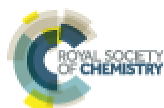


Analytical Methods

In situ measurement of pH in liquid chromatography systems using a colorimetric approach

Journal:	*Analytical Methods*
Manuscript ID	AY-TEC-11-2018-002496.R1
Article Type:	Technical Note
Date Submitted by the Author:	04-Dec-2018

Complete List of Authors:	Mazzi Leme, Gabriel; Gustavus Adolphus College, Chemistry Madigan, Benjamin; Gustavus Adolphus College, Chemistry Eikens, Joseph; Gustavus Adolphus College, Chemistry Harmes, David; Gustavus Adolphus College, Chemistry Richardson, Douglas; MErck & Co., Inc. Carr, Peter; University of Minnesota, Chemistry Stoll, dwight; Gustavus Adolphus College

SCHOLARONE™
Manuscripts

Please do not adjust margins

Analytical Methods

TECHNICAL NOTE

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

In situ measurement of pH in liquid chromatography systems using a colorimetric approach

Gabriel Leme,*[a] Benjamin Madigan[a] , Joseph Eikens[a], David C. Harmes[a], Douglas Richardson[b], Peter Carr[c] and Dwight Stoll[a]

In liquid chromatography differences between the pH of an injected sample and the pH of the mobile phase can have a significant impact on retention times, peak widths, and resolution. When the injection volume is small relative to the column volume this is typically not a problem. However, when the injected volume becomes large enough there will be a zone of sample that travels through the column without mixing with the surrounding mobile phase, and thus the pH of this zone will be that of the sample rather than the column eluent itself. We have studied situations like this in detail, specifically in the case of two-dimensional liquid chromatography where the composition (pH and concentration) of the first dimension eluent which carries the sample is quite different from the second dimension eluent into which it is injected. In this paper we describe a colorimetric approach for the *in situ* determination of the pH in LC systems thus enabling more detailed studies of pH changes at different points inside the system. We find that this approach is complementary to existing technologies for inline pH measurement (e.g., ion selective electrodes) in that it can be implemented with a UV detector, can be used at high pressures, is easy to use, and is sufficiently reproducible to be useful in this context.

Introduction

Mobile phase pH is an important method parameter in liquid chromatography (LC) separations for many different types of analytes ranging from small molecule pharmaceuticals (e.g., ibuprofen) to proteins (e.g., immunoglobulins)[1]. The mobile phase pH can affect the ionization states of both analytes and stationary phases, and cause increases or decreases in retention depending on the functional groups and particular retention mechanism involved (e.g., reversed-phase, ion-exchange, etc.).

In our research on two-dimensional liquid chromatography (2D-LC) we have become acutely aware of the potential for mismatch between the pH conditions of the first (^{1}D) and second dimension (^{2}D) mobile phases to negatively affect the performance of ^{2}D separations[2]. In conventional one-dimensional LC (1D-LC) injection volumes are typically reasonably small relative to the volume of the LC column itself and injected samples quickly mix with surrounding mobile phase after they are injected into the column. However, in 2D-LC the volume of ^{1}D effluent injected into the ^{2}D column is often a significant fraction of the void volume of the ^{2}D column itself,

[a.] Department of Chemistry, Gustavus Adolphus College, Saint Peter, Minnesota 56082, United States
[b.] Analytical Research and Development, Merck & Co., Inc., Kenilworth, New Jersey 07033, United States
[c.] Department of Chemistry, University of Minnesota, Minneapolis, Minnesota 55104, United States.

This journal is © The Royal Society of Chemistry 20xx

Please do not adjust margins

and can even exceed the ^{2}D column volume. In these cases, mixing of the sample pulse with the ^{2}D eluent can be quite incomplete, and from the point of view of analytes injected into the column, the sample solvent effectively *is* effectively the mobile phase, at least for a short period of time. From a mechanistic point of view, a more interesting case is one in which the injected sample is on the order of 10% of the column volume. In this case, the injected volume is too big to mix quickly with the surrounding mobile phase, but not so big that the zone of pH corresponding to the sample buffer persists all of the way to the column exit. In a case like this we would like to answer the question – what is the mobile phase pH profile inside of the column as a function of length between the column inlet and exit?

