Journal of Chromatography A, 1460 (2016) 68–73

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Monitoring gradient profile on-line in micro- and nano-high performance liquid chromatography using conductivity detection

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ARTICLE INFO

Article history:
Received 11 February 2016
Received in revised form 2 July 2016
Accepted 4 July 2016
Available online 5 July 2016

Keywords:
Nano-flow high performance liquid chromatography
Gradient profile monitoring
Capacitively coupled contactless conductivity detector
Electroosmotic pump

ABSTRACT

In micro- or nano-flow high performance liquid chromatography (HPLC), flow-splitters and gradient elutions are commonly used for reverse phase HPLC separations. When a flow splitter was used at a high split-ratio (e.g., 1000:1 or higher), the actual gradient may deviate away from the programmed gradient. Sometimes, mobile phase concentrations can deviate by as much as 5%. In this work, we noticed that the conductivity (σ) of a gradient decreased with the increasing organic-solvent fraction (φ). Based on the relationship between σ and φ, a method was developed for monitoring gradient profile on-line to record any deviations in these HPLC systems. The conductivity could be measured by a traditional conductivity detector or a capacitively coupled contactless conductivity detector (C^4D). The method was applied for assessing the performance of an electroosmotic pump (EOP) based nano-HPLC. We also observed that σ value of the gradient changed with system pressure; $a = 0.0175\Delta P$ ($R^2 = 0.964$), where a is the percentage of the conductivity increase and ΔP is the system pressure in bar. This effect was also investigated.

1. Introduction

Nano-flow HPLC has attracted increasing attention due to its advantages over conventional HPLC systems [1,2]; particularly when coupling with a mass spectrometer (MS), nano-flow HPLC offers very high efficiencies converting analytes to MS signals. For proteins/peptides analyses, gradient elutions are commonly used [3]. However, a limited number of pumps are available for delivering gradient eluents at high pressures (e.g., hundreds of bars) and low flow rates (e.g., tens of nL per minute). A frequent approach is to incorporate a conventional HPLC pump with a flow splitter [1]. If a very low flow rate is desired, the splitting ratio can be as high as 1000 or greater. That is one part of the gradient solution is utilized for analyte elution, while 1000 parts are wasted. More importantly, in such a system the residence time of eluent flowing through the column can be different from that flowing through the splitter restrictor. Because the eluent composition is a function of time, the different residence times can lead to different flow restrictions between the column and the splitter restrictor and consequently a varying splitting ratio. As a result, gradient can deviate away from the programmed profiles (e.g., delay, dispersion, etc.) in any HPLC system having a flow-splitter [4,5]. It will be beneficial if such deviations can be monitored on-line.

Measuring the actual gradient for reverse phase HPLC is performed frequently via a spiking procedure. In our lab, acetone is introduced into the organic phase, and absorbance (A) at 265 nm is then measured as the function of time. Because the acetone concentration is directly proportional to the organic solvent concentration, the organic solvent fraction (φ) can be computed [4,5]. However, acetone degrades C18 columns [5], and in addition, the molar absorptivity of acetone changes with increasing acetonitrile content (the organic solvent).

In another method [6], uracil is used as a tracer for monitoring the gradient profile. It is believed that uracil does not interact with column packing and its molar absorptivity does not change with acetonitrile. However, uracil can cause baseline drifting. A method named "Measure Your Gradient" [5] is proposed recently. In this method, a sample mixture containing 20 standards (known analytes) is first separated under both isocratic and gradient conditions; producing a set of functions exhibiting the elution behaviors for all these standards. In a real separation, these standards are co-eluted with sample analytes. The actual gradient is calculated

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http://dx.doi.org/10.1016/j.chroma.2016.07.005
0021-9673/

retrospectively based on the standard-elution functions. This strategy was tested for tracing linear gradient [5], but the process was tedious and inconvenient for practical applications.

Our laboratory has been engaged in developing miniaturized HPLC for several years. Nano-flow gradient elution has been achieved by a number of unconventional approaches [7–9]. To get reliable and repeatable results, it is preferable to know the actual gradient of each separation. In this study, we establish an approach to address this issue. We first determine the relationship between eluent conductivity (σ) and organic solvent fraction (φ). We employ a capacitively coupled contactless conductivity detection (C^4D) [10–13] scheme to monitor σ, and we use the σ values to calculate φ and then the gradient profile. Because C^4D actually measures the admittance of a solution, it is also termed admittance detection [12,13]. C^4D has been used for characterizing stationary-phases during elutions [14,15], but few have reported using it for monitoring eluent gradient profiles in micro- or nano-HPLC systems.

2. Experimental

2.1. Materials and reagents

Acetonitrile (MeCN) and methanol (MeOH) were purchased from Macron Fine Chemicals (Center Valley, PA, USA). Trifluoroacetic acid (TFA), formic acid (FA) and acetic acid (HAc) were HPLC grade and purchased from EMD (Billerica, MA, USA). All solutions were prepared with fresh ultrapure water purified by a Nanopure infinity ultrapure water system (Barnstead, Newton, WA, USA). All fused silica capillaries (FSC) were from Polymicro Technologies (Phoenix, AZ).

