

## PTH Amino Acid Analyses on Deactivated 2.1mm ID and 4.6mm ID HPLC Columns

In analyses of the products of automated Edman sequencing of proteins and peptides, reversed phase HPLC is the only means of reliably resolving all PTH amino acids in one analysis. Narrowbore SUPELCOSIL LC-18-DB columns containing a deactivated 3 $\mu$ m silica-based packing offer rapid, sensitive results for this analysis, and analytical conditions for using these columns are examined here. Because some investigators continue to use 4.6mm ID columns for this application, we also evaluated 25cm x 4.6mm SUPELCOSIL LC-18-DB columns with a 5 $\mu$ m packing, analyzing 20 PTH amino acids and 4 sequencer impurities and characterizing the method with respect to pH, buffer ionic strength, and temperature. Either a 2.1mm or 4.6mm ID deactivated SUPELCOSIL column and a triethylamine/acetic acid buffer system provided excellent separations and peak shapes for the recovered amino acids, ensuring reliable identification of quantities as small as 1 picomole.

### Key Words:

- amino acids ● PTH-amino acids ● peptides ● proteins
- narrowbore HPLC

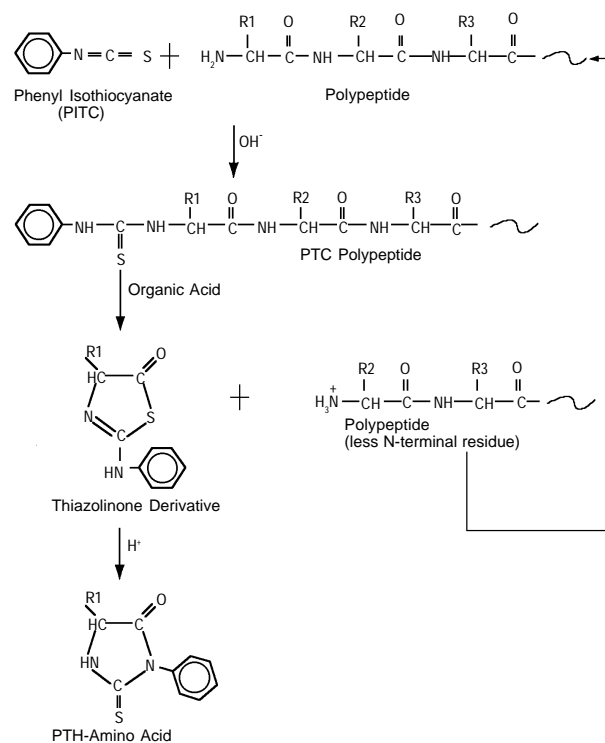
### Introduction

The amino acid sequence of a peptide or protein is routinely determined through Edman degradation (Figure A, references 1 and 2). In this three-step process, phenylisothiocyanate (PITC) is coupled to the amino-terminal amino acid, the derivatized amino acid is cleaved from the molecule, and the cleaved amino acid is converted to the more stable phenylthiohydantoin (PTH) form for analysis. The process can be repeated for as many as 50 amino acids, using an automated N-terminal protein sequencer coupled with HPLC. On-line analysis is the preferred and more sensitive method, but the analysis also can be performed off-line.

Because the analytical approach must be sensitive to picomole levels of analytes, reversed phase HPLC currently is the method of choice for identifying the products of automated Edman sequencing. Reversed phase HPLC allows rapid, sensitive analysis and is the only means of reliably resolving all PTH amino acids in a single chromatographic separation.

2.1mm ID SUPELCOSIL™ LC-18-DB columns containing a 3 $\mu$ m silica-based packing offer excellent performance for PTH amino acid analyses. Independent researchers have observed that these columns are superior narrowbore reversed phase columns for on-line PTH amino acid analysis (Figure B and references 3 and 4).

Figure A. Edman Degradation



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In this bulletin, we have addressed column type, mobile phase composition and pH, and other conditions for optimizing a PTH amino acid separation.