Inline pH measurement cells based on the same principles as benchtop pH meters (i.e., ion selective electrodes) are commercially available for LC systems and deployed in cases where real-time determination of mobile phase pH is valuable.[3] Previous work in the area of supercritical fluid chromatography (SFC) has demonstrated the utility of pH indicator dyes for determination of apparent pH in eluents typically used in SFC, namely supercritical carbon dioxide and small molecule modifiers. The ability to determine pH in situ in the case of SFC is particularly useful because the properties of SFC eluents are obviously very different under the operating conditions of the chromatography (e.g., several hundred bar of pressure) than they are at ambient pressure where most pH measurements are made. Wen and Olesik measured UV absorption spectra of several pH indicator dyes dissolved in mixtures of carbon dioxide and eluent additives using a high pressure UV flow cell. More recently, West and coworkers measured UV absorbance spectra of several indicator dyes following injection of the dyes into flowing SFC eluents with the goal of determining the effects of various SFC eluent modifiers on the apparent eluent pH[5]. This group used Principal Component Analysis to calculate the apparent pH based on changes in the spectra of the dyes.

In our work described here we have studied the use of a universal pH indicator solution (i.e., a cocktail of pH-sensitive dyes) to determine the mobile phase pH *in situ* at specific points in a LC system. This approach is different from previous work and based on the work of Blair and co-workers that described the use of the hue of a solution (calculated mathematically from absorbances of red, green, and blue light) to determine solution pH under static (i.e., no convective flow) conditions[6]. Specifically, we have used this approach to determine the local pH at the inlet and outlet of LC columns under different conditions. This complements the previous work of Olesik and West in that it enables precise determination of changes in local pH over distance (i.e., location between injector and detector) and time under chromatographically meaningful conditions. The potential advantages of this colorimetric approach over the use of electrode-based cells in this context include: 1) very fast response – response is limited by the acquisition rate of the spectroscopic detector used to determine the hue of the mobile phase at the point of measurement; 2) pH can be determined at high pressures – the pressure limit of the measurement is limited by the detection cell of the spectroscopic detector (e.g., cells with 400 bar capabilities are commercially available); 3) less extra-column peak broadening - typical UV-Vis absorbance flow cells are much smaller in volume than electrode-based flow cells; and 4) a dedicated measurement cell is not required – hue of the indicator solution can be determined using an existing UV absorbance detector.

Materials and methods

Solvents, salts and solutions

Water was purified in-house using a Milli-Q water purification system (Billerica, MA). Ethanol (HPLC grade), phosphoric acid (85%), sodium phosphate monobasic monohydrate, sodium phosphate dibasic, sodium phosphate dibasic heptahydrate, sodium phosphate tribasic dodecahydrate, sodium hydroxide (50% w/w), sodium chloride (≥99%) and benzylamine (99%) were obtained from Sigma-Aldrich (St. Louis, MO) and used as received. Universal pH indicator solution was obtained from Ricca Chemical Company (p/n: 8870-15, Arlington, TX).

