Determination of Taxanes in *Taxus sp.* with the Agilent 1290 Infinity 2D-LC Solution

Suitable for Agilent
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Application Note

Small Molecule Pharmaceuticals & Genetics

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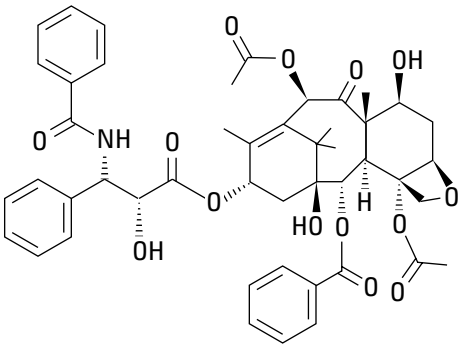
Abstract

Taxane standard solutions and *Taxus sp.* extracts were analyzed with comprehensive two-dimensional liquid chromatography (LC×LC) using the Agilent 1290 Infinity 2D-LC solution with DAD and MS detection. Reversed-phase liquid chromatography was applied in both the first and second dimension. Orthogonality was achieved by using different column chemistries and mobile phases. Qualitative and quantitative aspects of the method were investigated and tested with real samples.



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Introduction

Taxanes are a group of diterpenes useful in cancer treatments because of their mitotic inhibitory activity[1]. Taxol was first isolated from the bark of *Taxus brevifolia* (Pacific yew tree) in 1971[1].

The paclitaxel content of the bark is low; therefore, attempts have been made to synthesize the product. The synthesis proved to be possible, but difficult, and not commercially interesting[2]. At present, semisynthesis from more abundant precursors (10-Deacetylbaccatin-III, Baccatin-III, and Cephalomannine) and plant cell fermentation are the main sources of therapeutic paclitaxel[3].

The analysis of the taxane content of various species of yew trees is important for selection of the correct type and growing conditions. Taxanes are generally analyzed by (U)HPLC and LC/MS, and their separation is challenging. Analyzing these compounds in a complex matrix such as *Taxus sp.* extracts is even more difficult due to the lack of peak capacity in a one dimensional system. The matrix constituents interfere with detection and quantitation of the target compounds.

An efficient way to increase peak capacity significantly is to perform comprehensive two-dimensional liquid chromatography (LC×LC). This Application Note presents data on the analysis of taxanes with the Agilent 1290 Infinity 2D-LC solution. On-line mass spectroscopic detection was additionally employed with 2D-LC to confirmation of the identity of detected taxanes in *Taxus sp.* extracts.

Experimental

Solutions and chemicals

All solvents were HPLC gradient grade from Biosolve B.V. (Valkenswaard, the Netherlands). A standard solution of 14 taxanes consisting of paclitaxel, precursors, and analogues (20 µg/mL each) was purchased from Chromadex (Irvine, CA, USA). The composition of this mixture is shown in Table 1. This solution was further diluted in methanol with 0.1 % acetic acid to the appropriate concentration.

Software

- Agilent OpenLAB CDS Chemstation revision C.01.04 with 2D-LC add-on software
- GC Image LC/LC Edition Software for 2D-LC data analysis (GC Image, LLC., Lincoln, NE, USA)

Samples and extraction

Samples of two different yew tree species were taken and extracted according to the procedure below (based on the procedure by Wang, *et al.*[4]).

Table 1. Composition of the standard solution.

Peak	Name	Molecular weight
1	10-Deacetylbaccatin-III	544
2	Taxol side chain methyl ester	299
3	Baccatin-III	586
4	7-Xylosyl-10-deacetyltaxol B	921
5	Taxinine M	703
6	7-Xylosyl-10-deacetyltaxol	943
7	7-Xylosyl-10-deacetyltaxol C	937
8	10-Deacetyltaxol	811
9	7-Xylosyltaxol	985
10	Cephalomannine (Taxol B)	831
11	7-epi-10-Deacetyltaxol	811
12	Paclitaxel (Taxol)	853
13	Taxol C	847
14	7-Epitaxol	853

Instrumentation

An Agilent 1290 Infinity 2D-LC solution was used. The configuration is shown below:

