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

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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 (C⁴D). 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; $\sigma = 0.0175\Delta P$ ($R^2=0.964$), where σ is the percentage of the conductivity increase and ΔP is the system pressure in bar. This effect was also investigated.

Keywords: nano-flow high performance liquid chromatography, gradient profile monitoring, capacitively coupled contactless conductivity detector, electroosmotic pump

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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 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) and formic acid (FA) 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 x 380 μ m

ID x 1.6 mm OD) between two HPLC stainless steel unions (1/16 inch). 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 C4D (Innovative Sensor Technologies GmbH, Strasshof, Austria) was used and its detection head was inserted with FSCs (360 μ m 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 μ m ID FSC, where C⁴D was placed. A 6-port selector (Valco Instruments) equipped with 5 restrictors (20 μ m 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 x 250 μ m ID x 360 μ m OD).

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 μ L/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 x 50 μ m ID x 360 μ m OD), and another end was connected to a FSC tubing (10 cm length x 50 μ m ID x 360 μ m OD). Following the FSC tubing were a nano-injector (50 nL, Valco Instruments) and a separation column (3.5 μ m Symmetry C18, 100 μ m x

150 mm, Waters). In some tests, the separation column was replaced with a FSC loop (300 cm length x 20 μm ID x 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 x 100 μm ID x 360 μm OD FSC, then a 160 cm length x 250 μm ID x 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 x 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 eluent having the same φ , σ depended on the additive property and concentration. For example, eluent with TFA ($\text{p}K_a = -0.3 \sim 0.25$ [16-19]) was more conductive than that containing FA ($\text{p}K_a = 3.77$ [20]). MeCN or MeOH was used as the organic solvent in a gradient eluent, and its concentration could be traced by the conductivity detection method. If

TFA concentration was within a normally used range, e.g., 0.05-0.1% (v/v), the sensitivity of this conductivity method was adequate for measuring φ from 0 to 1. However, if the acid additive was weak and its concentration was low, σ value at a high φ number could be too low to be accurately measured. For example, a MeCN/water mixture with FA in Fig. 2a could not be accurately determined at $\varphi > 0.7$. To address this issue, the sensitivity of the conductivity detector needed to be increased. The relationship between TFA concentration and molar conductivity (Λ_m) was investigated in solutions with a varying φ (see Fig. S2). The concentration of TFA was added from 0.05% to 0.4% (v/v), equaling to 6.7 to 53.6 mM. When the φ value varied from 0 to 0.3, TFA behaved as a strong acid, Λ_m of which was almost constant with increasing TFA concentration in dilute solution due to near complete dissociation. However, if φ value was ≥ 0.4 , an apparent decreasing of Λ_m with TFA concentration could be seen. Ostwald's Dilution Law Equation was used to calculate the limiting molar conductivity (Λ_m^∞) in S·cm²/mol, and acid dissociation constant (K_a):

$$\frac{1}{\Lambda_m} = \frac{1}{\Lambda_m^\infty} + \frac{c\Lambda_m}{K_a(\Lambda_m^\infty)^2}$$

where c is the concentration of TFA in mol/L. When $1/\Lambda_m$ was fitted with $c\Lambda_m$ linearly (Table S1), the intercept was $1/\Lambda_m^\infty$, and the slope was $1/(K_a(\Lambda_m^\infty)^2)$. Λ_m^∞ values were calculated from the intercept while pK_a values were computed from Λ_m^∞ values and slopes. Fig. 3 shows that the pK_a value being increased with φ while Λ_m^∞ being changed in a reverse trend. Note, because Ostwald's Equation was not accurate for strong electrolyte, the pK_a value at lower part had large uncertainty but still fell within the reported range (-0.3~0.25) [16-19].

3.2. Measurement with contactless conductivity detection

To monitor the conductivity of nano-flow, as a non-invasive method, contactless conductivity detection was preferred. The conductivity of above-mentioned solutions in FSCs was measured with C⁴D as well. The response signal is presented in Fig. 2b. The trends of all solutions were well agreed with contact conductivity measurement, but with lower sensitivity at the higher conductivity region. The response comparison of the two detectors is presented in Fig. 2c; a nonlinearity relationship was observed. This could be attributed primarily to the limitation of

C⁴D hardware for dealing with high signal responses. Another possible reason was that the C⁴D was not an absolute conductivity detector, and the capacitance part of solution also affected its response [12]. The permittivity changing of organic solvent/water mixture could also give response to the detector. At the high conductivity region, the contribution to signal from solution capacitance changing could be neglected.

