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Hold-and-flush, a novel fraction collection method in semi-preparative subcritical and supercritical fluid chromatography

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Abstract

In supercritical fluid chromatography, the partial phase separation that occurs in the dead volume between automatic backpressure regulator and collection vessels causes significant peak tailing and delayed arrival of compounds to the collectors. As a result, when two peaks are barely baseline separated, it becomes very difficult to correctly set fraction collection triggers, which in turn results in fractions being collected with lower purity and lower yield. The problem can be solved with a simple addition of a four-port, two-position switching valve between UV detector and automatic back pressure regulator. The valve acts as a timed gate to release each peak out of the back pressure regulator and into the collection vessel, while at the same time it holds later peaks inside the UV detector and the column in a closed loop.

Keywords: Fraction collection; Preparative chromatography; Subcritical fluid chromatography; Supercritical fluid chromatography; Instrumentation

1. Introduction

In the past few years supercritical fluid chromatography (SFC) has gained wider acceptance as a tool of choice for semi-preparative chiral separations, especially in drug discovery research. As reviewed by various authors [1–5], it is clear that, compared to normal-phase HPLC, SFC offers distinctive advantages including much faster runs, often better enantiomer separations, and much lower costs in organic solvent purchase, disposal and evaporation. It has been shown [6] that these distinctive advantages are retained under certain specific subcritical conditions (e.g., for CO_2 and 15% methanol mobile phase, at 30 °C and 100 bar). Herein, we will use the abbreviation "SFC" to also include chromatography under such specific subcritical conditions.

During a semi-preparative SFC run, the mobile phase (e.g., CO_2 and methanol) is kept under high pressure (e.g., 100–200 bar) during separation, aided by an automatic backpressure regulator (aBPR) after the separation column and UV detector. Upon exiting the aBPR, the mobile phase is depressurized to facilitate liquid–gas phase separation for fraction collection and subsequent CO_2 exhaust. Usually the collection vessels are above atmospheric pressure held by a manual backpressure regulator (mBPR) (Fig. 1). Fraction collection start and stop triggers can be set either by the UV signal or simply by time (after certain calibration process). Berger and coworkers have thoroughly reviewed the various methods used to collect the resulting liquid phase, such as using a cyclone separator, a trapping solvent reservoir, or a controlled heating/depressurization device to avoid aerosol formation [6]. SFC manufacturers have generally reported high numbers for both collection efficiency (>95%) and collected sample purity (>99%), presumably for well separated peaks and using time-based collection triggers.

In reality, enantiomers sometimes cannot be baseline resolved; or, to increase sample throughput, overloading is employed to allow two peaks separated just to baseline. In such cases, in our experience with a Thar Technologies' SFC-50 instrument using the standard UV threshold triggering method, the second fraction was usually found impure with the first fraction as the contaminant. In addition, compound recovery (after solvent evaporation) was typically lower than expected. Such problems have been observed and reported [7] but to our knowledge no general solution is available. Logically, these problems are caused by the dead volume between UV detector and the fraction collectors, which creates a time delay. Unfortunately, it is not an easy task to compensate for this time delay because it depends on

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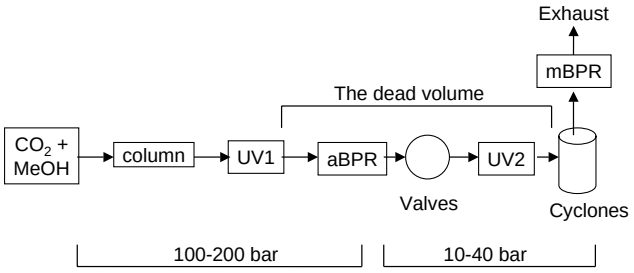

Fig. 1. The mobile phase flow diagram of Thar SFC-50 used in this study. A second UV detector (UV2) was added to monitor *the dead volume* (as shown) effects. Typical pressure settings are indicated. The sudden pressure drop at the automatic BPR prompts gas–liquid phase separation and aerosol formation.

the total flow rate and the percentage of liquid in the mobile phase. As such the delay must be calibrated under individual conditions. Furthermore, one would expect this dead volume to result in peak broadening which leads to problems in both purity and yield as well. Notably, the peak broadening effect cannot be corrected by a simple time shift.

Here we report a simple modification to the conventional UV triggered fraction collection method to achieve higher collection yield and sample purity for closely separated chromatographic peaks.

2. Experimental

2.1. Reagents

All reagents were purchased from Aldrich (Milwaukee, WI, USA), including HPLC-grade methanol, isopropanol, triethylamine (TEA), and trans-stilbene oxide (TSO, 98%). Coolant grade CO_2 (99.99%) cylinders were purchased from Airgas (San Diego, CA, USA).