Instrument	Part number
Agilent 1290 Infinity binary pump (1st dimension)	G4220A
Agilent 1290 Infinity binary pump (2nd dimension)	G4220A
Agilent 1290 Infinity autosampler	G4226A
Agilent 1290 Infinity autosampler thermostat	G1330A
Agilent 1290 Infinity thermostatted column compartment	G1316C
Agilent 1290 Infinity diode array detector with standard flow cell	G4212A
Agilent 1290 Infinity valve drive	G1170A
2-position/4-port duo valve for 2D-LC	G4236A
Agilent Single Quadrupole LC/MS with APCI source	G6130B

Extraction

1. 5 g chopped cuttings
2. Add 15 mL hexane, vortex, and sonicate for 10 minutes.
3. Discard hexane and repeat once.
4. Dry needles at room temperature overnight.
5. Add 35 mL methanol and mix using Ultra-Turrax, then allow to stand for at least 10 hours.
6. Collect methanol.
7. Add 25 methanol to the needles, vortex, then allow to stand for 30 minutes.
8. Collect methanol and repeat once.
9. Dry the combined methanol phases under nitrogen at 45 °C.
10. Redissolve in 10 mL methanol.

Purification of extract

1. 1 mL of extract + 5 mL water
2. Solid-phase cartridge: AccuBond II ODS-C18, 500 mg, 6 cc
   - Condition cartridge.
     - Methanol, 5 mL
     - Water, 2.5 mL
   - Load diluted extract.
   - Wash cartridge.
     - Water, 5 mL
     - Water/methanol 75/25, 5 mL
   - Dry cartridge for 5 minutes.
   - Elute cartridge.
     - Methanol, 5 mL
     - Methanol/ethylacetate 50/50, 5 mL
3. Combine elution phases.
4. Take 4 mL aliquot and dry under nitrogen at 45 °C.
5. Redissolve in 4 mL methanol/ acetonitrile and filter.

Method

1st dimension		
Column	Agilent ZORBAX Eclipse Plus C18, 2.1 × 100 mm, 3.5 µm (p/n 959793-902)	
Solvent A	Water	
Solvent B	Methanol	
Flow rate	60 µL/min	
Gradient	30 % B at 0 minutes 55 % B at 1.5 minutes 85 % B at 36 minutes 100 % B at 37 minutes 100 % B at 45 minutes	
Post time	5 minutes at 30 % B	
Column temperature	30 °C	
2nd dimension		
Column	Agilent ZORBAX Plus Phenyl-Hexyl RRHT, 4.6 × 50 mm, 1.8 µm (p/n 959941-912)	
Solvent A	Water with 0.008 % formic acid	
Solvent B	Acetonitrile with 0.004 % formic acid	
Flow rate	4 mL/min	
Idle flow rate	0.4 mL/min	
Initial gradient	20 % B at 0 minutes 33 % B at 0.3 minutes 20 % B at 0.31 minutes	
Gradient modulation	20 % B at 0 minutes to 55 % B at 37 minutes to 85 % B at 42 minutes 33 % B at 0.3 minutes to 85 % B at 37 minutes	
Column temperature	40 °C	
Modulation		
Modulation on	9 to 37 minutes	
Loops	Two 40 µL loops, co-current configuration	
Modulation time	0.40 minutes	
Loop filling	60 %	
Injection		
Volume	5 µL (injection program, mixed with 10 µL water plug)	
Sample temperature	12 °C	
Needle wash	5 seconds flush port (methanol)	
Detection DAD		
Wavelength	Signal 228/8 nm, Reference 370/60 nm	
Data rate	80 Hz	
Detection MSD		
Ionization mode	APCI	
Source settings	Drying gas flow Drying gas temperature Nebulizer pressure Vaporizer temperature Capillary voltage Corona current	5 L/min 320 °C 50 psi 380 °C 3,000 V (pos and neg mode) 4 µA (pos mode) and 15 µA (neg mode)
Detection mode	FastScan Scan 250–1,000 *m/z* Fragmentor 120 V	

The effluent from the second-dimension column was split by a T-piece. The DAD was connected to the T-piece by a 70 mm × 0.12 mm id stainless steel capillary, and the inlet of the APCI source was connected to the other outlet of the T-piece by a 340 mm × 75 µm id stainless steel capillary. The 75 µm capillary acts as a restriction and routes the largest part of the flow towards the DAD.