A working solution of pH indicator was prepared by diluting 5 mL of the solution as purchased with 157 g of ethanol and 793 g of water (1:200, v/v). Buffer solutions were prepared as follows. A pH 3 mobile phase solution was prepared by dissolving 13.8 g of sodium phosphate monobasic monohydrate in approximately 800 mL of water. The pH was measured using a glass electrode (Orion 8101BNWP ROSS Half-Cell Electrode, from Thermo Scientific (Waltham, MA), calibrated using pH 1.68 and 4.00 standards, VWR, West Chester, PA; p/n BDH5006-500mL and p/n BDH5022-4L, respectively) and adjusted to 3.0 by adding 225 µL of phosphoric acid (85% w/w), and then the volume was finally brought to 1.00 L by adding water. The pH 7.0 mobile phase solution was prepared by dissolving 4.5 g of sodium phosphate monobasic monohydrate, 18.0 g of sodium phosphate dibasic heptahydrate and 5.8 g of sodium chloride in approximately 800 mL of water. The pH was measured using a glass electrode (calibrated using pH 4.00 and 7.00 standards, VWR, West Chester, PA; p/n BDH5022-4L and p/n BDH5050-4L, respectively) and the volume was brought to 1.00 L by adding water. The pH 2 calibration solution was prepared by dissolving 2.1 g of sodium phosphate tribasic dodecahydrate and 1.7 g of phosphoric acid in 1 L of water. The pH 11 calibration solution was prepared by dissolving 2.1 g of sodium phosphate dibasic and 5.7 g of sodium phosphate tribasic dodecahydrate in 1 L of water. The pH was measured using a glass electrode. The 50 mM sodium carbonate mobile phase was prepared by dissolving 5.30 g of sodium carbonate in 1.00 L of water; the 10 mM phosphoric acid was prepared by adding 1.15 g of concentrated phosphoric acid (85% w/w) to 1.00 L of water. All of these buffer solutions were filtered through a 0.2 µm nylon filter membrane before use.

Analytical instrumentation and columns

This journal is © The Royal Society of Chemistry 20xx

The chromatographic system employed for the experiments was composed by modules from the 1200 series from Agilent Technologies (Waldbronn, Germany): a 1260 Bio Quaternary Pump (Model G5611A), a 1290 Binary Pump (Model G4220A), a Binary Pump SL (Model G1312B), a Diode-Array Detector (DAD) (Model G4212A, 1 µL flow-cell) and two multiport valves (Duo-valve and 6-port/2-position, p/n 5067-4214 and p/n 5067-4117, respectively) installed in a Flexible Cube module (model G4227A.) The Duo-valve was set up with two nominally identical sample loops (i.e., matched pairs of 7, 40, 80 or 120 µL). OpenLab Chromatography Data System (C.01.07), with a 2D-LC add-on (rev. A.01.04), was used to control the instrument. Absorbance signals were acquired from 190 to 650 nm and the signals at 636, 520 and 452 nm were exported to .CSV files for further processing. The acquisition rate was 40 Hz. Agilent Buffer Advisor (rev. A.01.01) was employed to establish the buffer composition needed to produce the pH gradient needed for the calibration of hue vs. pH as shown in Figure 3.

XBridge Protein BEH SEC columns (30 mm x 4.6 mm i.d., [illegible] µm) from Waters (Milford, MA) were connected in series to make a SEC column with a total length of 90 mm. A Poroshell HPH C18 column (50 x 2.1 mm, 2.7 µm, Agilent Technologies) was used for the benzyalmine analysis.

Methods

Hue vs. pH Calibration. Using the Buffer Advisor software, a method for a pH gradient from 2.4-10.4 was developed as follows: pH 2.4 from 0-10 min, increasing in steps of 0.2 pH and held at each step for 5 min (10 to 200 min), pH 10.4 from 200-215 min. At the beginning of an analysis the six-port valve shown at the lower left of Figure 1 was set as it is shown in the figure, so that the indicator dye would flow to waste. This enabled setting of the baseline absorbance to zero at the beginning of each analysis in a reproducible way. Then, at 3 min, the six-port valve was switched allowing the indicator dye to mix with the mobile phase through a “T-piece” and reach the detector. The flow-rate was 0.9 mL/min for the mobile phase and 0.1 mL/min for the indicator dye, so that the total flow exiting the T-piece was 1.0 mL/min (unless stated otherwise). These experiments were carried out at ambient temperature (~ 23 °C).

INSERT FIGURE 1 HERE

Figure 1. Instrument setup employed for calibration of hue vs. pH.