2.2. Instrumentation and chromatography

Semi-preparative SFC experiments were performed on a Thar Technologies (Pittsburgh, PA, USA) SFC-50 unit, equipped with three 500 mL cyclone collectors and a Finnigan SpectraSystem UV1000 detector (Fig. 1). The following modifications were made: The 1/8 in. o.d. stainless steel inlet intruding into each cyclone was replaced with a 1/16 in. o.d. × 0.04 in. i.d. tubing with the tip bent in the tangential direction along the cyclone inner wall (1 in. = 2.54 cm). The collection efficiency was increased by this modification. The standard mBPR (rated 250 psi maximum; 1 psi = 6894.76 Pa) was replaced with a mBPR (700 psi maximum) purchased from Tescom (Elk River, MN, USA). The standard SSI-2000 injection pump was replaced with a MicroLab 500A dispenser from Hamilton (Reno, NV, USA) for accurate injection volume delivery. Column temperature was controlled by a CTO-10ACvp column oven made by Shimadzu (Columbia, MD, USA). The Thar liquid pump was replaced with a SSI-2000 pump for accurate solvent delivery at low flow rates. A Jasco (Easton, MD, USA) UV-1570 M detector with a preparative flow cell (2 mm flow path) was used as the second UV detector, added after the fraction collection valve to monitor the dead volume effects *for the purpose of this study only*. A computer-controlled 8-relay board was added (R85, from National Control Devices, Osceola, MO, USA) to allow easy switching of all devices and adding injection UV markers to chromatograms.

Semi-preparative SFC conditions: CO_2 flow rate 30 g/min; methanol with 0.2% (v/v) TEA flow rate 3 mL/min; column temperature 30 °C; aBPR 100 bar, 50 °C; collectors 35–40 bar, 35 °C; UV detectors 214 nm. Test sample was prepared by dissolving 500 mg of TSO in a final volume of 10 mL methanol, of which 300 μL was injected each time in an overlapping, repetitive fashion. A 250 mm × 21.1 mm (R,R)-Whelk-O2 column (Regis Technologies, Morton Grove, IL, USA) was used. The chiral stationary phase is based on 1-(3,5-dinitrobenzamido)-1,2,3,4-tetrahydrophenanthrene covalently bound to spherical Kromasil silica.

A four-port two-way switching valve (model EC4UW) was purchased from Valco Instruments (Houston, TX, USA). Stainless steel fittings and connectors were purchase from Western Analytical Products (Murrieta, CA, USA).

Analytical SFC was performed on a Jasco SFC unit with a UV-1570 M detector. Total mobile phase flow was 3 mL/min [85% CO_2 and 15% (methanol + 0.2% (v/v)TEA)] on a 250 mm × 4.6 mm (R,R)Whelk-O2 column. Sample injection volume was 20 μL. Needle wash was used to avoid any possible carryovers.

Analytical HPLC was performed on an Agilent 1100 system (Wilmington, DE, USA) with 100% isopropanol as the mobile phase on a 250 mm × 4.6 mm Chiralcel OD-H column purchased from Chiral Technologies (Exton, PA). The chiral stationary phase is cellulose tris(3,5-dimethylphenyl carbamate) coated on silica-gel. Flow rate was 0.4 mL/min. Sample injection volume was 20 μL. Needle wash was used to avoid any possible carryovers.

3. Results and discussion

In order to directly observe the effects produced by the dead volume, a second UV detector was placed right before the collection vessel, after the fraction collection valve (Fig. 1). UV absorbance at 214 nm was recorded from the two detectors (in two identical sequential runs) and compared, as shown in Fig. 2. F1 and F2 indicate the fractions that would be collected based on the UV1 signal. It is noted F2 contained more than one component, but for our purpose the additional components are of no concern.

First, the time difference between UV1 and UV2 signals at the center of F1 maximum were measured to be 8, 7, 5, and 5 s, for mBPR pressure settings at 10, 20, 30, and 40 bar, respectively. Using a Hamilton Gastight 5 mL syringe the

