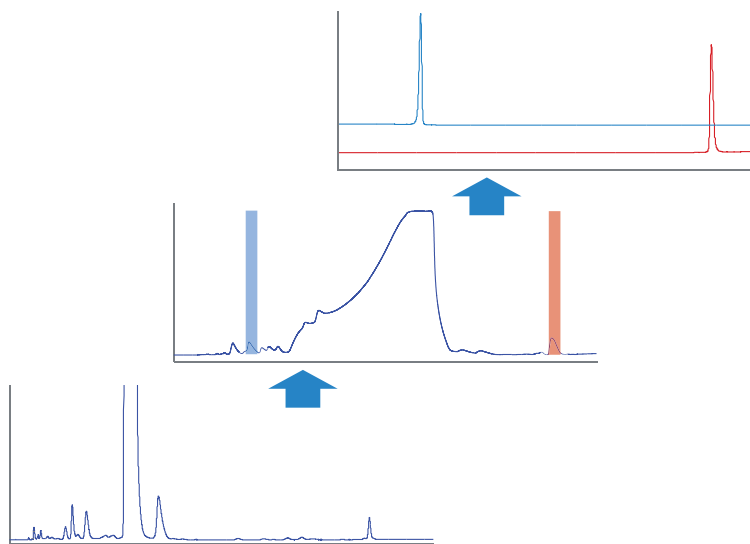


Mass-Based Purification of Natural Product Impurities Using an Agilent 1260 Infinity II Preparative LC/MSD System



Authors

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Abstract

Organic impurities can be more than a minor inconvenience in the making of high-quality products such as food additives or dietary supplements. Preparative-scale high performance liquid chromatography (HPLC) is frequently applied to separate and isolate unknown by-products for further characterization. This Application Note demonstrates the workflow from analytical scouting through scale-up to preparative conditions and purification of a natural product extract by mass-based fraction collection.

Introduction

Monitoring organic impurities is a critical issue in the pharmaceutical industry, as the presence of unwanted chemicals may, even in small amounts, influence the efficacy and safety of pharmaceutical products. According to regulations, for example the ICH Guidance for Industry Q3A, impurities need to be identified and characterized when exceeding a certain threshold¹. However, it is not only the pharmaceutical industry that needs to be aware of organic impurities. To deliver safe and high-quality products, dietary supplements and foodstuffs based on extracts of natural products need to be monitored in a similar manner.

The isolation of unknown impurities is crucial for identification and thorough characterization concerning biological activity and toxicity of a compound. Separation and isolation of impurities can be carried out with preparative-scale HPLC, coupled to an ultraviolet (UV) detector and a mass spectrometer (MS).

This Application Note describes the isolation of impurities of a natural product extract using an Agilent 1260 Infinity II Preparative LC/MSD System. The extract contains a primary compound of interest associated with various known impurities. Routine quality control analyses, however, revealed two unknown impurities that needed characterizing. With an estimated concentration of less than 0.5 % of that of the main compound, multiple runs with large injection volumes were necessary to collect sufficient amounts of the impurities for further characterization. A mass selective detector (MSD) was used for highly specific detection and fraction triggering of the unknown compounds.

Experimental

Instrumentation

The Agilent 1260 Infinity II LC/MSD System used for analytical scouting and fraction reanalyses consisted of the following modules:

- Agilent 1260 Infinity II Binary Pump (G7112B)
- Agilent 1260 Infinity II Vialsampler (G7129A)
- Agilent 1260 Infinity II Diode Array Detector WR (G7115A)
- Agilent LC/MSD XT (G6135B)

Preparative purification was carried out on an Agilent 1260 Infinity II Preparative LC/MSD System comprising the following modules:

- Agilent 1260 Infinity II Preparative Binary Pump (G7161A)
- Agilent 1260 Infinity II Preparative Autosampler (G7157A)
- Agilent 1260 Infinity II Column Organizer (G9328A)
- Agilent 1260 Infinity II Variable Wavelength Detector (G7114A), equipped with a 3-mm preparative flow cell (G1314-60024)
- Agilent 1260 Infinity II Isocratic Pump (G7110B)
- Agilent 1290 Infinity II MS Flow Modulator (G7170B)
- Agilent 1260 Infinity II Delay Coil Organizer (G9324A)
- Agilent 1290 Infinity II Preparative Open-Bed Fraction Collector (G7159B)
- LC/MSD XT (G6135B)