***in Situ* Measurement of pH under Chromatographic Conditions.** Mobile phase pH was determined immediately before and after chromatography columns used in two very different situations. As an example, one of these is shown in Figure 2. In this particular case the system under study is the second dimension of a 2D-LC system where the ^{1}D mobile phase is buffered at pH 3 and the ^{2}D mobile phase is buffered at pH 7. As the figure is drawn the pH is being determined post-column in this case. The pH can be determined pre-column by simply removing the column and connecting the pre-column capillary to the T-piece. Aside from the addition of the column, the setup and its use is similar to that shown in Figure 1 and discussed above. The flow-rate was 0.5 mL/min for the mobile phase and 0.05 mL/min for the indicator dye, so that the total flow exiting the T-piece was 0.55 mL/min (typically, the ratio of mobile phase and indicator flow rates was 10:1). Typically, the ratio of mobile phase and indicator flow rates was 10:1. As with the conditions for the hue vs. pH calibration each method used here started with the six-port valve diverting the indicator dye to waste to establish a baseline absorbance of zero at the beginning of the analysis. Then, at 0.5 min, this valve was switched to direct the indicator dye to the T-piece and joining the mobile phase flow. In the case of the configuration shown in Figure 2 that was used to mimic the second dimension of a 2D-LC system, the two-position/eight-port valve connecting the pH 3 and pH 7 buffer streams was set to start switching at 2 min with 1 min intervals (modulation time). The results were plotted using 1 min scale considering the valve switch as time zero.

The second chromatographic system studied in this work was similar to that shown in Figure 2, with the following exceptions: 1) Instead of the two-position/eight-port valve, sample injections were made into the mobile phase and column under study using a conventional autosampler; 2) The mobile phase flowing through the column was buffered at pH 11.5 with 50 mM sodium carbonate in water, at a flow-rate of 0.5 mL/min, and the injected sample was 10 mM phosphoric acid in water.

INSERT FIGURE 2 HERE

Figure 2. Instrumental setup employed for *in situ* pH measurement under real LC conditions. (A) Initial condition with the indicator dye diverted to waste; (B) Indicator dye is combined with the mobile phase after the LC column. In this position the contents of Loop 1 are injected and travel through the column. (C) In this position the contents of Loop 2 are injected.

Data Processing

Solution hue (H) was calculated at each point in chromatographic time using Eqn. 1, where R, G, and B are the absorbances of red (636 nm), green (520 nm), and blue (452 nm) light, and *max* and *min* are the greatest and least absorbance values for the set of three wavelengths at each time point[6].

$$H = \begin{cases} \left(\frac{G-B}{max-min}+0\right)/6\,; & if\ max = R \\ \left(\frac{B-R}{max-min}+2\right)/6; & if\ max = G \\ \left(\frac{R-G}{max-min}+6\right)/6; & if\ max = B \end{cases} \qquad Eqn.\ 1$$

The technical details associated with the establishment of the relationship between hue and pH are described in Methods section. Representative absorbance data for the calibration process are shown in Figure 3A. Four calibration curves for hue vs. pH are shown in Figure 3B. These curves were acquired on different days with two different batches of indicator dye solution. Calibrations #1-3 were acquired with mobile phase and indicator flow rates of 0.90 and 0.10 mL/min., respectively. Calibration #4 was acquired with mobile phase and indicator flow rates of 0.945 and 0.055 mL/min. Calibration #1 was acquired with the first batch of indicator solution, and calibrations #2-4 were acquired with a second batch. We observe that the shape of the calibration curve is nominally

Please do not adjust margins

independent of the mobile phase/indicator flow rate ratio, as expected, which is practically convenient.

INSERT FIGURE 3 HERE

Figure 3. pH profile used during calibration of hue vs. pH and the resulting absorbance (A) and hue profiles (B) calculated using Eqn 1.

