‘Green’ HPLC is Better and Easier Than You Think

by Joe Pesek, San Jose University and Bill Ciccone, Microsolv
Joseph.pesek@sjsu.edu bill.ciccone@mtc-usa.com

Being environmentally responsible is easy with small changes to our daily routine that can really add up. Each and everyone of us should look to make small changes in our daily habits and work flow to contribute to a cleaner and healthier environment. For chromatography laboratories, instituting modern and recent technology can be very green and contribute to the overall green effort, more than one might think. For example, using Cogent TYPE-C™ silica based HPLC columns can easily save costs on the use of solvents and disposal of contaminated HPLC water. Also, the longer lifetimes of the TYPE-C™ columns mean less steel to be disposed of; smaller sample sizes; faster equilibration times and less sample preparation, all helping a laboratory’s budget. Not only is being green responsible, it also can and should help your lab and company meet financial and throughput goals when properly instituted.

The increasing cost of most organic solvents as well as the expense of disposing or recycling them has been a major concern of all laboratories using chromatographic methods. In addition, the possibility of not being able to acquire sufficient amounts of mobile phase solvents at reasonable prices (such as the recent supply crisis for acetonitrile) has become a major factor in the drive for laboratories to adopt a more green approach, both for existing analyses and in the development of new methods. This review highlights some of the unique chromatographic properties of TYPE-C™ columns (based on silica hydride (Si-H))[1-15] that can help laboratories solve challenging analytical problems using methods that are consistent with minimising the amount and type of organic solvent used.

One of the unique features of the TYPE-C™ phases that has a dramatic impact on developing green methods is the rapid equilibration times between gradient runs. Since the analysis of most complex samples utilise gradient elution, this property can lead to substantial savings in solvent usage. An illustration of this property is shown in Figure 1 where a separation of polycyclic aromatic hydrocarbons (PAHs) is done on a TYPE-C C18 stationary phase. As shown in the figure, there is no detectable difference between the chromatograms using a column equilibration time of 25 min (31 column volumes) or 1 min (1.25 column volumes). Equilibration times of 5 to 10 column volumes are frequently recommended for many traditional reversed-phase materials.

Another aspect of TYPE-C columns is that every phase has the capability of retaining both polar and nonpolar compounds depending on the mobile phase conditions selected. At high aqueous content, reversed-phase properties are dominant while at high organic content normal phase retention is obtained. This combination has been designated aqueous normal phase (ANP). It is often confused with hydrophilic interaction liquid chromatography (HILIC) because of the common feature that both methods can retain polar compounds with a high organic content mobile phase. However, HILIC phases lack the ability to provide strong retention in the reversed-phase domain and it has been reported that HILIC can suffer from lack of precision after gradients.

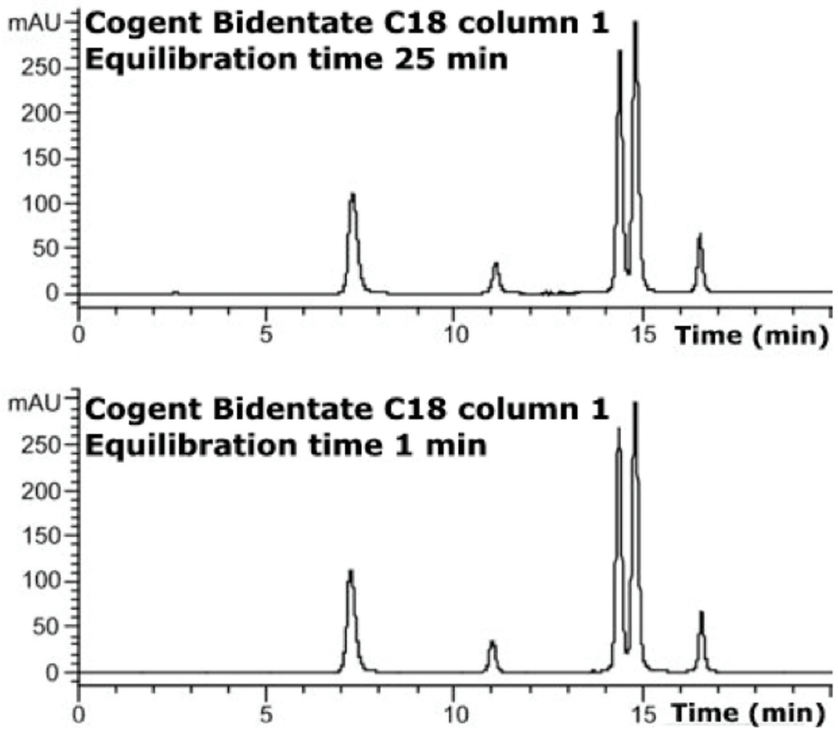
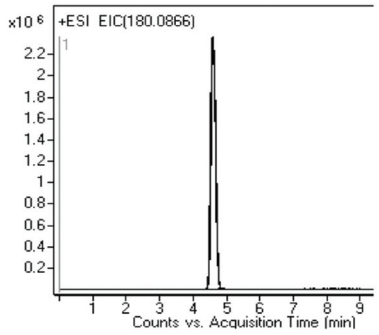