The Agilent OpenLAB CDS Chemstation with 2D-LC add-on software allows complete control of the multidimensional method settings in one panel. Figure 1 shows the setup of this control panel. The system allows the use of idle flow settings to save solvents whenever multidimensional separation is not performed. In this method, LC×LC was only activated from 9 to 37 minutes, that is, 28 minutes out of 45 minutes analysis time (50 minutes with re-equilibration time included). Before and after this window, and between injections, an idle flow of 0.4 mL/min was used, resulting in a reduction of solvent usage in the second dimension of 40 % (120 mL instead of 200 mL/analysis).

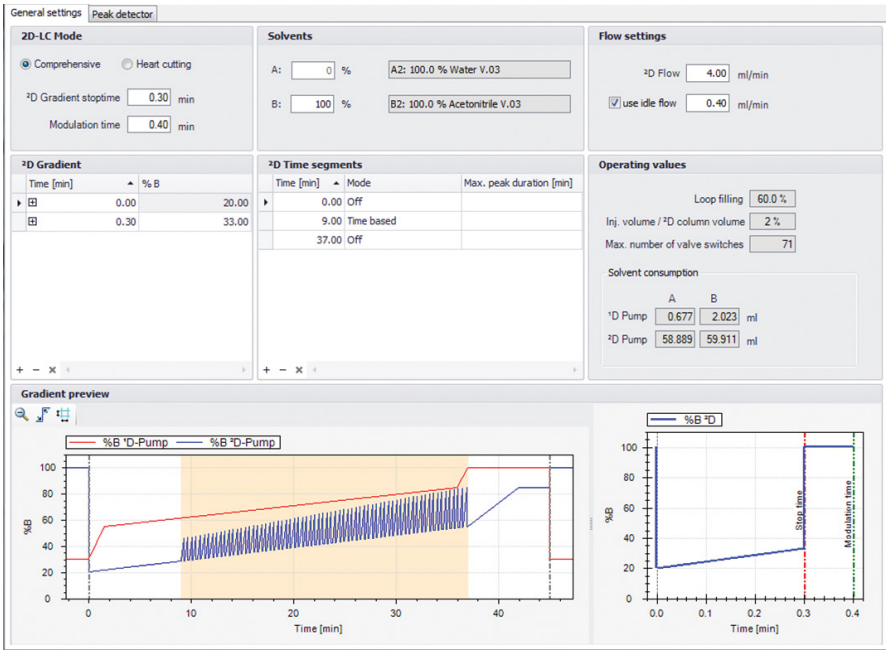

Figure 1. Screenshot of the 2D-LC method (OpenLAB CDS Chemstation).

Results and Discussion

A commercially available set of 14 taxanes consisting of paclitaxel, precursors, and analogues was used as a test sample. Paclitaxel, its precursors, and its analogues are very similar in structure but can be separated under optimized conditions. A previous Application Note describes the complete separation of these compounds using an Agilent Zorbax Eclipse Plus Phenyl-Hexyl column and a water/acetonitrile mobile phase[4]. The analysis becomes more challenging when these compounds have to be determined in real samples, for example *Taxus sp.* extracts. Figure 2 illustrates the problems associated with interfering matrix constituents when extracts are analyzed with one-dimensional LC and DAD. The separation shown was carried out on a ZORBAX Eclipse Plus C18 column (first dimension column), and the separation for the standard mix was not complete. This would have been the case if a Phenyl-Hexyl stationary had been used. The peak capacity (calculated by dividing the gradient time with the average peak width) in this separation was approximately 70. It is clear, however, that the analysis of extracts is significantly more complex and identification and quantitation of the standards in real samples is difficult.

One way to overcome this problem would be to perform a high resolution method with a longer column or smaller particles. This would increase the resolution based on efficiency and consequently the separation probability. For example, using a 2.1 × 150 mm column packed with 1.8 µm particles should give a peak capacity of approximately 120. The gain in resolution and peak capacity is not high enough to ensure complete separation of the taxanes from each other and from matrix constituents in every sample that is analyzed. Based on theory, the probability to separate, for example, 150 compounds with this high-resolution configuration would be less than 10 %. To obtain a significant increase in peak capacity, and consequently separation probability, comprehensive two-dimensional liquid chromatography (LC×LC) can be applied. Figure 3 shows the analysis of the same standard mixture with the Agilent 1290 Infinity 2D-LC solution using a Phenyl-Hexyl column in the second dimension. All compounds were separated and satisfactory orthogonality was achieved with the applied combination of stationary phase chemistries and mobile phases.

