Application Note

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Accurate quantification of oleanolic acid and ursolic acid in traditional chinese medicine

High-resolution sampling 2D-LC

Authors

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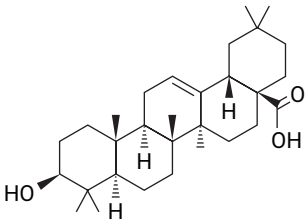
Abstract

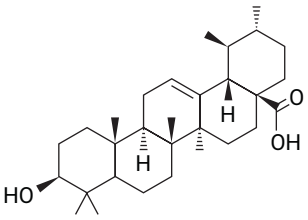
Quality control is one of the most important tasks within the Traditional Chinese Medicine (TCM) industry. It is required that levels of the main compounds in TCMs are determined using HPLC methods. Due to the complexity of TCM samples, the target compounds are likely to be coeluted with other minor compounds within an HPLC separation. A 2D-LC system could be a powerful method to separate complex samples using orthogonal column chemistries and mobile phases. This Application Note details the development of a high-resolution sampling 2D-LC method for the quantitative analysis of oleanolic acid and ursolic acid in *Pterocephali Herbra*.

Introduction

Traditional Chinese Medicine (TCM) analysis is a challenging task due to the complexity of the components in a single herbal medicine or combined preparation. To control the quality of TCMs, the amounts of main compounds are required to be determined using HPLC methods according to China Pharmacopeia (CHP) regulations. Often, other compounds will coelute with the target compounds, influencing the accuracy of their quantification. *Pterocephali Herbra* is one of the CHP examples that requires the amounts of two target compounds, oleanolic acid and ursolic acid (Figure 1), to be determined by HPLC. This study found that several other compounds coelute with oleanolic acid and ursolic acid, making the existing method inadequate for the quantification of these compounds.



Oleanolic acid
$C_{30}H_{48}O_3$
CAS: 508-02-1



Ursolic acid
$C_{30}H_{48}O_3$
CAS: 77-52-1

Figure 1. Target compounds separated in this study.

To achieve orthogonal separation, this Application Note used two Agilent InfinityLab Poroshell 120 columns with different selectivities (EC-C18 and Phenyl-Hexyl) and different mobile phase conditions in high-resolution sampling 2D-LC. The two target compounds were determined by collecting several small fractions over a selected time range, covering the entire area of a peak from a 1D chromatogram. Each cut was parked in a sampling loop, and all cuts were consecutively analyzed in the second dimension. This mode ensured that all the selected compounds were transferred to, and analyzed in the second dimension. Thus, selected coeluting compounds were subjected to the high-resolution sampling process for analysis in the second dimension. This enabled a reliable quantification, as shown in a previously issued Technical Overview[1].

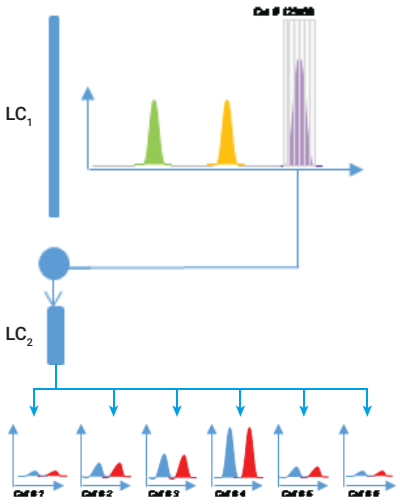


Figure 2. Illustration of high-resolution sampling 2D-LC.

Experimental

Chemicals and samples

All reagents and solvents were HPLC grade. Acetonitrile and methanol were purchased from JT Baker, USA. Ammonium acetate was purchased from J and K, Beijing China. Fresh ultrapure water was obtained from an ELGA water purification system, Lane End, UK. The extracts of *Pterocephali Herbra* and standards of oleanolic acid and ursolic acid were provided by a local pharmaceutical company in China. To achieve a concentration of 2.3 mg/mL and 2.0 mg/mL respectively, the stock standard solutions were made by dissolving oleanolic acid and ursolic acid together in methanol.

