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SHORT COMMUNICATION

Simultaneous determination of oleanolic acid and ursolic acid by RP-HPLC in the leaves of *Eriobotrya japonica* Lindl.

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KEYWORDS

RP-HPLC;
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Abstract Oleanolic acid (OA) and ursolic acid (UA) are isomeric triterpenic acids and only one methyl's position is different between them. OA and UA always exist in the same plant, so it is difficult to separate them when determining contents by RP-HPLC. In this study, a very simple mobile phase for HPLC was developed to simultaneously determine UA and OA, and the factors affecting separation were also discussed. The mobile phase is methanol: water (95:5) with flow rate 0.4 mL/min. The retention time for OA and UA was 20.58 and 21.57 min, respectively, the resolution was 1.61. The average contents of OA and UA of three Loquat leaves sets were 1.4 mg/g and 5.6 mg/g, respectively. Regarding the HPLC, we found that changing mobile phase, adjusting the pH value or adding ion-pairing agent could not affect the separation between UA and OA greatly. While adjustment of the flow rate and column temperature could improve the resolution greatly.

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1. Introduction

Eriobotrya japonica Lindl. (Loquat) is a native plant in southeastern China. Many parts of the Loquat, such as leaves, flowers, kernels, have been used for the treatment of cough, cold, anti-inflammatory, tumors, liver problems, chronic bronchites, nephropathy etc. At present, studies of pharmacological activities and application of Loquat are mainly focused on leaves for its cost and convenient collection. *E. japonica* leaves contain many bioactive components, such as flavnoids, phenolics, amygdalin, triterpenic acids and carotenoids, among them, the isomeric pentacyclic oleanolic acid (OA) and ursolic acid (UA) (Fig. 1) are predominant triterpenoids found in it.

OA and UA were reported to have biological activity including anti-inflammatory [1,2], antiprotozoal [3], antimicrobial properties

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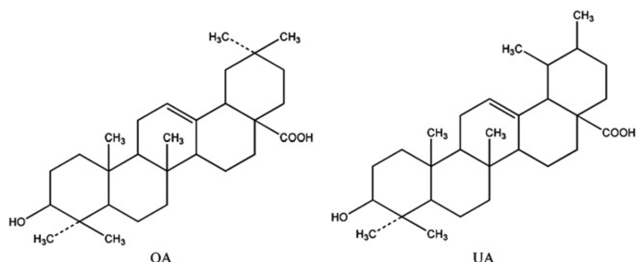

Figure 1 Chemical structures of oleanolic acid and ursolic acid.

[4] and cytotoxicity to cancer cells [5]. OA also possesses hepatoprotective [6] anti-ulcer activities [7], and UA exerts anti-tumor activity through enhancing the production of both nitric oxide and tumor necrosis factor-α via nuclear factor-kappa β activation in the resting macrophage [8].

OA and UA are isomeric triterpenic acids and always simultaneously exist in the same plant. When determining the contents of them by HPLC, it was difficult to separate them. In this study, a simple and rapid HPLC method was developed to simultaneously determine UA and OA in the leave of *Eriobotrya japonica* Lindl.

2. Methods

2.1. Chemicals

OA and UA standards were purchased from National Institute for the Control of Pharmaceutical and Biological Products. Methanol (HPLC grade) was Fisher ChemAlert® Guide obtained from Fisher Chemicals Company (USA). All the other organic solvents used in the study were of analytical grade. Ultrapure distilled water used for all HPLC work was prepared in-house using Aquaplus ultra water purification system.

2.2. Preparation of standard solution

Stock standard solutions of OA and UA were prepared by dissolving an appropriate amount of OA and UA in methanol to give a final concentration of 1 mg/mL. A serial dilution was made on each stock solution with methanol to prepare standard solutions at concentrations of 25, 50, 100, 200 and 300 μg/mL, from each of which 20 μL was used for plotting the standard curves for OA and UA, respectively.

2.3. High-performance liquid chromatography system

HPLC was performed on a Doinex Summit 2000 system (USA), equipped with a quaternary pump and degasser, an automated sample injector, a column compartment, a variable-wavelength UV detector, and Chromeleon software. The analytical column used was Alltech Apollo C18 (250 mm × 4.6 mm, 5 μm), which was coupled to a Dikma C18 guard column (10 mm × 4.6 mm, 5 μm).

The mobile phase was composed of methanol and ultra purewater (95:5, v/v). Before use, the mobile phase was filtrated through a 0.45 μm filter (Nylon66). The flow rate was 0.4 mL/min and elute was monitored at 215 nm. The temperature of the column was kept constant at 20 °C. The flow rate and temperature management is important in this method.

2.4. Preparation of sample solution

Loquat leaves were collected from Chengdu city, Sichuan province, China. The plant materials were dried using a microwave oven until the weight was unchanged and then ground into powder and stored at −20 °C until analysis.

Plant powder of 1 g was dissolved in 25 mL methanol for 30 min followed by 30 min ultrasonic extraction by AS5150A ultrasonic machine (Autoscience Instrument Co. Ltd., Tianjin, Hebei Province, China). The samples were extracted twice, both extracts were combined and diluted with methanol to accurately 50 mL. The solution was filtered through a 0.45 μm syringe filter prior to HPLC analysis.