Columns

- **Analytical column:**
Agilent Prep-C18 Scalar
4.6 × 100 mm, 5 μm
(p/n 449905-902)
- **Preparative column:**
Agilent Prep-C18
21.2 × 100 mm, 5 μm
(p/n 449905-702)

Software

Agilent OpenLab CDS ChemStation edition for LC and LC/MS Systems, version C.01.08 [210]

Solvents

All solvents used were LC grade. Fresh ultrapure water was obtained from a Milli-Q Integral system equipped with a 0.22-μm membrane point-of-use cartridge (Millipak).

Fraction collection

Peak detection and fraction collection were performed using the UV and MSD signals with the AND trigger combination. Using the 1290 Infinity II Preparative Open-Bed Fraction Collector enabled selection of fraction tubes that suited the flow rate and expected fraction volumes of the method. The connection of the preparative solvent flow with the MSD was implemented with the 1290 Infinity II MS Flow Modulator. This module of the InfinityLab LC family integrates seamlessly into the Agilent InfinityLab LC Purification Solutions, both from a software and hardware point of view. Split ratio and dilution, as well as start and stop times, are set and saved directly within the LC method. Depending on the make up and main solvent flow rate, split ratios can be set between 100:1 and 500,000:1 in 14 separate modes. To avoid overloading of the MSD, the 1290 Infinity II MS Flow Modulator timetable was programmed to stop splitting during the solvent front and around the elution point of the primary ingredient (see Table 1: Split ratio to MSD).

Method settings

Table 1. Chromatographic conditions of analytical and preparative runs.

Parameter	Analytical runs	Preparative runs
Mobile phase	A) 0.1 % formic acid in water B) 0.1 % formic acid in methanol	
Flow rate	1 mL/min	20 mL/min
Gradient	0 minutes – 30 %B 15 minutes – 45 %B 20 minutes – 80 %B	
Stop time	20 minutes	
Post time	5 minutes	
Injection volume	20 µL	900 µL
Temperature	Ambient	Ambient
UV detection	297 nm Peak width >0.025 minutes (0.5 seconds response time) 20 Hz data rate	
MS detection	Signal 1: positive scan <i>m/z</i> 500 to 1,000 Signal 2: positive SIM <i>m/z</i> 823.4 Signal 3: positive SIM <i>m/z</i> 839.4	
Split ratio to MSD	Full flow	1,000:1 (mode M1) 3.00 minutes ON 5.00 minutes OFF 15.0 minutes ON 18.6 minutes OFF
Fraction collection	Not applicable	Peak-based: 3.00 to 4.20 minutes 16.50 to 18.00 minutes

Table 2. MSD spray chamber and fraction collection settings.

Parameter	Value
Make up solvent	0.1 % Formic acid in methanol:water (70:30)
Make up flow	1.5 mL/min
Ionization source	Agilent Electrospray
Nebulizer pressure	35 psig
Drying gas temperature	350 °C
Drying gas flow	12.0 L/min
Capillary voltage	+3,000 V
Target mass	822.4; 838.4
Ion species	[M+H] ⁺

Results and Discussion

Analytical scouting

The concentrated extract was diluted tenfold with water, and separated on an analytical LC/MSD system. Under these conditions, the main compound was still overloading the column, whereas all impurities were sufficiently resolved (Figure 1). Using the positive scan signal of the MSD, target ions of m/z 823.4 and m/z 839.4 were identified for unknown impurities 1 and 2, respectively. These two target ions were then constantly monitored as signals 2 and 3 of the MSD, which was set to selected ion monitoring (SIM) mode.