Instrument

The Agilent 1290 Infinity II 2D-LC was composed of the following modules:

- Two Agilent 1290 Infinity II high speed pumps (G7120A)
- Agilent 1290 Infinity II multisampler (G7167B) with sample cooler (Option #100)
- Two Agilent 1290 Infinity II MCTs (G7116B)
- Two Agilent 1290 Infinity II DADs (G7117B) with a 10-mm Max-Light cartridge cell (G4212-60008) and a 60-mm Max-Light cartridge cell (G4212-60007)
- Agilent 1290 Infinity valve drive (G1170A) with 2-position/4-port duo valve (2D-LC valve head, G4236A)
- Two Agilent 1290 Infinity valve drives (G1170A) with multiple heart-cutting valves (G4242-64000) equipped with 40-µL loops

Method setup for high-resolution sampling

The method setup for high-resolution sampling referred to the previously issued Technical Overview[1]. Figure 3 shows the high-resolution sampling configuration of the Agilent 1290 Infinity II 2D-LC system, consisting of a 2-position/4-port duo valve connected to two multiple heart-cutting valves holding 12 sampling loops. With this setup, up to 10 consecutive cuts can be sampled and stored until analysis. For high-resolution sampling, a maximum loop filling of 80 % is recommended to prevent any loss of sample. Figure 4 shows the method setup used for the 2D pump. First, a 1D-LC separation of the sample was run, and the chromatogram was loaded as the reference signal preview window. High-resolution sampling was time-based, according to the peak of interest, with eight cuts covering the entire peak width. Under the given 1D conditions, a sampling time of 8.63 seconds equals a loop filling of 72 %.

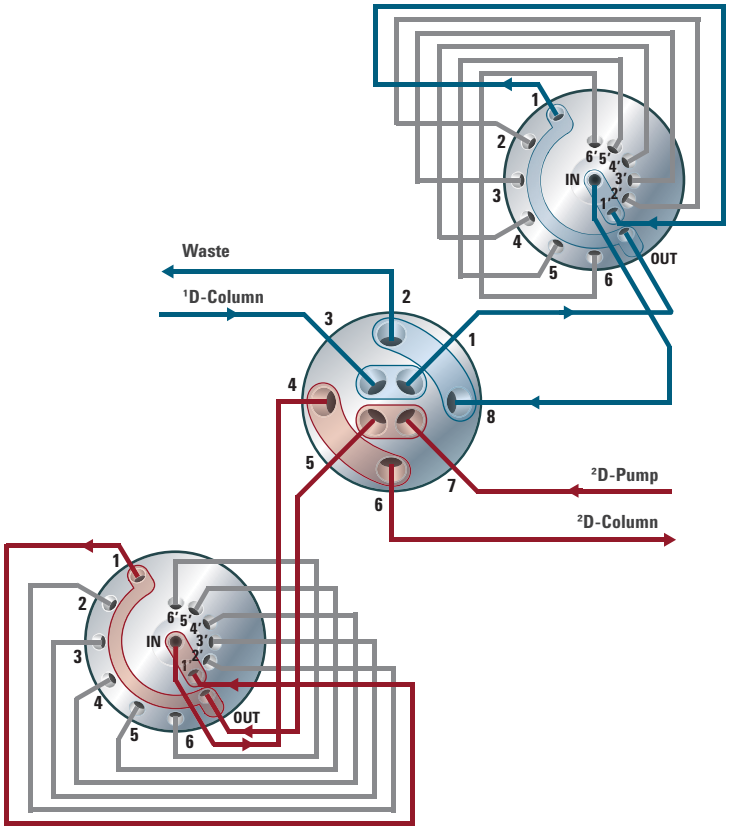


Figure 3. Setup of the Agilent 1290 Infinity II 2D-LC system, holding 12 sampling loops.

