Determination of oleanolic acid and ursolic acid in Chinese medicinal plants using HPLC with PAH polymeric C18

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ABSTRACT

Background: The RP-HPLC resolution of two triterpenic acid isomers was unstable. Objective: To separate the oleanolic acid (OA) and ursolic acid (UA) simply within RP-HPLC. **Materials and Methods:** The separation ability of five stationary phases was studied with the retention effect of their carbon loads. Also the resolution effects of mobile phase composition and different column temperatures were systematically investigated by using Drylab® (Rheodyne LLC.) after evaluating chromatograms automatically. **Results:** The best available resolution of two bioactive isomers was achieved (r = 3.4) via using PAH (polycyclic aromatic hydrocarbons) polymeric C18 bonded phase column. The chromatographic system was applied to the quantification in ten Chinese medicinal plants and the validation was carried out and the precision (RSD ≤1.34%), the linearity (r ≥0.9998) and the recovery (range from 92.1% to 102.6%) were acceptable. **Conclusion:** It is clear that the method was simple, rapid and reliable for the quantification of two compounds in new HPLC method within PAH polymeric C18.

Key words: HPLC, oleanolic acid, PAH polymeric C18, ursolic acid

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INTRODUCTION

Two naturally occurring pentacyclic triterpene acids, oleanolic acid (OA) and ursolic acid (UA) coexist in many widespread food and medicinal plants as the free acid form or as aglycone for triterpenoid saponins, which have been reported beneficial and notable effects on anti-inflammatory, anti-hyperlipidemia, anti-tumor and hepatoprotection.[1,2] For the analysis of two compounds, thin layer chromatography (TLC) and high performance TLC (HP-TLC) have previously been used but required iodine or sulfuric acid as visualizing regent;[3,4] cyclodextrin-modified capillary micellar electrokinetic chromatography was a hybrid technique to high-speed separate them but difficult to scale up;[5] gas chromatography (GC) has once been used but required silylation or methylation derivatisation.[6,7] Up to date, only reverse phase liquid chromatography (RPLC) has widely been used to determinate them and proved to be the effective and convenient separation method. However, in the most common octadecyl silane (ODS) stationary phase system the resolution of OA and UA was unstable, ranging from 1.3 to 1.6 due to the sample composition was complex and the couple substances were position isomer;[8-11] the low selectivity between OA and UA can be explained by similar log P (log of the octanol/water partition coefficient) values (6.468 and 6.427, respectively).[12] How to separate them simply was still a recognized problem.

Recently some good separation methods of OA and UA have been reported, such as ODS phase with β- and γ-cyclodextrins as mobile phase modifier.[13,14] But this method cannot be coupled to mass spectrometry (MS) due to the presence of non-volatile buffer; Also HPLC using porous graphitic carbon as stationary phase,[12] but the separation by this technique cannot be scaled-up to isolate and cannot be widely applied since the mobile phase including chloroform.

In this study, a simple, efficient and rapid chromatographic technique *via* PAH (Polycyclic Aromatic Hydrocarbons) polymeric C18 bonded phase to improve the separation, was successfully developed for separation and quantitative determination of OA and UA. The name 'PAH' reflects

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that this stationary phase was particularly evident for the separation of isomer mixtures, such as widespread trace detection of several isomeric polycyclic aromatic hydrocarbons in environmental analysis.[15] As we know the most common ODS phase we used was monomeric C18, but PAH polymeric phase resulted from the reaction of trifunctional silanes with silica in contrast with monomeric phase with synthesis of monofuctional silanes.[16] In general, polymeric stationary phases exhibit greater shape discrimination of long chained molecules than monomeric phases since multipoint attachment and bridged ether linkage. For sample-to-sample comparison at the similar assay analytical level, ten kinds of commonly used Chinese traditional medicinal plants in Chinese pharmacopoeia guidance [17] were selected for quantitative determination of OA and UA using RPLC with PAH column. The results show that when using the PAH stationary phase the triterpenic acid isomers of the vast majority of natural products can meet the requirements of separation.