Preparative run and fraction collection

Based on the analytical results, the separation of the concentrated sample was scaled up to preparative conditions using the same column type as the analytical run, but with a larger inside diameter. This linear scale-up allowed a reproduction of all relevant retention times within a margin of 0.2 minutes. Injecting 900 μL of the concentrated extract per run, the column was overloaded with the main compound; both targeted impurities, however, were well resolved, enabling peak-based fraction collection (Figure 2). With a mass-based trigger using the SIM signals on the two target ions, connected with the UV signal by the AND trigger combination, fraction collection could be triggered with high specificity and sensitivity. Fraction review is carried out in the Purify view in ChemStation Data Analysis, which has been improved in revision C.01.08. Collected fractions are now displayed in a comprehensive view, connecting the tick marks in the chromatogram with a graphical representation of the fraction collector bed, and *vice versa*, by a single mouse click (Figure 3).

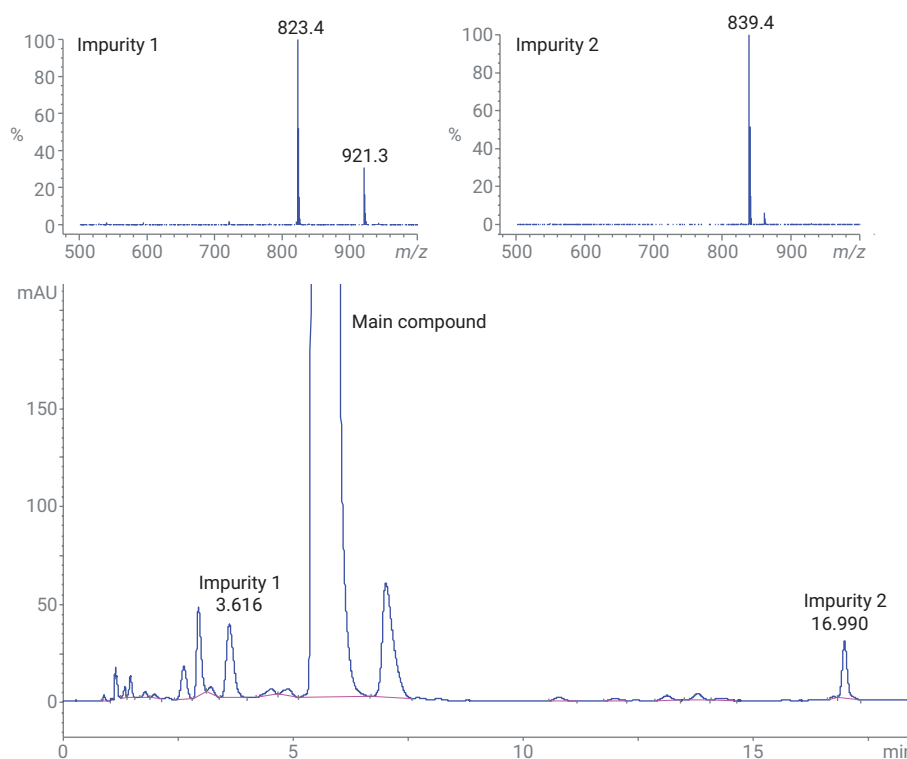


Figure 1. Diluted sample separated on an analytical system. Target ions for both impurities were extracted from the positive scan signal of the MSD.

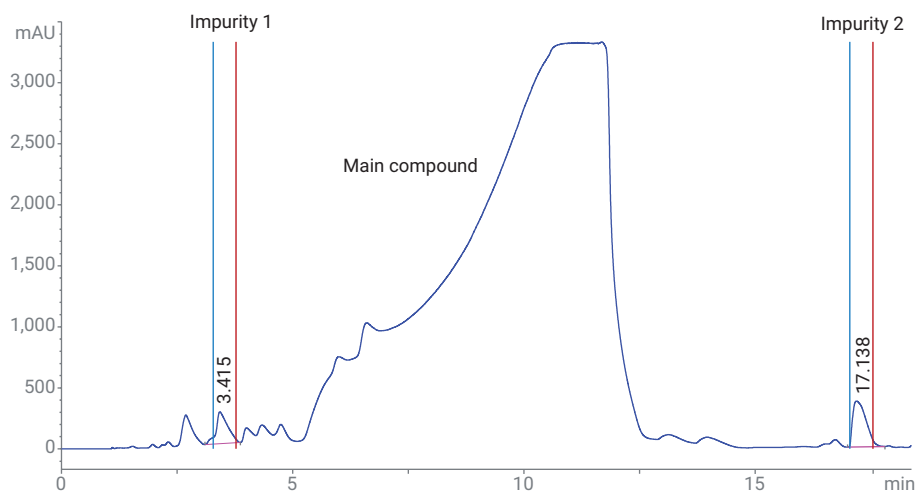


Figure 2. Preparative purification run with 900 μL injection. Both impurities were separated and collected.