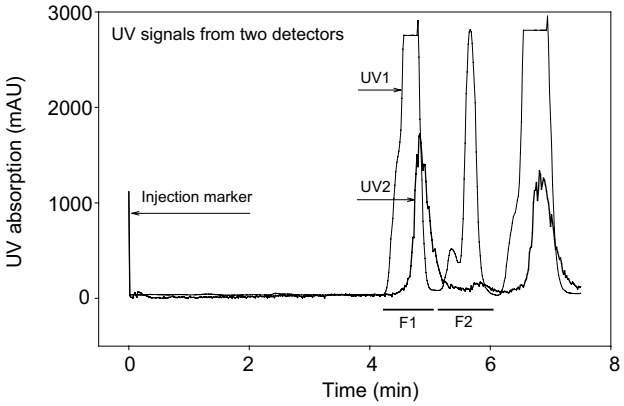

Fig. 2. UV signals at 214 nm as observed by two detectors in two identical sequential runs of TSO on Thar SFC-50. Fractions 1 and 2 that would be collected if triggered by UV1 are indicated by two horizontal bars (F1, F2). See text for experimental conditions. Compared to that observed by UV1, UV2 shows delaying and significant tailing for all peaks.

dead volume was measured as 2.5 mL. Thus, at (30 g/min of CO_2 + 3 mL/min methanol) flow rate, one would expect the time needed for the mobile phase to travel this dead volume to be about 5 s, which agrees with the observation. Note that the sensitivity of UV2 was much lower than that of UV1, a result of the fact that partial liquid gas phase separation took place within the dead volume. This shows, unfortunately, that the UV2 signal cannot be used as the collection trigger.

Second, using F2 intensity maximum as a height reference, it is apparent that significant peak broadening also occurred (F1 peak is more than two times wider as recorded by UV2 compared to that by UV1). In addition, the peaks are now apparently tilted with long tails, as can be seen clearly if a vertical line is drawn through the center of F1 on the UV2 trace. Thus, with (partial) phase separation, CO_2 gas expands in volume and flows at a much faster rate than liquid drops, as the latter has a stronger surface tension with the stainless steel tubing inner wall.

Since this differential in flow rate between CO_2 and methanol occurs only after aBPR, one can imagine that the best way to eliminate the dead volume problem is to locate the fraction switching valve right after the UV1 unit. This would require multiple independent aBPR units (one for each of the collectors), which is economically not feasible. However, a similar outcome can be achieved by adding a four-port, two-way valve right after the UV1 detector, as shown in Fig. 3. In this arrangement, as the first peak completely exits the UV1 detector, the valve switches such that later peaks are held in a closed loop inside the column and UV1, while at the same time the mobile phase "flushes" the first peak through the dead volume into the collector. We call this the "hold-and-flush" method. After a certain period of holding time (e.g., 1 min) the valve is turned back to its original position; And this process is then repeated for the second peak, and so on.

On the Thar SFC-50, TSO was separated by three regular runs, followed by three runs with a 30 s holding time, and

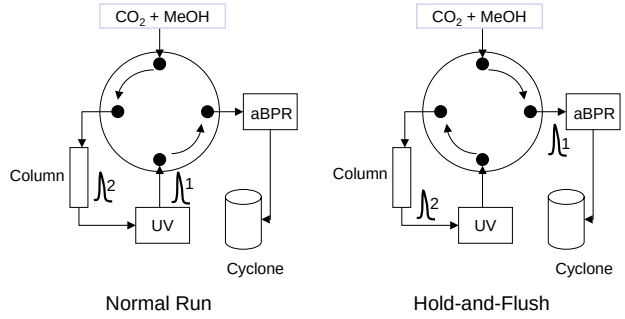

Fig. 3. Schematic diagram of the implementation of hold-and-flush method with a Valco 4-port, two-position valve. In the hold-and-flush mode, peak 1 is being flushed to the cyclone while peak 2 is held in a closed loop upstream of the UV detector.

then three runs with a 1 min holding time, in an overlapped injection fashion. The first run from each series is shown in Fig. 4. Six fractions were collected (F1a, F1b, F1c, F2a, F2b, F2c). One surprising observation was the absence of broadening for peaks held inside the column under high pressure. Indeed, for another compound tested, even after a 4 min holding time there was no apparent peak broadening for the second peak (data not shown). This indicates a slow molecular diffusion inside the column under high pressure but without flow, which is unexpected based on the belief that molecular diffusivity in SFC is relatively high.

Methanol was added to the six fractions described above so that the final volume of each fraction was 25 mL. Each fraction was then taken to the analytical SFC for purity and yield checks. Fig. 5 shows the analytical SFC runs, together with a TSO mixture sample. It can be seen that fraction F2b with 30 s holding time has significantly higher purity as well as yield compared to the normal run F2a. Increasing the holding time to 1 min (F2c) produced a slight further improvement over the 30 s counterpart (F2b).