Capillaries of different size were compared (see Fig. S3a), and obviously large cross section area would reduce the solution resistance leading to increased response. A large ID capillary produced lower resistance and higher signal that improved the detection sensitivity and resolution, but it created a large dead volume. In this work, the detection sensitivity using 50 or 100 μm ID FSC was adequate, and so 50 or 100 μm ID FSC were used. The probe frequency of the detector was tested (see Fig. S3b). A higher frequency could make more current through the capillary wall, while setting the frequency at “2x high” (310 kHz) would narrow the detection range. Probe frequency at “high” (150 kHz) was used for the rest of the experiments in this work.

3.3. Effect of pressure on conductivity

Interestingly, we noticed that the conductivity signal increased significantly with pressure (0-250 bar) (see Fig. S4a, b). Initially, we thought this was an error, but multiple tests indicated the same phenomenon. The conductivity data were obtained first by pressurizing a MeCN/water mixture with TFA through a flow cell of a conventional conductivity detector. To exclude the potential deformation of PEEK tubing at high pressure, the experiment was redone after the PEEK tubing was replaced with a rigid FSC. Additionally, a C⁴D detector was fixed at a position after the flow cell to measure the conductivity. All tested systems yielded similar results that confirmed our observation. If we define percentage conductivity increase (α) as

$$\alpha = \frac{\sigma_P - \sigma_{P0}}{\sigma_{P0}} \times 100\%,$$

where σ_P is the solution conductivity at given pressure, and σ_{P0} is that at atmosphere pressure, α increased linearly with pressure (see Fig. S4b). It has been reported that high pressure could facilitate weak acid dissociation [21], which might explain partially this α enhancement.

However, more investigation is required to achieve better understanding of the conductivity enhancement mechanism. The important point was that this effect needed to be corrected when conductivity was measured at high backpressures.

3.4. Calibration curve and method validation

Neither the response of C^4D nor that of contact conductivity detector with φ was linear. Different regression approaches, including exponential, logarithmic, polynomial and power, were tested to get best fitting of the curve. Polynomial equations (with a fitting order of 4) were finally chosen because the highest correlation coefficient (r^2) values were obtained. The regression results were presented in Table S2 and Fig. S5.

Two gradient profiles, one linear and the other stepwise, were measured by this conductivity method and an acetone-spiked method. Fig. 4 presents the results; the two methods exhibited good agreements.

3.5. Gradient in HPLC with flow-splitter

The gradient profile from an HPLC with a flow splitter was tested in this study. To simplify the calculation, the separation column in Fig. 1 was replaced with a FSC loop (300 cm length x 20 μm ID x 360 μm OD). A C^4D cell was placed respectively at four different locations as indicated in Fig. 1. Repetitive measurements at each location showed excellent repeatability of the system. Three linear and two stepwise gradient profiles at P1 were presented in Fig. S6.

Under isocratic elution conditions, the splitting ratio was constant because the backpressure was stable. Under gradient elution conditions, the viscosity of eluent varied with solvent φ and consequently the backpressure, leading to an unstable splitting ratio. A simple model was developed and a software program written in LabVIEW (National Instruments) was used to simulate the process. To simplify the simulation process, diffusion and laminar flow dispersion were not taken into account in this model. A gradient profile at restrictor (P0) was used to predict the profiles at P1, P2 and P3. The pressure drop of the whole system and the flow rates at the flow splitter were also calculated. Detailed process simulation was discussed in Support

Information (see attendant text and Fig. S7, S8). The experimentally measured and the model-predicted data are presented in Fig. 5.

The pump we used was not a constant-pressure pump. The pressure reading on the HPLC (see Fig. 5b) showed some fluctuations. Gradient eluent flowing through the capillary produced a varying pressure drop. Flow rates through the column and the restrictor were function of both pressure drop and viscosity. Our model successfully predicted the pressure drop and flow rate with time. Large deviations yielded for calculating the gradient profile at P2; these errors might have been caused by neglected diffusion and dispersion.

Attention should also be paid to ensure all joints were properly connected to avoid leaking and minimize dead volumes. An example of poor connection is presented in Fig. S9; a nut connection to the Tee created a large dead-volume in this test, and the dead volume caused additional profile deviations.

3.6. Post-column gradient detection

The conductivity of post-column gradient solution was measured. In Fig. 6, the post-column gradient profiles appeared to be different from the pre-column gradient profiles. An abnormal dip could be seen in a linear gradient profile. Obviously, the φ of MeCN could not be lower than zero. This “dip” indicated a sharp rise of the conductivity signal, and was not caused by the decrease of MeCN concentration. Multiple dips in the stepwise gradient were observed at $\varphi < 0.4$, but disappeared at higher φ values. At the time being, we do not have a satisfactory explanation of this phenomenon. This effect, however, will limit the application of our conductivity-measurement method for post-column gradient profile detection.

