Two Dimensional HPLC using a High pH Stable Superficially Porous Column

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Agilent Technologies
(P-W-1923) , HPLC 2014. New Orleans, USA

Introduction

The analysis of impurities is an important part of the development process in chemical industries. Due to the fact that impurities can be structurally similar to the main compound it can often be difficult to separate them chromatographically. A solution to this challenge can be the use of the Agilent 1290 Infinity 2D-LC. In a heart cutting experiment, the peak of interest is sampled from the first column and eluted into a loop capillary. This is then transferred by a switching valve to a second dimension column. In this way co-eluting peaks can be resolved. In this work the importance of the second dimension stationary phase is demonstrated.

Silica HPLC columns have a useful pH range between 2 and 9. The lifetime of these columns can be compromised by exposure to phosphate and bicarbonate buffers at elevated temperature or higher pH. In this regard, there are very limited options in superficially porous particles compared to totally porous particles. Unlike conventional superficially porous particles, Poroshell HPH columns are designed to be stable over a wide pH range. Using these columns, chromatographers can enjoy the ultrahigh efficiency of these superficially porous particles using phosphate or bicarbonate mobile phases making more selectivity options to facilitate method development.

In this work, we present a column packed with superficially porous particles that are stable in a wider pH range. Different selectivity can be achieved on the Poroshell HPH C18 column for various applications at low, and high pH. After an initial separation at high pH, a second dimension gradient is chosen, using a low pH buffer. The peak is sampled using a counter-current capillary filling, (Fill direction and de-fill direction are opposed, compressing the peak). The peak is sampled using a precise time sampling, so reproducible chromatography on the first dimension is critical. The heart cutting experiment requires no additional software for visualization of the data.

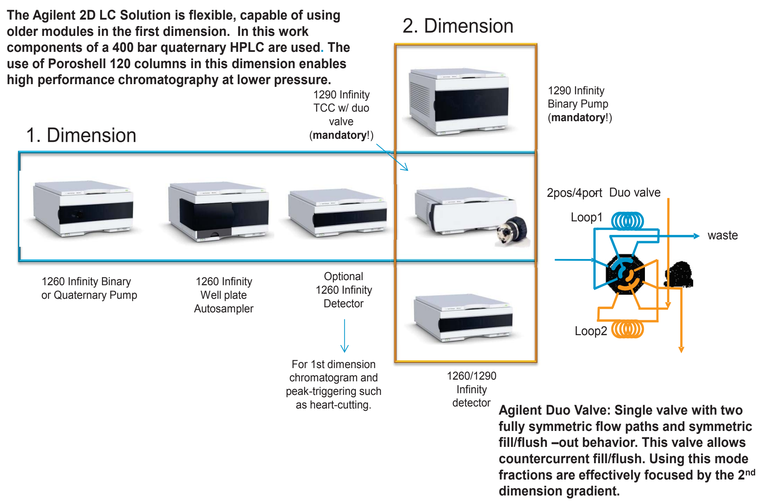
Experimental

The Agilent 1290 Infinity 2D-LC consisted of the following modules:

- Agilent G1329A 1200 Auto-sampler
- Agilent G1311A 1200 series Quaternary Pump (400 bar)
- Agilent G1315C 1200 Diode Array Detector (1st dimension detector)
- Agilent G4220A 1290 Infinity Pump
- Agilent G1316C 1290 Infinity Thermostatted Column Compartment (TCC) with a 2-position/4-port –duo valve (G4236A) for 2D-LC
- Agilent G4212A Infinity Diode Array Detector (2nd dimension detector).
- Open Lab version C 1.04 with 2D control software.

The first three components were recycled from an older system and were integrated with the 1290 Infinity Pump, 1290 Infinity DAD, 1290 Infinity TCC and valve by upgrading firmware.

Figure 1 Agilent 2DLC Solution



75 Acidic, basic and neutral compounds were injected individually or as a mixture on a 2.1 x 50 mm Poroshell HPH-C18 or Poroshell 120 EC-C18 column on an Agilent 1260. The Agilent 1260 consisted of the following modules:

- Agilent G1367D Hi-ALS +
- Agilent G1312B Binary Pump (600 bar)
- G1315C Infinity Thermostatted Column Compartment (TCC)
- Agilent G4212A Infinity Diode Array Detector
- Open Lab version C 1.05

Gradient: A: 10 mM Buffer (Ammonium Formate pH 3, Ammonium Acetate pH 4.8, Ammonium Bicarbonate pH 10) B: Organic (MeOH or ACN) Gradient: 5% B at t_0, ramp to 95% B in 6 min., hold at 95% B for 1 min. Flow Rate 0.42 ml/min 25 C. The data was transferred to Excel.