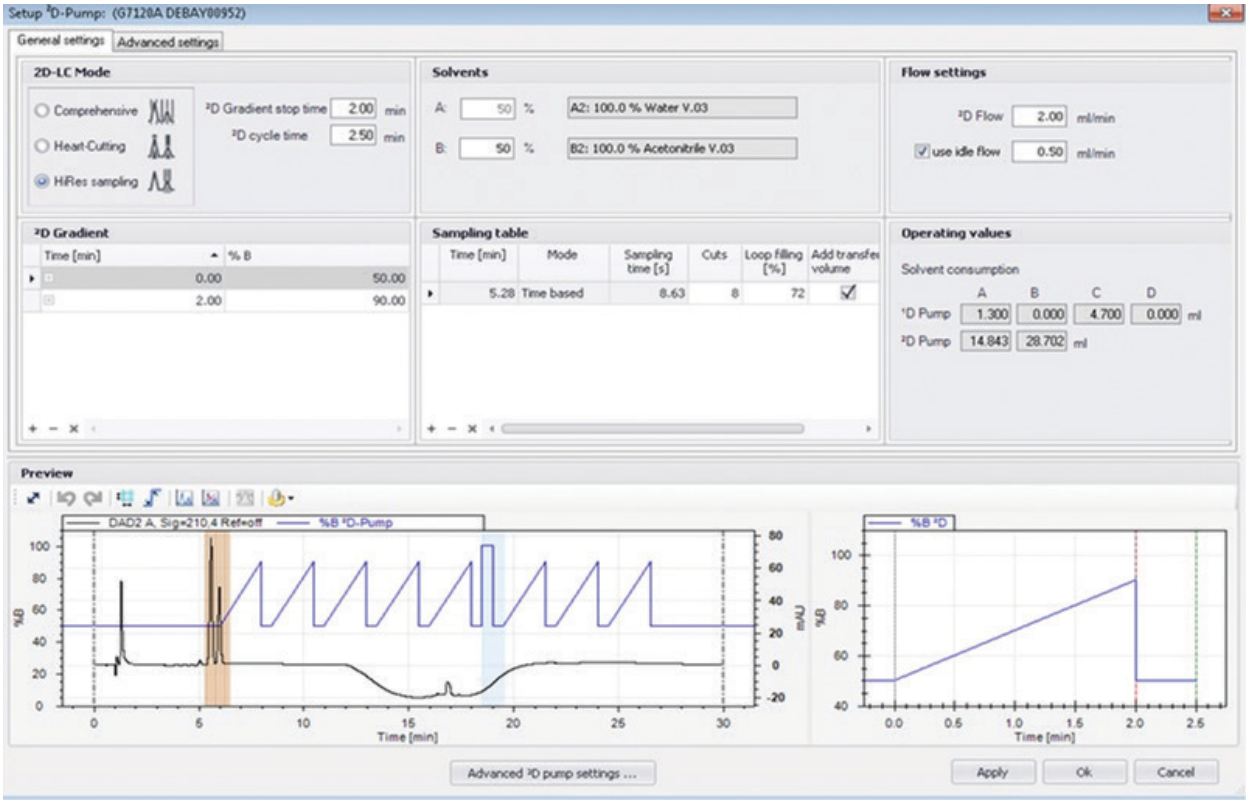


Figure 4. Method setup for the 2D pump.

This Application Note used the following LC columns:

- Agilent InfinityLab Poroshell 120 Phenyl-Hexyl, 2.1 × 100 mm, 1.9 µm (p/n 695675-912)
- Agilent InfinityLab Poroshell 120 EC-C18, 3.0 × 50 mm, 2.7 µm (p/n 699975-302)

Software used for system control and data analysis included:

Agilent OpenLab CDS ChemStation Edition Rev. C.01.07 SR2 [255] with Agilent 2D-LC Software add-on, Product Version A.01.03 [025].

Results and discussion

Separation

Figure 5 shows a typical chromatogram for oleanolic acid and ursolic acid analysis in *Pterocephali Herbra* using an optimized method according to a CHP method. While this method, using the InfinityLab Poroshell 120 4 µm, provided good resolution for the standards, the extreme complexity of the sample did not allow adequate separation of the target analytes from interfering compounds. Therefore, the target analytes were not able to be quantitated using a traditional 1D-LC separation.

To properly quantify these two target compounds, high-resolution sampling 2D-LC was used to separate them from interfering compounds. This was achieved using column chemistries that differed from one another in their selectivity. The introduction of many chemistries for 2.7 and 1.9 µm superficially porous particles makes InfinityLab Poroshell 120 columns ideal for achieving the separation orthogonality required in 2D HPLC separations. This work used a Phenyl-Hexyl column for the first dimension. The two target compounds, oleanolic acid and ursolic acid, were baseline separated on an Agilent InfinityLab Poroshell 120 Phenyl-Hexyl, 2.1 × 100 mm, 1.9 µm column, shown in Figure 6A. The sub-2 µm superficially porous particle Poroshell column gave high efficiency and good resolution for the two compounds with narrow peak widths, making it easy for 2D separation sampling. The second dimension separation used an Agilent InfinityLab Poroshell 120 EC-C18, 3.0 × 50 mm, 2.7 µm column. The low backpressure of these columns allows the use of a higher flow rate to achieve a quick separation of coeluted compounds away from target compounds. Using high-resolution sampling, eight cuts in the time range of the peak containing oleanolic acid and ursolic acid were sampled (Figure 6A) and injected to the second dimension for analysis (Figure 6D).

Table 1. High-resolution sampling 2D-LC method for the analysis of oleanolic acid and ursolic acid.

