Investigation of Peptide Separation on a Bioadvantage base-deactivated column with TFA- and acetic acid-modified mobile phase

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Introduction

Commercially available reverse-phase analytical columns that are commonly for peptide and protein separation are coated with octadecylsilane (C-18) stationary phase. During the manufacturing process, complete coverage of the silica particles with the stationary phase has proven difficult. Exposed silanol groups on the silica surface have deleterious effects on the elution of certain organic compounds. Particularly, basic compounds such as pyridine can interact with the surface silanols which results in peak tailing. The use of base-deactivated silica particles can minimize silanol interactions with eluting organic compound and improve peak shape.

Trifluoroacetic acid (TFA) is a commonly used as a mobile phase additive that serves as an ion-pairing reagent. TFA pairs with charged sites on organic compound and helps to further minimize solute interaction with the silica surface. If the silanol groups on the silica surface have been effectively deactivated, an ion-pairing reagent is no longer necessary. This is advantageous for the analysis biologically active compounds with LC-MS. The presence of TFA reduces the ionization efficiency of the MS detector, ultimately reducing its detection sensitivity.

A collaboration was setup between Skip Amburn of Thomson Instruments Inc., Arthur Mosely of Glaxo Wellcome Inc., and Kamlesh Patel of James Jorgenson's Research group at UNC-Chapel Hill. The goal for the short term collaboration is to investigate peptide separation on a Bioadvantage base-deactivated column with TFA- and acetic acid-modified mobile phase.

Experimental Setup

All chromatographic runs were performed on the same setup consisting of the following modules. A Hewlett Packard 1050 quaternary gradient pump was used to deliver the mobile phase. Four solvent bottles were used: two optima grade acetonitrile and two nanopure deionized water. Each mobile phase pair had either trifluoroacetic acid or acetic acid modifiers added. To remove dissolved gasses from the mobile phases, helium sparging was used. The base-deactivated Bioadvantage column used was 1.0 mm i.d. x 50 mm long with 5 µm 100 Å pore sized silica particles with C-18 stationary phase. Due to the small internal volume of the column, all precolumn tubing used was 0.005" PEEK tubing of the shortest length possible. Sample injections were made by using a rotary manual injector with a specific volume loop. Two inline filters with 0.5 µm stainless steel frits were used in the solvent line from the pump and before the column. Postcolumn tubing was

fused-silica capillary with a 1 cm detection window burned in the polyimide coating. The variable Linear UV/Vis detector was fitted with a capillary flow cell mount. Wavelengths used were 254 nm and 215 nm.

For the isocratic runs, the inline filter before the column was removed to minimize extra-column volume. Also a 4-port Rheodyne 7410, 0.5 µL internal loop injector was used. The injections were performed in a typical manner. A 50 µL syringe was used to flush and fill the loop. The postcolumn capillary was 75 µm i.d. x 5 cm long to the detection window. The ball lens of the capillary flow cell was approximately 2 mm in diameter resulting in an approximate detection volume of 30 nL. Wavelength detection was set for 254 nm with a rise-time of 0.3 seconds. The fullscale setting was either 0.002 or 0.005 AU. An Isocratic run on a pyridine/phenol mixture and a Metachem test mixture were performed. All samples were available in lab and used without any further purification or modification.

Gradient runs employed the solvent programming capabilities of the HP 1050 pump. A 6-port 1125 Rheodyne injector with a homemade 1µL loop was used. Injections were made in a manner similar to the isocratic runs except for one difference. Because of the low flow rates and large precolumn volume, approximately 4 minutes was needed for the gradient to reach the head of the column. Thus, injections were made 4 minutes after the gradient programming had started, allowing for preconcentration of the peptides on the front of the column. The postcolumn tubing used for the gradient runs was a 250 µm i.d. x 5 cm capillary. The larger diameter capillary increased the detection volume to approximately 0.1 mL. Wavelength detection was set for 215 nm with a rise-time of 0.3 seconds. The fullscale setting was set to 0.005 AU. Gradient runs of two peptide standard mixtures and two tryptic digest with both TFA- and acetic acid- modified mobile phase were performed. Peptide standards and tryptic digests of Myoglobin and Cytochrome C were readily available in lab.

Data from the UV/Vis detector were collected with a PC computer using a 16 bit analog/digital converter. The collection rate for all chromatograms was 5 Hz. Gaussian fit analysis was performed using Igor Pro version 3.0.

Results and Discussion

Provided by Thomson Instruments, the chromatograms in figure 1 show an isocratic separation of a pyridine/phenol mixture on three commercially available base-deactivated columns (BDS) with no mobile phase modifier added. The Bioadvantage column clearly exhibits the best pyridine peak shape when compared

to the other BDS columns. Attempts to reproduce similar results with the 1.0 mm x 50 mm column are shown in figure 2a and 2b. The major difference between the two columns is the size. The column used to generate the chromatogram in figure 1 is 4.6mm x 250mm, whereas the column in figure 2a,b is only 1.0mm x 5.0mm. The later column has significantly fewer theoretical plates due to the reduced length. As a result, a lower percent organic mobile phase (20% ACN and 80% H20) is necessary to increase retention. As mentioned in the experimental section, extra-column volume must be minimized to achieve maximum column efficiency. The use of a capillary flow cell minimizes peak broadening but detection sensitivity is sacrificed. In order to maintain a good signal to noise ratio, relatively concentrated samples were used. Figure 2a and 2b show the separation of a 2mM pyridine/7mM phenol mixture and 4mM pyridine/7mM phenol mixture, respectively. Although pyridine is not completely baseline resolved in either figure, pyridine does fit a Gaussian profile well and suggests only minor peak tailing. The small unknown peak after pyridine is an impurity in the mixture.

To further evaluate the column and the system setup, a separation of a MetaChem test mixture comprised of uracil (dead-time marker), acetophenone, methyl benozate, toluene and napthalene was performed (figure 3). Individual peaks were fitted to a Gaussian profile to calculate the number of theoretical plates. Acetophenone has a k' of 2.87 with 2,334 plates per column length. This corresponds well to the number plates calculated for fluorene (k'=2.67 and N=2,366) in the test chromatogram provided with the column by Thomson Instruments (figure 4).

With confidence that the system and column are operating well, gradient analysis of peptide mixtures and tryptic digest is investigated. The first peptide mixture is comprised of the peptides: met-enkephlin, met-leu-phe, and angiotensin 1. The sequences of these peptides are shown in table 1. met-enkephlin and met-leu-phe are chosen because they are relatively small and are known to behave well in RPLC. Their good behavior in RPLC can be explained by the fact that neither peptide has any basic or acidic amino acid residues. angiotensin, on the other hand, is much larger and has an acidic (aspartic acid) and basic (araginine) residue near one end of the peptide fragment

Figure 4a and 4b show two runs under similar gradient programs, injection volumes, concentrations and flow rates. In figure 4a, 0.1% (v/v) TFA is added to the water and acetonitrile solvent bottle. The separation of all three peptides shows good peak shapes. In figure 4b, 0.1% (v/v) acetic acid is added to the mobile phase.

