Application Note

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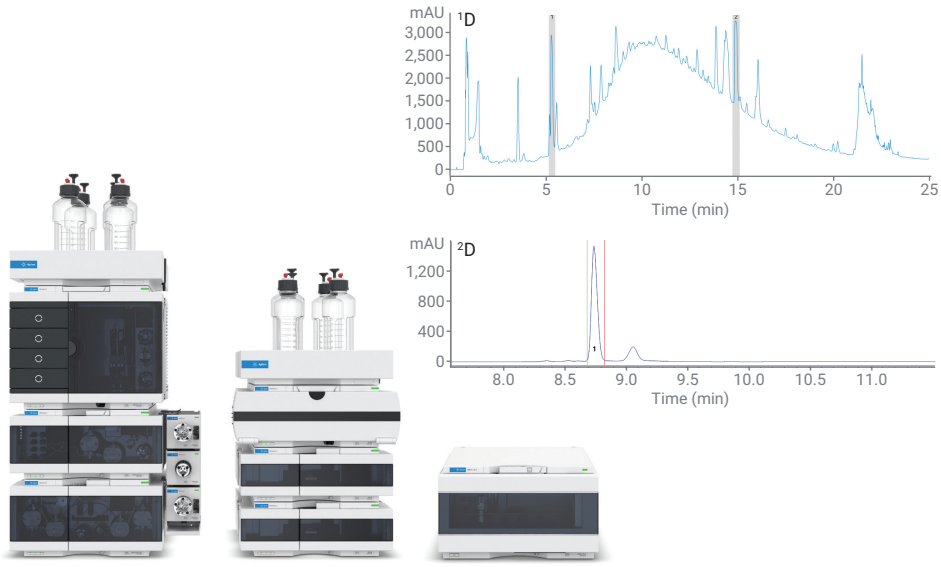
Two-Dimensional Isolation of Target Compounds from Complex Natural Products with Agilent InfinityLab 2D-LC Solutions



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Abstract

Two-dimensional liquid chromatography (2D-LC) allows the isolation of high-purity target compounds from complex natural samples. To date, mostly offline 2D-LC is used for two-dimensional purification, which is time-consuming, difficult to automate, and can incur sample loss or contamination. This Application Note shows the online 2D-LC isolation of high-purity target compounds from a complex natural sample using the Agilent 1290 Infinity II 2D-LC System with mass-based fraction collection. The target compounds salidroside and rosavin are isolated from the natural product *Rhodiola rosea*, and highest purity is obtained. The online 2D-LC purification requires no extra time compared to a one-dimensional LC (1D-LC) purification and avoids manual work, saving time and reducing errors.

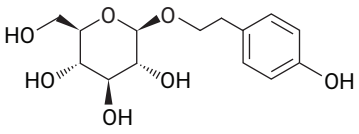
Introduction

Natural products present a major resource for the investigation of naturally occurring biologically active compounds, for example, during lead identification in drug discovery.[1,2] Pure target compounds need to be isolated from natural products for bio-activity research, for potential structure confirmation using NMR analysis, and to obtain standard reference material for quantitative purposes.[2-4]

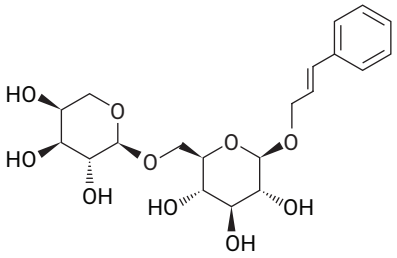
The complexity of natural products complicates the isolation of high-purity target compounds,[2] and 1D-LC might not provide adequate separation.[1,2] To facilitate isolation of high-purity target compounds from complex natural products, 2D-LC is a valuable tool.[2] 2D-LC enables the application of orthogonal separations or separations with different selectivity in the first (^{1}D) and second (^{2}D) dimension. This allows separation according to different compound properties. The most common approach for two-dimensional purification is offline 2D-LC; however, the offline approach is time-consuming, difficult to automate, and can incur sample loss or contamination.[1,3]

This Application Note shows the online 2D-LC purification of high-purity target compounds from a complex natural sample using the 1290 Infinity II 2D-LC System with mass-based fraction collection. The online approach is faster and requires less manual work compared to an offline approach, saving time and avoiding errors.

The purification of the target compounds salidroside and rosavin (see Figure 1) from an extract of *Rhodiola rosea* were used to exemplify isolation of high-purity target compounds from a complex natural sample. Biological activity has been described for *Rhodiola* species[5,6] and *Rhodiola* has been used in Chinese traditional medicine.[6] For *R. rosea*, three classes of bioactive constituents have been described: phenylethanoids, phenylpropanoid glycosides, and monoterpenes. Salidroside belongs to the class of phenylethanoids, and rosavin to the class of phenylpropanoid glycosides.[6] This Application Note compares the purity and recovery of salidroside and rosavin purified using 1D-LC and 2D-LC.



