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Determination of Oleanolic acid and Ursolic acid in loquat leaf extract By Liquid Chromatography-Tandem Mass Spectrometry

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Introduction

Ursolic acid (UA) and oleanolic acid (OA) belong to a class of triterpenic acid, which play significant bioactive roles in anti-inflammatory, antitumor as well as enhancing the cellular immune system. However, due to the lack of suitable chromophoric or ionization groups in molecular structures, both UV and MS responses are far from satisfaction. In this study, a highly sensitive method based on derivatization and LC-MS/MS has been developed.

After being extracted, a pair of stable isotope probes, 2-dimethylaminoethylamine (DMED) and d4-2-dimethylaminoethylamine (d4-DMED) were added for derivatization. The generated heavy labeled triterpenic acids were used as internal standards for quantification, the detection sensitivities of analytes improved sharply, the detection limits of OA and UA were 0.92 and 1.06 ng/L, respectively.
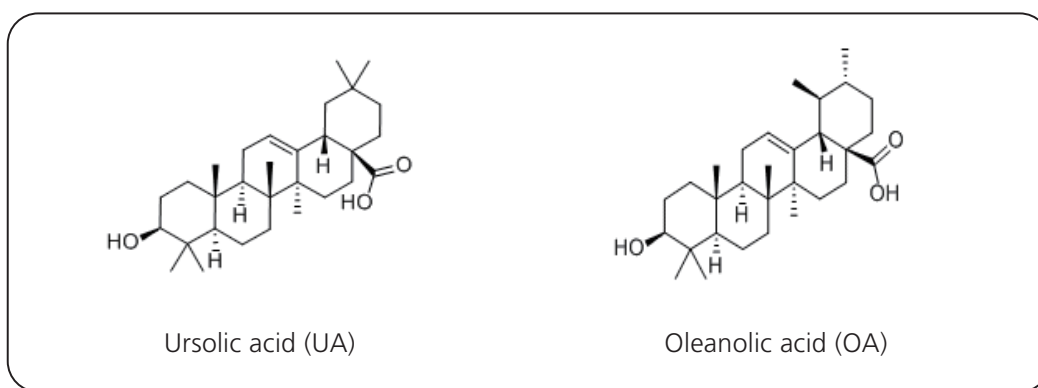

Ursolic acid (UA)   Oleanolic acid (OA)

Figure 1   Structure of Ursolic acid (UA) and oleanolic acid (OA)

Methods

The derivatization of standard solution

Take 200 µL of working standard solution, after adding 10 µL 20 µmol/mL of triethylamine (TEA) and 10 µL 20 µmol/mL of 2-chloro-1-methylpyridinium iodide (CMPI), the solution was vortexed for 5 min. Then 10 µL 40 µmol/mL of DMED was added, and the mixture was vibrated at 1500 rpm for 1 h at 40 °C. The sample was evaporated to dryness under a mild nitrogen steam at room temperature. The residue was dissolved in 100 µL of mobile phase, and then analyzed by LC-MS/MS.

Preparation of Samples

0.5 g of sample were added into 50 mL volumetric flask, and then diluted with saturated salt water to the required volume. 1 mL above solution was withdrawn and added 200 µL of ethyl acetate. After being vortexed and centrifuged, and the resulting supernatant was used for derivatization. The procedure of derivatization was the same as that of standard solution.

Preparation of Internal Standard

The derivatization process of internal standard was almost the same as that of standard solution except that the DMED was replaced with d4-DMED. The evaporated residue was dissolved in 1 mL of mobile phase, took 10 µL of that to each standard solution and real samples, and then used for analysis.

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extract By Liquid Chromatography-Tandem Mass Spectrometry

High Speed Mass Spectrometer

Ultra Fast Polarity Switching

• 5 msec

Ultra Fast MRM

• Max. 555 transition /sec

Figure 2   LCMS-8050 triple quadrupole mass spectrometer

The LC-MS/MS system were Prominence LC-20A and triple quadrupole mass spectrometry (Shimadzu Corporation, Kyoto, Japan). Shimadzu LC-20A system consists of a CBM-20A system controller, two LC-20AD pumps, a SIL-20AC autosampler, a CTO-20AC column oven, and a DGU-20A3 online degasser. MS/MS detection was performed by LCMS-8050. Data acquisition and processing were performed with Labsolution software Version 5.72. Electrospray ionization was operated in multiple-reaction-monitoring (MRM) mode .