In this run, met-enkephlin and met-leu-phe fit the Gaussian profile well with little peak tailing. However, angiotensin peak is tailed and broadened in comparison to its counterpart TFA run. This suggests that basic residue near the end of angiotensin still interacts to some degree with the surface of the silica. Three other observations are noted in figure 4. First, the peptides elute in different order between the two runs. This could be a result of the pH difference between the two mobile phase pairs or an unknown selectivity with the modifiers. Secondly, the peaks in acetic acid chromatogram eluted in less organic than in the TFA chromatograms, resulting in lower k' values. Lastly, the baseline width (4σ) for the peaks in this particular peptide mixture in acetic acid-modified mobile phase are approximately 0.7 to 1.7 larger than TFA runs. The exact reason for this broadening of the peaks are unclear. These three trends are noted with all chromatograms when comparing between TFA- and acetic acid- modified mobile phase.

To further investigate the interactions of basic residues of larger peptides, a second peptide mixture is compared between the two modifiers. The second peptide mixture consists of dynorphin fragment A, bradykinin and neurotensin. The peptide sequences are shown in table 1. The first two have sequences that end in lysine and araginine, respectively. Neurotensin serves as a reference since it has no basic end residue. The chromatograms are shown in figure 5a and 5b. With TFA modifier, all three peaks show good symmetry and good Gaussian fit. Only slight peak tailing can be seen on all three peaks with TFA. In acetic acid runs, dynorphin A and bradykinin peak shapes suffer significantly compared the TFA run, yet neurotensin remained similar to the TFA run. The standard deviation of neurotensin in acetic acid is only 0.27 larger, resulting in 1.08 second increase in the baseline width (4σ). However, the standard deviation for dynorphin fragment A and bradykinin are significantly larger.

With these two peptide mixtures, it can be concluded that separation of peptides with neutral amino acids have limited interactions with the silica surface and do not degrade in peak shape when used with acetic acid-modified mobile phase. Peptides with basic residues near the peptide ends do degrade when used with acetic acid-modified mobile phase. One note to consider is that bradykinin and dynorphin fragment A were chosen because they tail on standard analytical columns that are not base-deactivated, even when used with TFA-modified mobile phase. The fact that these peptides show excellent peak symmetry in TFA suggest that these compounds have limited interaction with the surface silanols due to base deactivation of the silica surface. However, an ion-pairing regent is still necessary

for good peak shape of these extreme peptides. Qualitatively in looking at figure 6b, the peaks shapes in acetic acid are baseline resolved and still usable for chromatographic analysis.

The peptide standards provided insight to the performance of the column. In order to test a wide range of peptides of varying sizes,, and interactions, a tryptic digest of a protein is necessary. The two proteins digests conveniently available in lab are those of myoglobin and cytochrome C. Figure 7a and 7b compare TFA and acetic acid runs of the tryptic digest of myoglobin. TFA and acetic acid concentrations were dropped to 0.05% with no noticeable effect on the separation. Furthermore, the gradient program used had the same slope (1% organic per min). Acetic acid runs started at 1% ACN where TFA runs started at 5% ACN. The shift in lower organic for acetic acid is necessary since peaks elute in lower organic mobile phase than TFA. Both showed complete separation of the chromatograms in the 30-minute gradients. Different selectivity and shifting of the peaks prevent direct comparison of the peaks between the two chromatograms. However, many similarities exist between the two chromatograms. Even with the different selectivity, the 0.05% acetic acid run has good separation of the peptide fragments from the myoglobin digest. The resolution in either run is limited by the short column length. One percent change per minute for 30 minutes is the optimum gradient slope for this column. Figure 8a and Figure 8b show an expanded view of 10 minutes of each gradient run. The peaks are fitted to a profile for comparison. The four peaks of interest in the TFA run are all baseline resolved. Only two of the four peaks in the acetic acid run are baseline resolved. Assuming that these compounds are similar in nature and are not shifted, the peaks in acetic acid run compare well with the TFA run. Standard deviations are similar and have excellent peak shape. Figures 9a and 9b show another tryptic digest of cytochrome C. Similar trends as those seen in myoglobin digest can be seen in the cytochrome C digest. Again, acetic acid-modified mobile phase provides good separation of the peptide fragments.

The column clearly shows good peak shape with small basic organic compounds such as pyridine without any added modifier to the mobile phase. This is a direct result of the deactivation of the silanol groups on the silica surface. For separation of peptides, only peptides with charged basic groups suffered poor peak shape and tailing. For the best separation of basic amino acids such as lysine and araginine, some amount of TFA seems necessary. The minimum TFA concentration needed is hard to exactly determine. Varying the concentration of

TFA from 0.1% to 0.05% to 0.01% did not effect the peak shape significantly but did slightly increase the peak widths at the baseline (figure not shown). More test are necessary to accurately determine the specific TFA concentration effects on peak shapes with peak widths. Nonetheless, the digest of myoglobin and cytochrome C shows good separation even with acetic acid as the mobile phase modifier. Between the two modifiers, a noticeable shift in peaks elution times compared to TFA, elution in less organic, and slightly larger peaks width are three general conclusion that can be make when comparing the two modifiers.

In conclusion, the base-deactivation of the silanol group of the silica is a significant improvement in column technology From the peptide chromatograms, TFA concentration can be significantly reduced with BDS columns and even possibly eliminated for peptide separations. Making the BDS columns well suited for use with mass spectrometry. Between the two modifiers, shifts in elution times for peptides are noticed. Furthermore, less organic is needed for runs with acetic acid-modified mobile phase. The only observable disadvantage of using acetic acid over TFA is that basic residues suffer from tailing. An ion-pairing agent is still needed these peptide.

Acknowledgments

I would like to thank Thomson Instruments for the opportunity on collaborating of this short term project. Furthermore, I would like to acknowledge the support and purchase of the Bioadvantage column by Glaxo Wellcome.