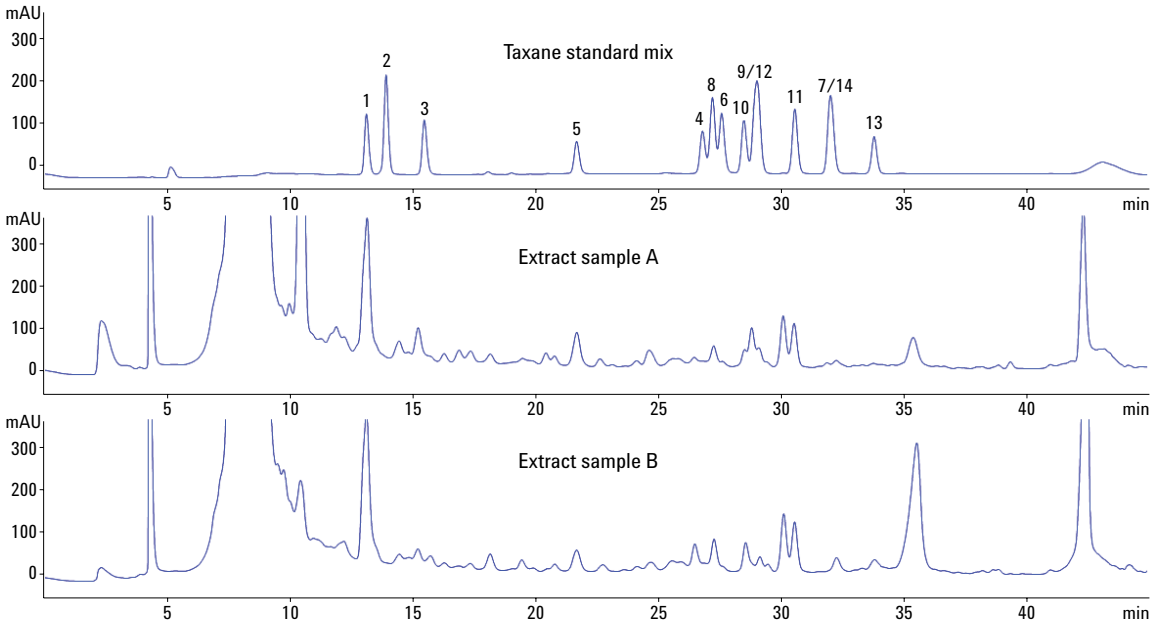

Figure 2. Comparison of a one-dimensional analysis of the taxane standard mixture (10 µg/mL each) and *Taxus sp.* extracts. Conditions: see 1st dimension in method section. Detection: DAD, 228 nm, the detector was installed after the 1st dimension column. Peak identification: see Table 1.