Salidroside



Rosavin

Figure 1. Structure of the target compounds salidroside and rosavin contained in *R. rosea*.

Experimental

Equipment

The Agilent 1290 Infinity II 2D-LC System comprises the following modules:

- Two Agilent 1290 Infinity II High Speed Pumps (G7120A)
- Agilent 1290 Infinity II Multisampler (G7167B) with cooler (option #100)
- Two Agilent 1290 Infinity II Multicolumn Thermostats (G7116B)
- Two Agilent 1260 Infinity II Diode Array Detectors WR (G7115A) with Standard Flow Cell (G1315-60022)
- Agilent 1290 Infinity Valve Drive (G1170A) with 2D-LC Valve, Active Solvent Modulation (G4243A)
- Two Agilent 1290 Infinity Valve Drives (G1170A) with Multiple Heart-Cutting Valves (G4242-64000) equipped with 180 µL loops (part number 5067-6647)

The following modules were added to the system to enable mass-based fraction collection:

- Agilent 1260 Infinity II Bio-Inert Fraction Collector (G5664B)
- Agilent 1290 Infinity II MS Flow Modulator (G7170B)
- Agilent 1260 Infinity II Isocratic Pump (G7110B)
- Agilent LC/MSD XT (G6135B)

Software

Agilent OpenLab CDS ChemStation Edition Rev. C.01.10 [201] with 2D-LC Software version A.01.04 SR3.

Columns

- Agilent ZORBAX Eclipse Plus Phenyl-Hexyl, 3.0 × 100 mm, 1.8 µm (part number 959758-312)
- Agilent ZORBAX Eclipse Plus C18, 4.6 × 100 mm, 3.5 µm (part number 959961-902)

Chemicals

All solvents were LC grade. Acetonitrile and methanol were purchased from Merck (Darmstadt, Germany). Fresh ultrapure water was obtained from a Milli-Q Ultrapure Lab Water System equipped with a Millipak 0.22 µm membrane point-of-use cartridge (Millipore, Merck (Darmstadt, Germany)). Formic acid was purchased from VWR (Darmstadt, Germany). Salidroside and rosavin were obtained from Sigma-Aldrich (Steinheim, Germany).

Sample and sample preparation

R. rosea plant extract capsules were purchased at a local pharmacy. The content of six capsules, approximately 1.0 g, was weighed and extracted with 5 mL of methanol in an ultrasonic bath for five minutes. The resulting extract was filtered using a 1 mL plastic syringe with Captiva Premium Syringe Filters, nylon, 15 mm, 0.45 µm (part number 5190-5091) before injection into the HPLC System.

For purification of salidroside and rosavin, the undiluted extract was used. The extract was diluted 1:10 with methanol for quantification of salidroside and rosavin for determination of recovery.

Standards

Individual stock solutions of salidroside and rosavin were prepared at a concentration of approximately 1 mg/mL in methanol. Combined calibration standards for the determination of recovery were prepared in the concentration range of 5 to 500 µg/mL by dilution of the stock solutions with methanol.

Method for 1D-LC purification

For 1D-LC purification, the outlet of the UV detector was connected through the MS flow modulator to the fraction collector.

Parameter	Value
Column	Agilent ZORBAX Eclipse Plus Phenyl-Hexyl, 3.0 × 100 mm, 1.8 µm
Solvent	A) Water B) Methanol
Gradient	0 minutes – 5% B 20 minutes – 50% B 21 minutes – 100% B Stop time: 25 minutes Post time: 5 minutes
Injection	Injection volume: 3 µL; sandwich injection with 6 µL water plugs; 3 seconds needle wash in water/methanol (50/50; v/v)
Flow Rate	0.500 mL/min
Temperature	40 °C
Detection	UV and MSD
UV	210/4 and 254/4 nm, reference 360/100 nm, 20 Hz
MSD	
Spray Chamber	Agilent Jet Stream Electrospray
Make-Up Solvent	0.1% Formic acid in methanol/water (70/30, v/v)
Make-Up Flow Rate	0.50 mL/min
Ms Flow Modulator	Mode M4, split ratio 50:1, dilution factor 1:50
Signal 1	Positive scan 100 to 600 *m/z* Fragmentor 125 V
Signal 2	Negative scan 100 to 600 *m/z* Fragmentor 125 V
Drying Gas Flow	12 L/min
Nebulizer Pressure	35 psig
Drying Gas Temperature	300 °C
Sheath Gas Temperature	350 °C
Sheath Gas Flow	11 L/min
Capillary Voltage	±4,000 V
Nozzle Voltage	±600 V
Fraction Collection	Peak-based, starting from 0.0 minutes UV and MSD connected with AND condition UV: Peak trigger: 210/4 nm, reference 360/100 nm; peak detection mode: threshold and slope; threshold: 400 mAU; up- and down slope: 5 mAU/s MSD: FC mode – Use sample target mass; MS signals and adducts: positive scan (sodium adduct) and negative scan (formate adduct); threshold 100,000 cps

Method for 2D-LC purification and analysis of fractions

For 2D-LC purification, the outlet of the ^{2}D UV detector was connected to the fraction collector through the MS flow modulator and a 1 m × 0.5 mm (0.2 mL) delay coil (part number 5500-1341).