Results and Discussion

The stability of Poroshell HPH is discussed further in Poster P-M-0204. In that work it is demonstrated that the column is stable at pH 10 for 40,000 column volumes.

In Figure 2, the retention time of individual compounds with the pH 3 gradient are plotted against pH 4.8 on a Poroshell 120 EC-C18 or pH 10 on a Poroshell 120 HPH C18 gradient using Methanol as an organic modifier. In the top figure plotting pH 3 and pH 4.8 on the Poroshell 120 EC-C18 yields highly correlated data, most of the data falls on the trend line with an R^2 value of 0.94 indicating very little difference between the two conditions., In the lower figure the data is more scattered, the R^2 value of 0.46 indicates the data is non correlated. Figure 3 shows a similar plot with Acetonitrile as the organic modifier. In the top figure plotting pH 3 and pH 4.8 we observe a highly correlated data set, not as correlated as the methanol set with an R^2 value of 0.79. In the lower plot (pH 3 vs. pH 10) the data is more scattered with an R2 value of 0.35. This data indicates that pH can be used to decrease the correlation of a separation. In this work the decreasing R^2 values indicate that the separations become more orthogonal as the pH differences are greater and will yield very different separations.

Figure 2 Correlation in MeOH

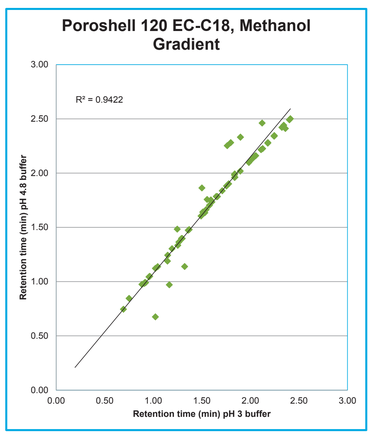
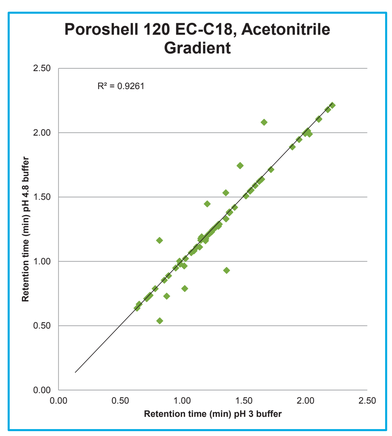
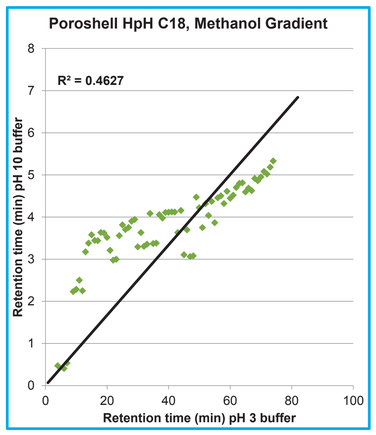
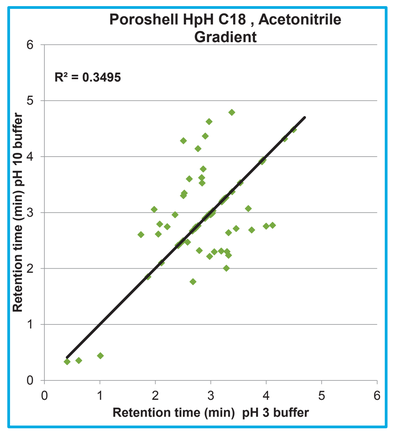


Figure 3 Correlation in MeCN

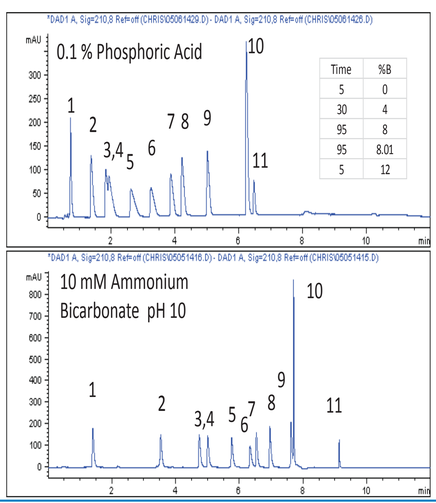






The benefit of high pH orthogality is shown in a real example. Amphetamines are basic compounds. These compounds are better retained at high pH and have better peak shape using Ammonium Bicarbonate Buffer. Increased retention can be an advantage when injecting large volumes. Better peak shape is an advantage in minimizing overlapped peaks (selectivity) and increased sensitivity (sharper peaks are taller)

