Constant Pressure Mode of Operation in the Second Dimension of Two-Dimensional Liquid Chromatography: A Proof of Concept

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Abstract

The use of two-dimensional liquid chromatography (2D-LC) continues to grow as the advantages over 1D-LC become increasingly clear in specific application areas, and the number of experienced 2D-LC users increases. As with any technique, however, there is always room for innovation that could improve the performance of 2D-LC. In recent years the technical aspects and potential benefits of a volume-based mode of operation were studied in detail for 1D-LC. The salient features of this approach that are immediately interesting for use in 2D-LC are two-fold. First, the ability to maintain a nominally constant pressure in the second dimension by dynamically adjusting the flow rate to compensate for changes in the viscosity of the fluid in the ^{2}D flow path provides a means to more fully utilize the pressure capability of the pumping system, and accelerates separations in the second dimension (^{2}D). Second, constant pressure operation minimizes physical stress on the system components and the ^{2}D column. In this paper we discuss the aspects of volume-based operation of LC that are particularly relevant to 2D-LC systems. The proof-of-concept experiments illustrate the viability of the constant pressure mode of operation for the second dimension of 2D-LC. In the described separations the throughput improvement is on the order of 10%; this gain will be strongly application-dependent, and may be as large as several tens percent in some cases. Future work will involve a detailed investigation of the impact of the constant pressure mode on robustness of ^{2}D separations.

Introduction

Increasingly, 2D-LC is being used to improve upon the performance of conventional one-dimensional LC, and is being applied in diverse application areas ranging from environmental science to biopharmaceutical analysis [1,2]. In some cases this improved performance appears as an increase in resolving power compared to 1D-LC separations without significantly increasing analysis time [3,4]. In other cases the benefits of 2D-LC manifest in other ways; for example, replacing two 1D-LC methods with a single 2D-LC method, or providing additional selectivity to resolve a particular set of analytes in a mixture that would otherwise be very difficult to resolve [5,6].

In recent years the number of 2D-LC users has increased significantly due to the increasing capabilities and robustness of commercially available instrumentation and creative demonstrations of problem-solving using these technologies [7,8]. However, there is still ample room for new concepts and technologies that can aid in further improving robustness and performance, which would help users to more fully realize the benefits of 2D-LC separations in routine analysis, regulated environments, and for processing of large numbers of samples.

About a decade ago a volume-based operational concept (VOBA) for modern liquid chromatography was introduced [9,10]. It was shown that in the context of 1D-LC, VOBA can yield meaningful analysis time reductions on the order of 25%, along with improvements in separation robustness. The goal of this paper is to demonstrate that VOBA – particularly a special case of VOBA we refer to as a constant pressure (cP) mode - can be implemented in the second dimension of 2D-LC separations. We envision two major potential benefits of this capability.

First, VOBA/cP can enable significant improvements in the throughput of 2D-LC in situations where the second dimension is the primary determinant of the total analysis time. Such scenarios could be encountered when storing of samples from ^{1}D occurs, before they become sequentially analyzed in the ^{2}D (e.g. in selective comprehensive (sLCxLC, a.k.a. HiRes sampling) or multiple heartcutting (MHC) mode). These gains can be realized by increasing the ^{2}D flow rate in regions of the analysis where in the conventional constant flow (cF) operational mode the ^{2}D pressure would be well below the limit of the pump. In the following we explain when and how these situations arise in 2D-LC. We anticipate that the analysis time savings as much as several tens percent can be achieved in some cases.

Second, cP operation can significantly reduce pressure variations experienced by the ^{2}D column during each ^{2}D separation. Given that prior work [11] has shown that repeated pressure cycling can be detrimental to the lifetime of chromatographic columns used in the second dimension of 2D-LC systems, this has the potential to significantly improve the robustness of 2D-LC operation, particularly in cases where many ^{2}D separations are done per analysis (i.e., in comprehensive mode (LCxLC) and sLCxLC with fast ^{2}D separations).

Experimental

Chemicals

LC/MS-grade acetonitrile (ACN) and methanol (MeOH) were from Merck (Germany) and water from a Milli-Q system (Millipore, Germany). Formic acid (FA) was from Honeywell (Fluka). Samples were the

phenone check out sample (Agilent p/n 5188-6529) and a dilution of the 2D-LC checkout standard (Agilent p/n 5190-6895), containing a suite of pesticides [12].

Instrumentation

Data were acquired using two similar but different 2D-LC systems.