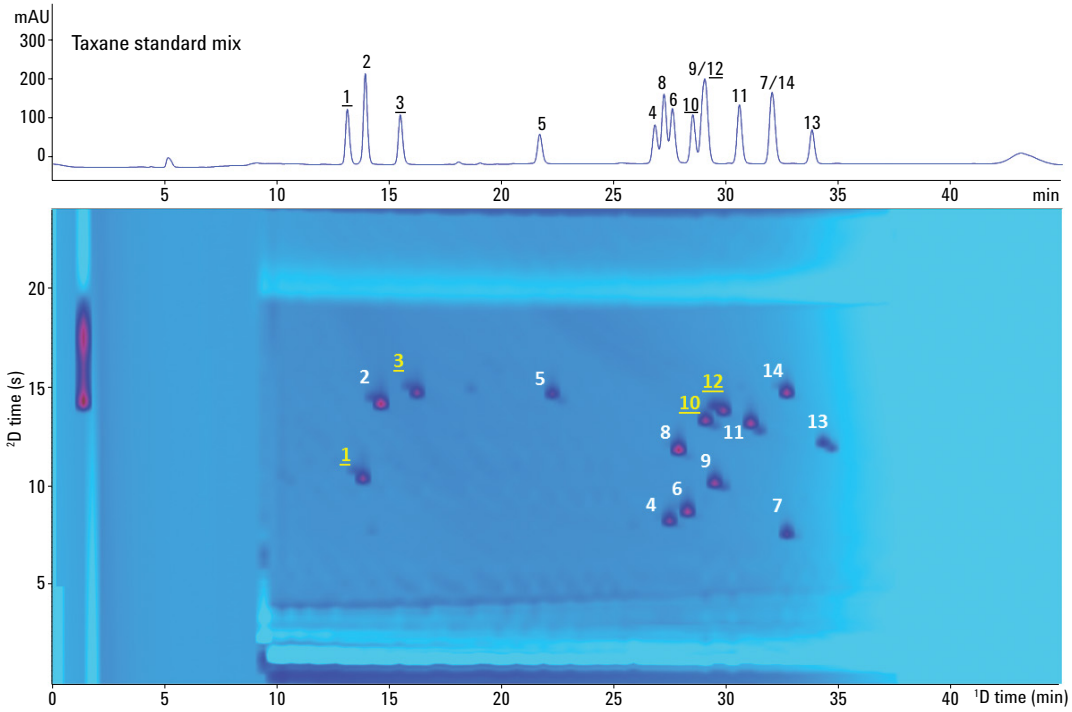

Figure 3. Comparison of a one-dimensional (LC) and comprehensive two-dimensional (LC/LC) analysis of the taxane standard mixture (10 µg/mL each). Detection: DAD, 228 nm.

The precision of the method was determined by six consecutive injections of the 10 µg/mL standard mixture and a calibration was performed by single injections of standard solutions containing 0.4 to 30 µg/mL of the taxanes. The data are summarized in Table 2.

The method was applied to the analysis of extracts of two *Taxus sp*. samples. The composition of both samples was different, but in both samples a number of taxanes were detected. The most interesting taxanes, 10-Deacetylbaccatin-III (1), Cephalomannine (10), and Paclitaxel (12) were detected, whereas Baccatin-III (3) was absent in both samples.
A comparison of the standard solution and the samples is shown in Figure 4. The calibration data was applied on both samples to calculate the taxane content in the extracts. Additionally, both samples spiked with 4 µg/mL taxane standards were analyzed to calculate the recovery. Acceptable recovery was obtained for both samples with values approximating 100 % for most compounds. The quantitative data are shown in Table 3.

Table 2. Precision and calibration data of the method.

Peak	Name	R^2	Peak volume precision (RSD %)
1	10-Deacetylbaccatin-III	0.99999	0.35
2	Taxol side chain methyl ester	0.99997	0.34
3	Baccatin-III	0.99995	0.41
4	7-Xylosyl-10-deacetyltaxol B	0.99992	0.49
5	Taxinine M	0.99996	0.29
6	7-Xylosyl-10-deacetyltaxol	0.99997	0.20
7	7-Xylosyl-10-deacetyltaxol C	0.99999	0.33
8	10-Deacetyltaxol	1.00000	0.15
9	7-Xylosyltaxol	0.99999	0.17
10	Cephalomannine (Taxol B)	0.99994	0.80
11	7-epi-10-Deacetyltaxol	0.99997	0.34
12	Paclitaxel (Taxol)	0.99995	0.54
13	Taxol C	0.99999	0.47
14	7-Epitaxol	0.99998	0.32