Results

The objective of the method described here is to measure pH as a function of time at different physical locations inside of a chromatograph. We refer to the resulting data as "pHgrams". Figure 4 shows the pHgrams obtained in the scenario where pH 3 buffer is injected into a pH 7 mobile phases as shown in Figure 2. Panel A shows the results for four different injection volumes ranging from 2 to 120 µL. Panel B shows the pHgrams obtained at the outlet of a 90 mm x 4.6 mm i.d. SEC column (1.7 µm; the 90 mm length is composed of three 30 mm long segments coupled together). Panel C shows four replicates of the pHgrams obtained at the column outlet for the 120 µL injection. It is striking that for all injection volumes except 7 µL the local mobile phase pH at the column inlet dips all of the way down to pH 3. This suggests that at the level of 7 µL there is sufficient mixing of the injected fluid with the surrounding mobile phase between the injection valve and the measurement point that the injected pH 3 buffer is almost entirely neutralized by the pH 7 mobile phase. On the other hand, for the larger injection volumes the length of connecting tubing occupied by the injected sample is simply too large to allow complete physical contact of the two buffers and a zone of pH 3 buffer persists all of the way from the injection valve to the detection point. This observation is consistent with studies of injection profiles made under other conditions[7,8]. Turning to Panel B we see that the pHgrams are very different from those in Panel A. The zone of low pH is considerably wider in time units because it has been broadened by dispersion inside the column. The biggest difference is that the minimum pH in the center of the injected sample does not drop all of the way to 3 as it does at the column inlet. We believe this is due to incomplete neutralization of the pH 3 buffer by pH 7 mobile phase as the injected pulse travels through the column which acts as a static mixer (albeit a poor one!). Although there is a measureable difference between the pHgrams obtained at the inlet and outlet of this column, these results clearly show that analytes injected in a large (i.e., > 20 µL) sample may experience pH conditions below 4 for the entire time they are in the column, unless there is a mechanism to retain them and pull them out of the plug of injected buffer as it travels through the column. Residence time at low pH should be considered during method development for compounds that are pH sensitive.

INSERT FIGURE 4 HERE

Figure 4. pH profiles showing the pH variation measured at the inlet (A) and outlet (B) of a 90 mm x 4.6 mm i.d. SEC column for different injection volumes, and (C) four replicate measurements at the outlet for 120 µL injections..

Results from a second scenario where a sample buffered at pH 2.4 is injected into a mobile phase buffered at pH 11.5 are shown in Figure 5. Whereas in the previous example only blank buffer solutions were injected, in this case the sample contained the analyte benzylamine and the separation conditions involve a reversed-phase column (HPH C18) and a mobile phase containing acetonitrile (ACN) and aqueous buffer. The pHgrams obtained at the column inlet and outlet are shown in Panels A and B for injection volumes of 2 or 20 µL. These results are qualitatively consistent with those in the previous example. However, here there is a zone of low pH buffer that persists all of the way to the outlet of the column even when only 20 µL of sample is injected. This is probably because the volume of the column itself is much smaller than in the previous case (85 vs. 1000 µL) and thus is less effective as a mixer. It is also possible that in the case of the 90 mm SEC column, which consisted of three 30 mm columns coupled together, there is additional mixing in the inlet and outlet frits of the column segments that does not exist in the case of the smaller column that has just one inlet and outlet frit. Panels C and D show the chromatograms obtained for the analyte benzylamine under these conditions. From other work not shown here we know that under the conditions of this experiment the retention of benzylamine in the deprotonated state (high pH, neutral) is about ten times higher than in the protonated state (low pH, positively charged). When the small 2 µL injection is used there is sufficient mixing of the injected sample inside the connecting tubing and column that the analyte experiences a local pH that is very close to the pH of the mobile phase and elutes as a single symmetrical peak (Panel C). However, when 20 µL of the same sample buffer is injected (this time with 10X less benzylamine so that the analyte mass is constant), the *in situ* pH measurement shows us that a zone of low pH that is the same as the sample persists all of the way to the column outlet. This in turn has devastating effect on the chromatography. Panel D shows that the peak is very broad and split. Part of the analyte elutes much earlier than in Panel C because it travels at a high velocity with the low pH zone, and part of the analyte is retained as the trailing edge of the injected sample is neutralized and the benzylamine is more retained in its deprotonated state. In previous work we've shown this peak splitting phenomenon and in fact proposed practical solutions to resolve the problem[2]. However, this *in situ* pH measurement approach now provides definitive evidence that zones of pH mismatch can persist inside of LC columns for a very long time, sometimes all of the way to the column exit.