Result

Method development for UA and OA

HPLC conditions	
Column	: InertSustain C18 Column (2.1 mm I.D.×250 mm L., 3 μm)
Mobile phase A	: 0.1% formic acid aqueous solution B: Acetonitrile
	A:B=47:53 (v/v)
Flow rate	: 0.25 mL/min
Injection vol.	: 10 μL
Column temperature	: 35 ℃
MS conditions (LCMS-8050)	
Ionization	: ESI, Positive MRM mode
Nebulizer Flow	: 2.0 L/min
Heating Gas Flow	: 10.0 L/min
Interface Temperature	: 300 ℃
DL Temperature	: 250 ℃
Heat block Temperature	: 400 ℃
Dry Gas	: 10.0 L/min

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Table 1. MRM transition

Compound	MRM transition	Q1 Pre Bias (V)	CE	Q3 Pre Bias (V)
Oleanolic acid	527.30>482.30*	-20	-34	-23
	527.30>203.20	-20	-41	-20
Ursolic acid	527.30>482.30*	-20	-34	-23
	527.30>203.20	-20	-41	-20
d4-Oleanolic acid	531.30>482.30	-20	-34	-23
d4-Ursolic acid	531.30>482.30	-20	-34	-23

OA and UA are pentacyclic triterpenoids, the structural difference is merely a methyl group at C-20 on the OA shift to C-19 position. It is very difficult to separate these two compounds that belong to the geometric isomers. In this study, 3 μm of InertSustain C18 column was used, the resolution is 1.110, which is better than the results reported in the literature. Figure 3 shows MRM chromatograms of 0.1 μg/mL standard solution of UA and OA(a), as well as 0.1 μg/mL standard solution of d4-UA and d4-OA(b). It took 12 minutes per one LC-MS/MS analysis, and excellent separation and high sensitive detection were obtained.
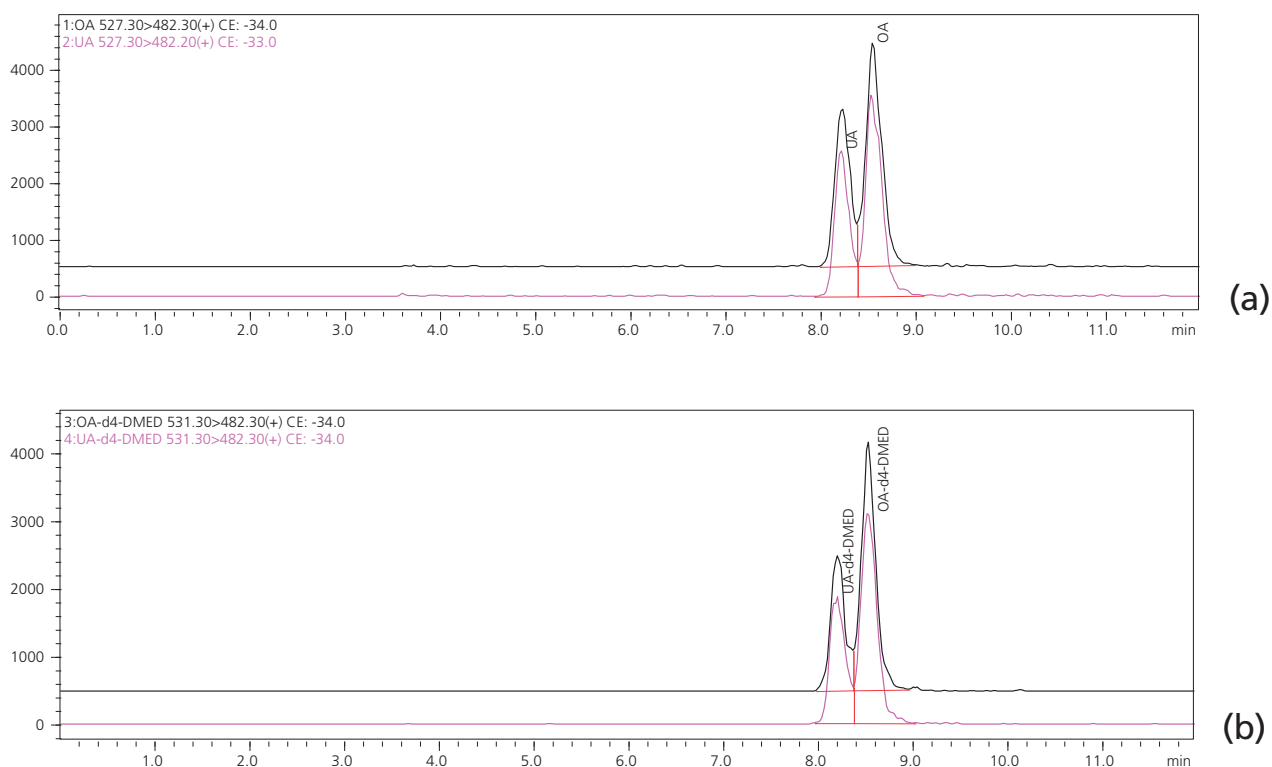

(a)

(b)

Figure 3   MRM chromatograms of standard solution of UA and OA(a), as well as internal standard solution (Concentration of each compound were 0.1 μg/mL)