System A

The first and second dimensions incorporated 1290 Infinity II modules (Agilent, Germany) including binary pumps (Model G7120A), multisampler (G7167B), multi-column thermostats (MCT) (G7116B), and DAD detectors (G7117B). A multiple heartcutting (MHC) 2D-LC interface was used with 40 µL loops. Detailed chromatographic conditions are given in the captions of Figs. 1-3.

Agilent LC ChemStation C 01.10 was used for instrument control and data acquisition and processing.

System B

All LC modules were from the 1290 series from Agilent (Germany): ^{1}D and ^{2}D pumps (G7120A), both with 35 µL JetWeaver mixers; multisampler (G7167B); ^{1}D and ^{2}D multicolumn thermostats (G7116B); ^{1}D (G7114B) multiple wavelength UV absorbance detector, and ^{2}D (Model G4212A; ultralow dispersion flow cell G4212-60038) diode-array (DAD) UV absorbance detector. The active solvent modulation (ASM) valve interface (p/n: 5067–4266) used to connect the two dimensions, was set up with two nominally identical 20 µL sample loops.

Agilent ChemStation software (C.01.07 SR3 [465]), with a 2D-LC Add-on (rev. A.01.04 [025]), was used for instrument control and data acquisition and processing. Detailed chromatographic conditions are given in the caption of Fig. 4.

VOBA operation

VOBA operation was supported by an experimental prototype firmware for a 1290 Infinity binary pump and prototype Chemstation add-ons, which enabled method execution and chromatogram presentation referring to delivered eluent volume, as explained in more detail below.

Results and Discussion

Before explaining how VOBA/cP works in 2D-LC and discussing initial results obtained under these conditions, it is instructive to review the conceptual differences between VOBA and conventional operating conditions for HPLC based on the cF mode.

Conventional LC, Constant Flow (cF) Operation

Conventional LC systems operate in a constant flow (cF) mode. Although most contemporary LC systems allow changes in flow rate during an analysis, this is seldom implemented during the separation period itself, and sometimes during column re-equilibration, for example. The pressure drop from the pump outlet to the column outlet depends on eluent composition (which determines its viscosity), the system permeability, and the preset, fixed flow rate. Use of more viscous eluents leads to higher pressure drops at a given flow rate. Consequently, in the gradient elution mode, the pressure changes during the analysis in response to changes in the eluent viscosity caused by the eluent composition change. This type of pressure trace is illustrated in Fig. 1A, which shows a chromatogram obtained from a separation of a mixture of alkylphenones using a solvent gradient elution (20/80 to 80/20 ACN/water). The pressure at the pump outlet (normalized to the maximum pressure recorded during the analysis) is plotted in addition to the chromatogram, with the pressure scale shown on the right Y-axis. The maximum viscosity of ACN-water mixtures is at about 20% ACN [7], which is why the pressure trace is at its highest point at the beginning of the separation. As the separation proceeds, the %ACN increases toward 80%, the viscosity drops, and the pressure follows.

If for the moment we assume that the analytical instrument or the column impose a pressure limit close to the maximum pressure in the plot (normalized value = 1) it becomes evident that for the majority of the analysis there is a significant, underutilized pressure capability; this is highlighted by the blue hatched area in Fig. 1A. This means that at any time during the analysis except for the initial phase where the pressure is at its maximum it is possible to increase the flow rate and accelerate the analysis, without exceeding any pressure limitations of the instrument or column.

Volume-Centric View of Chromatography

Description of an elementary step in an ideal chromatographic process (i.e., the mass transport of an analyte within a mobile phase through a column) does not require “time” as parameter to explain retention behavior. Indeed, at every step associated with perfusion of an elementary volume of eluent through a column, each analyte travels a certain distance equivalent to a fractional portion of the elementary volume. This distance is determined by a local distribution ratio of the analyte between the mobile and stationary phase – that is, by a local retention factor (k). Thus, the chromatographic process can be adequately described in a volume-domain (i.e., the chromatogram can be plotted versus volume instead of time without altering or losing any information).

In order to produce stable and interpretable chromatographic results, it is sufficient to execute a method (i.e., solvent gradient program, event tables, wavelength switching, valve switching) using the volume of eluent pumped into the column as the basis for progress (rather than time), and to relate the detector signal to a volume axis representing the volume of eluent pumped (rather than an axis representing elapsed time). However, LC separations are conventionally executed in a time-based operational mode. This means that methods, utilizing a strictly constant (or at least invariably programmed) flow rate are executed referring to time and the resulting chromatograms are recorded in relation to a time axis. Prior work has shown that for 1D-LC results can be obtained using the VOBA approach with variable flow rate, that are comparable to those obtained using conventional cF operation [9],[10].