INSERT FIGURE 5 HERE

Figure 5. Effect of sample/mobile phase pH mismatch on the peak shape for benzylamine analyzed at high pH under reversed phase conditions. In this case, the injected sample solution was buffered at pH 2.4 and the mobile phase at pH 11.5. The sample and mobile phase contained 13 and 23% ACN, respectively. The left two panels (A and B) show pHgrams at the column inlet and outlet, and the right two panels (C and D) show the chromatographic peaks for benzylamine observed under these conditions for injection volumes of 2 or 20 µL. In this case a hue vs. pH calibration curve different from those shown in Figure 3 was used, based on fewer pH buffer standards, but running all of the way up to pH 12.

This journal is © The Royal Society of Chemistry 20xx

Conclusions

A method for *in situ* pH measurement in LC systems based on colorimetric pH indicators was developed and demonstrated under two very different chromatographic conditions. This method differs from the use of ion selective electrodes for pH determination that rely on dedicated instrumentation and are far from ideal from a chromatographic point of view. The colorimetric approach described here can be implemented using a conventional UV-Vis absorbance detector used in most LC systems, and the data analysis involves a simple transformation of absorbance at three wavelengths into a single hue value. We find that the process is sufficiently reproducible to be useful for studying pH changes inside of LC systems on the timescale of chromatographic separations. Under conditions commonly used in 2D-LC we observe that a difference of 9 pH units between the mobile phase and the injected sample zone can persist all of the way to the column exit and significantly affect the separation of ionogenic solutes. This approach should enable a more detailed understanding of the effect of sample and mobile phase pH on chromatographic performance in a wide variety of situations.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

JE, BM, DH, and DS were supported by a grant from the United States National Science Foundation (CHE-1508159). GL was supported by a Thought Leader Award from Agilent Technologies. All of the instrumentation used in this work was provided by Agilent Technologies.

Notes and references

1 L. R. Snyder, J. J. Kirkland and J. W. Dolan, in *Introduction to modern liquid chromatography*, Wiley, Hoboken, N.J, 3rd ed., 2010, pp. 320–321.
2 D. R. Stoll, K. O'Neill and D. C. Harmes, *Journal of Chromatography A*, 2015, **1383**, 25–34.
3 F. Füssl, K. Cook, K. Scheffler, A. Farrell, S. Mittermayr and J. Bones, *Analytical Chemistry*, 2018, **90**, 4669–4676.
4 D. Wen and S. V. Olesik, *Analytical Chemistry*, 2000, **72**, 475–480.
5 C. West, J. Melin, H. Ansouri and M. Mengue Metogo, *Journal of Chromatography A*, 2017, **1492**, 136–143.
6 B. Pearson, R. Kelly and B. Ryan, *Chemistry Undergraduate Publications, Presentations, and Projects*.
7 D. R. Stoll, *LCGC North America*.
8 D. R. Stoll, R. W. Sajulga, B. N. Voigt, E. J. Larson, L. N. Jeong and S. C. Rutan, *Journal of Chromatography A*, 2017, **1523**, 162–172.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

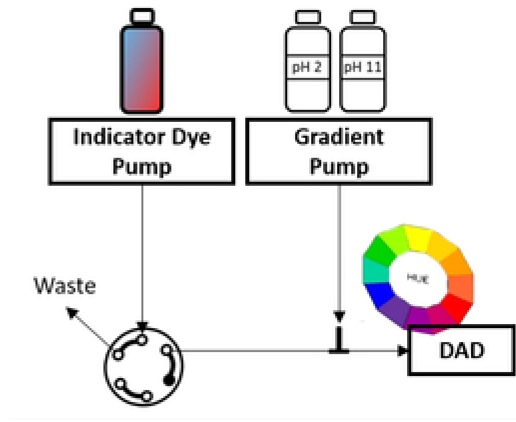


Figure 1. Instrument setup employed for calibration of hue vs. pH.