MATERIALS AND METHODS

Chemical reagents and materials

OA and UA standards were purchased from Shanghai Standard Biotech Company (Shanghai, China), HPLC grade acetonitrile and methanol were purchased from Tedia (USA). High purity water was prepared by Milli-Q reagent water purification system (Millipore Corporation, MA, USA). The Chinese medicinal plants, namely *Crataegus pinnatifida* Bge. (Crataegi fructus), *Forsythia suspensa* (Thunb.) Vahl (Forsythiae fructus), *Prunella vulgaris* L. (Prunellae spica), *Verbena officinalis* L.(Verbenae herba), *Eriobotrya japonica* (Thunb.) Lindl. (Eriobotryae folium), *Ligustrum lucidum* Ait.(Ligustri lucidi fructus), *Diospyros kaki* Thunb.(Kaki calyx), *Chaenomeles speciosa* (Sweet) Nakai (Chaenomelis fructus), *Ziziphus jujube* Mill.(Jujubae fructus), *Cornus officinalis* Sieb. Et Zucc.(Corni fructus), were purchased from Guoyitang TCM Store(Hefei, China) and were authenticated by Professor Zhou(Anhui University of Traditional Chinese Medicine) according to morphological characteristics. The plant materials were air-dried and stored in the dark at room temperature.

Preparation of standard substances solution

Stock solutions (365 μg/mL and 332 μg/mL) of the OA and UA standards were prepared by dissolving in methanol accurately weighed amounts of the standards, stored in freezer (-20°C). Otherwise, working standard solutions were prepared by dilution of the stock standard solutions with methanol and stored under refrigerator (4°C).

Preparation of samples

The air-dried plant materials were powdered by a disintegrator and then sieved (40-60 mesh). The powders were accurately weighed by CP225D electronic balance (Sartorius, Germany) and were ultrasonic extracted with 50mL methanol by KQ-200KDB ultrasonic processor (220 V, 50 Hz, Kunshan, China). All the solutions were filtered through 0.22 μm nylon membrane filter before injection.

Equipment and chromatographic conditions

The HPLC apparatus was Waters Acquity H-Class UPLC series, (Waters, USA) consisting of QSM (Quaternary Solvent Manager), SM-FTN (Sample manager with flow-through needle), TUV (UV-Vis detector) and Empower 2 chromatography data software. The stationary phases used within the study were Ultimate XB-PAH(4.6 × 250 mm, 5 μm, Welch Materials, USA), Ultimate XB-C18(4.6 x 250 mm, 5 μm), Phenomenex Luna-C18(4.6x250 mm, 5 μm, Phenomenex, USA), Shimpack CLC-C18(4.6 x 250 mm, 5 μm, Shimadzu, Japan), Ultimate XB-C8(4.6 x 250 mm, 5 μm), Ultimate XB-Phenyl(4.6 x 250 mm, 5 μm), Hypersil BDS-Cyano (4.6 x 250 mm, 5 μm, Elite, China).

The detection wavelength was 210 nm and the column temperature was kept at 25°C; the injection volume was 10 μL; and the flow rate at 1.0 mL/min.

RESULTS AND DISCUSSION

Influence of different stationary phase

A suitable stationary phase may possess the selectivity, efficiency and reproducibility to provide adequate separation of OA and UA in reasonable time. In this case, seven different commercially available packing columns, including five commonly used reverse phases such as C18 (octadecyl silane), C8 (octyl silane), phenyl, cyano and PAH (polymeric octadecyl silane) were conducted to separate working standard mixture of OA and UA with using the acetonitrile/water (85: 15, v/v) as the mobile phase. They were chemically different bonded phases and demonstrate significant changes in the selectivity using the same mobile phase.

Figure 1 displayed the variation in retention time and resolution of OA and UA in contrast with different stationary phase column. OA and UA had the same retention time and thus eluted together as a single peak by cyano, phenyl bonded phases and they were partially separated by octyl silane bonded phase. With the ODS bonded phase columns, the resolution was better than above three phases, but not separated exactly (r <1.1). In contrast, the PAH polymeric C18 bonded stationary phase showed the high specific selectivity for the analytes as compared to monomeric C18 phases, and the resolution

reach 3.0. Studies have shown that the retention time of the couples (OA, UA) become stronger with the growth of carbon load of stationary phase, as well as the increase of separation. The results showed that the PAH polymeric C18 bonded stationary phase can reach high resolution due to the multipoint attachment and bridged ether linkage with pentacyclic compounds. In addition, high carbon contents could ensure more degree of hydrophobic interactions between analytes and bonded phase and therefore the most retention.

Influence of different composition of mobile phase

With the PAH phase column, different composition of acetonitrile/water and methanol/water were performed as mobile phase on the analysis of different medicinal plants. Although the peaks shape of the couples were more symmetry on methanol/water system, the baseline noise was higher than acetonitrile/water system since the absorbance of methanol at the short wavelength UV region was large in comparison with acetonitrile. Typically under acetonitrile/water system OA and UA could ensure more baseline resolution with other compounds in plant samples than methanol/water system due to different selectivity of solvents.