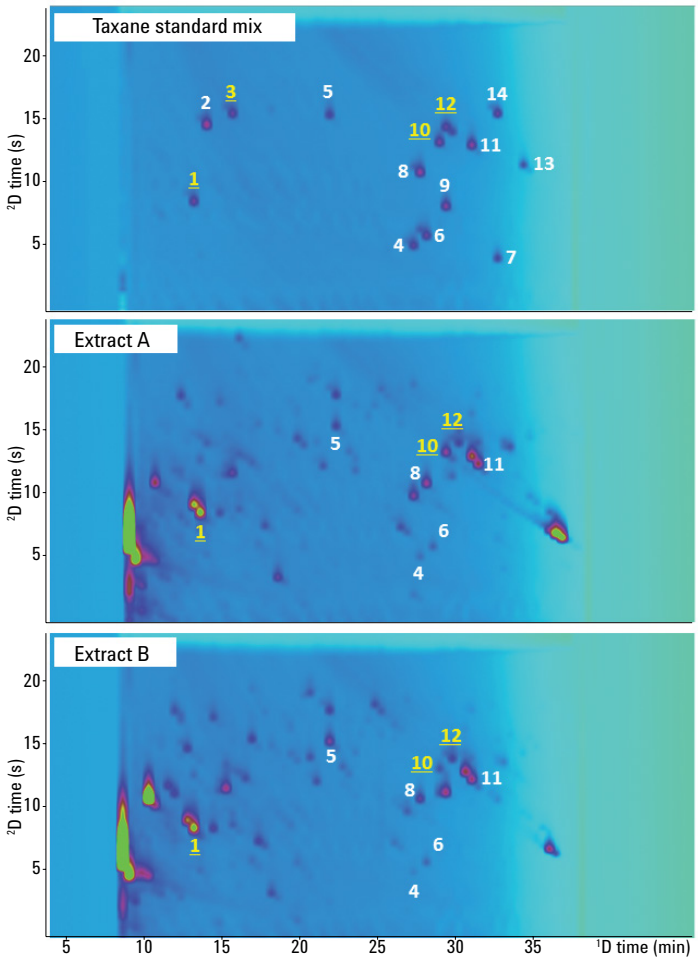

Figure 4. Comparison of comprehensive two-dimensional (LC×LC) analysis of the taxane standard mixture (4 µg/mL each) and extracts. Detection: DAD, 228 nm. Peak identification: see Table 1. (Paclitaxel and the most important precursors have bold/underlined labels).

Table 3. Quantitative data.

		Concentration (µg/mL)		Recovery (%)	
Peak	Name	Extract 1	Extract 2	Extract 1	Extract 2
1	10-Deacetylbaccatin-III	42.492	31.293	102.3	118.8
2	Taxol side chain methyl ester	0.000	0.000	76.2	72.9
3	Baccatin-III	0.000	0.000	73.9	78.7
4	7-Xylosyl-10-deacetyltaxol B	0.662	0.192	96.6	104.1
5	Taxinine M	3.653	5.178	99.3	121.4
6	7-Xylosyl-10-deacetyltaxol	0.777	0.543	95.4	102.4
7	7-Xylosyl-10-deacetyltaxol C	0.000	0.000	96.4	102.4
8	10-Deacetyltaxol	4.208	2.040	99.0	108.2
9	7-Xylosyltaxol	0.000	0.000	96.8	102.9
10	Cephalomannine (Taxol B)	5.839	2.079	99.6	102.9
11	7-epi-10-Deacetyltaxol	17.004	13.062	92.4	98.9
12	Paclitaxel (Taxol)	3.488	3.740	95.5	86.5
13	Taxol C	0.000	0.000	95.3	101.3
14	7-Epitaxol	0.000	0.000	96.5	102.0

The identity of these compounds was confirmed by spiking experiments, UV-spectra, and mass spectra. For example, the mass spectra for Peak 10 (Cephalomannine, Taxol B) taken from the standard solution and from Sample 1 are shown in Figure 5. The similarity between the spectra is excellent in both polarities. In positive mode, the molecular ion (832 *m/z*) and multiple fragments can be detected in both solutions. In negative mode a formate adduct (876 *m/z*) is the dominating ion.