Parameter	Value
First Dimension (^{1}D)	
Column	Agilent ZORBAX Eclipse Plus Phenyl-Hexyl, 3.0 × 100 mm, 1.8 µm
Solvent	A) Water B) Methanol
Gradient	0 minutes – 5% B 20 minutes – 50% B 21 minutes – 100% B Stop time: 25 minutes Post time: 5 minutes
Injection	*R. rosea* extract and calibration standards: Injection volume: 3 µL; sandwich injection with 6 µL water plugs; 3 seconds needle wash in water/methanol (50/50; v/v) Fractions collected after 1D-LC purification: Injection volume: 10 µL; 3 seconds needle wash in water/methanol (50/50; v/v) Fractions collected after 2D-LC purification: Injection volume: 20 µL; 3 seconds needle wash in water/methanol (50/50; v/v)
Flow Rate	0.500 mL/min
Temperature	40 °C
Detection	UV
UV	210/4 nm and 254/4 nm, reference 360/100 nm, 20 Hz
Second Dimension (^{2}D)	
Column	Agilent ZORBAX Eclipse Plus C18, 4.6 × 100 mm, 3.5 µm
Solvent	A) Water B) Acetonitrile
Temperature	40 °C
Detection	UV and MSD
UV	210/4 nm and 254/4 nm, reference 360/100 nm, 20 Hz
MSD	
Spray Chamber	Agilent Jet Stream Electrospray
Make-Up Solvent	0.1% Formic acid in methanol/water (70/30, v/v)
Make-Up Flow Rate	0.50 mL/min
Ms Flow Modulator	Mode M4, split ratio 200:1, dilution factor 1:50
Signal 1	Positive scan 100 to 600 *m/z* Fragmentor 125 V
Signal 2	Negative scan 100 to 600 *m/z* Fragmentor 125 V
Drying Gas Flow	12 L/min
Nebulizer Pressure	35 psig
Drying Gas Temperature	300 °C
Sheath Gas Temperature	350 °C
Sheath Gas Flow	11 L/min
Capillary Voltage	±4,000 V
Nozzle Voltage	±600 V
Fraction Collection	2D-LC purification: Peak-based, starting from 0.0 minutes UV and MSD connected with AND condition **UV:** Peak trigger: 210/4 nm, reference 360/100 nm; peak detection mode: threshold and slope; threshold: 20 mAU; up- and down slope: 5 mAU/s **MSD:** FC mode – Use sample target mass; MS signals and adducts: positive scan (sodium adduct) and negative scan (formate adduct); threshold 100,000 cps Analysis of fractions: None

Results and discussion

Natural products possess a high complexity that can complicate the isolation of high-purity target compounds using one-dimensional purification. Figure 2 shows the 1D-LC analysis of *R. rosea* and of the target compounds salidroside and rosavin. The complexity of *R. rosea* is apparent, particularly with UV detection at 210 nm (Figure 2A).

2D-LC	
Configuration	2D-LC valve ASM + 2 MHC valves; 180 µL loops; countercurrent
2D-LC Mode	Heart-cutting
Gradient	0.00 minutes – 3% B 0.85 minutes – 3% B 0.86 minutes – 5% B shift to 15 minutes – 5% B shift to 15.01 minutes – 13% B 5.86 minutes – 15% B shift to 15 minutes – 15% B shift to 15.01 minutes – 23% B 5.87 minutes – 100% B ^{2}D gradient stop time: 6.87 minutes ^{2}D cycle time: 8.87 minutes
Flow Rate	2.000 mL/min
ASM	ASM capillary: 5500–1301 (0.12 × 170 mm) ASM enabled (ASM factor: 3) Flush sample loop 3.0 times (0.85 minutes)
Sampling Table	5.15 minutes, time-based 14.71 minutes, time-based

