Technical Overview

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Injection of Highly Concentrated Samples Using an Agilent 1290 Infinity II Preparative LC System with Independent Flow Channels

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Abstract

Solubility is often the limiting factor for sample concentration in reversed-phase preparative chromatography. Highly concentrated samples tend to precipitate in aqueous mobile phases. An approach to overcome this obstacle is to separate the aqueous and organic mobile phase flowpaths and connect the autosampler to the organic flowpath. This Technical Overview demonstrates the principle of organic phase injection using an Agilent 1290 Infinity II Preparative LC System equipped with an Agilent 1290 Infinity II Preparative Binary Pump with independent flow channels.

Introduction

Increasing sample throughput is one way to optimize the productivity on a high-performance liquid chromatography (HPLC) purification system. To purify more sample per run, the preparative column can be overloaded either by volume or concentration. Both techniques are successfully applied in practice, but can also have specific disadvantages. Volume overloading can lead to dispersion and unfavorable peak shapes, whereas concentration overloading is often limited by the solubility of the sample. To maintain a high sample concentration and keep the injection volume necessary for complete purification low, preparative samples are often dissolved in strong solvents such as dimethyl sulfoxide (DMSO). Under typical reversed-phase HPLC starting conditions, for example, 90% aqueous mobile phase, highly concentrated samples tend to precipitate when they first come in contact with the mobile phase. The location of precipitation is usually in the injection valve of the autosampler, where the sample encounters the mobile phase. This precipitation causes a massive pressure increase, and can clog the valve, leading to severe damage. Expensive and time-consuming maintenance is the consequence, which in turn causes unproductive downtime of the system.

One approach to overcome the obstacle of sample solubility is to modify the flowpath between the pump and the column. By separating the aqueous and organic mobile phase flowpaths and connecting the autosampler to the organic path, the sample only comes in contact with the strong organic mobile phase.[1–3] The mixing point with the aqueous mobile phase is shifted to a T-piece, located directly upstream of the column. The dwell time is kept as low as possible so that the sample is likely to reach the column without precipitating.

In addition, the T-piece is less prone to clogging, in case part of the sample precipitates. A beneficial side-effect of this setup is that the concentrated sample is gradually diluted with aqueous mobile phase at the column head, permitting higher sample loads compared to a standard setup.[3]

This Technical Overview demonstrates how to replumb and configure a 1290 Infinity II Preparative LC System to enable sample injection into the organic flowpath.

Experimental

Instrumentation

The Agilent 1290 Infinity II Preparative LC System used consisted of the following modules:

- 1290 Infinity II Preparative Binary Pump (G7161B)
- 1260 Infinity II Preparative Autosampler (G7157A) with multidraw loop kit (option 020)
- 1260 Infinity II Column Organizer (G9328A)
- 1260 Infinity II Variable Wavelength Detector (G7114A) with 0.3 mm flow cell (option 024)
- 1290 Infinity II Preparative Open-Bed Fraction Collector (G7159B)

Column

Agilent Prep-C18 30 × 50 mm, 5 µm (part number 446905-302)

Software

Agilent OpenLab CDS ChemStation Edition for LC and LC/MS Systems, version C.01.09 [144]

Solvents

All solvents 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).

Sample

Caffeine, ethylparaben, biphenyl, phenanthrene, and pyrene were purchased from Merck and dissolved in DMSO to a final concentration of 25, 250, 50, 18, and 5 mg/mL, respectively.

System setup

In a standard RP-HPLC configuration, the mobile phase eluents are joined directly downstream of the two pump channels and directed through a common pressure sensor, purge valve, and mixer before entering the autosampler and the chromatographic column. The sample is transported by the mobile phase at starting conditions of the gradient, which usually has a high water content, leading to precipitation of highly concentrated samples inside the autosampler.