When separations are carried out using the VOBA approach, it is possible to present the resulting chromatograms on an artificial time basis linearized in relation to the pumped volume, which is

compatible with the native representation of the data obtained in constant flow mode. To do so, one replaces eluent volume on the X-axis by the combination of time with a fixed flow rate. In fact, the conventional time-based representation of the chromatographic process is already a description of a volume-based process, but one that implicitly assumes data are collected at a fixed eluent flow rate. Thus, the use of real time as basis for data collection and review is not a fundamental requirement and the flow rate can be varied during the separation period without compromising the quality of the results if the LC operation is related to the pumped eluent volume.

A comparison of chromatograms obtained using VOBA and conventional cF approaches is shown in Fig. 1. Panel A shows the chromatogram obtained using the cF approach. Panel B shows the chromatogram vs. real time, obtained using the VOBA approach with variable flow rate, for the same sample and elution conditions (with the solvent gradient program translated from a time basis to a volume basis). The chromatograms in Panels A and B look different, as expected given that the flow rates were different. It is important to bear in mind, that in both panels A and B solvent composition gradient is linear with eluent volume. Consequently, the composition is changing linearly with time in the panel A but not linearly with time in the panel B (because the eluent volume is not linear with time if the flow rate is not constant). However, when the chromatogram in Panel B is stretched so that its volume base is linked to that in panel A, the resulting chromatogram, which is shown in the Panel C is indistinguishable from that in the panel A. Also, the solvent composition, which is changing linearly with volume, is linear in the coordinates of the Panel C plot. Such treatment of volume based data provides a representation over an artificial time axis enabling direct comparison to data collected in the cF mode [9].

Constant Pressure (cP) Operation as a Special Case of VOBA

A special execution mode of VOBA is constant pressure volume-based operation (cP). It is possible to operate a chromatograph in such a way that the pump dynamically adjusts the flow rate during the analysis to maintain a user-specified system pressure at all times. The lower the eluent viscosity is, the higher the flow rate will be, and vice versa. In fact, the chromatogram shown in Fig. 1B was obtained by execution of the method used for the Fig. 1A, but this time in cP mode with the pressure set to the maximum value observed in Panel A. In the initial phase of both separations the flow rate was the same. Whereas in Panel A the pressure decreased as the viscosity of the eluent was decreasing, in Panel B the eluent flow rate was increasing (right Y-axis in Fig. 1B) in response to this viscosity decrease so that the pressure was nominally constant throughout the analysis. Consequently, the analysis time is markedly reduced due to full utilization of the available pressure capabilities over the entire analysis. (i.e., the analysis time reduction is related to the size of the blue area in the Fig. 1A); this illustrates one of the most prominent benefits of the cP mode operation.

In addition to the benefit of enabling more effective use of the available system pressure, the cP mode can also improve method robustness by avoiding overpressure situations (e.g., due to slow pressure creep, or a partial flow obstruction), and reducing physical stress on components of the LC system by reducing the number of major pressure cycling events that occur over time. While these benefits are attractive in conventional 1D-LC, they may be even more advantageous in the context of 2D-LC.

2D-LC – Conventional Operation

To the best of our knowledge 2D-LC separations are always carried out using constant flow rates in the first and second dimensions, at least during the actual separation (i.e., not the re-equilibration periods). Given that significant amounts of the mobile phase used in the first dimension must be transferred into the second dimension, the pressure variations during a 2D-LC separation can be more complex compared to those in 1D-LC. The mobile phase viscosity varies in the second dimension not only due to eluent composition changes during a gradient elution, but also due to mismatch between the viscosity of fractions collected from the first dimension and the ^{2}D eluent itself. This is clearly illustrated in a 2D-LC separation run using a typical 2D-LC separation method with the cF mode in both dimensions.