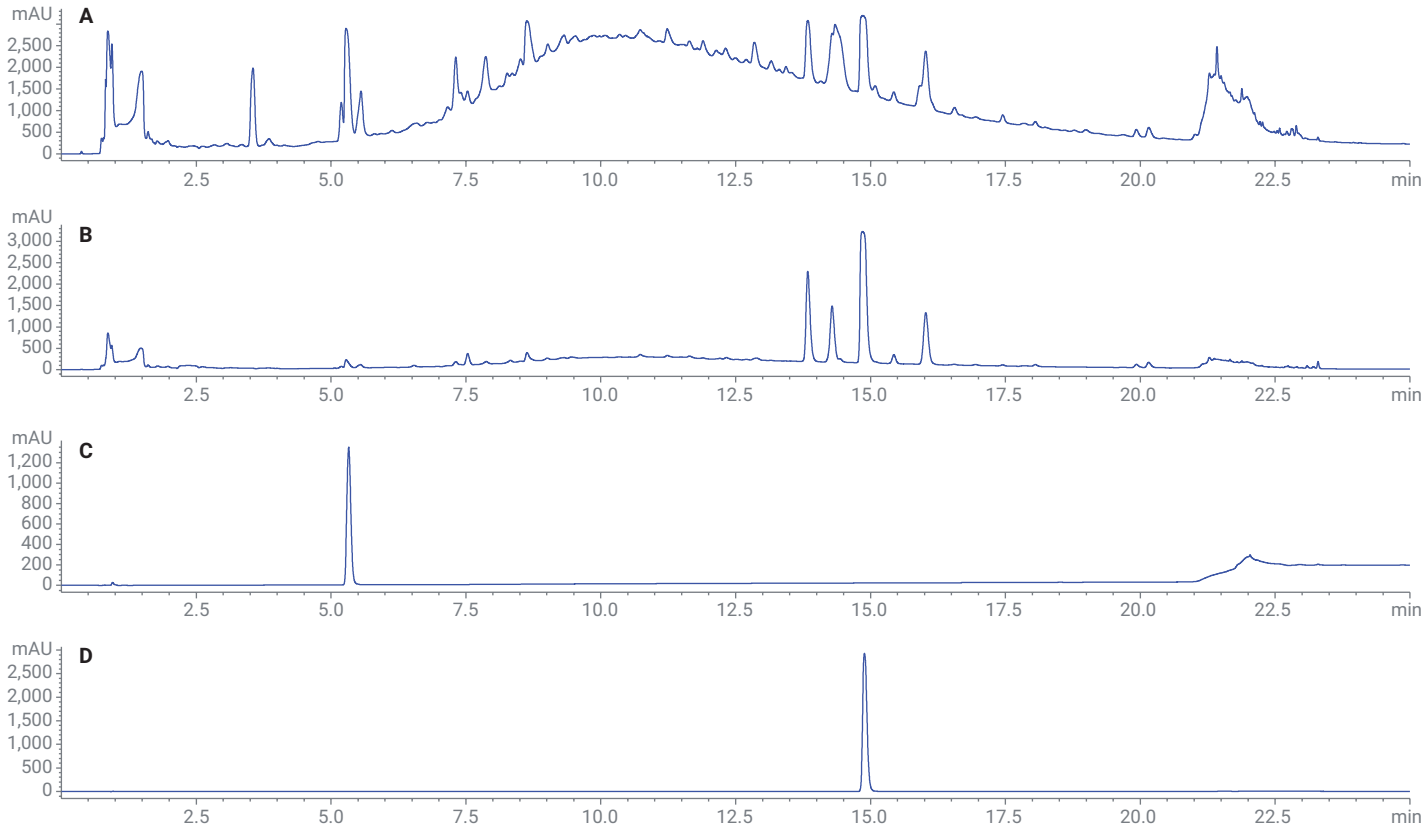


Figure 2. 1D-LC analysis of (A) *R. rosea* with UV detection at 210 nm; (B) *R. rosea* with UV detection at 254 nm; (C) Salidroside with UV detection at 210 nm; (D) Rosavin with UV detection at 254 nm.

In Figure 3, the 2D-LC analyses of *R. rosea*, salidroside, and rosavin are displayed. The described 1D-LC separation was used in the ^{1}D, and the entire ^{1}D peaks of salidroside and rosavin were transferred to the ^{2}D separation in heart-cuts 1 and 2, respectively. Matrix constituents of *R. rosea*, which were not separated from salidroside and rosavin in the ^{1}D, were resolved in the ^{2}D separation.

Isolation of salidroside and rosavin from *R. rosea* was performed using both 1D-LC and 2D-LC separation. Mass-based fraction collection was used to enable highly specific peak triggering with the masses of salidroside (300.1 Da) and rosavin (428.1 Da) set as the target masses. Peak-based fraction collection triggered by the UV and MSD signals, connected by a logical AND combination, was used. This combination triggers a fraction start when both detector signals have exceeded the user-defined threshold/slope settings. Collection continues until one of the detector signals drops below the settings.