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Analytical Performance

Linearity

The determination of UA and OA was verified using an internal standard for quantification. The internal calibration was performed by plotting peak area ratios versus concentration ratios of DMED derivates and d4-DMED derivates (As seen in Figure 4). The sample solutions were spiked with stock solution to get final concentrations of UA and OA at 0.01, 0.05, 0.1, 0.5, 1.0, 5.0 and 10 ng/mL.
The detailed calibration curves, ranges, correlation coefficients and precisions were shown in Table 2.
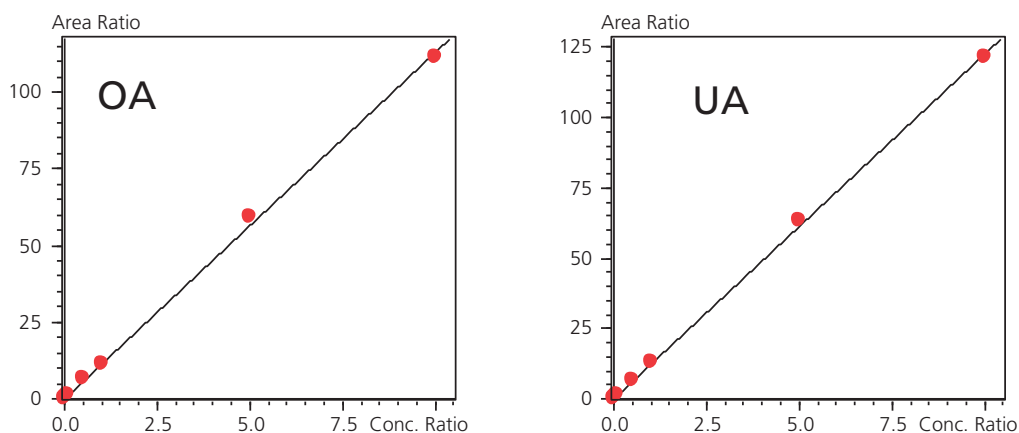

Figure 4   Calibration curve of OA and UA

Table 2. Parameters of Calibration Curves

Compound	Calibration Curves	Range (ng/mL)	Coefficient (r2)	Precision (%)
OA	Y=(11.230) X + (0.480)	0.01~10.0	0.9995	81.7~117.8
UA	Y=(12.197) X+ (0.467)	0.01~10.0	0.9998	94.6~119.7

Sensitivity

Detection and quantification limits were calculated as the concentration corresponding to a signal 3 and 10 times of the baseline noise, and the limit of detection oleanolic acid and ursolic acid were 0.92 and 1.06 ng/mL, the quantification limits were 3.07 and 3.53 ng/L, respectively.

Recovery

Preparation of blank loquat leaf extract samples as well as blank loquat leaf extract samples spiked at 100 mg/kg. According to the mentioned method before, each sample was measured three times in parallel. The recovery is calculated by subtracting the content of OA and UA in blank samples. The results showed that the recoveries of OA and UA were in the range of 98.7~102.7 % and 97.2~105.0 %, respectively.

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Quantitative Analysis of real sample

According to the sample preparation method, loquat leaf extracts from market were analyzed. The sample was determined parallel twice. The content of oleanolic acid and ursolic acid in this sample were 7.40 and 23.6 mg/Kg, respectively. The results were shown in Figure 5.
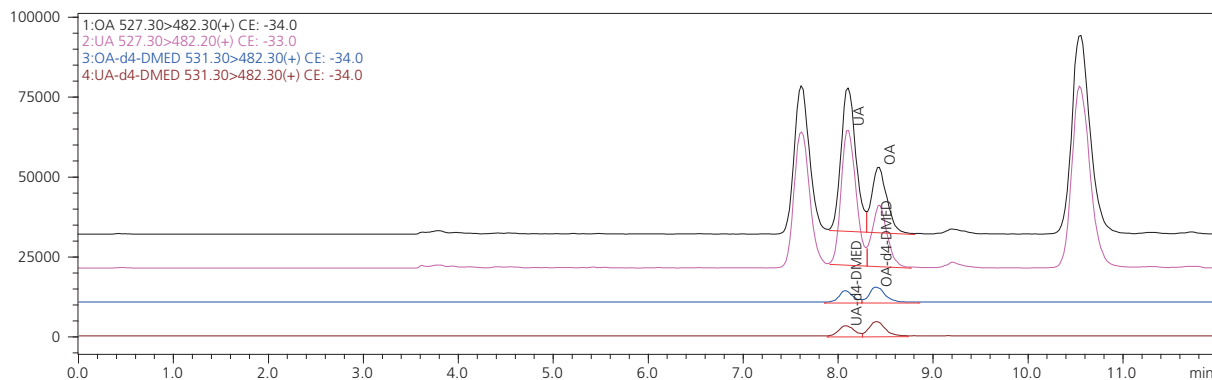

Figure 5   MRM chromatograms of real samples

Conclusions

In this paper, a highly sensitive method based on derivatization of oleanolic acid and ursolic acid coupled with LC-MS/MSdetection was established.
This method has good linearity, with correlation coefficient greater than 0.999, the limit of detection were 0.92 and 1.06 ng/mL, the quantification limits were 3.07 and 3.53 ng/L, respectively. The recoveries were between 97.2~105.0 %. This method is simple, rapid, accurate and can be applied to detect OA and UA in loquat leaf extract.

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