Reduced Solvent Consumption and Labor, and Improved Laboratory Safety, when Performing Rapid HPLC Method Optimization of Buffer pH and Molarity in Reversed Phase Method Development

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Abstract

When optimizing pH in the development of HPLC separations, particularly in gradient mode using constant buffer molarity, the preparation of binary aqueous and organic/aqueous endpoint mobile phases is tedious, error-prone and costly, especially when one considers the labor for each preparation, and reagent and disposal cost of unsuccessful mixtures. A much more favorable approach is to use a quaternary blending pump with water, organic, an acidic modifier and a basic modifier. The pH modifiers are prepared in substantially higher concentration than the desired mobile phase, usually 5-10X greater, and water and organic mix with the needed ratio of acidic and basic modifier. This results in independent control of aqueous/organic ratio, buffer molarity and pH. It dramatically reduces labor and waste when compared to binary gradient experiments and allows many experiments to be done in an unattended fashion. This capability, along with available tools to assist users in designing robust pH control experiments, is described and demonstrated in the work here.

Introduction

Quaternary blending during HPLC method development offers greater programmatic access to mobile phase variables and generates far less unused waste mobile phase than similar experiments with binary blending. Independent control of pH, ionic strength and aqueous to organic ratio are readily available for gradient or isocratic method development.

A mixture of phenolic and acidic aromatic compounds was optimized, using acetate buffer, to develop a gradient (complex analyte mix) or isocratic (simplified analyte mix presented here) method with optimized organic/aqueous ratio, buffer molarity and overall pH. Mobile phase components (A) Water, (B) ACN, (C) 1 M Acetic Acid and (D) 1 M Ammonium Acetate, pH 7, are blended in appropriate ratios to develop a stable separation with adequate resolution of all analytes. Acetate molarity on column was 100 mm, and tests with 50 mm and 200 mm buffer produced comparable results. The experiments described focus on the sole effect of pH on the separation.

Experimental

Agilent 1200 series Infinity LC, consisting of:

G1311B 1260 or G4204A 1290 quaternary pumps with integral vacuum degassers

G1367E1260 or G4226A 1290 high performance autosamplers

G1316C thermostatted column compartment

G4212A or G4212B UV/VIS Diode Array Detector (DAD)

ZORBAX Eclipse Plus C18, 3 x 100 mm, 3.5 um, 40 C

Experimental

A	B	C	D
Water %	ACN %	1M HOAc %	1M AmmOAc %
70	20	10	0
70	20	9	1
70	20	8	2
70	20	7	3
70	20	6	4
70	20	5	5
70	20	4	6
70	20	3	7
70	20	2	8
70	20	1	9
70	20	0	10

Table 1. A set of 11 methods were constructed in the data system to reflect the variables shown above. Based on reference and empirical data, this set of experiments covers a pH range of approximately 3 to 7 and is robust in the fact that the pKa of acetate lies in the middle of the experimental range.

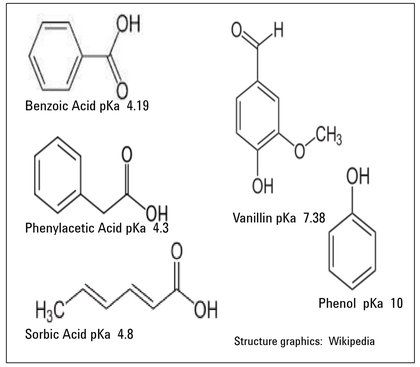

Figure1. The common names, structures and pKa values (various sources) are shown above. Two weakly ionic phenolic compounds are combined with three aromatic organic acids having similar pKa values . These five were selected from a more complex mixture, with a broader range of analyte polarity and pKa values, to facilitate a simple isocratic experiment.

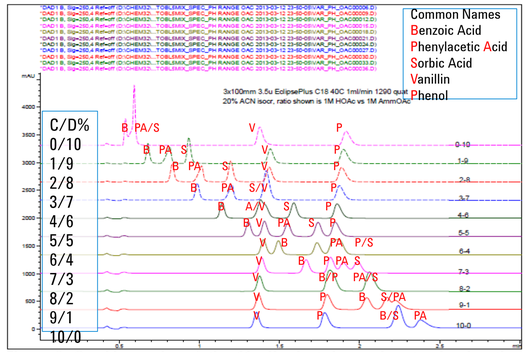

Figure 2. Graphic overlay of the 11 pH-oriented experiments showing the relative position of each of the five analytes and the ratio of modifiers C and D, 1 M Acetic Acid and 1 M Ammonium Acetate, respectively. As might be expected, rather large shifts in retention occur with the three acids and rather small shifts occur with the phenolics. Two separate zones of favorable resolution are discovered in this rapid survey, near 5/5 ratio and again at 2/8 – 1/9, however at the higher pH (5.5-6 in this range) phenylacetic acid begins to show poor peak shape. Vanillin does shift to earlier retention as the mobile phase pH nears its' pKa It also experiences a spectral shift at the higher pH, making library identification of the compound difficult unless high and low pH reference spectra have been loaded into the library. Conditions: 1 ml/min, 20% ACN, 70% water, (C) 1 M HOAc, (D) 1 M AmmOAc

Results and Discussion

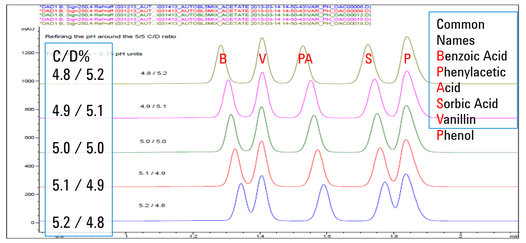

Figure 4. Refinement of separation pH. After the initial survey of the full pH range was completed, the 5/5 condition was refined by incrementing the composition in 0.1% units to see where the better resolution might be found. Baseline resolution is adequate at 5/5 and a newer column or smaller particle size might be a simple approach, however we found that a slight increase in the pH would also be very favorable. The estimated range of pH in these five experiments is 0.15 and the trend of the data suggest that a slightly higher pH, perhaps with 5.3/4.7 as the ratio, might be slightly better. It might be appropriate at this point, though, to evaluate small changes in the ACN % or column temperature, both of which can be done free of hands-on contact with the instrument.