Columns	
First dimension	Agilent InfinityLab Poroshell 120 Phenyl-Hexyl, 2.1 × 100 mm, 1.9 µm
Second dimension	Agilent InfinityLab Poroshell 120 EC-C18, 3.0 × 50 mm, 2.7 µm
1D Pump	
Solvent A	10 mM ammonium acetate in water
Solvent B	Methanol
Flow rate	0.2 mL/min
Gradient	75 %B at 0 minutes 75 %B at 8 minutes 100 %B at 8.1 minutes 100 %B at 12.0 minutes 75 %B at 12.1 minutes
2D Pump	
Solvent A	0.05 % H_3PO_4 in water
Solvent B	Acetonitrile
Flow rate	2 mL/min
Gradient	50 %B at 0 minutes 90 %B at 2 minutes
2D Gradient stop time	2.0 minutes
2D Cycle time	2.5 minutes
Stop time	30 minutes
High-resolution sampling	
Time based	5.28 minutes
Sampling time	8.63 seconds
Number of cuts	8
Multicolumn thermostat	
First dimension	30 °C
Second dimension	40 °C
Multisampler	
Injection volume	1 µL
Needle wash	5 seconds in methanol: water 50:50
1D Diode array detector	
Wavelength	210 nm/4 nm
Data rate	40 Hz
Flow cell	10-mm Max-Light cartridge cell
2D Diode array detector	
Wavelength	210 nm/4 nm
Data rate	80 Hz
Flow cell	60-mm Max-Light cartridge cell

HPLC Conditions

Parameter	Value
Mobile phase	15 % 10 mM ammonium acetate in water/85 % methanol
Flow rate	1 mL/min
Injection volume	5 µL
Temperature	25 °C
Detector	UV 210 nm

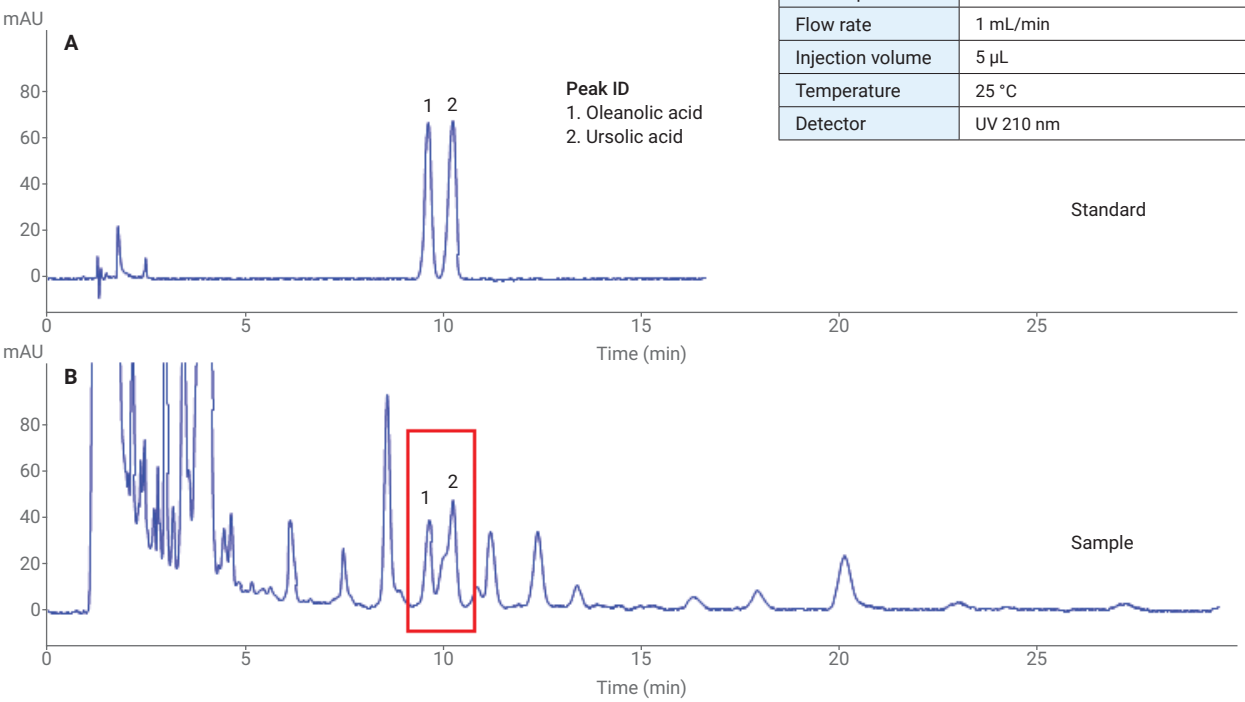


Figure 5. Chromatogram of oleanolic acid and ursolic acid analysis in *Pterocephali Herbra* with an Agilent InfinityLab Poroshell 120 EC-C18, 4.6 × 150 mm, 4 µm column.