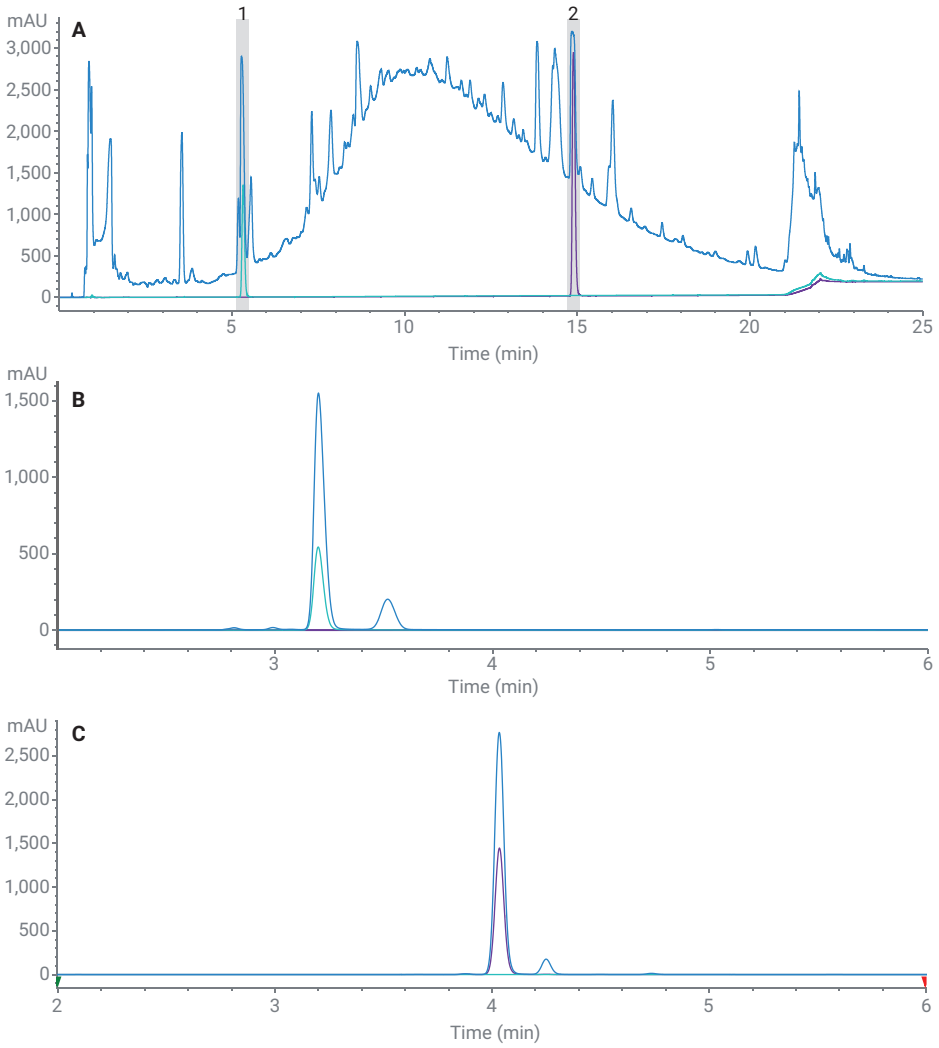


Figure 3. 2D-LC analysis of *R. rosea* (blue), salidroside (turquoise), and rosavin (purple). (A) ^{1}D chromatogram with UV detection at 210 nm; (B) ^{2}D chromatogram of cut 1 (salidroside) with UV detection at 210 nm; (C) ^{2}D chromatogram of cut 2 (rosavin) with UV detection at 254 nm.

Figure 4 shows the 1D-LC purification of salidroside and rosavin from *R. rosea*. Salidroside and rosavin are not completely separated from matrix constituents of *R. rosea* with the chosen 1D-LC method. This is reflected in the UV signal, and more than one fraction is collected during the detection of the target masses of salidroside and rosavin. In the extracted ion chromatogram (EIC) of the sodium adduct of rosavin, a second peak eluting at approximately 14 minutes is detected, which triggers fraction collection. This peak most likely originates from rosarin, an isomer of rosavin, which is also contained in *R. rosea*.