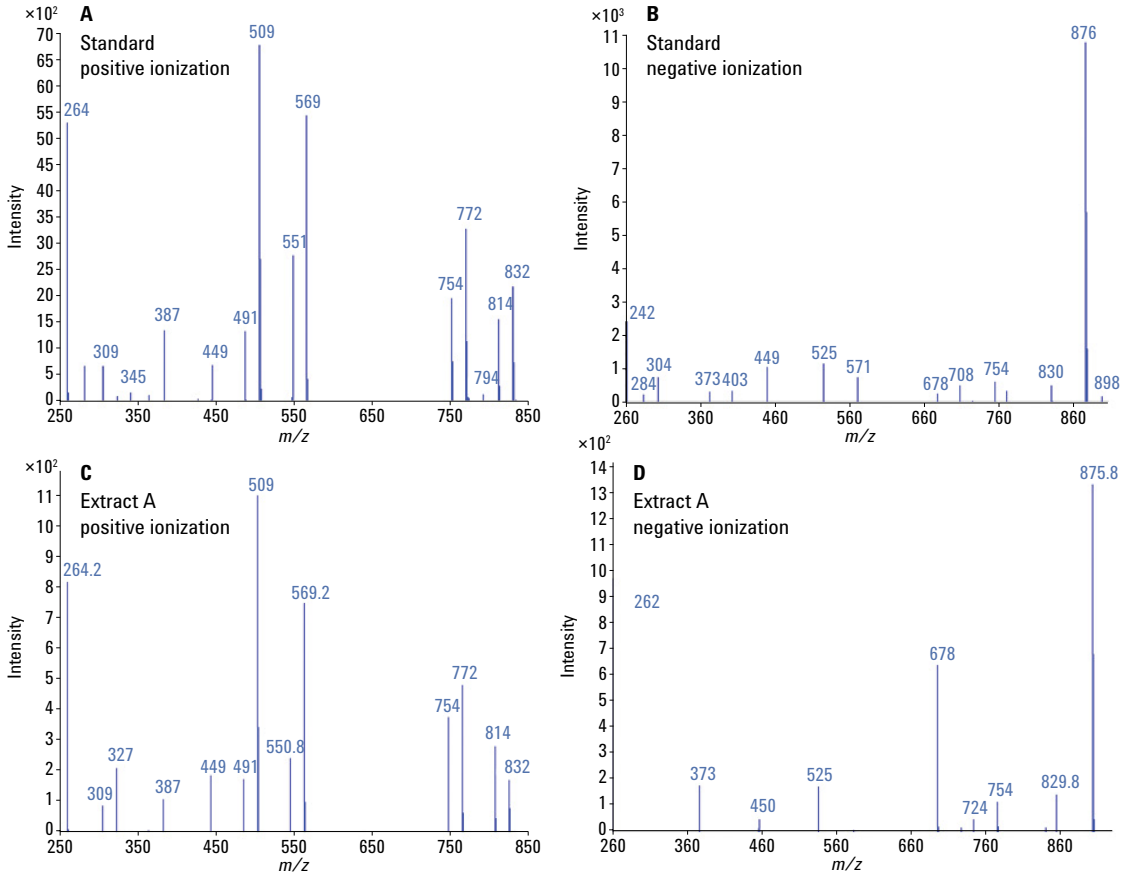

Figure 5. Mass spectra for Cephalomaninne taken from the 2D-LC plots of a standard solution and sample.

Figures 6 and 7 show a comparison of the 2D LC plot (DAD 228 and MS negative TIC and EIC 589, 631, 876, 898 *m/z*) for a standard solution and a sample, respectively. Although some peak broadening is observed in the second dimension for MS detection compared to DAD, the results are satisfactory and definitely useful for identification of the detected spots. The Agilent Series 6100 Single Quadrupole MSD used in this study can be used as a confirmation tool. High resolution MS systems such as Agilent Series 6200 TOF or Agilent Series 6500 Q-TOF would provide additional possibilities for identification of unknowns with this setup in *Taxus sp.* extracts and, by extension, other plant extracts.