22x18mm (300 x 300 DPI)

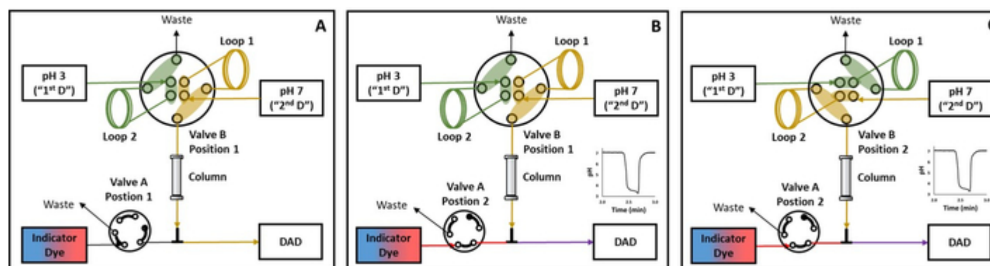
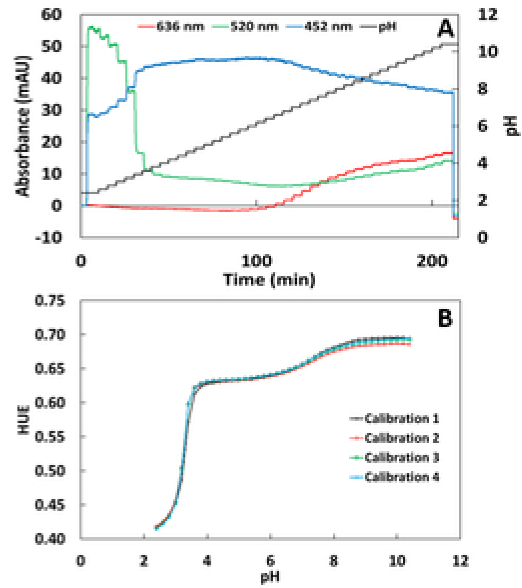


Figure 2. Instrumental setup employed for in situ pH measurement under real LC conditions. (A) Initial condition with the indicator dye diverted to waste; (B) Indicator dye is combined with the mobile phase after the LC column. In this position the contents of Loop 1 are injected and travel through the column. (C) In this position the contents of Loop 2 are injected.

60x16mm (300 x 300 DPI)

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25



26 Figure 3. pH profile used during calibration of hue vs. pH and the resulting absorbance (A) and hue profiles
27 (B) calculated using Eqn. 1.
28
29 21x25mm (300 x 300 DPI)

30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

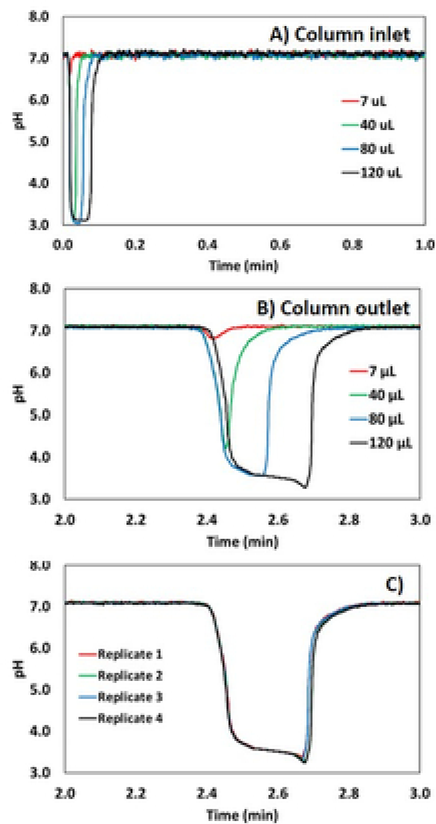


Figure 4. pH profiles showing the pH variation measured at the inlet (A) and outlet (B) of a 90 mm x 4.6 mm i.d. SEC column for different injection volumes, and (C) four replicate measurements at the outlet for 120 µL injections..