Table 1

Peptide Mixture 1

a) **Angiotensin 1 Human** Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu
F.W. 1296.5

b) **Met-Enkephelin** Tyr-Gly-Gly-Phe-Met
F.W. 573.7

c) **Met-Leu-Phe**
F.W. 391.53

Peptide Mixture 2

a) **Dynorphin A Fragment** Try-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-
F.W. 1640.0 Leu-Lys

b) **Bradykinin** Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg
F.W. 1060.2

c) **Neurotensin** pGlu-leu-Tyr-Glu-Asn-Lys-Pro-Tyr-Ile-Leu
F.W. 1672.9

Figure 1 Chromatogram of pyridine/phenol mixture provided by Thomson Instruments

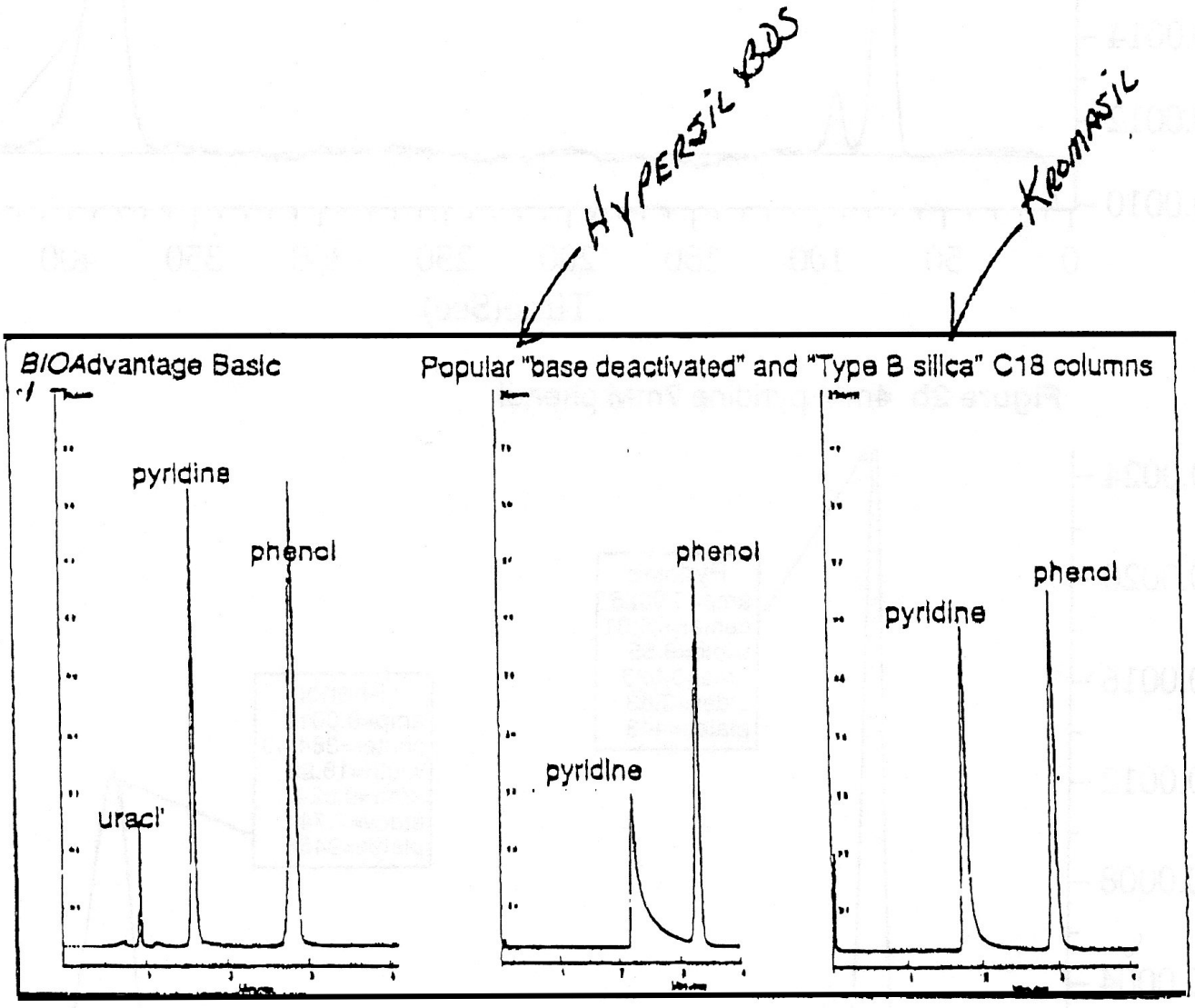
Column Type: *BIOAdvantage Basic C18*, 5 µm, 4.6 x 100 mm

Mobile Phase: A: 60% Water B: 40% Acetonitrile

Gradient: Isocratic.

Detection: 254 nm

Discussion: The new *BIOAdvantage Basic* has been designed to shield basic compounds, like pyridine, from any solute-silanol interaction. Unlike other manufacturers who illustrate the performance of their "Type B" silicas with buffered mobile phases, our *Basic* columns illustrates superior peak shape and efficiency ***without*** any mobile phase "helpers".



Isocratic runs of Pyridine and Phenol at 20/80 ACN/H20 0.5 uL inj at 0.1mL/min
0.005 AUFS at 254 nm (75 um i.d. flow cell) deadtime approx 33 seconds