Figure 1. Comparison of the chromatograms for the separation of benzene, naphthalene, phenanthrene, anthracene and pyrene using a column equilibration time of 25 min (top) and 1 min (bottom). Mobile phase: Solvent A, water; Solvent B, acetonitrle. Column: Cogent BD C18, 4.6 x 75 mm. Flow rate = 1 mL/min. Gradient: 0-3 min ACN/water (50:50); 3-18 min to 100% ACN; 18-23 min 100% ACN; Equilibration to 50:50 ACN/water



Dh 2.1 x 150 mm, 0.4 mL/min
Glucosamine, 180.0866 M/Z [M+H]+
A: DI water+0.1% FA
B: ACN+0.1% FA

Gradient: Glum-Gr1

Time (min)	%B	Flow rate (mL/min)
0.00	80.0	0.400
5.00	30.0	0.400
7.00	30.0	0.400
8.00	80.0	0.400

10 overlaid injections

Figure 2. Ten overlaid injections of glucosamine on the DH stationary phase with an equilibration time of one column volume between injections. Column: Cogent Diamond Hydride, 2.1 x 150 mm. Mobile Phase: Solvent A, DI water + 0.1% formic acid; Solvent B, acetonitrile = 0.1% formic acid. Flow rate = 0.4 mL/min. Gradient: 0 min 80% B; 0-5 min to 30% B; 5-7 min 30% B; 7-8 min to 80% B. Detection by Agilent 6210 TOF MS at m/z 180.0866.

More importantly, the rapid equilibration of TYPE-C materials observed in the reversed-phase is also present when operating under high organic content for the retention of polar compounds. An example of this property is shown in Figure 2 for the retention of glucosamine on the Diamond Hydride (DH) stationary phase. As can be seen there is no change in the retention time for the analyte with such a short equilibration time. This is in contrast to many HILIC applications which often require 20 or more column volumes between runs or for ion-exchange applications that may need as much as 100 column volumes for equilibration. All TYPE-C applications for polar retention with a normal phase mechanism require no more 2 to 3 column volumes between injections. The fast equilibration observed in both the reversed and normal phase modes is thought to be due to the fact that the more hydrophobic surface of the TYPE-C materials are based on silica hydride and have a minimal amount of silanols. Ordinary silica-based stationary phases have a layer of water on the surface due to the presence of silanols that are not present on the hydride surface. When mobile phase conditions change, this layer of water is slow to re-equilibrate in comparison to mimimal amount of water associated with the hydride surface.

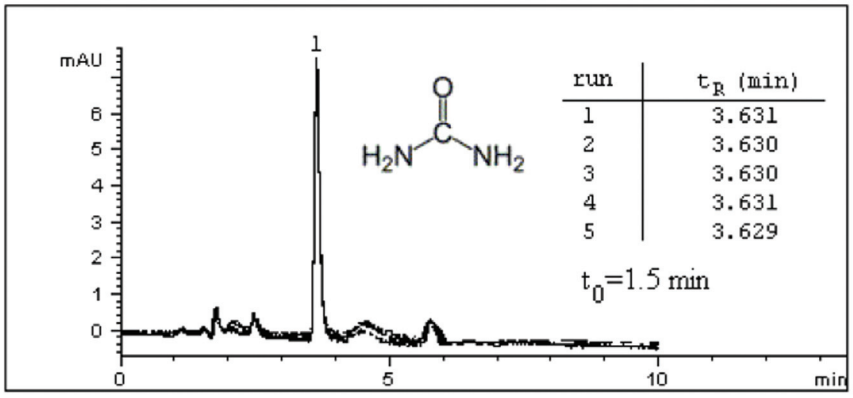
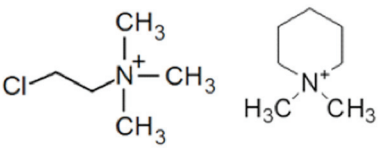