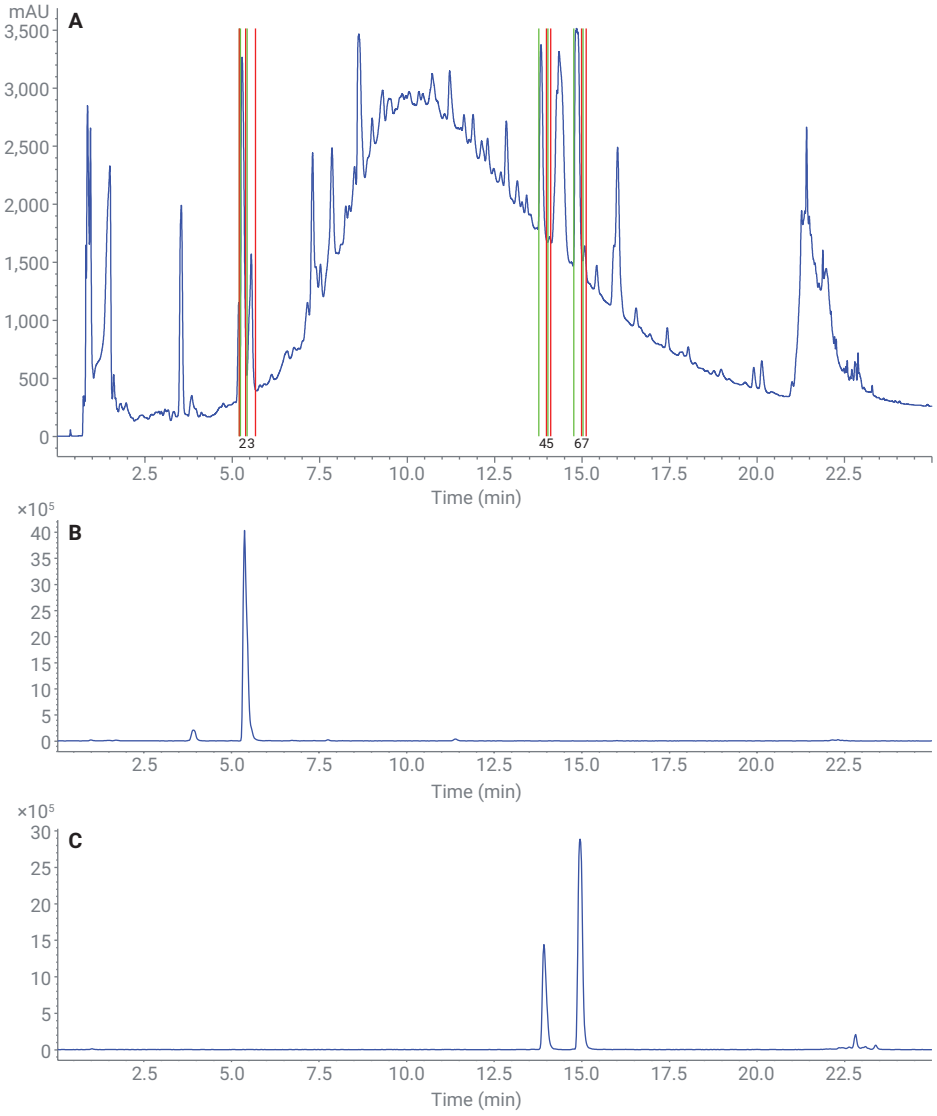


Figure 4. 1D-LC purification of salidroside and rosavin from *R. rosea*. (A) UV detection at 210 nm with fraction markers; (B) EIC of the sodium adduct of salidroside (*m/z* 323); (C) EIC of the sodium adduct of rosavin (*m/z* 451).

Figure 5 shows the 2D-LC purification of salidroside and rosavin from *R. rosea*. In the ^{2}D, salidroside and rosavin are further separated from matrix constituents of *R. rosea*. This should enable the collection of fractions with higher purity.

For determination of the purity and recovery of the isolated target compounds salidroside and rosavin, the collected fractions were vortexed and analyzed by 2D-LC. Salidroside and rosavin were quantified in the ^{2}D. The total amount of target compound injected for purification was determined from the 2D-LC analysis of the diluted *R. rosea* extract. Recovery was calculated as the percentage of the quantified amount of the target compound in the collected fraction with reference to the total amount of target compound injected for purification. The purity of the fractions was determined based on the ratio of the target compound peak area and the total peak area at 210 nm for salidroside and at 254 nm for rosavin.