After successful collection, fractions were reanalyzed on the analytical system. The typical purity of the collected fractions was found to be greater than 98 %.

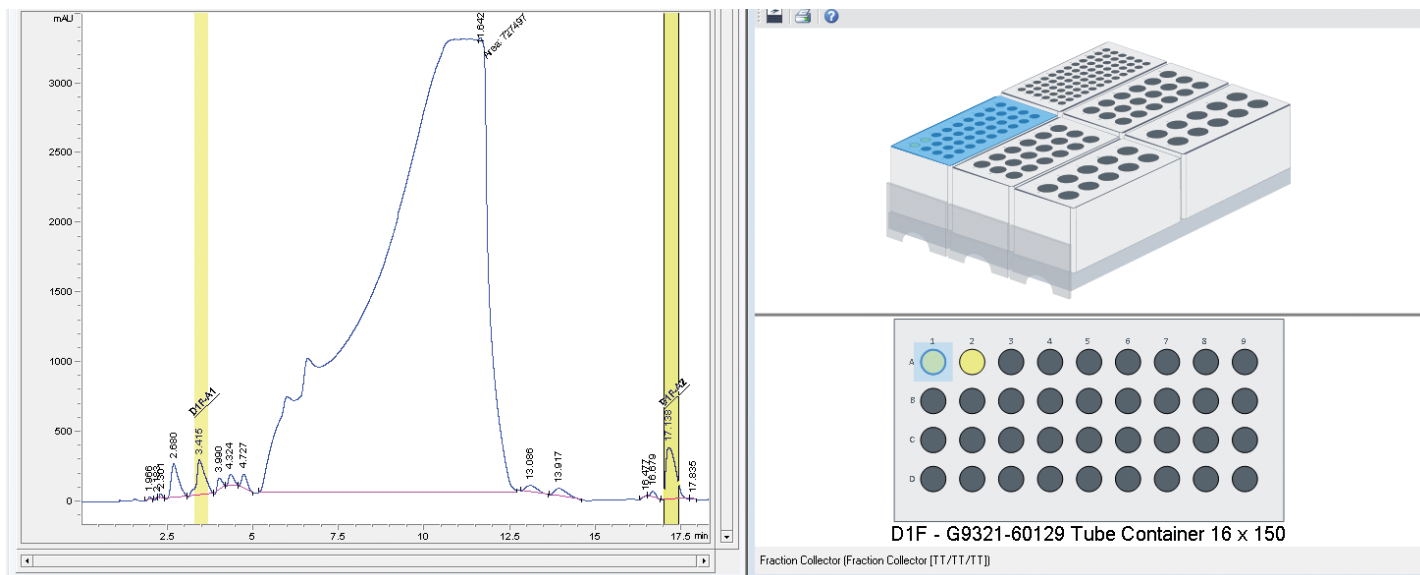


Figure 3. Purify view in Agilent OpenLab CDS ChemStation edition C.01.08. Click a collected peak in the chromatogram to display its position in the fraction collector, and vice versa.

Conclusion

This Application Note demonstrates a workflow from analytical scouting to preparative purification using a 1260 Infinity II LC/MSD System and a 1260 Infinity II Preparative LC/MSD System. A natural product extract with two unknown impurities was analyzed on an analytical system first. Highly loadable Agilent Prep-C18 stationary phases available in matching dimensions facilitated linear scale-up to preparative conditions with reproducible separation of the target compounds. Fraction triggering by UV and MSD enabled highly specific collection and yielded pure fractions suitable for further analyses to characterize the two unknown impurities of the extract.

Reference

1. ICH Guideline Q3A(R2): *Impurities in New Drug Substances*, **2006**.

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