In an organic phase injection setup, each of the two pump channels has a separate pressure sensor and purge valve (see Figure 1), enabling individual control of the A and B channels. Figure 2 illustrates the flowpath of an organic phase injection setup: The channel B outlet is connected to the autosampler, whereas channel A leads to a T-piece located in front of the column. The autosampler outlet is joined with this T-piece, where the two channels mix and enter the column. During the injection process, the sample is transported through the organic channel only, keeping it dissolved while passing the critical parts of the flowpath. To prevent the sample from precipitating before it is retained on the column head, the connection from the T-piece to the column is kept as short as possible.

Due to the low volume, the mixing performance of a T-piece is lower than that of a solvent mixer used in a standard HPLC setup. This might cause inferior peak shapes when using columns with inside diameters (ids) of 21.2 mm or lower.

Adding additional mixing volume, for example, using a guard column, helps increase mixing performance and peak shapes when using smaller columns.

Unlike a standard configuration, an organic phase injection setup transports the sample only with a fraction of the total pump flow rate, depending on the gradient starting conditions. For example, if the flow rate is 50 mL/min and the gradient starts at 5% B, the sample is transported at 2.5 mL/min. Assuming an injection and capillary volume of 5 mL, it would take two minutes for the sample to reach the column. While the sample is retained at the column head, the sample solvent should be flushed away at starting conditions. The combined column loading and flush time lead to a minimum isocratic hold of three minutes before the separation gradient starts. To enhance sample transport and flush-out, we recommend using sample loops and capillaries of 1 mm id (1/16 in outside diameter) or lower between autosampler and column.

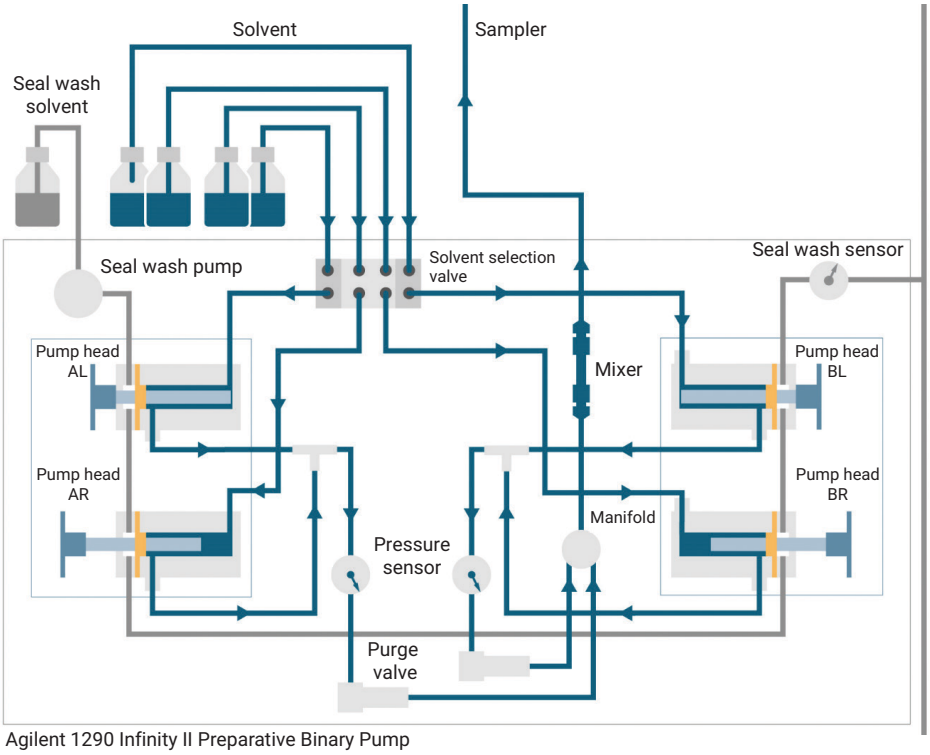

Figure 1. Hydraulic path of the Agilent 1290 Infinity II Preparative Binary Pump in standard configuration. The mixing point is located inside the pump module. The premixed gradient flows to the sampler.