### Analytical Concerns

Conventional abbreviations and pKa values for 20 common amino acids are presented in Table 1. The demands of separating complex mixtures of 20 PTH amino acids, plus by-products of the reaction, dictate the use of highly efficient, highly reproducible reversed phase columns. Because the quantity of sample being sequenced is very small, sequencer impurities and other contaminants can be present in significantly larger amounts than any of the PTH amino acids. Separation of the PTH amino acids from these impurities is of primary importance for reliable amino acid identification and quantification. Some columns cannot resolve the reaction by-product diphenylurea (DPU) from PTH-Trp. This limitation is especially serious when the quantity of sample is in the low picomole range. Because Trp is one of two amino acids having a

**Figure B. PTH Amino Acids on a Narrow Bore Deactivated Column, Using ABI Solutions**

Column: **SUPELCO SIL LC-18-DB, 25cm x 2.1mm ID, 3µm particles**  
 Cat. No.: **57943**  
 Mobile Phase: **A = tetrahydrofuran:3M sodium acetate, pH 3.9:water (4.5:0.55:94.95), plus 1mL 12.5% aqueous trimethylamine/L B = acetonitrile**  
 column washed with 900µL 80% B after analysis, reequilibrated with 11% B for 9 min  
 Flow Rate: **220µL/min**  
 Temp.: **55°C**  
 Det.: **UV, 270nm**  
 Inj.: **PTH-amino acid standards**

**Gradient Program**

Time (min)	% B
0	11
1.5	15
15	26
27	45
30	45

1. Aspartic acid
2. Asparagine
3. Carboxymethyl cysteine
4. Serine
5. Glutamine
6. Threonine
7. Glycine
8. Glutamic acid
9. DMPTU
10. Histidine
11. Alanine
12. ΔS (DTT adduct of PTH-Ser)
13. Arginine
14. Tyrosine
15. Proline
16. Methionine
17. Valine
18. DPTU
19. DPU
20. Tryptophan
21. Phenylalanine
22. Isoleucine
23. Lysine
24. Leucine

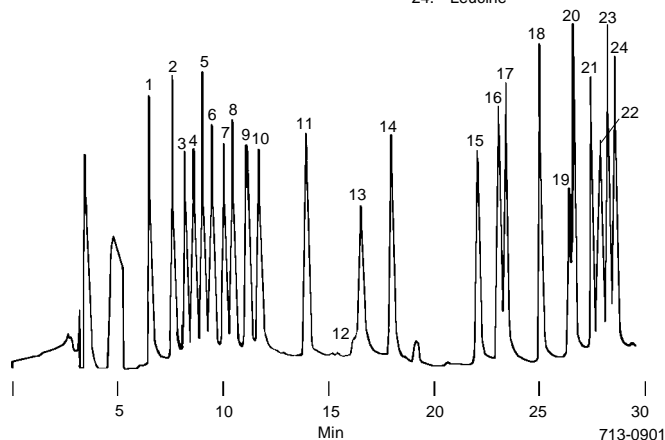


Figure provided by Dr. David Speicher, Wistar Institute, Philadelphia, PA, USA

single codon, its identification is exceptionally important when determining sequences for oligonucleotide probes.

Additionally, recovery of some PTH amino acids from the sequencer can be low, and recovery of all PTH amino acids decreases as the degradation proceeds. As a result, elution volumes must be small, to increase the PTH amino acid concentration and the detection sensitivity. Elution volumes can be minimized by using narrowbore (2.1 mm ID) columns containing small (3µm), uniformly sized silica supports and by minimizing extra-column effects that can lead to band broadening (e.g., improper connections and/or excessive tubing between injector and column or between column and detector). Additionally, the column must be suitably deactivated. Analyte interaction with residual exposed silanol groups on the packing particles will reduce the resolution of PTH amino acids. If a column is not suitably deactivated, the number of accessible silanol groups tends to increase significantly