20x35mm (300 x 300 DPI)

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

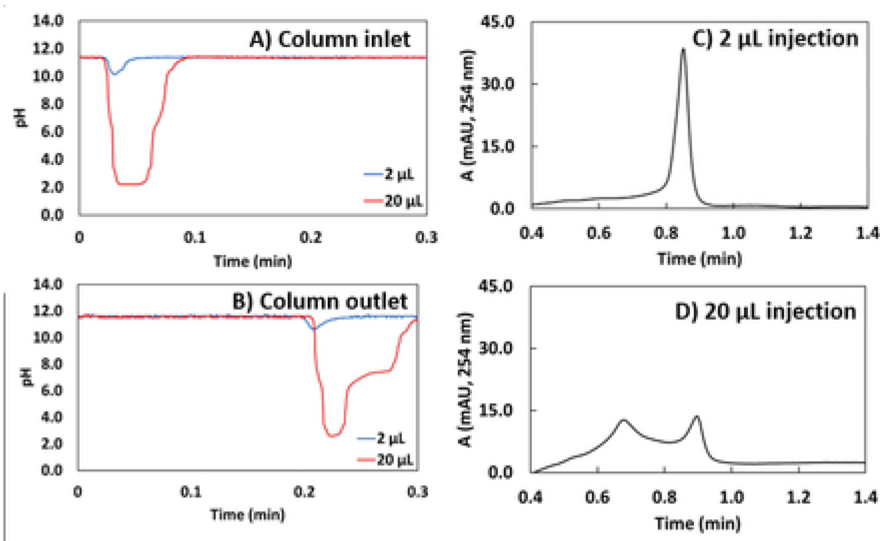
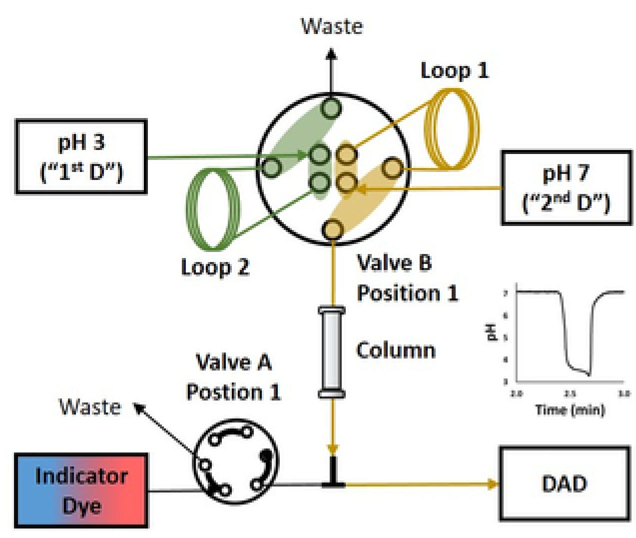


Figure 5. Effect of sample/mobile phase pH mismatch on the peak shape for benzylamine analyzed at high pH under reversed phase conditions. In this case, the injected sample solution was buffered at pH 2.4 and the mobile phase at pH 11.5. The sample and mobile phase contained 13 and 23% ACN, respectively. The left two panels (A and B) show pHgrams at the column inlet and outlet, and the right two panels (C and D) show the chromatographic peaks for benzylamine observed under these conditions for injection volumes of 2 or 20 µL. In this case a hue vs. pH calibration curve different from those shown in Figure 3 was used, based on fewer pH buffer standards, but running all of the way up to pH 12.

37x22mm (300 x 300 DPI)

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60



27x22mm (300 x 300 DPI)