Figure 2a 2mM pyridine 7mM phenol

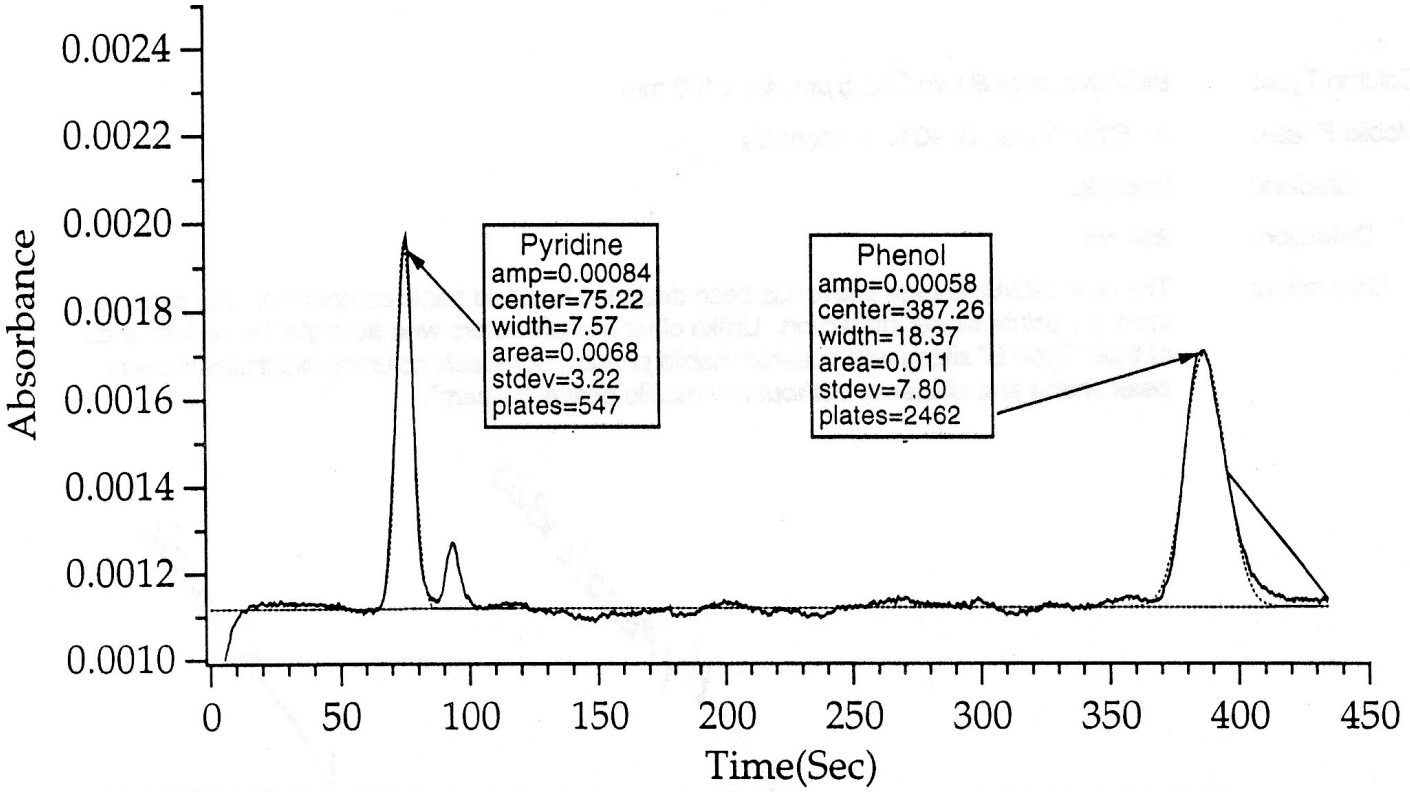


Figure 2b 4mM pyridine 7mM phenol

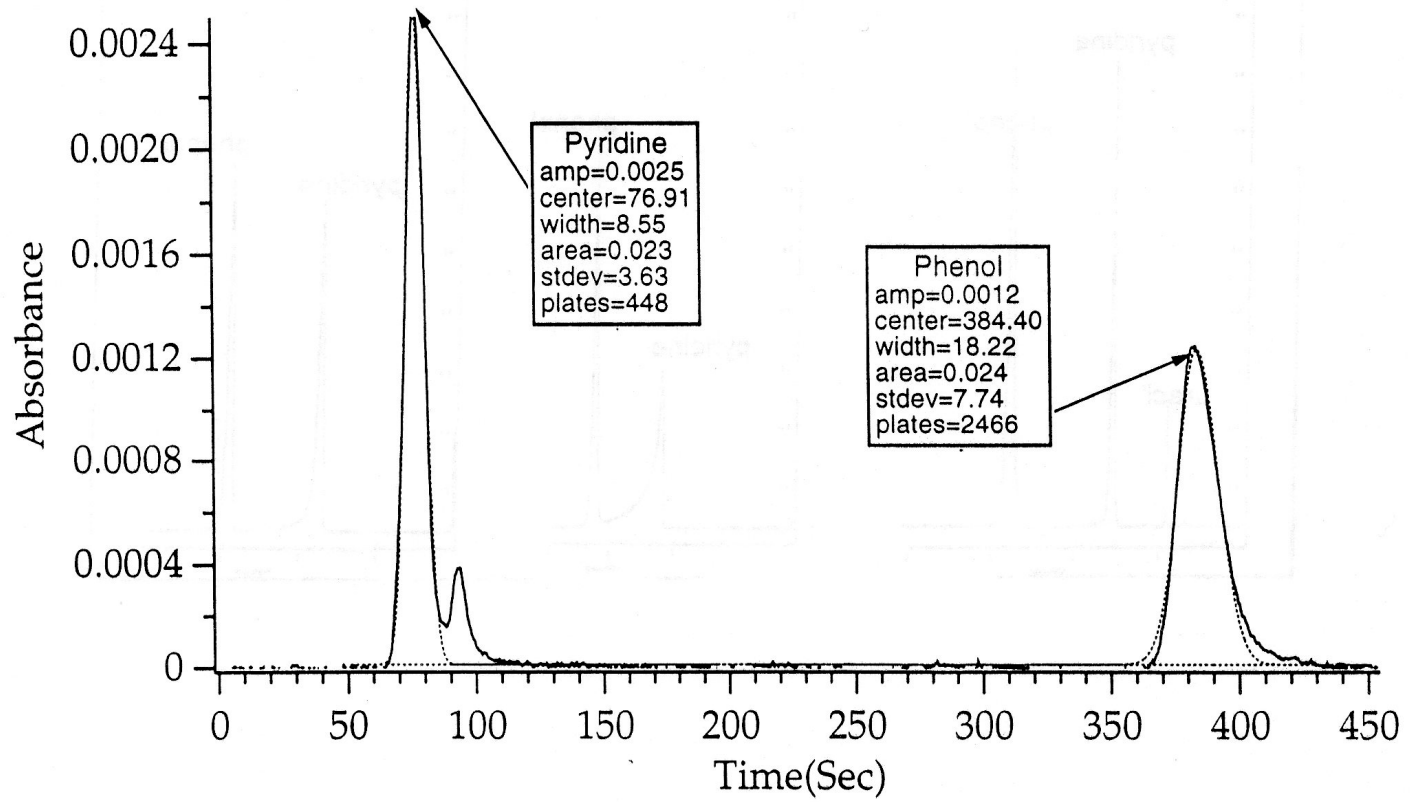


Figure 3 Isocratic run of Metachem test Mixture 40/60 ACN/H20
0.05 uL inj 0.1 mL/min at 0.002 AUFS at 254 nm