**Table 1. Common Abbreviations for Amino Acids**

Amino Acid/ Sequencer Impurity	Abbreviation	-COOH	pKa -NH <sub>3</sub>	-R
Alanine	Ala	A	2.35	9.87
Arginine	Arg	R	1.82	8.99 12.48 (guanidino)
Asparagine	Asn	N	2.10	8.84
Aspartic Acid	Asp	D	1.99	9.90 3.90 (β-COOH)
Carboxymethyl cysteine	CMC			
Cysteine	Cys	C	1.92	16.78 8.33 (sulfhydryl)
Glutamic Acid	Glu	E	2.10	9.47 4.07 (α-COOH)
Glutamine	Gln	Q	2.17	9.13
Glycine	Gly	G	2.35	9.78
Histidine	His	H	1.80	9.33 6.04 (imidazole)
Isoleucine	Ile	I	2.32	9.76
Leucine	Leu	L	2.33	9.74
Lysine	Lys	K	2.16	9.18 10.79 (C-NH <sub>3</sub> <sup>+</sup> )
Methionine	Met	M	2.13	9.28
Norleucine	Nleu	NL	2.39	9.76
Phenylalanine	Phe	F	2.16	9.18
Proline	Pro	P	2.95	10.65
Serine	Ser	S	2.19	9.21
Threonine	Thr	T	2.09	9.10
Tryptophan	Trp	W	2.43	9.44
Tyrosine	Tyr	Y	2.20	9.11 10.13 (phenol)
Valine	Val	V	2.29	9.74
Dimethylphenylthiourea	DMPTU			
Diphenylthiourea	DPTU			
Diphenylurea	DPU			
Dithiothreitol	DTT			

during the column's lifetime, necessitating frequent corrections to mobile phase and gradient conditions.

Reliability of the analysis is crucial. Many samples being sequenced are so small that only one sequencing run can be made. Most PTH amino acids have very similar chemical structures and thus elute closely. Thus, reproducibility of run-to-run and lot-to-lot retention times is vital. Detection of PTH amino acids at 270nm increases the signal 40-50% relative to detection at 254nm. The column temperature is usually maintained at 55°C.

### Optimizing a Separation

General conditions for PTH amino acid analyses on SUPELCO SIL LC-18-DB columns include using a mobile phase consisting of sodium acetate buffer, aqueous tetrahydrofuran (THF), trimethylamine or triethylamine, and an acetonitrile gradient. High purity THF should be used – peroxides in poor-quality THF will destroy low levels of PTH amino acids and will increase background. The solvent composition and gradient conditions recommended by most manufacturers of sequencing instruments are applicable for use with SUPELCO SIL LC-18-DB columns. Table 2 summarizes the effects of changes in mobile phase composition or the gradient program on specific PTH amino acids. The arrows in Table 2 indicate the direction of movement of a peak, toward or away from the injection point of the chromatogram, associated with the change.

**Table 2. Mobile Phase Changes to Optimize PTH Amino Acid Separations**

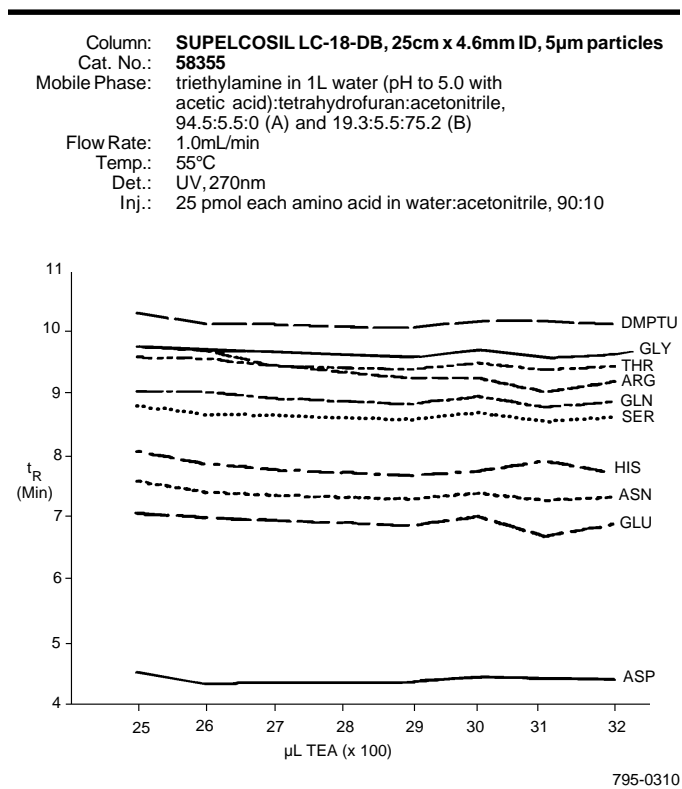
Desired Peak Movement (from Injection Point)	Mobile Phase Change
Glu → ← DMPTU CMC →	decrease initial % mobile phase B
Lys →	decrease final % mobile phase B
His → ← Arg	decrease pH of mobile phase A
Asp → Glu → CMC →	decrease pH of mobile phase A
His → Arg →	decrease ionic strength of mobile phase A
← Pro Met →	increase THF
Gln → ← Thr	increase THF