2.5. Recovery

To test the extraction recovery, dried plant powder (1 g) was added with 0.2 mg OA and 0.1 mg UA standards before extraction. The follow-up extractions and HPLC analyses were accomplished in the same manner as detailed above. The recovery was determined as follows

$$\text{Recovery}(\%) = (\text{A}-\text{B})/\text{C}*100\%$$

where A is the result after addition, B is the amount of sample without adding standards, C is the added amount of the standards.

2.6. Repeatability and precision

To assess the intra-day precision of the method 25, 100 and 300 μg/mL standard solutions of OA and UA were injected for several times ($n=5$) during the same day. These studies were repeated on different days ($n=5$) to determine the inter-day precision.

3. Results and discussion

3.1. HPLC condition

The detection wavelength was chosen at 215 nm for UA and OA because they have better absorption and sensitivity at this wavelength.

Several mobile phases based on methanol, acetonitrile, phosphate buffer with different pH, ion-pairing agents were tried to provide good chromatographic separation between UA and OA. We found that adjusting the pH or adding ion-pairing agent could not affect the separation of UA and OA greatly. However optimizing and adjusting the flow rate and column temperature could improve the separation greatly.

At last, the mobile phase consisted of methanol (A) and ultra water (B) with a ratio of 95:5 (A:B, v/v). The flow rate was 0.4 mL/min and column temperature was 20 °C. The retention time for OA and UA was 20.58 and 21.57 min, respectively. Under such HPLC conditions, HPLC chromatograms of OA, UA standards and the Loquat leaves extract are shown in Figs. 2 and 3, which indicate that OA and UA in the Loquat leaves were successfully separated and simultaneously identified, the resolution was 1.61.

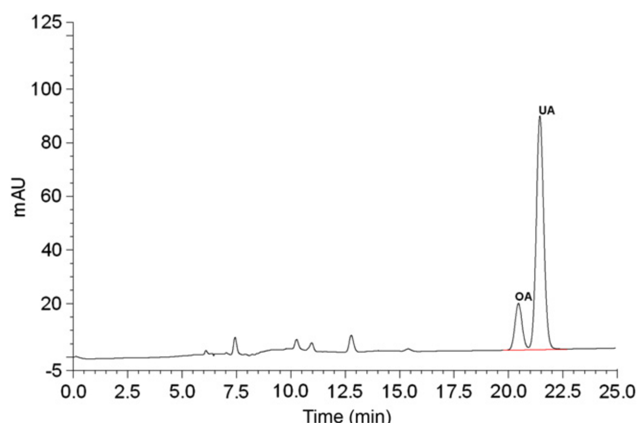

Figure 2 HPLC chromatograms of OA and UA standards.

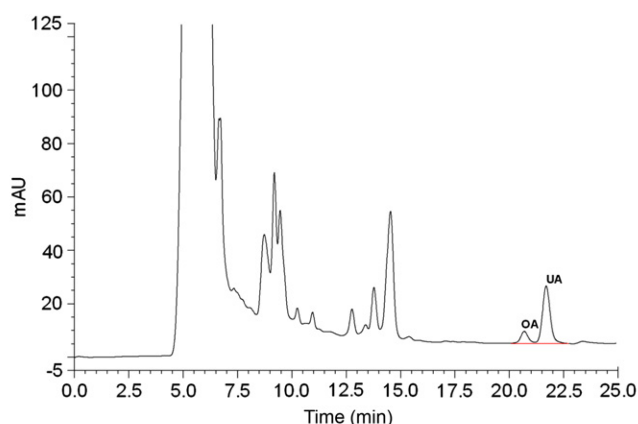

Figure 3 HPLC chromatograms of the Loquat leaves extract.

3.2. *Linearity*

Calibration graphs were constructed in the range of 25–300 μg/mL for OA and UA. The regression equation between the peak area (y) against concentration (x) and their coefficients of determination (R^2) were calculated as follows

$$\text{OA}: y = 2.6533x - 4.0803 (R^2 = 0.996)$$

$$\text{UA}: y = 2.7945x - 1.2515 (R^2 = 0.999)$$

3.3. *Repeatability and precision*

The inter-day and intra-day variations for the determinations of OA and UA were less than 3% at concentrations of 25, 100 and 300 μg/mL (Table 1).

3.4. *Recovery*

0.2 mg OA standard and 0.1 mg UA standard were added to the dried plant powder of 1 g and analyzed as described above ($n = 5$). The recoveries of OA and UA were 97.6% and 98.5%, respectively, with relative standard deviations (RSD) of 3.21 and 2.89 (Table 2).

Table 1 Inter-day and intra-day precision for OA and UA.

Standard	Concentration (μg/mL)	RSD (%)	
		Intra-day ($n=5$)	Inter-day ($n=5$)
OA	25	1.56	2.35
	100	1.38	1.89
	300	1.35	1.13
UA	25	1.67	2.31
	100	1.58	2.14
	300	1.54	2.45

Table 2 Recovery of OA and UA.

Standard	Amount added (mg)	Recovery ($n=5$)	
		Mean (%)	RSD (%)
UA	0.1	98.5	2.89
OA	0.2	97.6	3.21

Three sets of sample solutions were prepared as described above. When the sample solution was analyzed in the same manner, the peaks in the chromatograms were identified by comparison of their retention time with that of authentic standards of UA and OA. The average contents of OA and UA in three Loquat leaves sets were 1.4 mg/g and 5.6 mg/g, respectively, with RSD of 3.12 and 2.78.

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