

The Use Of A Novel Type-C Silica To Separate Amino Acids By Aqueous Normal Phase (ANP) With Electrospray (ESI) Detection

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Introduction

The development of new separation methods for hydrophilic compounds is being spurred by the interest in metabolomics. Many of the endogenous metabolites are very polar and are un-retained on standard reverse phase HPLC columns even in a 100% aqueous mobile phase. Chromatographers have tried many approaches to solving this problem such as: 1) chemical derivatization, 2) ion pairing chromatography, 3) normal phase chromatography and 4) HILIC (hydrophilic interaction chromatography). Of these, only HILIC offers the possibility of addressing the chemical diversity of the hydrophilic metabolites. The available HILIC materials have the following limitations: 1) slow re-equilibration, 2) non-reproducible chromatography and 3) require high levels of salts or buffers. Type-C silica is an ANP stationary phase with HILIC-like retention but without these disadvantages.

Amino acids were used in this study because they are an important class of metabolites and present interesting separation challenges. They were used in this study to demonstrate some of the properties of a silica hydride surface operated in the aqueous normal phase (ANP) mode. The principle of ANP is simple. Retention behavior is analogous to that found in normal phase chromatography but the mobile phase has water as part of the binary solvent. Normal phase implies that retention is greatest for polar solutes such as acids and bases. In addition, retention increases as the amount of the nonpolar solvent in the mobile phase increases. If the mobile phase consists of water and acetonitrile, retention will increase as the amount of acetonitrile increases. An interesting feature of ANP materials is that over some composition range of organic in the mobile phase it is possible for both polar and nonpolar compounds to have reasonable retention. This property distinguishes it from a HILIC column where only retention of polar compounds is obtained or an RP stationary phase where only nonpolar solutes are retained. ANP retention has been demonstrated for a variety of polar compounds on the hydride based stationary phases (J.J. Pesek, M.T. Matyska, J. Sep. Sci., 30 (2007) 637-647). Another important feature of the hydride-based phases is that for many analyses it is not necessary to use a high pH mobile phase to analyze polar compounds such as bases. The aqueous component of the mobile phase usually contains from 0.1 to 0.5% formic or acetic acid that are very compatible for mass spectral analysis.

Experimental

ANP Column

A high surface area, 4.0 μm particle size Type-B silica was converted to Type-C material having a silica hydride (Si-H) surface (Figure 1). To improve peak shape of the basic amino acids the Type-C silica was further reacted to reduce residual silanols (<5%). The material was packed into a 2.1 x 150 mm HPLC column format. This column packing is now commercially available from MicroSolv as Diamond Hydride¹.

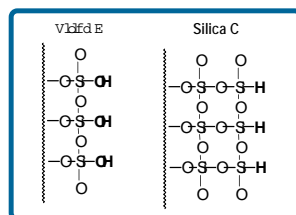


Figure 1. Topology of Silica B And Silica C

Amino Acid Standards

Individual amino acid standards were purchased from Sigma-Aldrich. The amino acids studied are in Table 1.

| Amino Acid | Empirical Formula | MW | (M) ⁺ | (M) ⁻ |
|-----------------|---|---------|------------------|------------------|
| L-Alanine | C ₃ H ₇ N ₂ O | 89.097 | 90.035 | 88.039 |
| L-Arginine | C ₆ H ₁₂ N ₄ O | 174.117 | 175.133 | 173.133 |
| L-Asparagine | C ₄ H ₈ N ₂ O | 132.055 | 133.055 | 131.052 |
| L-Aspartic acid | C ₄ H ₇ N ₂ O | 133.055 | 134.055 | 132.037 |
| L-Glutamine | C ₆ H ₁₂ N ₂ O | 146.075 | 147.075 | 145.072 |
| L-Glutamic acid | C ₆ H ₁₁ N ₂ O | 147.075 | 148.075 | 146.053 |
| L-Glutamine | C ₆ H ₁₂ N ₂ O | 146.075 | 147.075 | 145.072 |
| Cysteine | C ₃ H ₇ N ₂ O | 75.092 | 76.093 | 74.092 |
| L-Histidine | C ₆ H ₉ N ₃ O | 155.097 | 156.073 | 154.077 |
| L-Isoleucine | C ₆ H ₁₁ N ₂ O | 131.094 | 132.102 | 130.088 |
| L-Leucine | C ₆ H ₁₁ N ₂ O | 131.094 | 132.102 | 130.088 |
| L-Lysine | C ₆ H ₁₁ N ₃ O | 146.105 | 147.113 | 145.097 |
| L-Methionine | C ₅ H ₉ N ₂ S | 149.071 | 150.093 | 148.032 |
| L-Prolylamine | C ₆ H ₁₁ N ₂ O | 131.094 | 132.102 | 130.088 |
| L-Valine | C ₆ H ₁₁ N ₂ O | 115.093 | 116.075 | 114.059 |
| L-Serine | C ₃ H ₇ N ₂ O | 105.092 | 106.074 | 104.048 |
| L-Threonine | C ₄ H ₉ N ₂ O | 119.094 | 120.055 | 118.054 |
| L-Tyrosine | C ₉ H ₉ N ₂ O | 181.073 | 182.077 | 180.051 |
| L-Tryptophan | C ₁₀ H ₉ N ₂ O | 204.093 | 205.097 | 203.092 |