3.7. Real-time monitoring nano-HPLC gradient

The gradient of a nano-HPLC was measured on-line before the micro-injector. Five pre-mixed MeOH/water eluents ($\varphi = 0.5, 0.4, 0.3, 0.2, 0.1$ and 0) containing 0.1% (V/V) TFA were aspirated into a storage loop in sequence and then pushed out to a separation column by an EOP. Because the first aspirated solution experienced the longest travel in the storage tubing, it would have the most extended mixing with its neighboring solutions. Fig. 7 presents three

repetitive measurements and derivatives of the gradient profiles. The gradient generated by nano-HPLC was highly reproducible. The derivative data showed the level of diffusion and mixing between neighboring solutions. The boundary of $\varphi = 0$ and 0.1 solutions was obvious, indicating a low-degree of mixing of the two solutions, while the boundary became smoother and smoother with increasing φ .

Real-time monitoring gradient profile was extremely helpful for trouble shooting. In Fig. 8, two repetitive separations of a digested-BSA sample are exhibited. Significant mismatches were observed for the peaks between 20-35 min. By checking their gradient profiles (monitored at real-time), one could see that the mismatches were caused clearly by an abnormal increase of φ (blue trace in Fig. 8).

In summary, we have demonstrated the feasibility of using a conductivity detector for real-time monitoring the gradient profiles in a reverse phase HPLC. We have discussed potential deviations of the gradient profile from the programmed profile in an HPLC with a flow splitter, especially when the splitting ratio is high. We have observed a phenomenon that the conductivity of a solution can increased with pressure; this effect needs to be considered as one uses conductivity measurement method for gradient profile monitoring. In this work, the acid additive we used was primarily TFA. The method can actually be used when other acidic additives such as FA and HAc are used (see Fig. S10). We do not recommend utilizing conductivity measurement method to monitor the post-column gradient profile because eluted analytes interfere with the eluent conductivity measurement. When nano-flow HPLC is used, particles in all reagents and solvents must be filtrated to avoid column clogging.

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

Figure2

Figure 2. (a) Conductivity of MeCN and MeOH with TFA and FA. Measured by Dionex conductivity detector; cell constant, 4408.7 /cm. (b) TraceDec signal of HPLC eluents. FSC ID: 250 μm for FA test, and 100 μm for all other tests; Detector condition: frequency, high; Voltage, -18 db; Gain, 50%. (c) Comparison of signals obtained by Dionex detector and TraceDec.

Figure3

Figure 3. Limiting molar conductivity (Λ_m^∞) and dissociation constant (pK_a) of TFA in MeCN/water mixture.

Figure4

Figure 4. Result comparison between conductivity-measurement method and acetone-spiking method. Acetone of 1% (v/v) was added into MeCN solvent with 0.1% TFA, and then the solvent was mixed with water, containing 0.1% (v/v) TFA to generate gradient. A 100 μm ID x 360 μm OD FSC with removed coating was used as flow cell. 265 nm was used as detection wavelength, and 400 nm, where acetone has no absorption, was for correct the baseline changing due to reflection index varying.

Figure5

Figure 5. Comparison between experimentally measured and model-predicted results. (a) measured and predicted linear gradient profiles; all conditions were the same as in Fig. 1. Eluent A: H₂O + 0.1%(v/v) TFA; B: MeCN + 0.1%(v/v) TFA. Separation column was replaced by a FSC loop. Black dash line: programed gradient; solid line: measured profiles; green dash line: predicted profile at P2. (b) measured (solid) and predicted (dash) flow rate (red) and pressure (blue) profiles.

Figure6

Figure 6. Prior- and post-column linear gradient measurement. (a) linear gradient; (b) steep gradient.

Separation column was connected. P0 and P1 was prior-column; P2 and P3 was post-column. All conditions as Fig. 1. Eluent A: H₂O + 0.1%(v/v) TFA; B: MeCN + 0.1%(v/v) TFA.

Figure7

Figure 7. Three gradient (a) and their derivative (b) profiles of a EOP based nano-HPLC [9]. Conditions: MeCN/water mixture with 0.1% (v/v) TFA; ϕ = 0.5, 0.4, 0.3, 0.2, 0.1 and 0 and volume: 49, 3, 3, 3, 3, and 49 μ L, respectively. Flow rate, 610 nL/min.

Figure8

Figure 8. Two gradient profiles and chromatograms for separation of digested BSA sample by nano-HPLC [9]. Conditions: MeCN/water mixture with 0.1% (v/v) TFA ; $\phi = 0.5, 0.4, 0.3, 0.2, 0.1$ and 0 and volume: 49, 3, 3, 3, 3, and 49 μL , respectively. Flow rate, 230 nL/min. Detection wavelength, 210 nm.