In the formation of the PTH derivatives, the terminal amino and carboxyl groups lose their acid-base properties, so that only the properties of the side chain play a distinct role in the chromatography. PTH-Gln and PTH-Lys are likely to overlap with other PTH amino acids. Analytical conditions for these derivatives are optimized by adjusting the THF concentration or the acetonitrile gradient. The THF concentration should be adjusted to center PTH-Gln between PTH-Ser and PTH-Thr. Alternatively, PTH-Gln can be moved toward PTH-Thr by decreasing the percent of mobile phase B used during the initial gradient. PTH-Lys elutes between PTH-Ile and PTH-Leu. It can be moved toward PTH-Leu by reducing the increase in % B in the next gradient step.

After the neutral amino acids are suitably positioned, elution of the charged amino acids can be adjusted by modifying the pH and ionic strength of mobile phase A. PTH-Asp and PTH-Glu are controlled by adjusting the pH. Decreasing the pH causes both analytes to elute later. PTH-Asp should elute before PTH-Asn and well after oxidized DTT. PTH-His and PTH-Arg should be positioned between DMPTU and PTH-Pro, to prevent coelution with PTH-Ala, PTH-Tyr, or the DTT adduct of PTH-Ser ( $\Delta$ S). The positions of PTH-His and PTH-Arg are adjusted by modifying the ionic strength of mobile phase A. Increasing the ionic strength causes both amino acids to elute earlier. PTH-Arg can be a minor problem if it is one of the first few residues of a sequence – sodium chloride in the Polybrene carrier used to help bind proteins to the sequencer matrix will be partially extracted through the first few cycles. This will increase the ionic strength of mobile phase A and, on a base deactivated column such as SUPELCOSIL LC-18-DB, retention times may shift for charged PTH amino acids, especially PTH-Arg. However, since PTH-Arg is basic, it elutes with a characteristic asymmetrical peak shape and, because the retention shift is only significant in the first 1-3 cycles, the problem is minor.

Basic amino acids (His and Arg) are sensitive to column age and the ionic strength of mobile phase A. Over time, a gradual increase in the exposure of residual silanol groups on the surface of the packing particles will gradually increase retention times for basic amino acids. As long as the increase in silanol groups is gradual, these shifts can be easily dealt with by gradually increasing the ionic strength of mobile phase A throughout the column lifetime. Triethylamine (TEA) improves peak shape for basic compounds, and a small amount (for example, 100-200 $\mu$ L TEA/L of buffer) can be used to increase the ionic strength of mobile phase A and maintain preferred retention times. The effects of changing TEA concentration on analytes in the first half of the chromatogram are shown in Figure C. At pH 5.0, only PTH-Arg and, to a lesser extent,

**Figure C. Effect of Triethylamine Concentration on Retention Times of Early-Eluting PTH Amino Acids**



PTH-His show reduced retention times with increasing TEA concentration. All analytes in the second half of the chromatogram are independent of TEA concentration (data not shown). SUPELCOSIL LC-18-DB columns have a low and relatively stable residual silanol character, and thus minimize the need for mobile phase adjustments.

**PTH Amino Acid Separations: Applied Biosystems Conditions**

A high resolution SUPELCOSIL LC-18-DB column can be used with popular ABI (Applied Biosystems, Division of Perkin-Elmer Corp.) buffers on sequencers compatible with narrowbore columns, to obtain baseline resolution of all 20 common PTH derivatives (Figure B) and resolve DPU from PTH-Trp. In obtaining Figure B, the pH was adjusted by varying the ratio of two stock solutions of 3M sodium acetate buffer (pH 3.8 and pH 4.6). Ionic strength was adjusted by changing the total amount of buffer added. Backpressure typically was less than 1000psi at 200 $\mu$ L/min and thus was not a problem. As long as the gradient segments are adjusted to maintain a constant gradient volume, flow rates up to 400 $\mu$ L/min can be used to reduce analysis time without loss of resolution. Customer feedback has indicated that the life expectancy of the column can be at least 1000 injections.