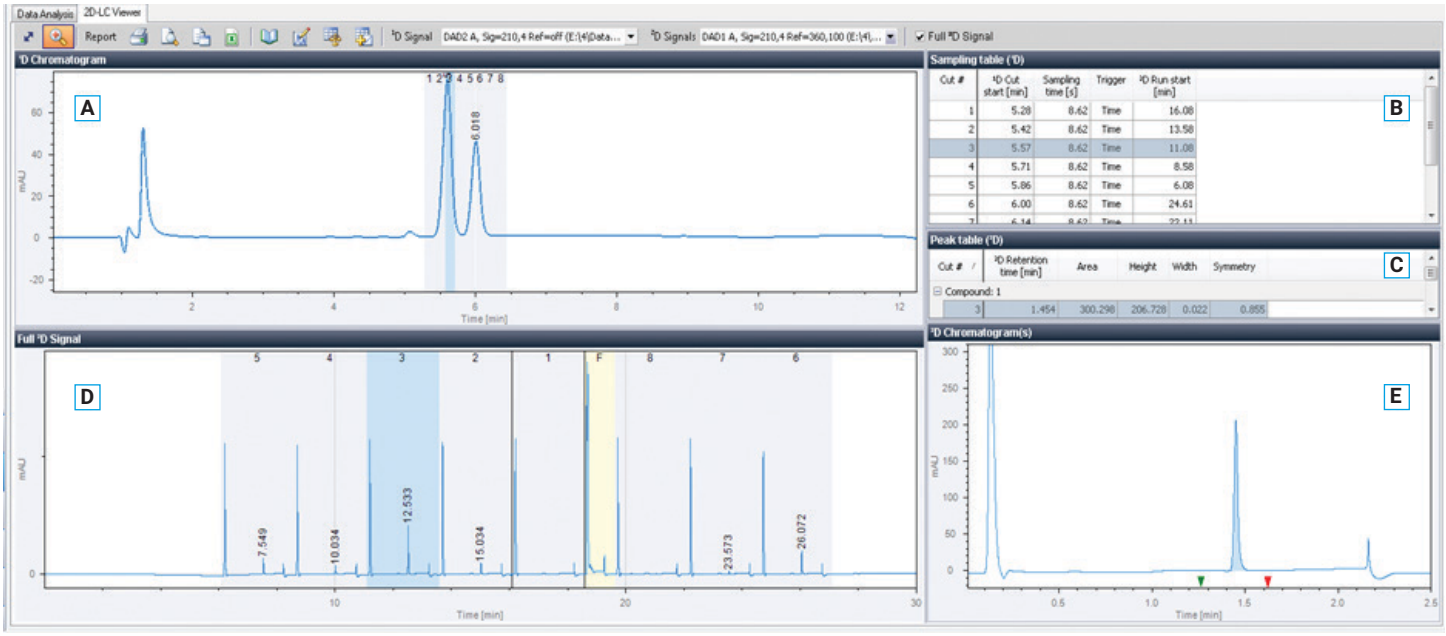


Figure 6. 2D-LC Viewer. A) 1D chromatogram with eight consecutive cuts over the entire width of the two peaks. B) Sampling table with eight cuts taken from the first dimension. C) Peak table of the selected cut and its peak area and other parameters. D) Overview of all 2D chromatograms of eight cuts. E) Chromatogram of each selected cut.

Figure 7 shows an overlay of 2D chromatograms of oleanolic acid standard (from cut 1 to cut 4), and Figure 8 shows the ursolic acid standard (from cut 5 to 8). The entire peaks of oleanolic acid and ursolic acid were sampled respectively, then transferred to, and analyzed in, the second dimension. In this manner, possible coeluting compounds in the sample can be separated in the second dimension. Figure 9 shows overlaid 2D chromatograms of a TCM sample isolating oleanolic acid. The sample showed a good separation of oleanolic acid, and some small peaks. In Figure 10, the 2D separation shows that ursolic acid was well separated from several other compounds observed in the 1D chromatogram of the TCM sample.