After selecting the most suitable column and mobile system, the composition of mobile phase and column temperature were optimized by using Drylab® 2000 plus software (Rheodyne LLC., USA). Regarding the examples shown in Figure 2 and Figure 3, the working standard solution was chromatographed at two temperatures (20°C and 30°C) and two contents of the acetonitrile (80% and 96%). Retention data of OA and UA were processed with Drylab® in order to find acceptable resolutions for the relevant peaks. Taking into account the influence of the retention time and peak shape, optimum separation (Rs ≥3.4) was found at 88% acetonitrile and at column temperature of 23°C.

Method validation

The HPLC method was validated by defining the precision; accuracy; limit of detection; limit of quantitation; linearity and range. The chromatographic method presents a good linearity in the concentration range considered. The regression equation of OA was Y = 516.69 X -459.82 (r = 0.9999) with linear range 0.73 μg/mL-36.5 μg/mL. And the regression equation of UA was Y = 446.59X-545.25 (r = 0.9998) with linear range 0.66 μg/mL-33.2 μg/mL. Intra-day and inter-day variations were used to determine the precision of the HPLC analysis method. The intra-day variation was determinated by analyzing in triplicate the same mixed standard solution for six times within one day. While for inter-day variability test, the standard solution was examined in triplicate for three consecutive days. The RSD of the peak area were taken as the measures of precision. As presented in Table 1, the overall intra-day and inter-day variations were less than

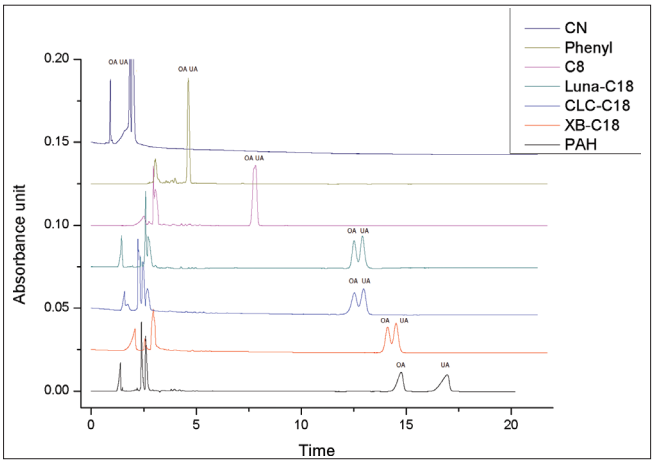

Figure 1: Resolution and retention time with different stationary phase

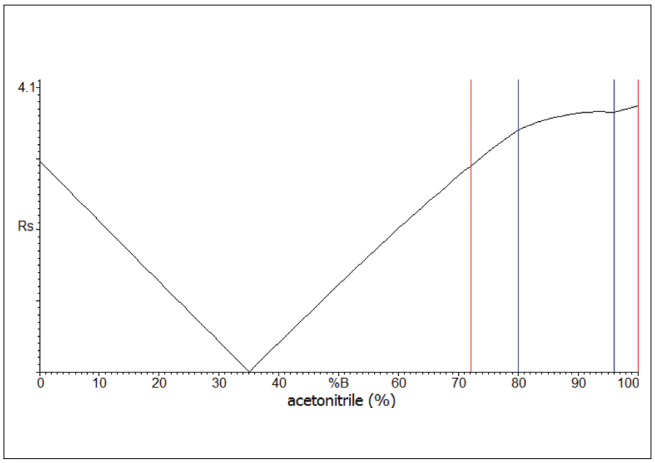

Figure 2: Resolution map for the separation of OA and UA in dependence of the acetonitrile content of the mobile phase

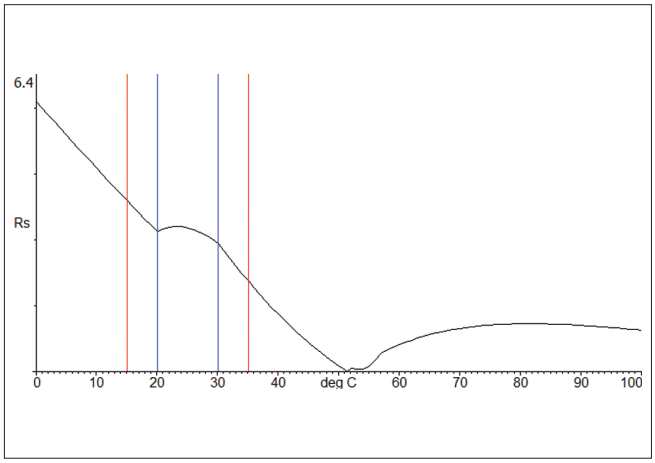

Figure 3: Resolution map for the separation of OA and UA in dependence of column temperature