Figure 3: Analysis of urea on a Cogent Bidentate C18 column with a 100% aqueous mobile phase. Column 4.6 x 150 mm. Flow rate 1 mL/min. UV detection at 210nm. Sample: 1 mg/mL in DI water.

A consequence of the dual retention capabilities of the TYPE-C phases is the possibility of more efficiently developing new analytical methods. This can be important when the samples contain analytes with a wide range of polarities. In such a case, it may not be obvious whether a reversed-phase or an aqueous normal phase method will provide the best selectivity in the shortest possible analysis time. However, running a single gradient in each direction (common reversed phase from high water to high organic and ANP from high organic to high water) it is possible to determine which approach is most likely to succeed. Ultimately this approach will lead to a shorter analysis time and fewer experiments needed to design the best analytical protocol thus resulting in considerable savings in solvent costs and time. This development process is done on a single column rather than two in order to determine the retention mode that is most suitable for a particular analysis which may provide an additional cost saving.

Another approach that minimises the use of organic solvents for some polar compounds is an RP method utilising 100% water as the mobile phase. An example of such an application is the analysis of urea on the Cogent™ Bidentate C18 column shown in Figure 3. Significant retention is obtained in this method and the ruggedness of the analysis is illustrated in the figure that consists of an overlay of five separate injections. The retention times are so close that the overlay appears to be a single chromatogram. The actual retention times for the five runs are shown in the table inset. Many traditional RP columns have poor reproducibility in high aqueous mobile phases due to the unstable configuration of the hydrophobic bonded material in a very polar environment.

One of the advantageous features of the TYPE-C phases in the ANP mode is that retention of polar compounds can occur at higher percentages of water in the mobile phase than with many HILIC applications. Figure 4 shows the separation of the two plant growth regulators chlormequat and mepiquat on the DH column. In this case the composition of the mobile phase is 70:30 water/acetonitrile. While this is unusual there



1. Chlormequat 2. Mepiquat

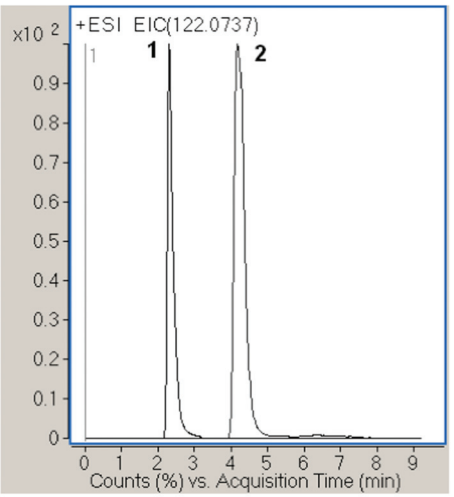


Figure 4. Separation of chlormequat and mepiquat on the DH column in the normal phase mode using a 70:30 water/acetonitrile mobile phase. Column: 2.1 x 150mm. Mobile Phase: Solvent A, DI water + 20 mM ammonium acetate adjusted to pH 3.3 with formic acid; Solvent B, acetonitrile. Detection by Agilent 6210 TOF MS at m/z 122.0737 (chlomequat) and m/z 114,1277 (mepiquat).