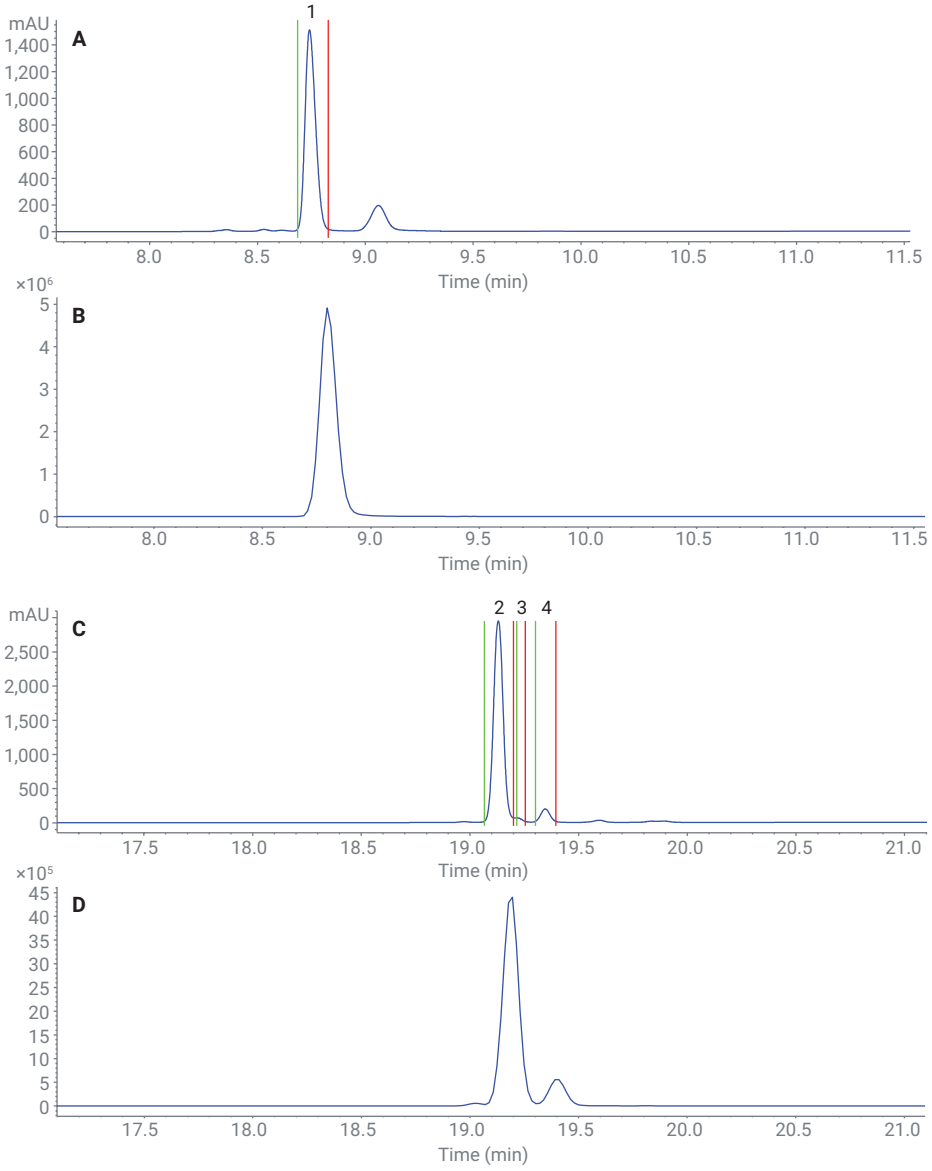


Figure 5. 2D-LC purification of salidroside and rosavin from *R. rosea*; (A) ^{2}D chromatogram of cut 1 (salidroside) with UV detection at 210 nm with fraction markers; (B) ^{2}D EIC of the sodium adduct of salidroside (*m/z* 323); (C) ^{2}D chromatogram of cut 2 (rosavin) with UV detection at 210 nm with fraction markers; (D) ^{2}D EIC of the sodium adduct of rosavin (*m/z* 451).

Figures 6 and 7 show the 2D-LC analyses of the salidroside fractions collected after 1D-LC and 2D-LC purification, respectively. The salidroside fraction purified using 1D-LC contains matrix constituents of *R. rosea*, which are detected in the ^{1}D and ^{2}D separation (Figure 6). In the salidroside fraction purified using 2D-LC, no matrix constituents of *R. rosea* are detected (Figure 7), indicating highest purity of the collected fraction.

Comparable observations were made during the 1D-LC and 2D-LC purification of rosavin (data not shown).

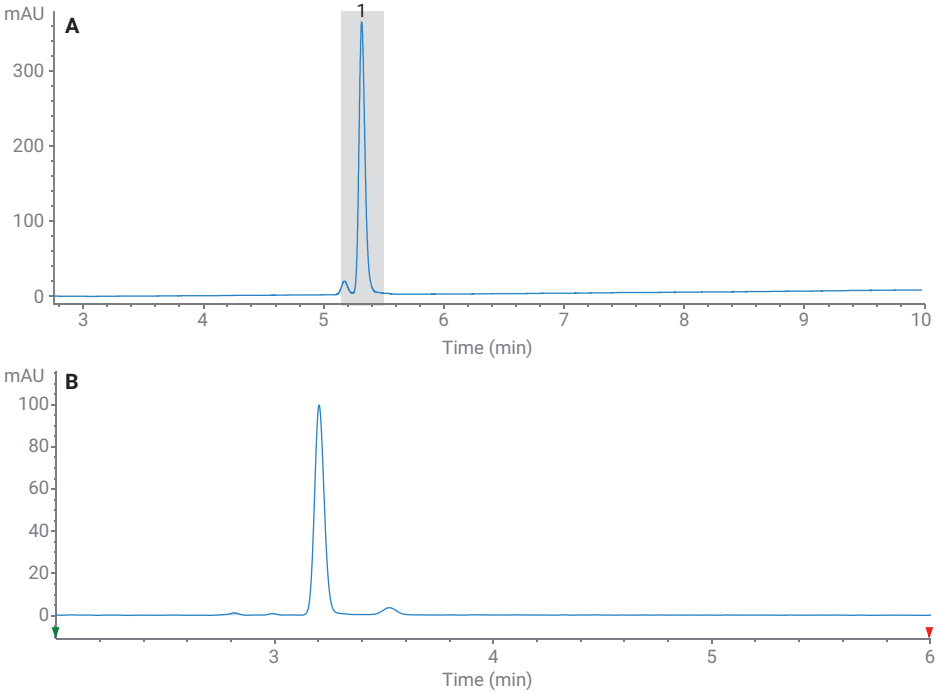


Figure 6. 2D-LC analysis of the salidroside fraction collected after 1D-LC purification (fraction 2 in Figure 4). (A) ^{1}D chromatogram with UV detection at 210 nm; (B) ^{2}D chromatogram of cut 1 (salidroside) with UV detection at 210 nm.