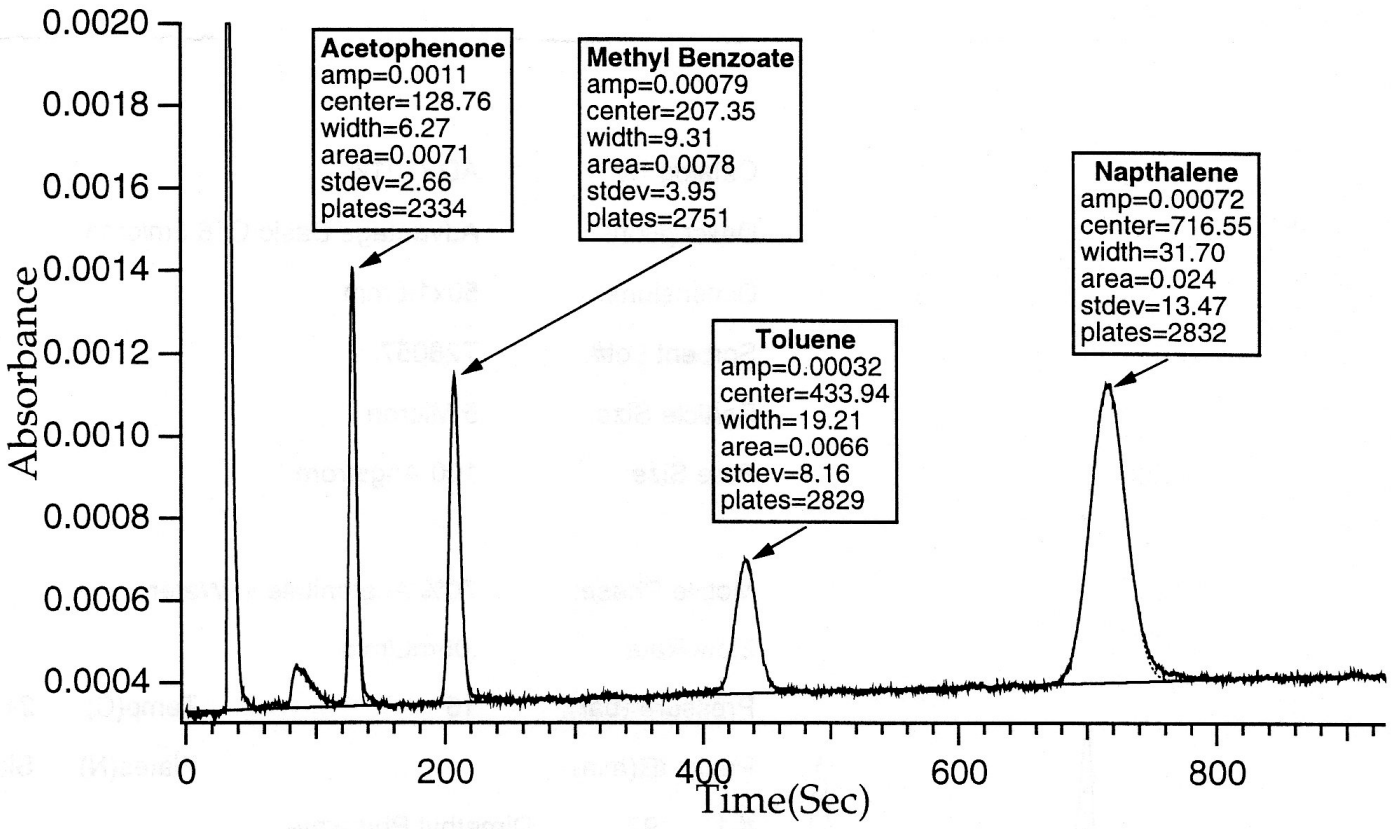
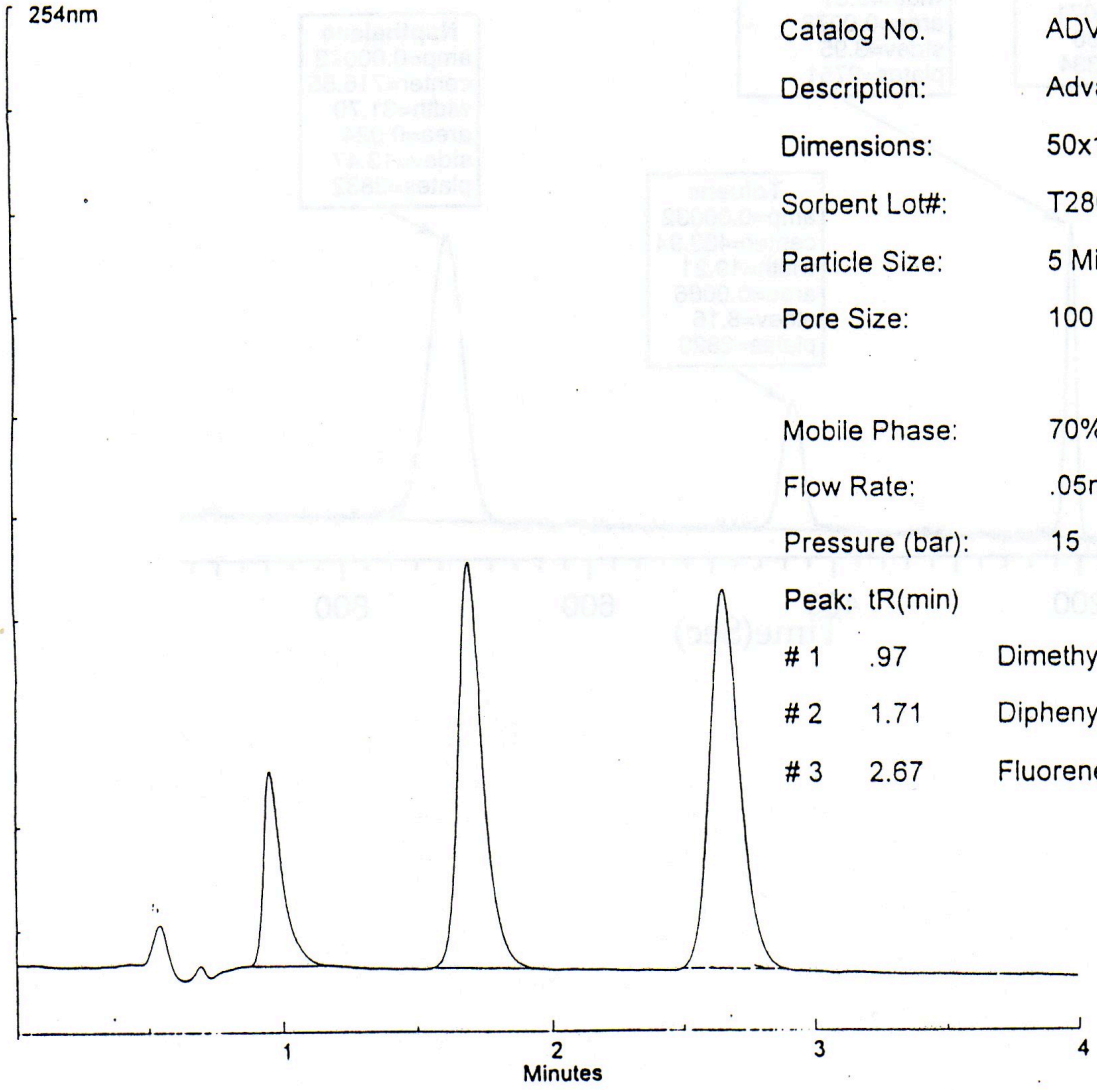