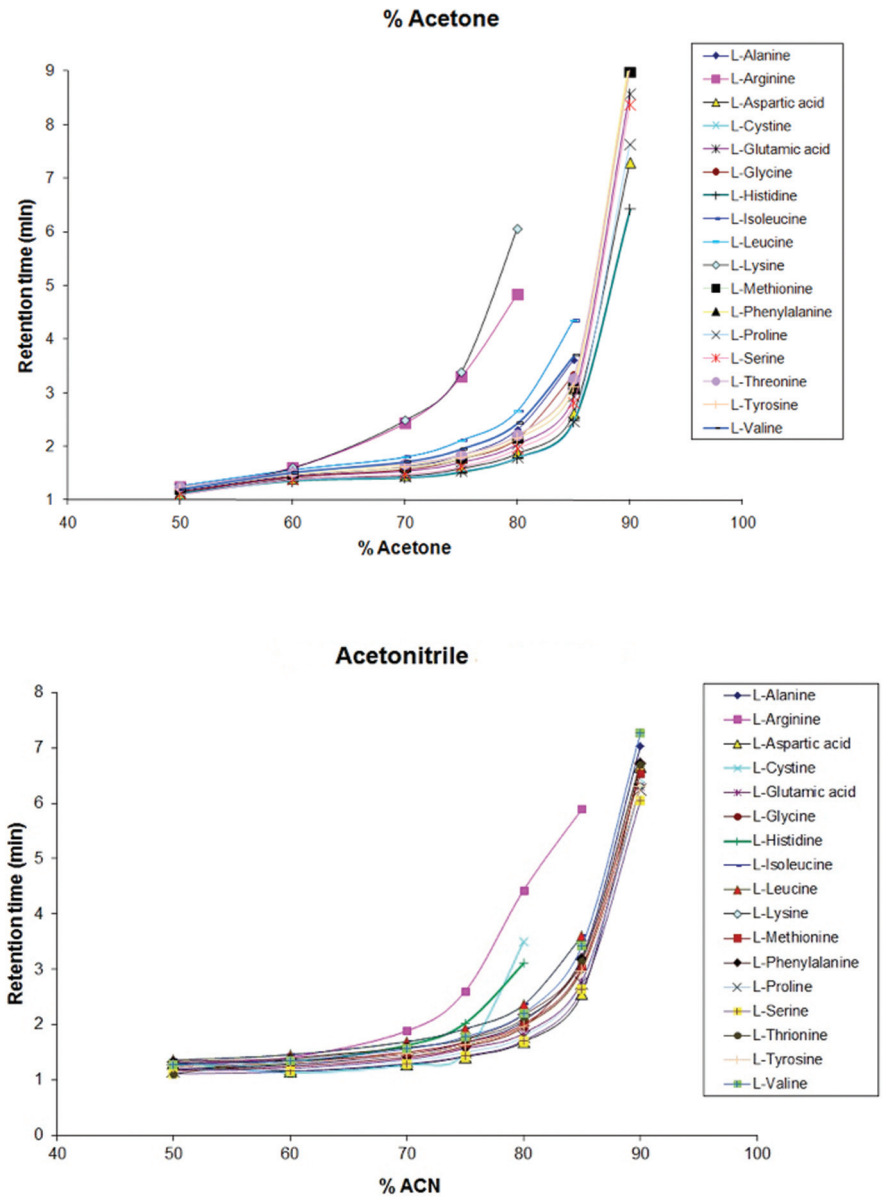


Figure 5: Retention map for 17 amino acids using acetone (top) and acetonitrile (bottom) as the organic component in the mobile phase. Column: Cogent Diamond Hydride, 2.1 x 150mm. Mobile phase: Solvent A: DI water + 0.1% formic acid; Sovent B; acetonitrile + 0.1% formic acid. Gradient: 0-5 min 100% B; 5-6 min to 90%B; 6-7 min 90% B; 7-9 min to 70% B; 9-10 min 70% B. Detection by Agilent 6210 TOF MS at appropriate m/z for each amino acid.

are many reported applications where the amount of acetonitrile required for adequate retention is only 60% while in most HILIC methods a minimum of 80% organic is needed. For example, the basic drug tobramycin can be retained on the TYPE-C cholesterol phase at this amount of acetonitrile in the mobile phase. For laboratories doing numerous analyses of drugs or metabolites, this difference can result in significantly less consumption of organic solvents that need to be recycled or incinerated.

Another option that will result in a greener analysis in the ANP mode is to switch from acetonitrile to the more environmentally friendly solvent acetone (easier to recover and reuse). Acetone has similar retention capabilities to acetonitrile, although in some instances there can be differences in selectivity. This effect is demonstrated in Figure 5 (top) that shows the retention map (retention time vs. % organic solvent in the mobile phase) for a series of common amino acids utilising acetone as the organic solvent while Figure 5 (bottom) is the same data utilising acetonitrile. Measurable retention begins in the range of 60-75% acetone in the mobile phase depending on the amino acid analysed. While acetone and acetonitrile can be used interchangeably when mass spectrometry is used for detection, this is not true for a UV detector. Significant absorption by acetone in the low wavelength region of the UV spectrum can significantly diminish sensitivity or eliminate detection for many compounds.