Different lots of 2.1mm ID SUPELCOSIL LC-18-DB columns have performed similarly, requiring only minor ionic strength and gradient adjustments to maintain separations (5). Three SUPELCOSIL LC-18-DB columns were evaluated against two other manufacturers' columns, and the SUPELCOSIL columns were deemed the best choice for PTH amino acid separations. Custom-

ers have confirmed our observations that the deactivated SUPELCOSIL columns have very low initial negative charge and that increases in charge with age are much smaller than for other popular columns. Near-baseline resolution has been achieved for all components except DPU and PTH-Trp. However, the greater than 50% resolution of this difficult-to-separate pair is quite adequate since DPU is present at very low levels in sequences.

The very important separation of DPU and PTH-Trp is difficult on many columns. These analytes can be partially separated by manipulating the gradient in the latter part of the chromatogram. A low proportion of mobile phase B permits baseline separation of DPU/PTH-Trp, and improves PTH-Met/PTH-Val resolution, but causes PTH-Lys to elute later relative to other hydrophobic amino acids (e.g., PTH-Leu). The best way to separate both DPU/PTH-Trp and PTH-Lys/PTH-Leu is to maintain a shallow gradient until DPU elutes, then quickly initiate a steep gradient so that the proportion of mobile phase B is sufficiently high to elute PTH-Lys before PTH-Leu. The steepness of the gradient is limited (approximately 5%/min at 200 $\mu$ L/min) by refractive index changes which can cause severe baseline problems. These conflicting requirements limit the resolution that can be obtained using this buffer system and make a highly efficient, reproducible column an absolute necessity for workable resolution of all components. This requirement is met by 2.1mm ID SUPELCOSIL LC-18-DB columns.

#### Alternative Conditions for PTH Amino Acid Analyses

Although 2.1mm and 1.0mm ID columns with 3 $\mu$ m packings are state-of-the-art technology for analyses of PTH amino acids, some investigators continue to use 4.6mm ID columns containing 5 $\mu$ m packings. We evaluated 25cm x 4.6mm SUPELCOSIL LC-18-DB columns with a 5 $\mu$ m packing for this application, using experimental buffers to analyze 20 PTH amino acids and 4 sequencer impurities and characterizing the method with respect to pH, buffer ionic strength, and temperature. The deactivated column and a triethylamine/acetic acid buffer system provided excellent peak shape and identification of the recovered amino acids, to quantities as small as 1 picomole. Figure D shows the 20 PTH amino acids and 3 sequencer impurities separated with nearly baseline resolution in less than 20 minutes. The fourth sequencer impurity, DTT, coelutes with PTH-Asp at pH 5.00. Clearly, this coelution does not interfere with identification of the PTH amino acids.

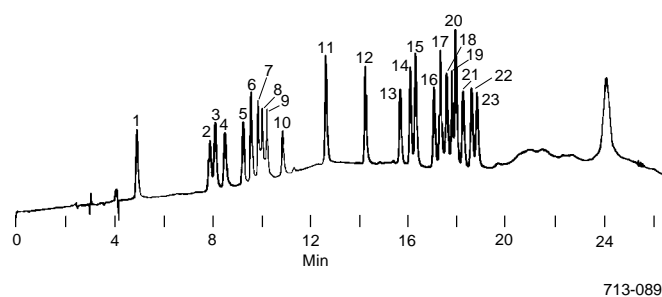
Because minor changes in pH will reposition the charged amino acids, we determined retention times at pH 4.5–5.1. Acidic PTH-Asp and PTH-Glu and basic PTH-His and PTH-Arg, in the first half of the chromatogram, are sensitive to changes in pH. The degree of -R group ionization changes over the pH range studied, and thus retention times for these analytes, is sensitive to the changes in pH (Figure E). As expected, the acids are retained less at higher pH, while PTH-His retention is longer. PTH-Arg is independent of pH changes within this range, as are all analytes in the second half of the chromatogram (data not shown). Thus PTH-Asp and DTT can be resolved, but care must be taken to prevent coelution of other peaks. Since DTT does not hinder identification of PTH-Asp, the conditions listed for Figure E are quite satisfactory.