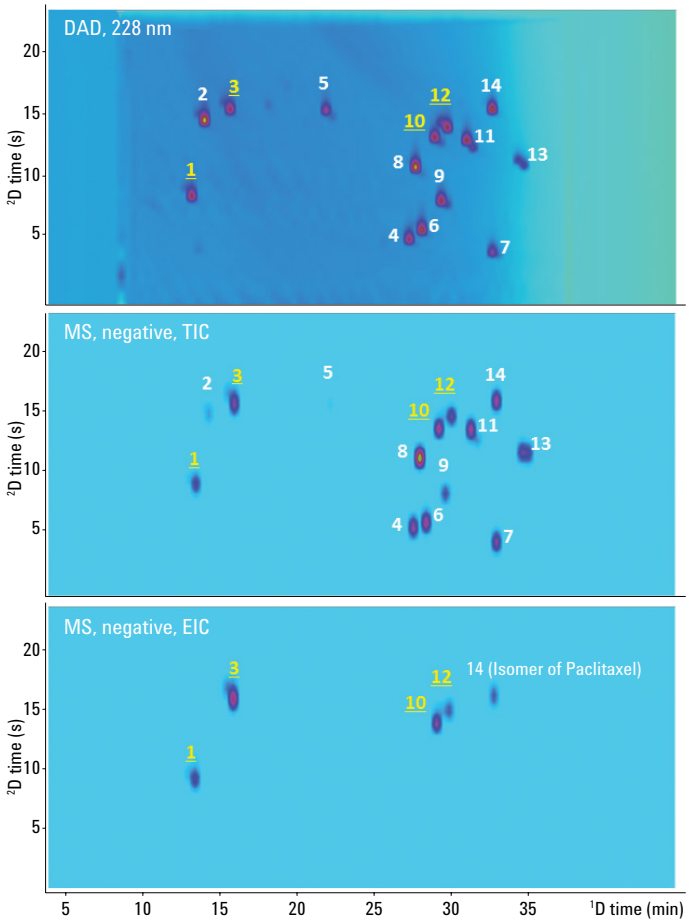

Figure 6. Overlay of DAD 228, MS negative TIC, and MS negative EIC (589, 631, 876, and 898 *m/z*) for a standard solution.

Conclusion

The combination of the Agilent 1290 Infinity 2D-LC solution with DAD and MS detection is a useful tool for screening of yew tree extracts for taxane content. A comprehensive two-dimensional liquid chromatography (LC×LC) method was developed using two reversed-phase columns with different stationary phase chemistries and different mobile phase composition. Adequate orthogonality was obtained using this approach. Method performance parameters such as linearity and repeatability of detection were investigated using a taxane standard mixture. The result was satisfactory, and the final method was applied to yew tree extracts. DAD could be used as a detector for general screening purposes. A successful hyphenation of the separation with an Agilent Series 6100 Single Quadrupole MSD was established that is useful for identity confirmation of the detected taxanes.

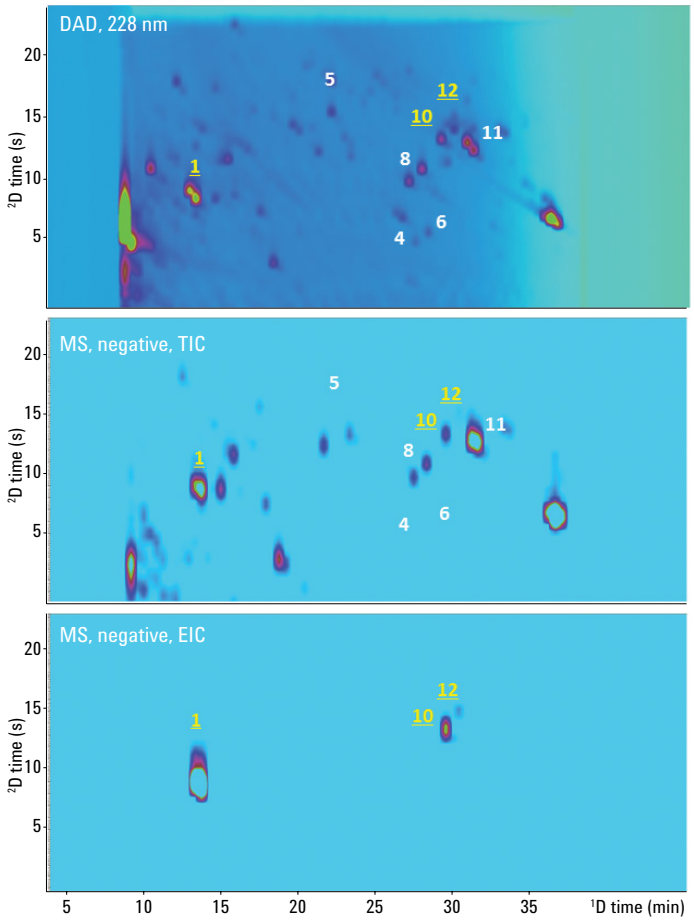

Figure 7. Overlay of DAD 228, MS negative TIC, and MS negative EIC (589, 631, 876, and 898 *m/z*) for Extract A.

References

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