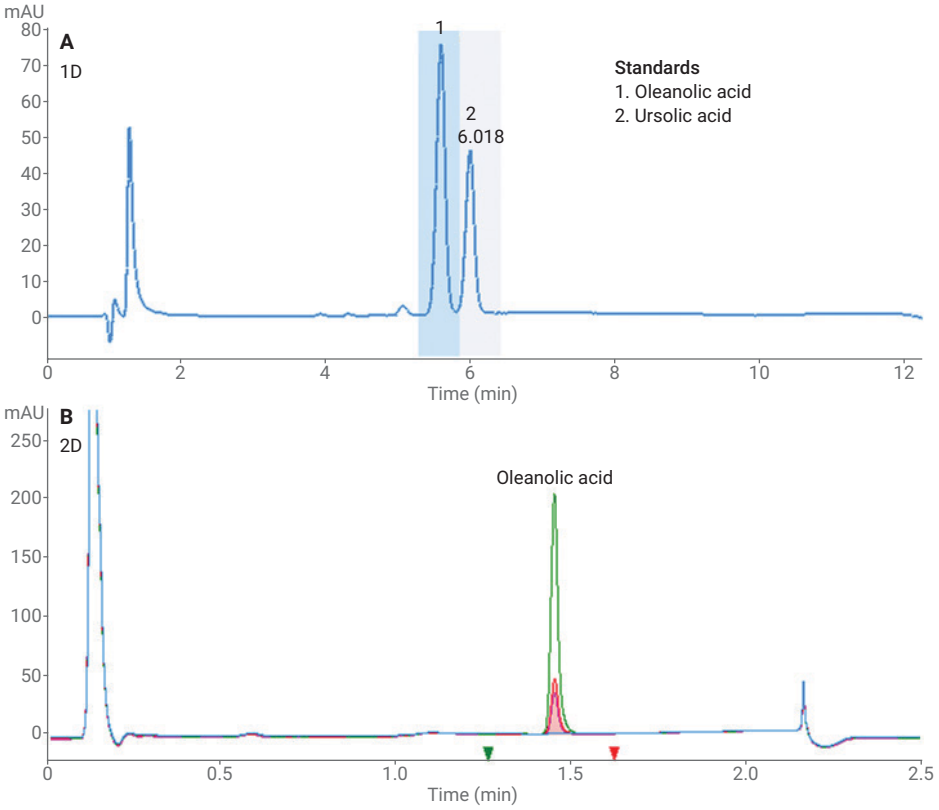


Figure 7. Overlay of 2D chromatograms of cuts 1–4 for oleanolic acid standard.

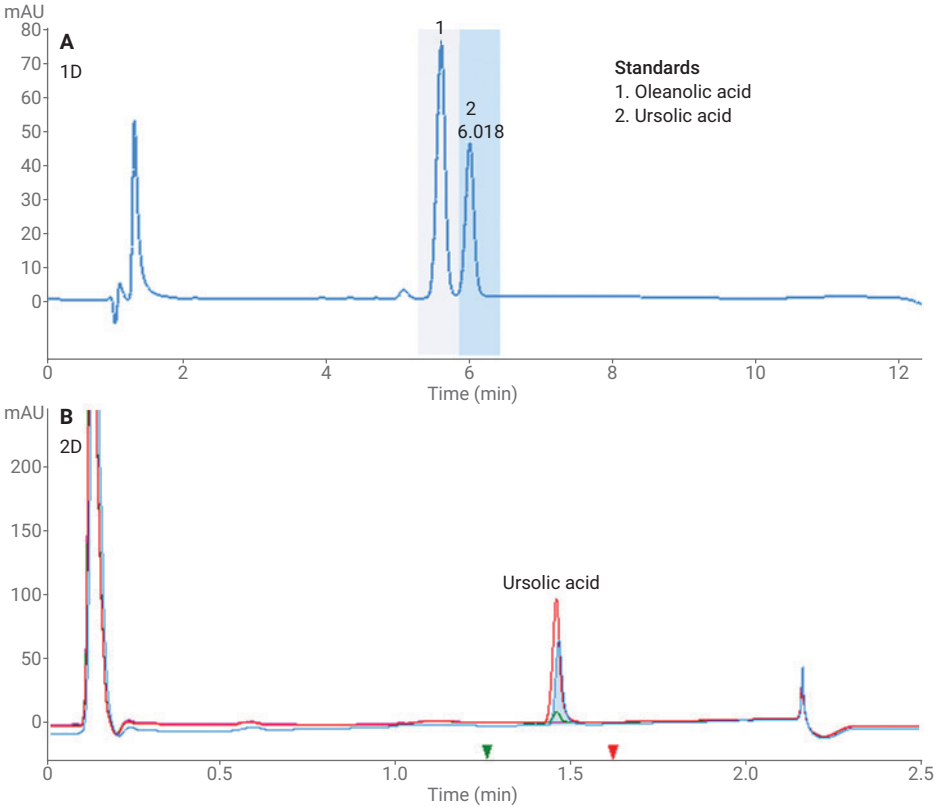


Figure 8. Overlay of 2D chromatograms of cuts 5–8 for ursolic acid standard.