Figure 4 Test Chromatogram for 1.0 mm x 50 mm Bioadvantage Column



Catalog No.	ADV7012
Description:	Advantage Basic C18 5micron
Dimensions:	50x1.0mm
Sorbent Lot#:	T28057
Particle Size:	5 Micron
Pore Size:	100 Angstrom
Mobile Phase:	70% Acetonitrile in Water
Flow Rate:	.05mL/min
Pressure (bar):	15
Temp(C):	24.8

Peak:	tR(min)		Plates(N)	Skewnes
# 1	.97	Dimethyl Phthalate		
# 2	1.71	Diphenylamine	1,361	0.244
# 3	2.67	Fluorene	2,366	0.124

Peptide mixture: Angiotensin, Met-Enkephlin, Met-Leu-Phe
Gradient: 5/99-35/65 ACN/H20 in 30 min 1% per min
1 uL inj 4 min after gradient start 0.005 AUFS at 215 nm

Figure 5a 0.1% TFA mobile phase pH=2.2

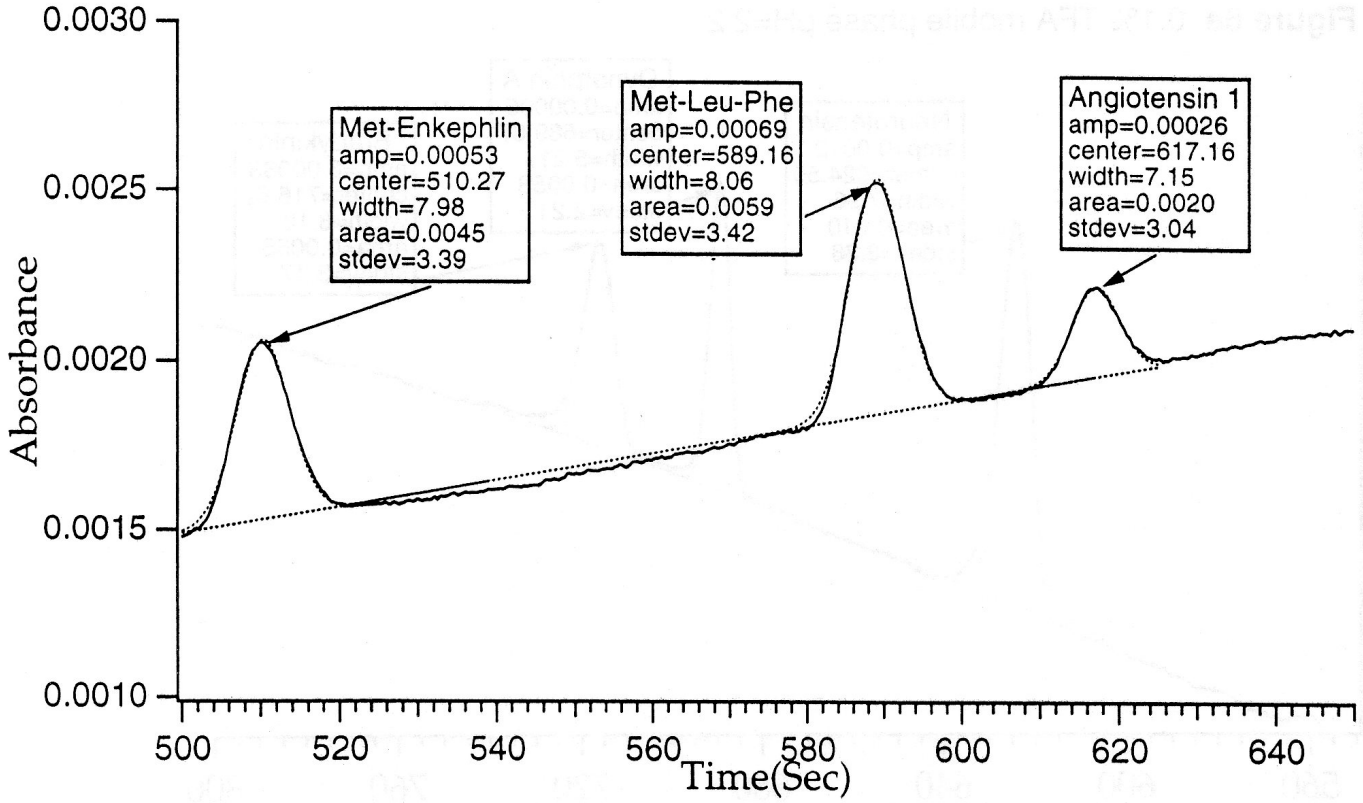
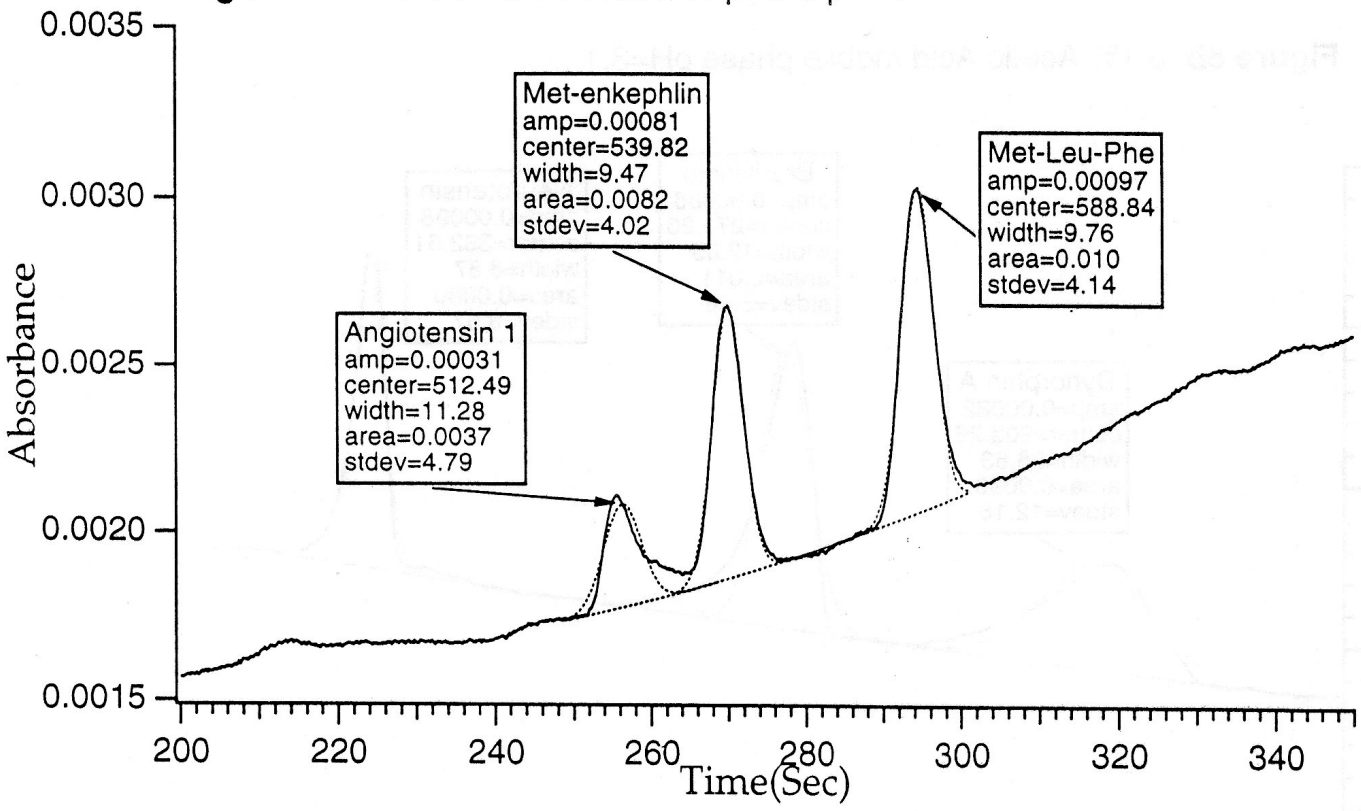