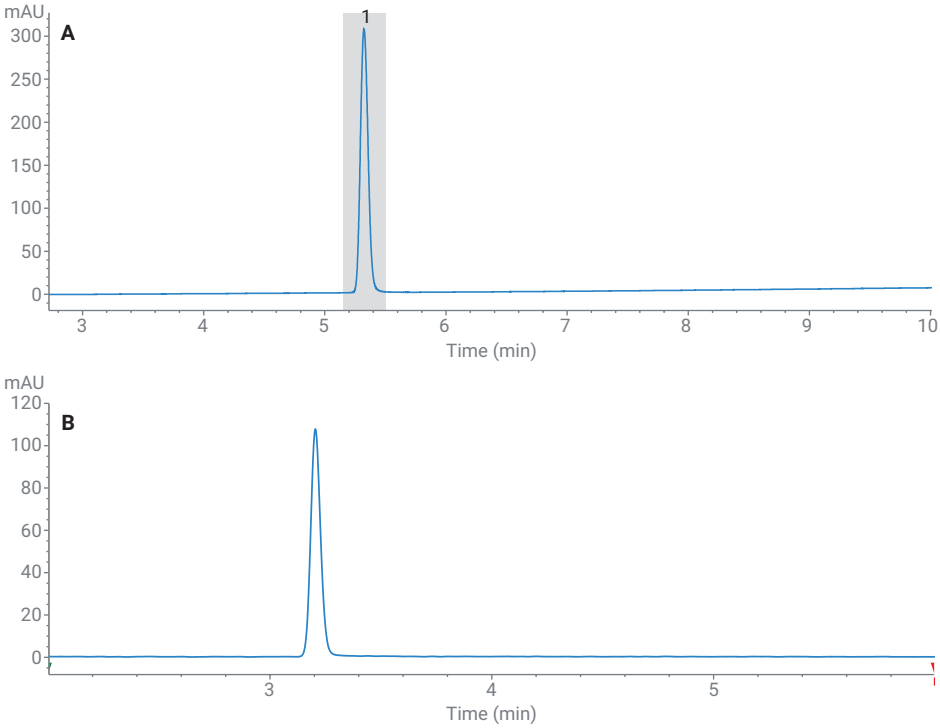


Figure 7. 2D-LC analysis of the salidroside fraction collected after 2D-LC purification (fraction 1 in Figure 5). (A) ^{1}D chromatogram with UV detection at 210 nm; (B) ^{2}D chromatogram of cut 1 (salidroside) with UV detection at 210 nm.

Table 1 compares the purity and recovery of salidroside and rosavin purified using 1D-LC and 2D-LC. For evaluation of purity, the ratio of the target compound peak area and the total peak area was calculated individually for the ^{1}D and ^{2}D chromatograms. For both target compounds, salidroside and rosavin, higher purities are obtained using 2D-LC purification. For fractions purified using 1D-LC, the purity calculated from the ^{1}D chromatogram is higher than the purity calculated from the ^{2}D chromatogram. The ^{2}D separation resolves matrix constituents from *R. rosea*, which coelute with the target compound in the ^{1}D separation. Using 2D-LC purification, higher recoveries are obtained compared to 1D-LC purification. The lower recoveries obtained after 1D-LC purification can likely be explained by the partial coelution of matrix constituents of *R. rosea* with the target compounds. The chosen collection settings use a combination of threshold and slope, which limits the collected fraction of the target compound in 1D-LC purification.

Table 1. Purity and recovery of salidroside and rosavin purified using 1D-LC and 2D-LC (N = 3); purity was determined at 210 nm for salidroside and at 254 nm for rosavin.

	1D-LC Purification		2D-LC Purification	
	Salidroside	Rosavin	Salidroside	Rosavin
Purity ^{1}D (%)	95.5	98.7	>99	>99
Purity ^{2}D (%)	93.6	92.9	>99	>99
Recovery (%)	53.5	56.6	86.4	84.0

Conclusion

The Agilent 1290 Infinity II 2D-LC System with mass-based fraction collection enables the online 2D-LC isolation of high-purity target compounds from complex natural products. For the isolation of salidroside and rosavin from *R. rosea*, highest purity was achieved using 2D-LC purification. In comparison to common offline 2D-LC purification, the online 2D-LC purification avoids manual work, saving time and reducing errors. 2D-LC purification also requires no extra time compared to 1D-LC purification, because the ^{2}D separation runs simultaneously with ^{1}D separation.

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