Fig. 2A shows the pressure profile from the ^{1}D separation, running a solvent gradient from 10/90 to 80/20 MeOH/water over the course of 30 min. As expected based on the known viscosities of MeOH/water mixtures [13], the pressure increases up to about 50% MeOH and then decreases as the MeOH fraction increases further. The narrow blue rectangles in Panel A (labelled Cut 1, 2, 3) indicate regions of this ^{1}D separation where the ^{1}D effluent was collected (using 40 µL loops) and transferred to the ^{2}D column for further separation. Panel B shows the pressure profiles measured at the ^{2}D pump during the injection and separation of each of the collected fractions, using a solvent gradient from 10/90 to 90/10 ACN/water. For reference, Panel Bi shows the ^{2}D pressure profile if no injection of ^{1}D effluent is made. Again, given the known viscosities of ACN/water mixtures, this pressure profile appears as expected, with a maximum that occurs around 20% ACN. Panels Bii-Biv show the pressure profiles obtained from the ^{2}D separations of Cuts 1-3. In Panel Bii we see that during the injection period (roughly the first 20 s of each ^{2}D separation) the pressure increases due to the higher viscosity of the MeOH/water mixture (^{1}D effluent) compared to the ACN/water mixture (^{2}D eluent). Looking further at Panels Biii and Biv we see that this pressure "bump" during injection of the ^{1}D effluent increases further due to the even higher viscosity of the injected ^{1}D effluent.

In this case the magnitude of the pressure "bump" at the beginning of each ^{2}D separation is most strongly influenced by the viscosity mismatch between the ^{1}D effluent and the ^{2}D eluent. It is important to note, however, that the magnitude of this pressure variation is further influenced by other factors such as the volume of the ^{1}D effluent fraction transferred, the ^{2}D column and connecting capillaries, and whether or not Active Solvent Modulation (ASM) is used [14]. The influence of ASM can be significant because this approach involves dilution of ^{1}D effluent with ^{2}D mobile phase as the effluent fraction exits a sample loop in the 2D-LC interface towards the ^{2}D column. The viscosity of the diluted fraction can be higher or lower than the viscosity of the ^{2}D mobile phase, depending on the particular combination of solvents. The total volume to be injected onto the ^{2}D column is higher with ASM, and thus a section of the column containing different viscosity solvent is larger and the pressure effect may be more pronounced. Whatever this pressure "bump" is due to, a pressure maximum experienced in the second dimension in the course of the entire 2D LC separation effectively determines the maximum flow rate usable in the ^{2}D method. For example, if at a ^{2}D flow rate of 1 mL/min. the pressure bump for only one of the aliquots is at 750 bar, and the instrument pressure limit is 800 bar, then a ^{2}D flow rate setting cannot be increased even if the pressure during the rest of the separation is 600 bar or less. This makes the underutilization of the pressure capability of the pump in the ^{2}D separation in cF mode even more pronounced. In the case of the separation in Fig. 2, the portion of underutilized pressure due to the effect of the mismatch between injected ^{1}D effluent and ^{2}D eluent is indicated by the red hatched area; the blue hatched area represents

the portion of underutilized pressure that results from viscosity changes during solvent gradient elution as discussed above for 1D-LC separations.

2D-LC - Operation in cP Mode

Since the principle of the cP mode of operation relies on the ability of the pump to dynamically respond to changes in viscosity by adjusting the eluent flow rate to keep pressure constant, it is does not matter which aspect of the system (e.g., ^{1}D eluent / ^{2}D eluent viscosity mismatch, eluent composition etc.) the viscosity change comes from - the pump will respond in any case. To demonstrate the capability of cP mode in the second dimension we have carried out the separation shown in Fig. 3, both in cF and cP modes. Panel A shows a portion of a ^{1}D chromatogram from the separation of several pesticide standards. This is a multiple heartcutting experiment, where five fractions of ^{1}D effluent (40 µL each) are transferred to the second dimension as indicated by the blue vertical rectangles. Fig. 3B shows three rows each with five ^{2}D chromatograms, coming respectively from the five cuts indicated in Panel A. In the first row (Fig. 3Bi) we have chromatograms obtained using the cF mode in the second dimension. In this case the ^{2}D flow rate was 600 µL/min., and the nominal pressure corresponding to the initial gradient conditions was 600 bar. Each cut yields two well separated peaks that partially overlapped in the first dimension. The chromatograms in the second row (Fig. 3Bii) were obtained using the cP mode in the second dimension, with the pressure setpoint at 660 bar corresponding to the maximum in the pressure "bump" observed when the second dimension was run in cF mode. We see that the chromatograms look very similar to those in row 1, with comparable resolution of each pair of peaks, but peaks elute faster because a higher flow rate is applied compared to the 600 µL/min. used in cF mode; the initial flow was 680 µL/min and dynamically changed to reach 1040 µL/min at the lowest mobile phase viscosity. Finally, in row 3 the same chromatograms from row 2 are shown, but plotted using an artificial time axis calculated as t = V/F (with F= 600 µL/min) as discussed above for the 1D-LC separation in cP mode. Comparing the chromatograms in rows 1 and 3 we cannot distinguish them by eye. That is, we obtain very similar separations, but in less time (as shown in row 2) by more effectively using the available pressure capability of the pump throughout the ^{2}D separation.