Table 1. Amino Acid Standards

Experimental

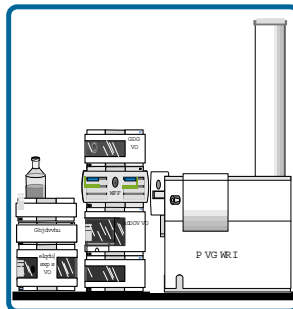


Figure 2. LC/TOF instrument configuration

Instrumentation

An Agilent 1100 Series HPLC system with binary pump and degasser, well plate autosampler with thermostal, thermostated column compartment, and an Agilent 6210 MSD TOF mass spectrometer was used (Figure 2).

Amino Acid Retention Data

Retention time data was collected under isocratic conditions of 50%, 60%, 70%, 75%, 80%, 85% and 90% organic solvent. The organic solvent was composed of 0.1% formic acid in acetonitrile (Figure 3), methanol (Figure 4) or acetone (Figure 5). Water containing 0.1% formic acid made up the difference. The mobile phase flow rate was 0.4 ml/min. The column temperature was 20°C. The column t_0 is 1.44 minutes.

Figures 3 - 5 are the retention plots for the amino acids investigated in this study. For acetonitrile and acetone there is a substantial increase in retention as the percentage of organic in the mobile phase exceeds 60%. For methanol only a small increase in retention is observed. Thus it appears that aprotic solvents induce greater ANP behavior than protic solvents. Use of acetone which appears to have good ANP properties is possible in LC/MS but not conventional UV detection. Another advantage of the use of ANP is the increase in sensitivity at high organic content in the mobile phase. Typical additives needed to promote ANP retention (acetic or formic acid) are very compatible with mass spectroscopy detection¹.

Results and Discussion

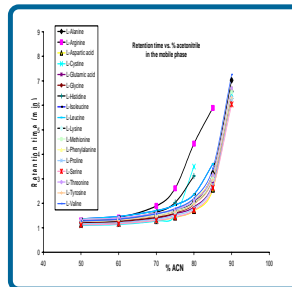


Figure 3. Retention Time With Acetonitrile

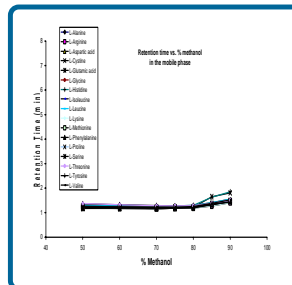


Figure 4. Retention Time With Methanol

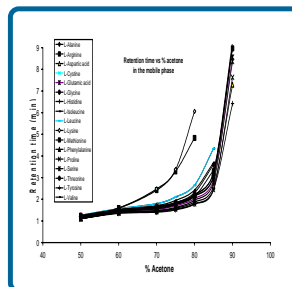


Figure 5. Retention Time With Acetone

Amino Acid Temperature Data

Temperature effect data was collected at 25% water / 75% acetonitrile with 0.1% formic acid in each. (Figure 6). The mobile phase flow rate was 0.4 ml/min. Data was collected at temperatures of 5, 15, 25, 35, 45, 55, 65, and 75°C.

Results and Discussion

Figure 6 shows the retention behavior of amino acids as a function of temperature ($\log t_R$ vs. $1/T$, vant Hoff plot). In all cases, retention increases as the temperature is increased indicating either a positive enthalpy for interaction of the solute with the stationary phase or substantial entropy contributions (proton activity). This temperature effect is opposite of what is typically observed under reverse phase conditions i.e. decreasing retention with increasing temperature¹.