1.34%. The limit of detection and limit of quantitation were used to evaluate the sensitivity of the analysis method, and the measured LOD and LOQ values of OA and UA see Table 2. The recovery of the method was determinate with the standard addition method for OA and UA in ten medicinal plant samples. The results are also listed in Table 3.

Application

Using the optimized stationary phase and mobile phase, two compounds were separated with good resolutions in all plant samples. In order to keep each sample in the similar concentration levels, different sets of sampling weights were employed. The identification of peaks was carried out based on the retention times of reference standards. The concentrations of the two target peaks encountered in the case samples were reported in Table 3 and the typical chromatograms of ten medicinal plants were shown in Figure 3. According to Figure 4, the peaks of OA and UA in all samples achieved complete separation (r>2.5), and the excellent chromatographic conditions could be recommended for reliable quality control evaluation of medicinal plants.

Table 1: Resolution and retention time with different stationary phase

Stationary phase	Carbon load	Retention time(min)		Resolution
		OA	UA	
Hypersil BDS-Cyano	5%	1.961	1.961	/
Ultimate XB-Phenyl	8%	4.656	4.656	/
Ultimate XB-C8	12%	7.745	7.825	/
Shimpack CLC-C18	14%	12.518	12.913	1.092
Phenomenex Luna-C18	15%	12.533	12.971	0.916
Ultimate XB-C18	17%	14.114	14.515	0.959
Ultimate XB-PAH	22%	16.945	19.115	3.426

CONCLUSION

In this study, the ability of five kinds of usual reverse stationary phases (seven commercial columns) to separate OA and UA under same mobile phase was systematically investigated and the layer-by-layer polymeric C18 bonded phase (PAH column) was the most suitable column to separate them. In order to separate OA and UA from other compounds in ten kinds of Chinese medicinal

Table 2: Precision, calibration curves, LOD and LOQ of OA and UA

Compounds	Intra-day precision (RSD %) n = 6	Inter-day precision (RSD %) n = 6	Calibration equation (n = 6)	Correlation coefficient (r)	Limit of detection (μg/ml)	Limit of quantitation (μg/ml)
OA	0.33	0.89	Y = 516.69x-459.82	0.9999	0.21	0.71
UA	0.73	1.34	Y = 446.59 X-545.25	0.9998	0.33	1.10

Table 3: Contents of OA and UA in ten medicinal plants and their accuracies

Samples (Latin name)	Samples (English name)	Sample weighed[a] (g)	Measured content of samples[b] (n = 3)		Average recovery (%, n = 6)	
			OA (mg/g)	UA (mg/g)	OA	UA
Crataegus pinnatifida Bge.	Crataegi fructus	1.7	0.593 ± 0.018	3.542 ± 0.071	98.4	96.6
Forsythia suspensa (Thunb.) Vahl	Forsythiae fructus	0.4	8.038 ± 0.072	3.595 ± 0.083	101.9	97.5
Prunella vulgaris L.	Prunellae spica	0.6	0.719 ± 0.019	2.211 ± 0.027	98.1	101.3
Verbena officinalis L.	Verbenae herba	1.2	0.696 ± 0.018	1.906 ± 0.057	96.5	94.1
Eriobotrya japonica (Thunb.) Lindl.	Eriobotryae folium	0.5	1.685 ± 0.025	7.807 ± 0.047	97.4	102.6
Ligustrum lucidum Ait.	Ligustri lucidi fructus	0.5	10.513 ± 0.242	3.293 ± 0.036	95.7	97.1
Diospyros kaki Thunb.	Kaki calyx	1.5	1.044 ± 0.021	2.833 ± 0.045	96.2	94.3
Chaenomeles speciosa (Sweet) Nakai	Chaenomelis fructus	1.0	4.492 ± 0.144	1.078 ± 0.013	98.2	94.5
Ziziphus jujube Mill.	Jujubae fructus	6.0	0.081 ± 0.001	0.023 ± 0.001	96.3	92.1
Cornus officinalis Sieb. Et Zucc.	Corni fructus	4.0	0.902 ± 0.020	2.632 ± 0.068	98.4	96.2