Quantification

Standard solutions of oleanolic acid in a concentration range of 57.5–1,150 µg/mL and ursolic acid in a concentration range of 50–1,000 µg/mL were analyzed three times with the above method as well as the sample solutions. For quantification, peaks of one compound were integrated in every 2D chromatogram by the software, and the sum of peak areas for each compound was calculated for the calibration curve. Figure 11 shows good linearity across the entire calibration range, with R^2 values greater than 0.999 for both compounds. The amounts (average of three analyses) calculated in the determined samples were 95.6 µg/mL oleanolic acid and 258.9 µg/mL ursolic acid.

Repeatability

To determine the repeatability of the high-resolution sampling 2D-LC method, six consecutive analyses of a standard mix containing 230 µg/mL oleanolic acid and 200 µg/mL ursolic acid were performed. Total peak areas of oleanolic acid and ursolic acid were determined from the 2D chromatograms. Relative standard deviations (RSDs) were 0.8 % for oleanolic acid and 1.3 % for ursolic acid.

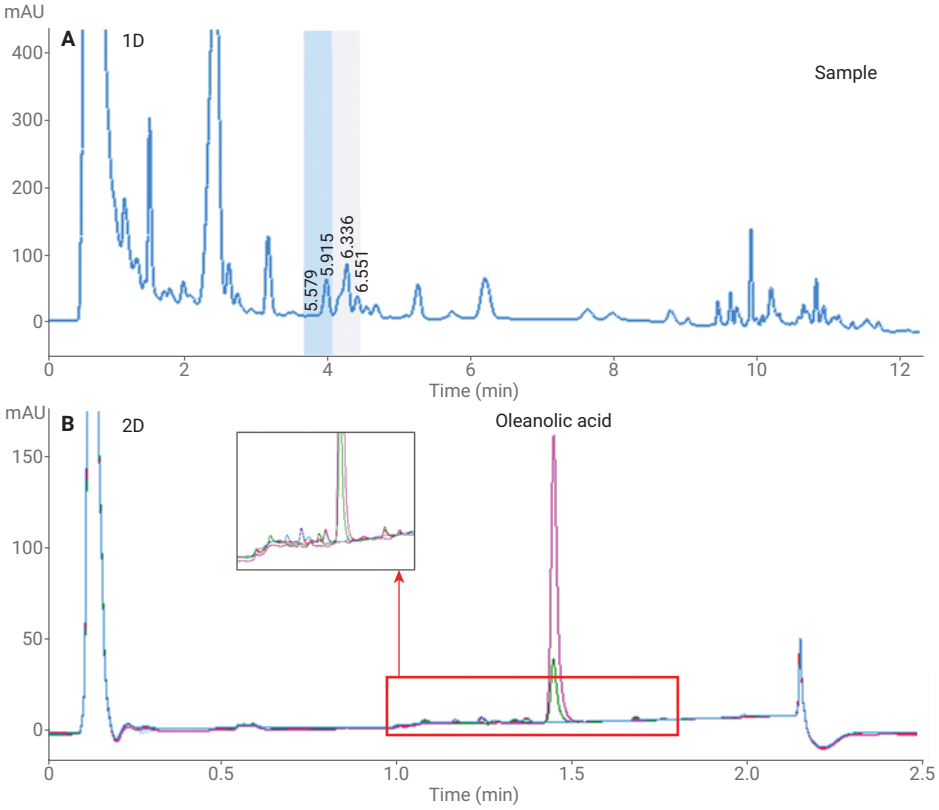


Figure 9. Overlay of 2D chromatograms of cuts 1–4 for oleanolic acid from sample.

