1.8 mm) was used in this experiment. With this rapid analysis column, the seven triterpenoids were completely separated within 5.0 min (Figure 3). Compared to the previous literature (13,14,16–19), this is one of the most rapid methods to separating triterpenoids.

### Selectivity and peak homogeneity

According to the literature (1,2), the major components in samples are flavone, organic acid, and triterpenoids. The polarities of flavone and organic acid are stronger than that of triterpenoids. Therefore, the flavone, organic acid, and triterpenoids can be separated completely on $C_{18}$ column. Flavone and organic acid do not interfere with the triterpenoids' determination. To ensure the triterpenoids were completely separated in every sample, the peak purity tests were carried out by HPLC–diode array detection and HPLC–mass spectrometry methods according to literature (9,20,21). Results showed that all seven peaks were separated completely. There were no impurities present in the peaks of triterpenoids. This method is highly selective.

### Calibration graphs

Under the optimum conditions, the regression equations of the seven triterpenoids were established based on the standard samples injected and their peak area. The residual standard deviations (s) were plotted. The limits of detection (LOD) are calculated by the ratio of signal-to-noise (S/N = 3). The results were shown in Table I. The reproducibility of this method was also examined for 10 mg/mL of the seven triterpenoids. The relative standard deviations (RSD) ($n$ = 9) were shown in Table I as well.

### Method recovery and precision

The recovery test was carried out by adding erythodiol, betulin, acetyl ursolic acid, ursolic acid, oleanolic acid, betulinic acid, and pomolic acid to the samples (three different concentrations of markers: 0.1, 0.5, and 2.0 mg). The sample was prepared like the previous procedure mentioned in the "Preparation of sample" section and injected for HPLC analysis to calculate the amount of the triterpenoids found. The results show that the recoveries ($n$ = 5) ranged from 96–104%. This method has high recovery.

The measurements of intra- and inter-day variability (determination of the same samples for seven times) were utilized to determine the precision of the developed method. The results shown that the RSD of overall intra-day variations was less than 2.2%, and the RSD of inter-day variations was less than 2.8%. This method has high precision.

**Table III. Determination Results (%) of the Triterpenoids in *Chaenomeles Sinensis* from Different Main Producing Areas**

| Components | Samples from Different Main Producing Areas | | | | | RSD% ($n$ = 5) | Recovery% ($n$ = 5) |
|---|---|---|---|---|---|---|---|
| | Yuxi | Dali | Chuxiong | Zhaotong | Leshan | | |
| Pomolic acid | 0.218 | 0.232 | 0.362 | 0.168 | 0.284 | 2.4 | 102 |
| Betulinic acid | 0.368 | 0.343 | 0.514 | 0.276 | 0.367 | 2.6 | 97 |
| Oleanolic acid | 0.946 | 1.07 | 0.83 | 1.19 | 1.43 | 1.8 | 104 |
| Ursolic acid | 0.638 | 0.670 | 0.626 | 0.514 | 0.852 | 1.5 | 98 |
| Acetyl ursolic acid | 0.147 | 0.268 | 0.184 | 0.212 | 0.216 | 2.3 | 97 |
| Betulin | 0.218 | 0.218 | 0.168 | 0.187 | 0.231 | 2.6 | 102 |
| Erythodiol | 0.112 | 0.198 | 0.138 | 0.118 | 0.141 | 2.1 | 96 |

**Table IV. Determination Results (%) of the Triterpenoids in Different Parts of *Chaenomeles Sinensis***

| Components | Samples from Different Parts(%) | | | | RSD% ($n$ = 5) | Recovery% ($n$ = 5) |
|---|---|---|---|---|---|---|
| | Fruit | Stem | Leaf | Root | | |
| Pomolic acid | 0.162 | 0.042 | 0.062 | 0.037 | 2.5 | 104 |
| Betulinic acid | 0.408 | 0.142 | 0.196 | 0.013 | 2.2 | 97 |
| Oleanolic acid | 1.24 | 0.181 | 0.227 | 0.187 | 2.1 | 102 |
| Ursolic acid | 0.867 | 0.297 | 0.357 | 0.313 | 1.8 | 97 |
| Acetyl ursolic acid | 0.132 | 0.103 | 0.126 | 0.114 | 2.2 | 102 |
| Betulin | 0.148 | 0.057 | 0.076 | 0.053 | 2.5 | 104 |
| Erythodiol | 0.113 | 0.019 | 0.037 | 0.019 | 2.9 | 94 |

### Analysis of pentacyclic triterpenoids in samples

This method was subsequently applied to simultaneous determination of the triterpenoids in different *Chaenomeles* variety samples, and samples of different areas, of different parts, and in different growth stages. The contents of triterpenoids are summarized in Table II (different varieties), Table III (different areas), Table IV (different parts), and Table V (different growing stage). The results demonstrated that all of the five different *Chaenomeles* varieties contain the triterpenoids, and *Chaenomeles sieneis* contained the highest triterpenoids content. The contents of triterpenoids in *Chaenomeles Sinensis* were varied in different

**Table V. Determination Results (%) of the Triterpenoids in Different Growing Stages of *Chaenomeles Sinensis***

| | Samples of Different Growing Stages (%) | | | | | | |
|---|---|---|---|---|---|---|---|
| Components | 1 month | 2 month | 3 month | 4 month | 5 month (mature) | RSD% ($n$ = 5) | Recovery% ($n$ = 5) |
| Pomolic acid | 0.076 | 0.107 | 0.126 | 0.149 | 0.181 | 2.2 | 97 |
| Betulinic acid | 0.162 | 0.184 | 0.278 | 0.336 | 0.432 | 1.8 | 96 |
| Oleanolic acid | 0.218 | 0.361 | 0.625 | 0.839 | 1.22 | 2.1 | 102 |
| Ursolic acid | 0.232 | 0.327 | 0.516 | 0.697 | 0.854 | 1.6 | 97 |
| Acetyl ursolic acid | 0.049 | 0.060 | 0.083 | 0.113 | 0.134 | 2.2 | 95 |
| Betulin | 0.052 | 0.064 | 0.082 | 0.104 | 0.148 | 2.7 | 103 |
| Erythodiol | 0.038 | 0.043 | 0.056 | 0.072 | 0.101 | 2.5 | 104 |

main producing areas, and the samples from Leshan Prefecture, Sichuan Province contain the highest triterpenoids content. In different part of *Chaenomeles Sinensis*, the fruit contain the highest triterpenoids content. In different growing stages of *Chaenomeles Sinensis*, the triterpenoids content increased markedly with the passing of time.

## Conclusions

In this manuscript, a Zorbax Stable Bound $C_{18}$ (4.6 mm × 10 mm i.d., 1.8 µm) rapid analysis column was used. The seven triterpenoids can achieve baseline separation with 5.0 min on this column. Compared to the normal column, 80% of separation time was saved. It is one of the most rapid methods for chromatographic analysis of triterpenoids. For the analysis of nonchromophoric compounds, ELSD has used in this method. The nortriterpenoids can directly be detected without derivation. The sample preparation for this method is simple. The triterpenoids was extracted from the samples with solvent and can directly undergo HPLC analysis. This preparation does not need a complex purification procedure.

In a word, this method is rapid, highly sensitive and selective, and provides good reproducibility and accuracy for the quantification of seven major pentacyclic triterpenoids in *Chaenomeles*.

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