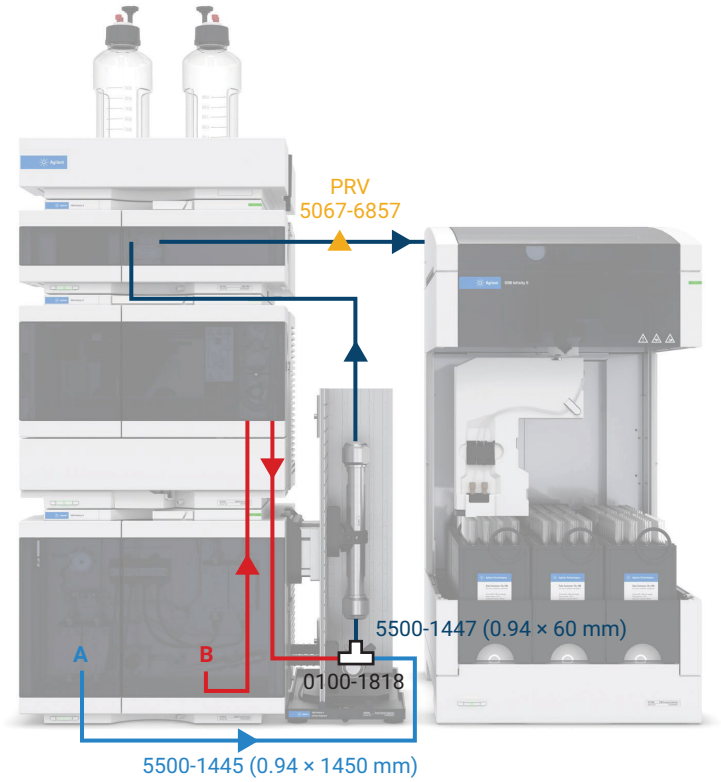

Figure 2. Plumbing scheme of an Agilent 1290 Infinity II Preparative LC System with independent flow channels. The aqueous mobile phase in channel A (blue) flows directly to the T-piece; the organic mobile phase in channel B (red) flows through the autosampler, transporting the sample without precipitation. A T-piece mixes the two phases and leads them to the column (dark blue). Part numbers refer to the accessory kit of the 1290 Infinity II Preparative Binary Pump. Capillaries not numbered are part of the system capillary kit, and need to be chosen according to the flow rate and stack setup. PRV: pressure relief valve.

Method settings

Table 1. Chromatographic conditions and method settings.

Parameter	Value
Mobile Phase	A) Water B) Acetonitrile
Flow Rate	42 mL/min
Gradient	Time (min) %B 0 10 2 10 4 50 11.5 80
Stop Time	12 minutes
Post Time	1 minute
Injection Volume	1,500 µL (522 mg sample)
Needle Wash	12 s with acetonitrile
Temperature	Ambient
UV Detection	274 nm Peak width >0.05 minutes (1 second response time) 10 Hz data rate
Fraction Collection	2 minutes: Peak-based by threshold Threshold: 30 mAU Limit peak duration: 78.5 seconds

Results and discussion

A highly concentrated mixture of five compounds with different polarities was analyzed using organic phase injection. The sample was successfully separated by a gradient from 10 to 80% B. Despite the wide range of polarity, all compounds were retained and well resolved without sample breakthrough. Fraction collection was successfully triggered by the ultraviolet-light (UV) absorption signal, see Figure 3.

The grey curve in Figure 3 shows the system pressure during the separation, which shows the typical profile of a linear gradient with increasing organic solvent percentage. At the beginning of the gradient, the pressure rises slightly before dropping gradually due to the increasing organic solvent composition towards the end of the gradient. Slight pressure drops around the elution point of the compounds are caused by the fraction collector diverter valve, which releases the flow into the fraction tubes. However, no sudden pressure increase has been recorded after injection, indicating that the sample has not precipitated and clogged the flowpath.