2.2. Conductivity measurement

Conductivity of MeCN/water/acid additive and MeOH/water/acid additive mixtures was measured with both contact and contactless conductivity detections. The flow cell for contact conductivity detection was built by connecting a piece of PEEK tubing (50 mm length × 380 μm ID × 1.6 mm OD) between two HPLC stainless steel unions (1/16 in.). The unions were soldered with two BNC cables and connected with a Dionex Model CDM-I conductivity detector (CA, USA). The above conductivity detection cell was calibrated by measuring a freshly prepared 1 mM KCl solution. In order to perform on-line and non-invasive measurement of the conductivity changing, a commercial TraceDec C^4D (Innovative Sensor Technologies GmbH, Strasshof, Austria) was used and its detection head was inserted with FSCs (360 μm OD). Pre-mixed solvent/water mixtures were pressurized by nitrogen gas to pass through the flow cell or capillaries for off-line conductivity measurement. All measurement was made under flowing conditions.

2.3. Conductivity measurement under pressure

The eluent conductivity (σ) was measured under a varying system pressure using an experimental setup as detailed in Fig. S1. Contact and contactless conductivity detectors were used for these measurements. An HPLC pump (LC30AD, Shimadzu,) was employed to deliver a pre-mixed eluent through a contact conductivity detection flow cell and then a 100 μm ID FSC, where C^4D was placed. A 6-port selector (Valco Instruments) equipped with 5 restrictors (20 μm ID FSCs of different lengths) was used to generate various backpressures (up to 250 bar). Each φ value was measured at a constant flow rate. The value of the system pressure was directly read from the HPLC pump. Initially, the flow cell was made from PEEK tubing coupled with two metal unions. To eliminate possible deformation of PEEK tubing under high pressure, the PEEK tubing was replaced with a piece of FSC (50 mm length × 250 μm ID × 360 μm OD).

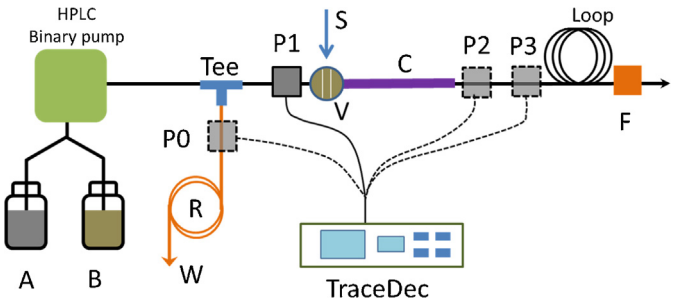

Fig. 1. Schematic diagram of capillary HPLC with flow-splitter. A: H_2O + additive; B: organic modifier + additive. R: restrictor, 50 μm ID × 360 μm OD × 500 mm length FSC; W: waste; S: sample; V: 50 nL injector; C: C18 capillary column; Loop: 250 μm ID × 360 μm OD × 160 cm length FSC; F: nano-flowmeter; Capillary @ P1: 50 μm ID × 360 μm OD × 10 cm length FSC; @P2 and P3: 100 μm ID × 360 μm OD FSC; distance between P2 and P3: 10 cm.

2.4. HPLC with flow splitter

Fig. 1 presents a schematic diagram of the HPLC with a flow splitter used in this experiment. A contactless conductivity detector was employed for this experiment. The eluent gradient was generated by a binary pump (Agilent 1200 Series Capillary Pump). The flow rate was kept at 100 μL/min. Flow was split by a PEEK Tee (P-727, Upchurch). One end of the Tee was connected to a restrictor coil (FSC, 50 cm length × 50 μm ID × 360 μm OD), and another end was connected to a FSC tubing (10 cm length × 50 μm ID × 360 μm OD). Following the FSC tubing were a nano-injector (50 nL, Valco Instruments) and a separation column (3.5 μm Symmetry C18, 100 μm × 150 mm, Waters). In some tests, the separation column was replaced with a FSC loop (300 cm length × 20 μm ID × 360 μm OD) that could generate a similar backpressure. With the FSC tubing, the backpressure as function of solution viscosity was more conveniently calculated. The column outlet was connected to a 20 cm × 100 μm ID × 360 μm OD FSC, then a 160 cm length × 250 μm ID × 360 μm OD FSC loop, and then a nano-flowmeter (Upchurch). The FSC loop were filled with DI water before each test to make sure that the liquid passing through the nano-flowmeter was water only. The flowmeter was calibrated by collecting and weighing the solution from its outlet.

The detection head of C^4D was placed at four different locations, as illustrated in Fig. 1. P0 was at the restrictor, P1 was at a position just prior to the injector, P2 was at a position ~20 mm away from the outlet of the column, and P3 was at a position ~10 cm away from P2 on the 100 μm ID × 360 μm OD FSC.

2.5. EOP based nano-HPLC

An EOP based nano-HPLC was assembled as described in our previous work [9]. Gradient profiles were monitored at a position prior to the nano-injector (50 nL). A digested-BSA sample was separated using this system.

3. Results and discussion

3.1. Conductivity of eluent

The σ value of a pre-mixed eluent (organic solvent/water mixture) with an acid additive was measured by a conventional conductivity detector. The acid additive concentration was kept constant in all solution. Fig. 2a presents σ as function of φ. A general trend was observed that σ decreased with φ. For a given