The six fractions were also assayed on chiral HPLC to obtain the percentage gains in purity and yield by using the hold-and-flush method (chromatograms not shown). In this

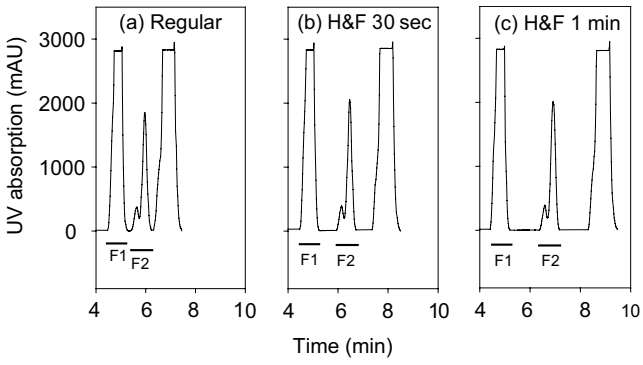

Fig. 4. Three otherwise identical runs of TSO on Thar SFC-50. Horizontal bars under the chromatograms show the time intervals for the two fractions collected (F1, F2) during each run. (a) The Valco valve is in the normal run mode as shown in Fig. 3. (b) Hold-and-flush for 30 s. (c) Hold-and-flush for 1 min. See text for experimental conditions.

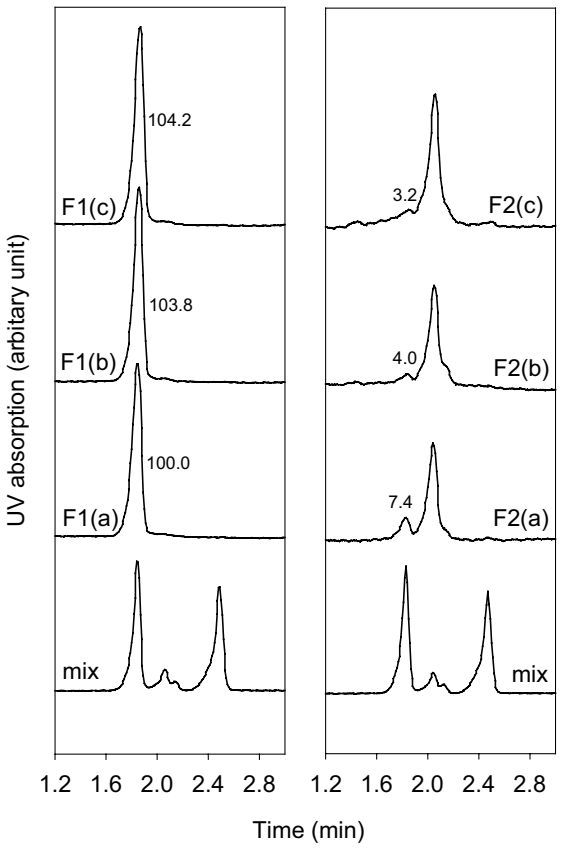

Fig. 5. Analytical runs on a Jasco SFC system for purity and yield assessment of the six fractions [F1(a,b,c) and F2(a,b,c)] collected on Thar SFC-50. The numbers near the centers of the chromatograms are *normalized peak areas for peak 1 obtained on an Agilent chiral HPLC*. Chromatograms F2(a,b,c) are scaled up vertically three times to show the F1(a,b,c) as contaminants.

case (using a Chiralcel OD-H column), HPLC runs provided much higher sensitivity and peak resolution compared to SFC runs and hence more reliable quantification data. F2 was eluted before F1 in HPLC runs. The relative areas for F1 are shown in Fig. 5 for the six fractions. The advantages of using the hold-and-flush method are obvious (e.g., a 4% increase in yield for F1 and more than 50% reduction of F1 contamination to F2).

The proposed hold-and-flush method is generally applicable to any UV-triggered SFC fraction collection. It is especially suitable to the following situations: when the separation is good but one would like to increase the loading until peaks are just baseline separated; when baseline separation cannot be achieved; when the first peak is much bigger than the second peak and thus the contamination of the second peak by the first will be severe; when there is a large dead volume (which should be minimized in the first place!); when the flow rate is slow compared to the dead volume; and, when it is actually desirable to increase the dead volume, e.g., to increase the time needed to heat the mobile phase inside the dead volume to minimize aerosol formation, or to allow time delays for MS- or evaporative light scattering detection-triggered fraction collection.

4. Conclusion

To overcome the dead-volume-induced peak broadening effect in semi-preparative SFC, we have devised a general method in which chromatographic peaks are allowed to exit the UV detector and be collected *one at a time*, separated by user-defined time intervals. The technique is simple to implement and produces no undesired side effects except that the run will be slightly longer (e.g., 2 min) than a normal run.

Acknowledgements

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