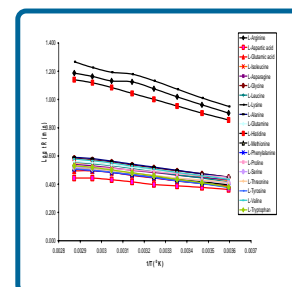


Figure 6. Vant Hoff Plot With Acetonitrile

Gradient Reproducibility

Retention time reproducibility data was collected for one gradient at 15 and 30°C. The flow rate was 0.4 ml/min and an equilibration time of 5 minutes. The gradient was:

| Time | %B | Time | %B |
|------|----|------|----|
| 0 | 50 | 6 | 50 |
| 5 | 50 | 9 | 50 |
| 6 | 80 | 14 | 50 |

Table 2 shows retention time reproducibility for nine amino acids at two temperatures. Four replicates were performed at each temperature. The reproducibility was 0.28% or better for the amino acids. This is a significant improvement over what is usually observed for most HILIC analyses, especially considering this is gradient data with only a 5 minute re-equilibration time between runs.

| Amino Acid | 5°C | 15°C | 5°C | 15°C | 5°C | 15°C | 5°C | 15°C | 5°C | 15°C | 5°C | 15°C |
|----------------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| Retention time | 15% | 15% | 15% | 15% | 15% | 15% | 15% | 15% | 15% | 15% | 15% | 15% |
| Alanine | 10.64 | 6.52 | 10.67 | 10.61 | 10.64 | 10.67 | 10.65 | 10.67 | 10.65 | 10.67 | 10.65 | 10.67 |
| Arginine | 11.76 | 11.96 | 11.96 | 11.96 | 11.76 | 11.76 | 11.76 | 11.76 | 11.76 | 11.76 | 11.76 | 11.76 |
| Asparagine | 11.76 | 11.76 | 11.76 | 11.76 | 11.76 | 11.76 | 11.76 | 11.76 | 11.76 | 11.76 | 11.76 | 11.76 |
| Aspartic acid | 11.76 | 11.76 | 11.76 | 11.76 | 11.76 | 11.76 | 11.76 | 11.76 | 11.76 | 11.76 | 11.76 | 11.76 |
| Glutamine | 8.90 | 8.90 | 8.90 | 8.90 | 8.90 | 8.90 | 8.90 | 8.90 | 8.90 | 8.90 | 8.90 | 8.90 |
| Glutamic acid | 10.64 | 10.64 | 10.64 | 10.64 | 10.64 | 10.64 | 10.64 | 10.64 | 10.64 | 10.64 | 10.64 | 10.64 |
| Leucine | 11.76 | 11.76 | 11.76 | 11.76 | 11.76 | 11.76 | 11.76 | 11.76 | 11.76 | 11.76 | 11.76 | 11.76 |
| Proline | 11.76 | 11.76 | 11.76 | 11.76 | 11.76 | 11.76 | 11.76 | 11.76 | 11.76 | 11.76 | 11.76 | 11.76 |
| Tyrosine | 11.76 | 11.76 | 11.76 | 11.76 | 11.76 | 11.76 | 11.76 | 11.76 | 11.76 | 11.76 | 11.76 | 11.76 |
| Valine | 10.64 | 10.64 | 10.64 | 10.64 | 10.64 | 10.64 | 10.64 | 10.64 | 10.64 | 10.64 | 10.64 | 10.64 |

Table 2. Gradient Reproducibility

Results and Discussion

Figure 7 shows the extracted ion chromatogram (EIC) of a nineteen amino acid mixture under one of the gradient conditions tested. All of the critical amino acid pairs (those that are isobaric or have masses within one mass unit) are separated under these conditions except for the Leucine / Isoleucine pair. At present, the maximum separation is approximately 0.15 min with 0.30 minutes needed for resolution with the peak widths obtained for these two amino acids. Additional gradient formats and mobile phases are under investigation to address this issue.

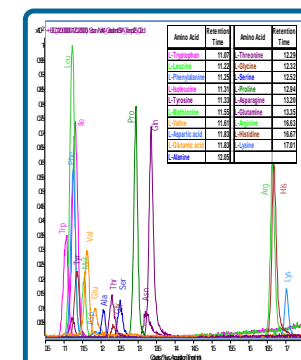


Figure 7. Extracted Ion Chromatogram Of Nineteen Amino Acid Separation

Conclusions

- Deactivated Type-C silica results in ANP retention for amino acids in acidic aqueous/acidic organic mobile phases when the mobile phase contains greater than 60% acetonitrile or acetone.
- Higher temperature results in increasing retention time.
- Excellent reproducibility was observed in isocratic or gradient elution with retention time RSD better than 0.3%
- Re-equilibration time is rapid (< 5 minutes) and similar to that observed on reverse phase separations.
- The use of Deactivated Type-C silica is a promising format for HPLC-MS analysis of metabolites.