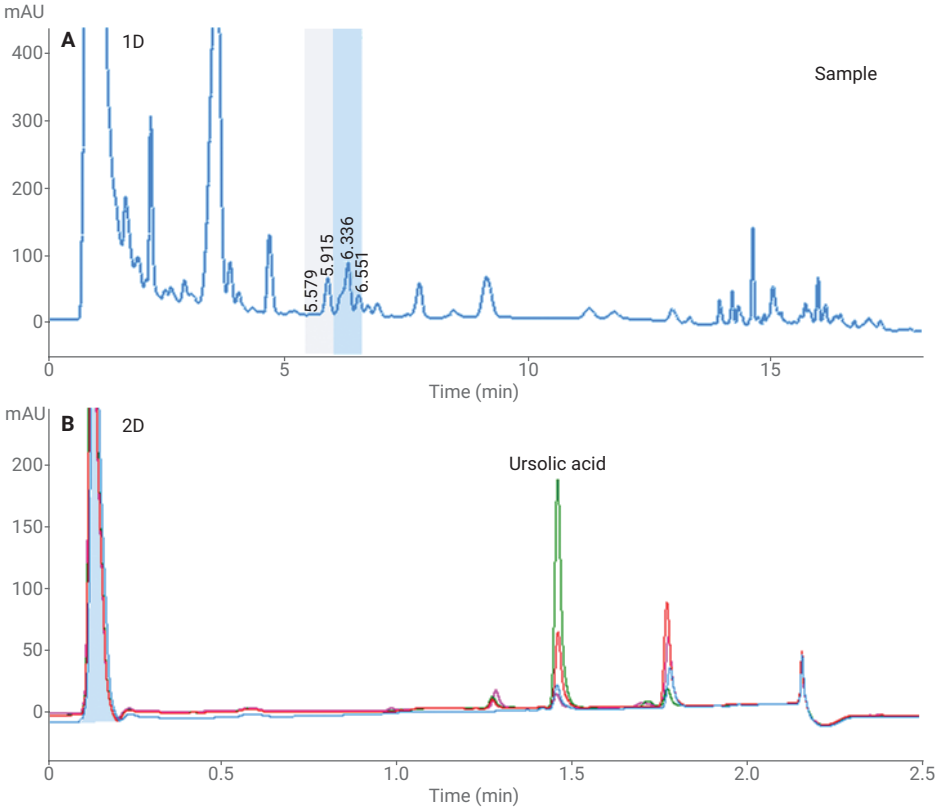


Figure 10. Overlay of 2D chromatograms of cuts 5–8 for ursolic acid from sample.

Conclusions

This Application Note demonstrates a high-resolution sampling Agilent InfinityLab 2D-LC solution for complex TCM analysis. Interfering compounds in the first dimension can be well separated so that target compounds are reliably quantified over high-resolution sampling 2D-LC within the same run. To achieve the required separations at each step of the 2D analysis, the Agilent InfinityLab Poroshell 120 columns provide ideal particle sizes. To provide the orthogonal separation required for a successful 2D-LC separation, these columns also offer wide selectivity options that can be used with different organic phases. The high-resolution sampling 2D-LC method developed in this Application Note enables reliable quantification for complex TCMs.

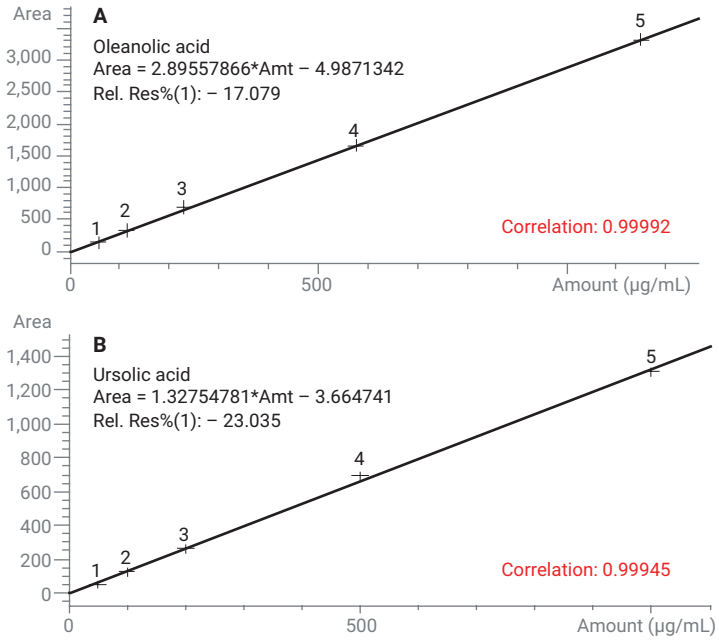


Figure 11. Calibration curves from the sum of 2D peaks for oleanolic acid and ursolic acid.

Reference

1. Stephan, S. High-Resolution Sampling 2D-LC with the Agilent 1290 Infinity II 2D-LC Solution. *Agilent Technologies Technical Overview*, publication number 5991-7637EN, **2016**.

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Printed in the USA, March 8, 2018
5991-9114EN

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