Sample preparation can often be a time-consuming process that involves the use of various organic solvents in extraction and concentration procedures. Many applications utilising TYPE-C columns have minimal or no sample preparation procedures for the analysis. The most common types of samples requiring a preparative step are those involving physiological matrices such as blood, plasma urine or saliva. For these types of analyses the only step needed is to precipitate out the proteins by adding a solvent such as acetonitrile and then to filter the resulting solution. After this, the sample can usually be injected directly for chromatographic analysis on a TYPE-C column. Other columns sometimes require additional sample preparation steps but more often are easily contaminated by the physiological matrix, thus requiring frequent cleaning steps or have limited lifetime. With Type-C phases, changing the A solvent from 100 % aqueous to 50:50 MeOH/water or 50:50 ispropanol/water keeps the column keep for extended periods of time and when cleaning is required it is often much faster resulting in a longer column lifetime.

An example of such a method is shown in Figure 6 for the determination of various polar metabolites in human urine using the DH column. In this case five metabolites were assayed after the proteins were precipitated by the simple acetonitrile procedure.

Another area where TYPE-C phases have the potential to provide selectivity enhancement in a format that would result in less consumption of organic solvents is in the separation of complex mixtures of peptides such as those found in proteomic analyses. Typically a complete proteomic analysis after digestion of the protein involves a two-dimensional separation, one in reversed phase and the other in ion-exchange or sometimes HILIC so that retention is obtained for both hydrophobic and hydrophilic peptides. This requires two separate chromatographic runs as well as equilibration times for the two columns. However, the Diamond Hydride column has been shown to have the ability to retain both polar and nonpolar peptides using a single run in the ANP mode[7] Figure 7 shows an example of such a separation with a series of peptides that span the range of strongly hydrophobic (Peak 7, calculated hydrophobicity = 11.34)[16] to very hydrophilic (Peak 1, calculated hydrophobicity = -2.31)[16]. While this represents a non-optimised

method on a capillary column, it demonstrates the potential for this specific TYPE-C stationary phase to provide a complete proteomic analysis in a single chromatographic run.

TYPE-C stationary phases have demonstrated a remarkable ruggedness in comparison to other comparable commercial columns. For example, the reversed-phase Bidentate C8 and Bidentate C18 columns are routinely used by many analysts for thousands of column volumes with no deterioration in performance. This feature is a result of the direct silicon-carbon bond that results in a stable attachment of the organic moiety to the surface. For the analysis of polar compounds, the DH can be compared to a wide variety of HILIC phases such as amino, cyano and bare silica. All of these materials have limited lifetimes under a wide variety of mobile phase conditions that are necessary for the challenging samples encountered when analysing hydrophilic compounds. These samples are often present in complex matrices such as physiological fluids that can easily foul traditional column phases due to the large amount of endogenous components present or as a result of deterioration of the column due to the mobile phase. The DH is much more resistant to both degradation and fouling due to the hydride surface. If the latter occurs, it is readily cleaned with water/methanol or water/isopropanol washing. Even the UDC cholesterol TYPE-C phase used for the analysis of steroids provides a shape selectivity mechanism that has excellent stability in comparison to many other specialty columns. These features result in less disposal of both column hardware and the phases themselves; another contribution to making a greener analytical lab.

Finally, the users of TYPE-C stationary phases are moving rapidly to smaller diameter columns, especially for use with mass spectrometry. Column diameters of 3.0, 2.1 and 1.0mm are becoming more common in the analytical lab. This change reduces the solvent consumption by as much as 40% at comparable linear flow rates to the normal 4.6mm column. Reducing the particle size will also result in less solvent consumption and this option will be available soon for TYPE-C columns.