Reproducibility of retention time was statistically analyzed for a total of 120 injections (Table 3). The data were gathered using 6 different mobile phase lots run on 5 different days and, therefore, indicate good run-to-run precision. The probability that a deviation from the mean will be within  $\pm 2$  standard deviations is 96%. Table 3 shows that if a retention window is defined as the retention time plus or minus 2 standard deviations, retention windows for

**Figure D. PTH Amino Acids on a 4.6mm ID Deactivated Column (pH 5.0)**

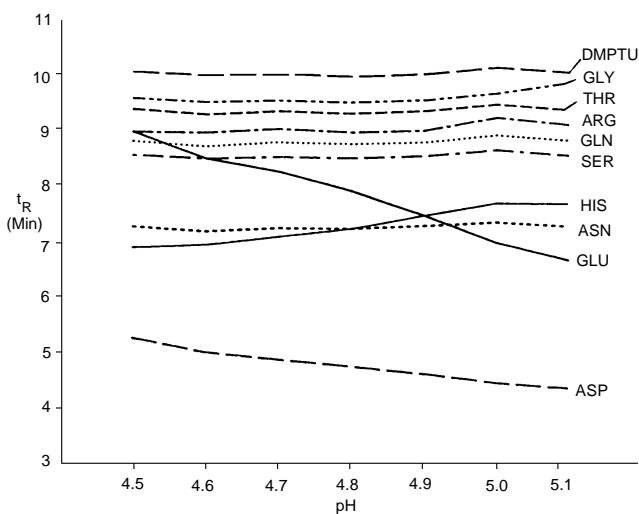
Column: SUPELCOSIL LC-18-DB, 25cm x 4.6mm ID, 5 $\mu$ m particles  
 Cat. No.: 58355  
 Mobile Phase: 2.65mL triethylamine in 1L water (pH to 5.0 with acetic acid):tetrahydrofuran:acetonitrile, 94.5:5.5:0 (A) and 19.3:5.5:75.2 (B)  
 Flow Rate: 1.0mL/min  
 Temp.: 55°C  
 Det.: UV, 270nm  
 Inj.: 25 pmol each amino acid in water:acetonitrile, 90:10

Gradient Program			
Time (min)	% B		
0	7	1. Aspartic acid	12. Tyrosine
5	40	2. Glutamic acid	13. Proline
10	63	3. Asparagine	14. Methionine
20	63	4. Histidine	15. Valine
21	7	5. Serine	16. DPTU
36	7	6. Glutamine	17. Tryptophan
		7. Arginine	18. DPU
		8. Threonine	19. Phenylalanine
		9. Glycine	20. Lysine
		10. DMPTU	21. Isoleucine
		11. Alanine	22. Leucine
			23. Norleucine



**Figure E. Effect of pH on Retention Times of Early-Eluting PTH Amino Acids**

Column: SUPELCOSIL LC-18-DB, 25cm x 4.6mm ID, 5 $\mu$ m particles  
 Cat. No.: 58355  
 Mobile Phase: 2.65mL triethylamine in 1L water (pH adjusted with acetic acid): tetrahydrofuran:acetonitrile, 94.5:5.5:0 (A) or 19.3:5.5:75.2 (B)  
 Flow Rate: 1.0mL/min  
 Temp.: 55°C  
 Det.: UV, 270nm  
 Inj.: 25 pmol each amino acid in water:acetonitrile, 90:10



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only 2 pairs of analytes (PTH-Arg/PTH-Thr and DPU/PTH-Phe) might overlap. As mentioned previously, the retention time for PTH-Arg is sensitive to column age and buffer strength and is easily adjusted. Separation of DPU and PTH-Phe (and of PTH-Ser and PTH-Gln) can be adjusted by changing the THF concentration. Although statistically possible at 96% probability, this kind of overlap is, in reality, much less likely to occur, because changes in retention due to variation in pump delivery usually occur in the same direction. Thus, routine analyses should present no identification problems, even for these two critical pairs.