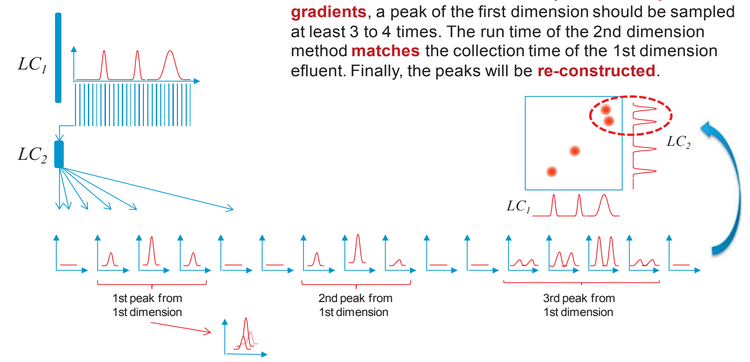
Figure 4 Separation amphetamine mix at low pH 0.1% Phosphoric Acid and pH 10 ammonium bicarbonate.



Results and Discussion

Comprehensive 2D-LC (LCxLC):

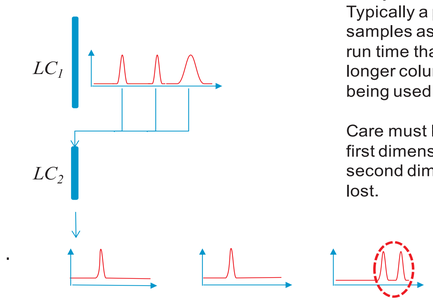
The **complete effluent** of the first column will be injected to the second column and will be analyzed with **very fast gradients**, a peak of the first dimension should be sampled at least 3 to 4 times. The run time of the 2nd dimension method **matches** the collection time of the 1st dimension effluent. Finally, the peaks will be **re-constructed**.



Heart-cutting 2D-LC (LC-LC):

Only **parts** of the effluent of the first column – the peaks eluted from the 1st dimension column - will be injected to the second column.
Typically a peak from the first dimension will be samples as a whole and a gradient with a **longer** run time than the collection time will be used. Also longer columns with higher seperation efficiency are being used in as 2nd dimension column.

Care must be taken if peaks are eluting from the first dimension column when a gradient on the second dimension is still running – this peak will be lost.



Using a first dimension separation at high pH an incomplete separation is generated using a 2.1 x 100 mm column. Between 3.2 and 3.3 minutes a large peak with a smaller impurity peak are found. By heart cutting the smaller eluted peak, it is separated fully from the major compound and a third compound is found. A small piece of the adjacent major piece is captured and separated as well. The software allows easy heart cutting, in this case by adjusting time. The two pumps are controlled in the same screen allowing quick optimization of the separation

Gradient Conditions 1st Dimension A: High pH, B: Acetonitrile

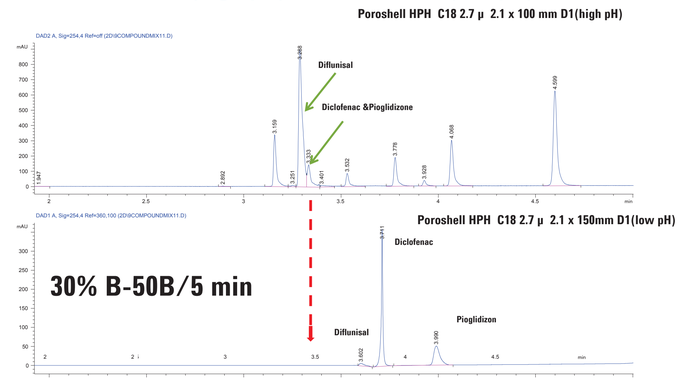
Time	% B	Flow rate (ml/min)
0	10	0.5
1	90	0.5
5	90	0.5

Gradient Conditions 2nd Dimension A: Low pH, B: Acetonitrile

Time	% B	Flow rate (ml/min)
0	30	1
5	50	1
6	50	1

50 mm

2D Separation Heart-cut
Poroshell HPH D1(high pH) Poroshell HPH (low pH) D2



Conclusions

- **Poroshell HPH is stable at pH 10 Bicarbonate Buffer**
- **Selectivity using pH can help generate highly non correlated separations**
- **Acidic compounds retain less at high pH than at low pH. Basic compounds are retained more at high pH than at low pH**
- **In 2D-LC separations it is possible to use pH to generate orthogonal gradients using the same column phase**

Thank you to Professor Dwight Stoll and David C. Harmes at Gustavus Augustus College for the Amphetamine Data.