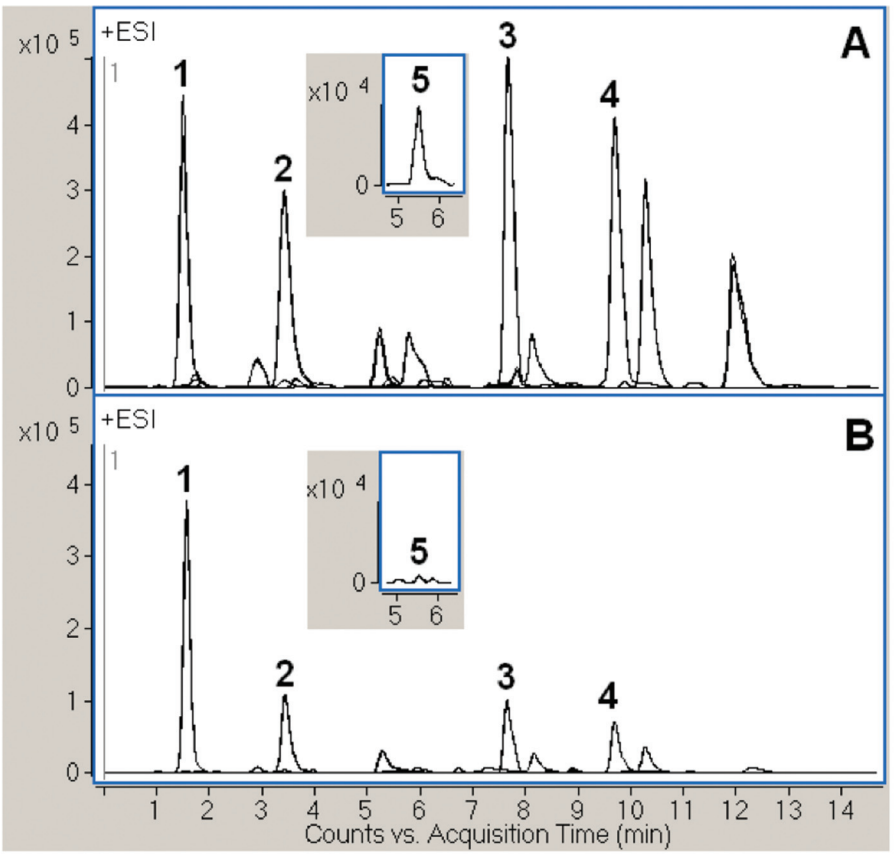


Figure 6: Composite EICs for various metabolites in human urine from two donors in the positive ion mode on the DH column. 1 = glucose, 2 = methionine, 3 = leucine; 4 = tryptophan, 5 = sucrose. Column: 2.1 x 150 mm. Flow rate = 0.4 mL/min. Mobile Phase, A = DI water + 0.1% formic acid; B = 90:10 ACN/DI water + 0.1% ammonium acetate. Gradient: 0.0 to 1.00 min 98% B; 1.00 to 16.00 min to 20% B. Detection by Agilent 6210 TOF MS at appropriate m/z for each analyte.

Conclusions

For a variety of reasons such as faster equilibration, the ability to reduce the amount of organic solvent needed for a particular type of analysis, the elimination of cumbersome and often wasteful sample preparation steps and longer column lifetime, TYPE-C stationary phases provide an attractive means for making many analytical laboratories greener. Many labs have already realised these advantages and have implemented them as part of their method development. Continued expansion of TYPE-C stationary phases and column formats will make their use even more desirable in the future.