A suggestion with respect to sample loading in preparative-scale HPLC is to inject a maximum of 1% of the amount of stationary phase to maintain acceptable resolution. The column used (30 × 50 mm) contains approximately 21 g of stationary phase, which would dictate a maximum sample load of 210 mg per run. Using the organic phase injection technique, a sample load of 522 mg, equaling 2.5% of the stationary phase, was injected and successfully separated. Injecting more than twice the usual amount means a massive increase in throughput, and proves the benefits of the organic phase injection technique.

To determine retention time (RT) precision, represented by the relative standard deviation (RSD), a series of six consecutive injections was carried out and evaluated. Although the peaks deviate from perfect Gaussian shape due to column overloading, all compounds were retained with excellent reproducibility, see Table 2.

Fraction collection was triggered in peak-based mode by the UV signal at 274 nm, applying a threshold of 30 mAU. Using these settings, all compounds were collected successfully, see Figure 3. After collection, all fractions were reanalyzed on an analytical HPLC. Fraction purity was higher than 99%, and recovery was higher than 91%, see Table 2.

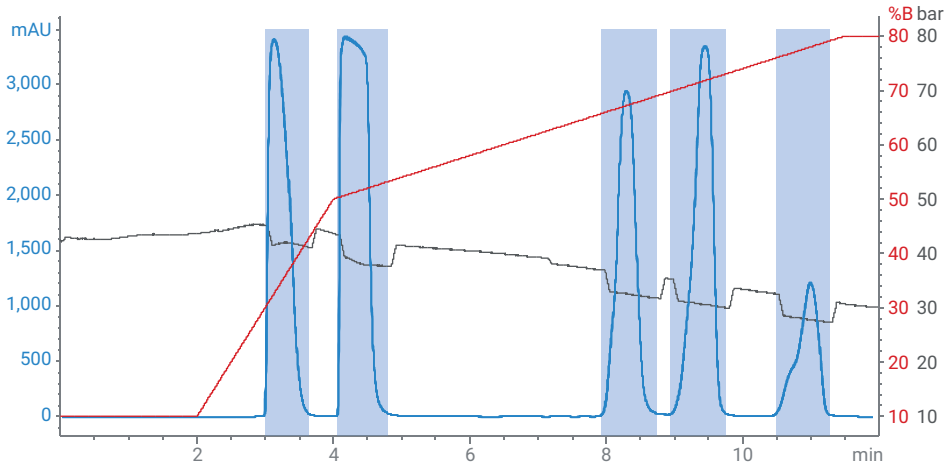

Figure 3. Chromatogram overlay (UV 274 nm) of six consecutive runs with 1,500 µL injection (blue), mobile phase composition (red), and system pressure (grey). Blue bars represent periods of fraction collection.

Table 2. Peak properties, fraction recovery, and purity of six consecutive injections.

Compound	RT (min)	RT RSD (%)	Recovery (%)	Purity (%)
Caffeine	3.122	0.072	92.2	>99
Ethylparaben	4.169	0.034	91.4	>99
Biphenyl	8.296	0.018	93.0	>99
Phenanthrene	9.452	0.010	91.7	>99
Pyrene	11.001	0.040	92.1	>99

Conclusion

This Technical Overview demonstrates an application of the Agilent 1290 Infinity II Preparative Binary Pump with independent flow channels. Using this setup, the challenges of sample solubility and concentration overloading can be alleviated by applying the technique of organic phase injection. A highly concentrated sample was injected into the organic phase without precipitation in the flowpath. All compounds, polar and unpolar, were separated with high reproducibility over a series of six consecutive runs (RT RSD ≤0.07%). Fraction collection was triggered by the UV signal, yielding pure fractions with a recovery higher than 91%. These data show that organic phase injection is a viable technique to increase throughput and minimize the risk of sample precipitation when working with highly concentrated samples in preparative-scale HPLC.

References

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Printed in the USA, May 20, 2019
5994-0576EN

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