The effects of the ionic strength of mobile phase A were discussed in conjunction with column aging. The positioning of basic compounds (PTH-Arg and PTH-His) can be adjusted by modifying the TEA concentration. As the column ages and PTH-Arg begins to coelute with PTH-Thr, a slight increase in TEA concentration (100-200µL/L buffer) will shorten the retention of PTH-Arg to the preferred position (between PTH-Gln and PTH-Gly, at pH 5.0).

**Table 3. Retention Times for PTH Amino Acids Are Consistent on 4.6mm ID SUPELCOSIL LC-18-DB Columns**

Amino Acid	Mean	Std. Dev.	Mean	Mean
			± 1 Std. Dev.	± 2 Std. Dev.
Aspartic Acid	4.42	0.04	4.38 – 4.46	4.34 – 4.50
Glutamic Acid	6.89	0.15	6.74 – 7.04	6.59 – 7.19
Asparagine	7.34	0.04	7.30 – 7.38	7.26 – 7.42
Histidine	7.68	0.05	7.63 – 7.73	7.58 – 7.78
Serine	8.59	0.05	8.54 – 8.64	8.49 – 8.69
Glutamine	8.82	0.04	8.78 – 8.87	8.74 – 8.92
Arginine	9.13	0.09	9.04 – 9.22	8.95 – 9.31
Threonine	9.38	0.03	9.35 – 9.41	9.31 – 9.44
Glycine	9.58	0.04	9.54 – 9.62	9.50 – 9.66
DMPTU	10.04	0.03	10.01 – 10.07	9.98 – 10.10
Alanine	12.05	0.03	12.02 – 12.08	11.99 – 12.11
Tyrosine	13.88	0.03	13.85 – 13.91	13.82 – 13.94
Proline	15.00	0.05	14.95 – 15.05	14.90 – 15.10
Methionine	15.64	0.04	15.60 – 15.68	15.56 – 15.72
Valine	15.84	0.04	15.80 – 15.88	15.76 – 15.92
DPTU	16.65	0.04	16.61 – 16.69	16.57 – 16.73
Tryptophan	16.92	0.04	16.88 – 16.96	16.84 – 17.00
DPU	17.18	0.04	17.14 – 17.22	17.10 – 17.26
Phenylalanine	17.30	0.05	17.25 – 17.35	17.20 – 17.40
Lysine	17.52	0.04	17.48 – 17.56	17.44 – 17.60
Isoleucine	17.75	0.05	17.70 – 17.80	17.65 – 17.85
Leucine	18.11	0.05	18.06 – 18.16	18.01 – 18.21
Norleucine	18.33	0.05	18.28 – 18.38	18.23 – 18.43

Data summarize 120 injections / 6 lots of mobile phase / 5 days

Although we do not present the data here, we also studied the effects of temperature, over a range of 25-55°C. As expected, retention of all analytes decreased with increasing temperature. In the first half of the chromatogram, PTH-Glu, PTH-His, PTH-Thr, and DMPTU are sensitive to temperature changes. At higher temperature, resolution improves for the later-eluting pairs PTH-Met/PTH-Val, DPU/PTH-Phe, and PTH-Lys/PTH-Ile. The optimum temperature for the separation is 55°C.

These studies indicate that a 4.6mm ID SUPELCOSIL LC-18-DB containing a 5mm packing will effectively and reproducibly separate 20 PTH amino acids and three sequencer impurities. Small modifications in the starting mobile phase composition and the gradient profile allow separation of all PTH amino acids and four sequencer impurities.

### Ordering Information:

#### SUPELCOSIL LC-18-DB Columns

25cm x 2.1mm ID, 3µm particles	57943
25cm x 4.6mm ID, 5µm particles	58355-U

#### Supelguard™ LC-18-DB Cartridge Columns (2/pk.)

2cm x 2.1mm ID, 5µm particles	59617
2cm x 4.6mm ID, 5µm particles	59565

#### Holder for 2cm Cartridge Columns 55205

For many other columns for protein and peptide analyses, please refer to the current Supelco catalog. 1.0mm ID columns can be custom packed with 3µm particles.

#### References

1. Edman, P. *Acta Chem. Scand.* 4: 283 (1950).
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  3. Reim, D., P. Heinback, and D. Speicher, *Techniques in Protein Chemistry III*, pp 53-60, Academic Press (1992).
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- References 1-4 not available from Supelco.

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