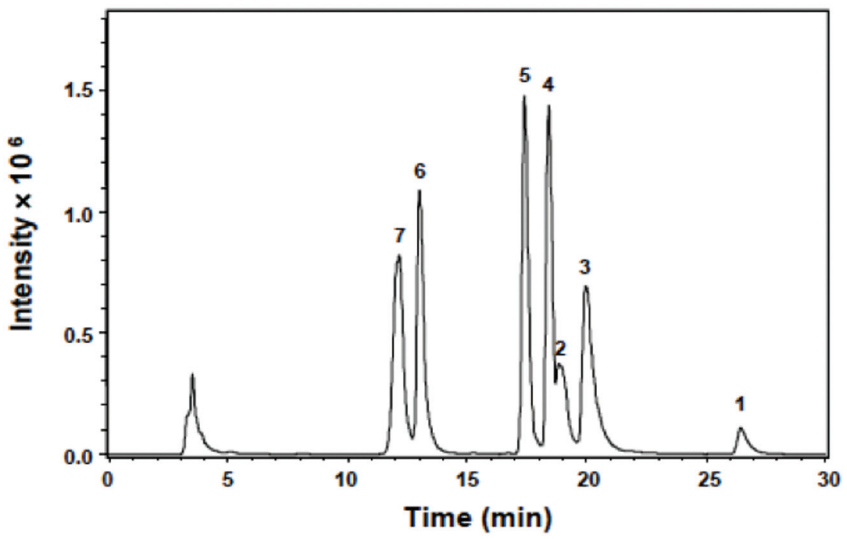


Figure 7: Separation of a peptide mixture on the DH column in the ANP mode. Elution order is from the most hydrophobic (7) to the most hydrophilic (1) peptide. Column: 0.3 x 150 mm. Mobile Phase: Solvent A, DI water + 0.5% formic acid; Solvent B, acetonitrile + 0.1% formic acid. Gradient: 0.0-5.0 min 90% B; 5.0-10.0 min to 70% B; 10.0-20.0 min to 60% B; 20.0-20.1 min to 30% B; 20.1-30.0 min hold 30% B. Detection by MS at appropriate m/z value for each peptide. Peaks: 1= H-Tyr-Tyr-Tyr-Tyr-Tyr-OH; 2 = H-Tyr-Gly-Gly-Phe-Leu-OH; 3 = H-Trp-His-Trp-Leu-Gln-Leu-OH; 4 = H-Tyr-Ile-Gly-Ser-Arg-OH; 5 = H-Lys-Gln-Ala-Gly-Asp-Val-OH; 6 = H-Arg-Gly-Asp-OH 7 = H-Gly-Arg-Ala-Asp-Ser-Pro-Lys-OH.

References

1. J.J. Pesek, M.T. Matyska, S.M. Fischer, T.R.Sana, J. Chromatogr. A, 1204 (2008) 48-55.

2. J.J. Pesek, M.T. Matyska, M.T.W. Hearn, R.I. Boysen, J. Chromatogr. A, 1216, (2009) 1140-1146.

3. J.J. Pesek, M.T. Matyska, J.A. Loo, S.M. Fischer, T.R. Sana, J. Sep. Sci., 32 (2009) 2200-2208.

4. J.J. Pesek, M.T. Matyska, J. Sep. Sci., 32 (2009) 3999-4011.

5. J.J. Pesek, M.T. Matyska, J. Duley, M. Zamzami, S.M. Fischer, J. Sep. Sci.,33 (2010) 930-938.

6. J.J. Pesek and M.T. Matyska, in Hydrophilic Interaction Chromatography (HILIC) and Advanced Applications, P.G. Wang, W. He, eds., CRC Press, Boca Raton, FL, USA, 2011, pp 1-26.

7. R.I. Boysen, Y. Yang, J. Chowdhury, M.T. Matyska, J.J. Pesek, M.T.W. Hearn, J. Chromatogr. A,. 1218 (2011) 8021-8026.

8. J.J. Pesek, M.T. Matyska, Bioanalysis, 4 (2012) 845-853.

9. J.J. Pesek, M.T. Matyska, J.E. Young, Separation Science, 4 (2012) 11-15.

10. J.J. Pesek, M.T. Matyska, R.I. Boysen, Y. Yang, M.T.W. Hearn, Trends in Analytical Chemistry (2012), doi: http://dx.doi.org/10.1016/j.trac.2012.09.016

11. J. Soukup, P. Jandera, J. Chromatogr. A , 1228 (2012) 125-134.

12. J. Soukup, P. Jandera, J. Chromatogr. A, 1245 (2012) 98-108.

13. D.L. Callahan, D. De Souza, A. Bacic, U. Roessner, J. Sep. Sci. 32 (2009) 2273-2278.

14. L.P.S. de Carvalho, H. Zhao, C.E. Dickinson, N.M. Arango, C.D. Lima, S.M. Fischer, O. Ouerfelli, C. Nathan, K.-Y. Rhee, Chem. Biol. 17 (2010) 323-327.

15. J. Marrero, K.Y. Rhee, D. Schnappinger, K. Pethe, S. Ehrt, Proc. Natl. Acad. Sci. USA

107 (2010) 9819-9824.

16. M.C.J. Wilce, M.I. Aguilar, M.T.W. Hearn, Anal. Chem. 67 (1995) 1210.-1216