[a]Samples were accurate weighed with electronic balance, [b]Values were the mean values with ± standard deviation

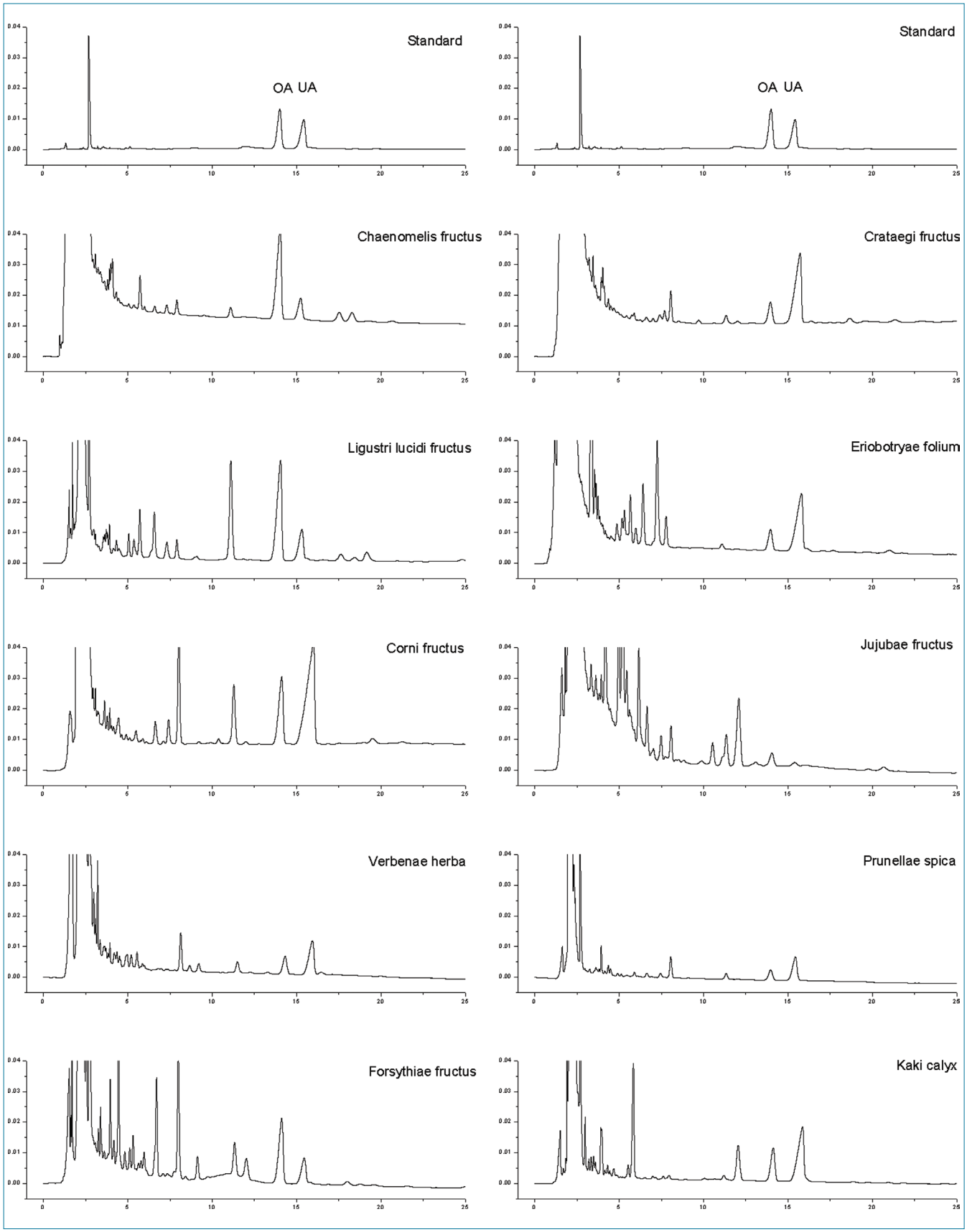

Figure 4: Determination of OA and UA in ten kinds of Chinese medicinal plants

plants, different compositions of mobile phase and different temperature were studied and acetonitrile/water (88:10, v/v) and 23°C was the optimal chromatographic conditions. This method offers a good alternative for routine analysis of triterpenic acid isomers due to its simplicity and sensitive separation.

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