Example Opportunity for Larger Time Savings in the Second Dimension

In the example discussed in the preceding section we see that the analysis time savings realized by switching from cF to cP mode in the second dimension was roughly 10%. The magnitude of this time savings is strongly dependent on the contrast between the viscosities of the ^{1}D effluent and the ^{2}D eluent, and sometimes can be much larger than 10%. To illustrate this point we show in Fig. 4 a pressure profile obtained from the second dimension of a 2D-LC separation of water- and fat-soluble vitamins. This application was first described by Bäurer, Lämmerhofer, and coworkers [15], who used isocratic elution in both dimensions. Recently, we have been studying the impact of ASM on this separation, which uses HILIC and RP separations in the first and second dimensions, respectively. The HILIC eluent contains 95% ACN, and the RP eluent contains 80% ACN, thus the viscosities of these eluents are similar. However, when ASM is used to mitigate the effect of the HILIC effluent on the RP separation, the ^{2}D pump is set to 100% water during the ASM step to act as a diluent for the fraction of effluent that comes from the ^{1}D separation. With an ASM factor of 2, this results in a fraction injected into the ^{2}D column that contains

about 47% ACN. The viscosity of the injected fraction is about 0.52 cP, whereas the viscosity of the ^{2}D eluent is about 0.31 cP (60 °C, 400 bar [13]). Thus, though the pressure is essentially constant during a ^{2}D separation step itself, the significantly more viscous aliquot matrix defines the nearly 1.5 times higher maximum pressure value in the ^{2}D analysis cycle. Given this contrast, we would expect a significant difference between the pressure during the ASM injection step, and the pressure during the rest of the separation. Indeed, this is what we observe in the pressure profile shown in Fig. 4, where the pressure during the ASM injection step is about 500 bar, compared to about 350 bar during the rest of the separation. This is an example of a real 2D-LC application where we would expect the implementation of the cP mode of operation to result in an improvement in the throughput of the second dimension on the order of 30%.

Effect of cP Mode on Robustness of 2D-LC Methods

Sudden pressure changes can be detrimental to the lifetime of analytical LC columns as has been demonstrated in prior investigations of this effect in 2D-LC [11]. It was found that pressure changes from ^{2}D operating pressure of about 500 bar to near zero that occurred upon switching the 2D-LC interface valve every 20 s caused rapid deterioration of the ^{2}D column performance during only two hours of operation; peak broadening, splitting, and tailing was observed. Such pressure changes can occur due to a short interruption of the flow towards the column during the valve switch [11]. In contrast, in the same experiment using an interface valve optimized to minimize these pressure changes, the column performance remained stable for at least 80 hours of operation.

Ongoing work suggests that repeated pressure cycling caused by the mobile phase gradient itself may also affect the lifetime of columns. Given these experiences, it seems likely that a cP mode of operation for the second dimension of 2D-LC should provide an avenue to further increase the robustness of 2D-LC by mitigating impacts of pressure variations on the performance of second dimension columns, particularly in cases where many ^{2}D separations are executed per 2D-LC analysis (i.e., LC×LC and sLC×LC with fast ^{2}D separations). Further work is needed to quantify this effect.

Conclusions

In this work we have carried out proof-of-concept experiments that demonstrate that a volume-based mode – and more specifically a constant pressure (cP) mode – of operation is viable in the second dimension of 2D-LC separations. This is an exciting new capability that opens new avenues for improving the throughput of second dimension separations, and possibly the robustness of 2D-LC separations in general. In the case of the separations shown here, the speed of each ^{2}D separation was improved about 10%. However, the magnitude of this speed improvement will be strongly dependent on the viscosities mismatch of the ^{1}D effluent that gets injected into the ^{2}D column, and the ^{2}D eluent. We expect that the upper end of the potential speed improvement is probably on the order of 30% or more. In addition to these potential improvements in separation speed, we also believe that the cP mode of operation may improve the overall robustness of 2D-LC by preventing overpressure situations in the second dimension, and by mitigating the negative effects of pressure variations on the lifetimes of ^{2}D columns.

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