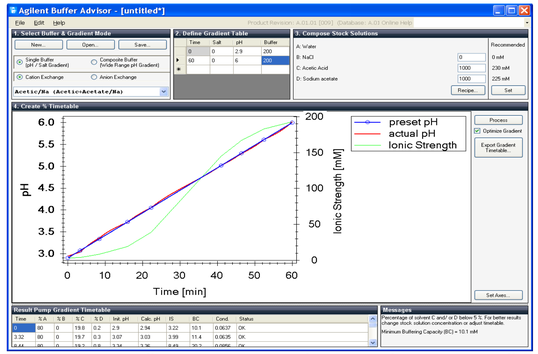

Figure 4. Agilent Buffer Advisor add-on software tool for assessing buffer performance. Buffer advisor, developed by Agilent in collaboration with Charles University in Prague, laboratory of Bob Gas. The original calculations are based on PeakMaster 5. It was developed primarily for the interest of protein chemists designing ion exchange pH, ionic strength and combined pH/ionic strength gradients for protein analysis and purification. It contains a broad selection of buffer choices for various pH ranges, using single or blended buffers for effective pH control at minimum ionic strength. It is readily adaptable to many small molecule reversed phase experiments, and demand for this will result in new buffers being introduced as interest grows. In the example above, we asked for an acetate gradient from pH 2.9 to 6.0 and a suitable multistep time program was devised. We can re-plot the time table as % D (alkaline buffer) vs. pH to see where the critical areas of the buffer might lie.

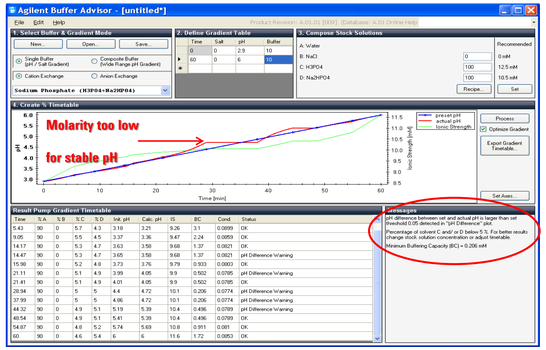

Figure 5. Phosphate gradient over the same pH 2.9 – 6 range. At a contrived low 10 mm phosphate molarity the software deduces that we are too far from the pKa values of the buffer for effective pH control. This is shown in the unstable graphic plot and by various warning messages in the time table. Increasing the phosphate to 50 mm, an easy task with the higher molarity stocks that were prepared, resulted in a new table with favorable results regarding stability.

Result Pump Gradient Timetable

Time	% A	% B	% C	% D	Init. pH	Calc. pH	IS	BC	Cond.	Status
0	90	0	10	0	2.9	2.88	1.37	6.28	0.0529	OK
9.05	90	0	9.6	0.4	3.37	3.39	4.44	10.9	0.051	OK
16.88	90	0	9	1	3.77	3.76	10.2	22	0.091	OK
23.22	90	0	8	2	4.1	4.09	20.1	38.2	0.166	OK
41.61	90	0	2.8	7.2	5.05	5.05	72	47.2	0.544	OK
47.04	90	0	1.7	8.3	5.33	5.32	83	32.8	0.62	OK
52.76	90	0	0.9	9.1	5.63	5.63	91	19	0.675	OK
60	90	0	0.4	9.6	6	6	96	8.86	0.709	OK

Table 2 Values calculated for a pH gradient with acetate buffer. The table shows composition requirements, buffer capacity and indicates if the buffering conditions are adequate to meet the required fit accuracy of 0.05 pH units.

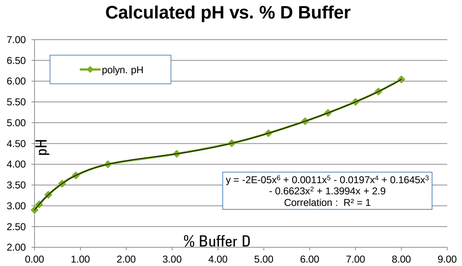

Figure 6. Plot of incremental pH values for an acetate buffer. By taking the data from the Buffer Advisor table and plotting it in Excel® it is possible to use the polynomial fit equation to iteratively calculate incremental pH values based on % D buffer. While it is also possible to derive a single number in the software, this approach allows a view of the buffer performance across the desired range. With this reference data it is straightforward to set up an optimized set of pH experiments that focus equal attention on critical areas of buffer performance.

Conclusion

Automatic buffer blending can save considerable time and solvent cost. In the examples discussed here, 3 buffer molarities and 11 pH values were evaluated using four prepared bottles, none of which required pH adjustment, to prepare 11 pH adjusted stock buffers and 66 different mobile phases for constant molarity for the tested organic/aqueous separations. Substantial labor and money were saved as a result, and misleading results due to human error in buffer preparation were avoided. When final conditions are optimized, it is then possible to prepare binary buffer/organic mixtures if method transfer to binary gradient systems is anticipated.

References: Agilent publications 5991-0565EN and 5990-9629EN, and user manual G5617-90000