Figure 5b 0.1% Acetic Acid mobile phase pH=3.1



Peptide mixture Dynorphin Frag A, Neurotensin, Bradykinin
Gradient 5/95-35/65 ACN/H20 in 30 min
1uL inj 4 min after gradient start 0.005 AUFS at 215nm

Figure 6a 0.1% TFA mobile phase pH=2.2

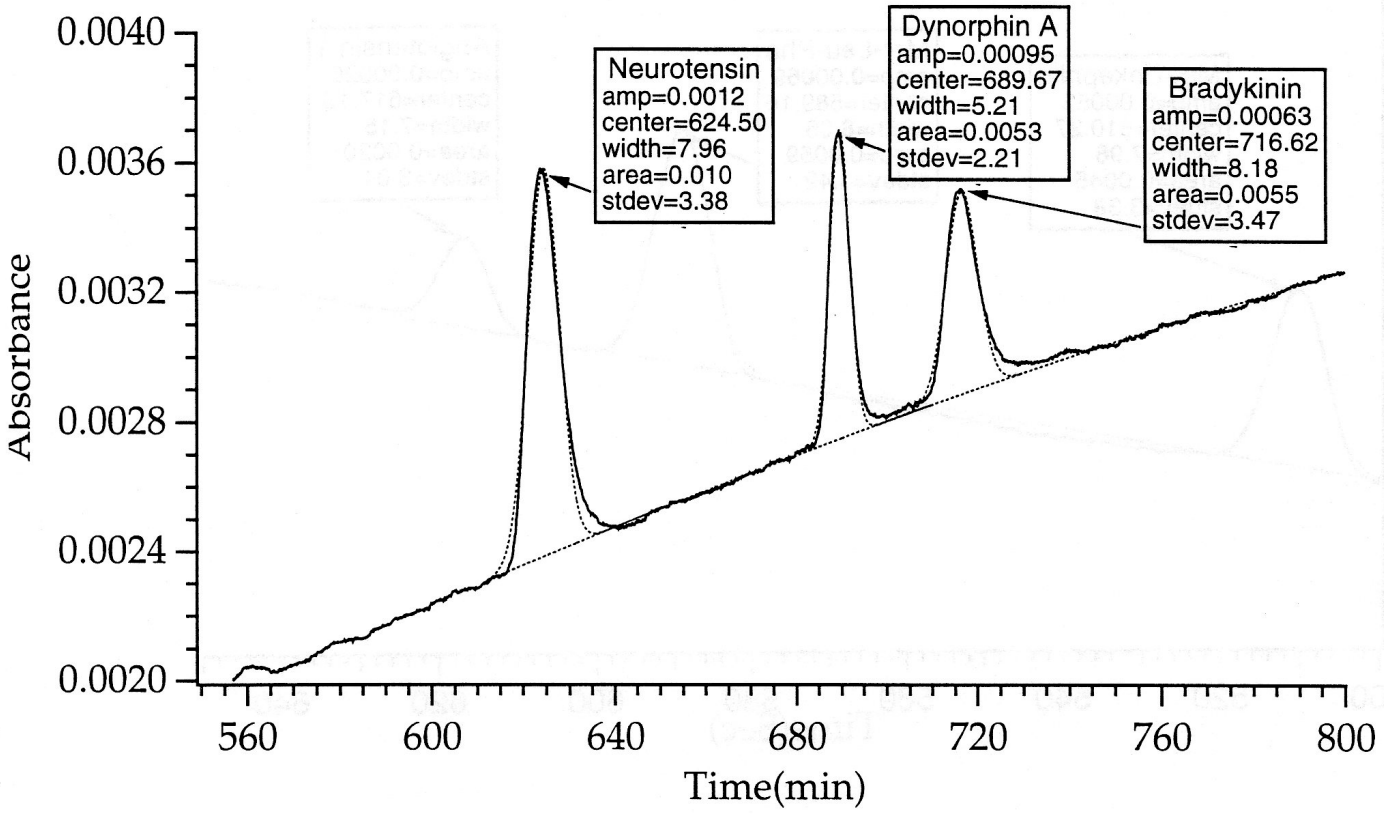


Figure 6b 0.1% Acetic Acid mobile phase pH=3.1

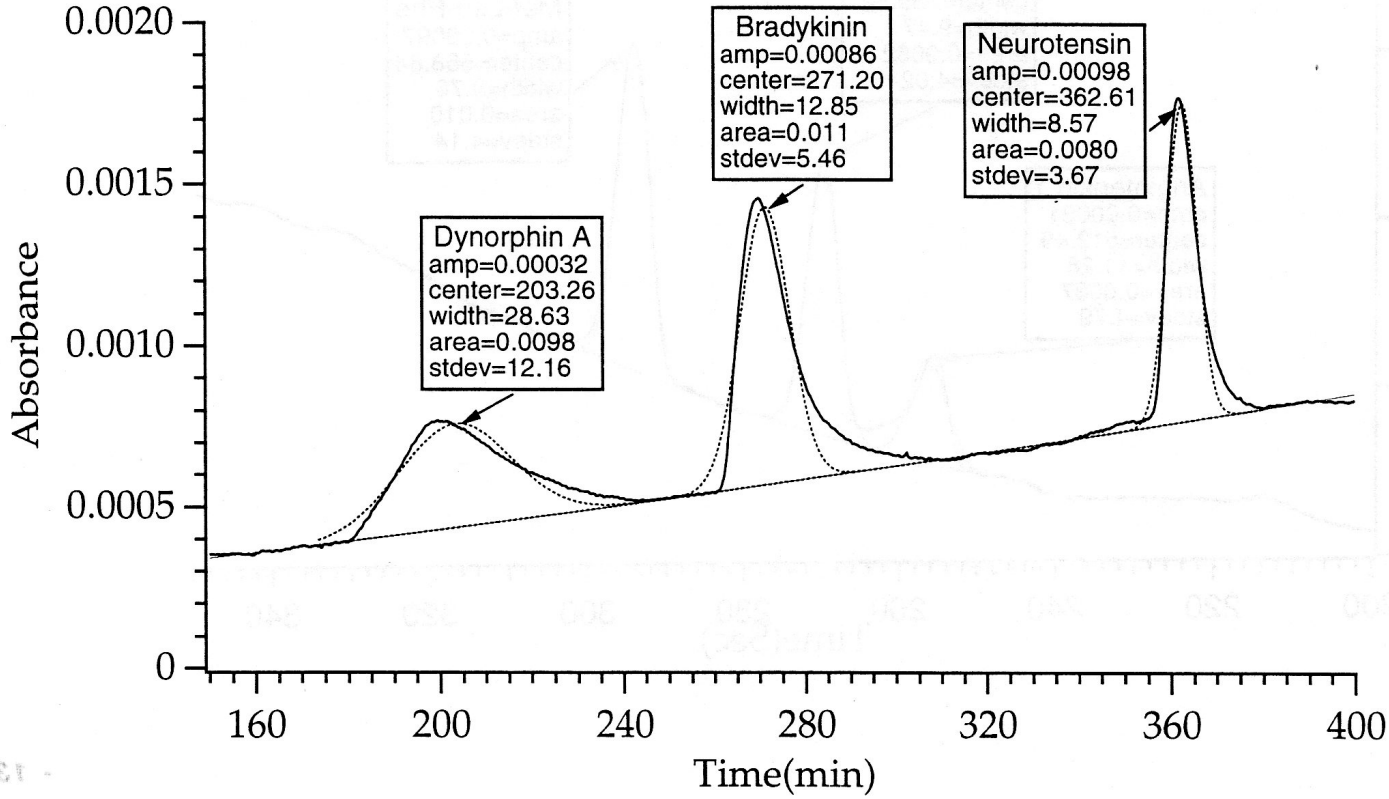


Figure 7a Gradient 5/95-35/65 ACN/H20 in 30 min 1% per min
0.05% TFA pH=2.2

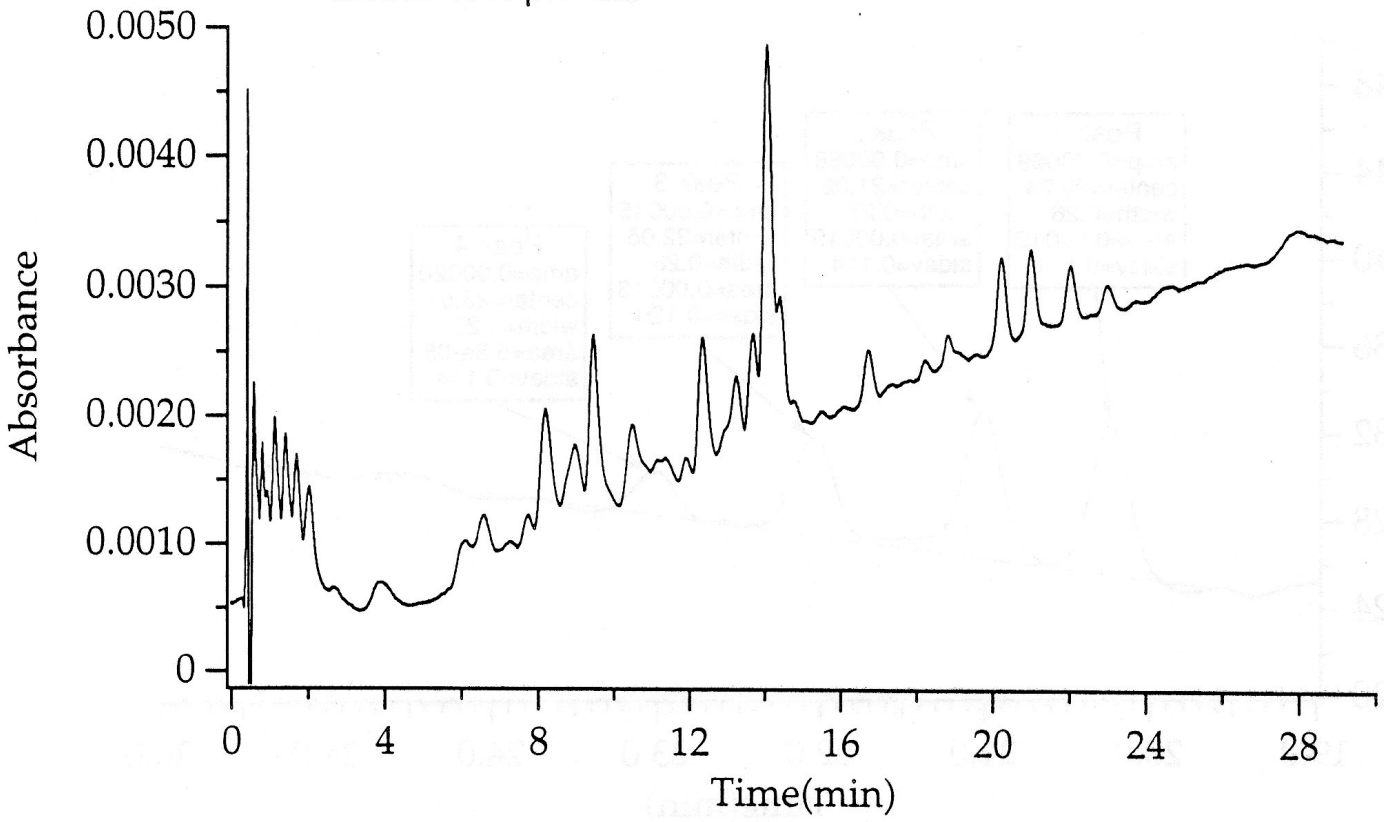
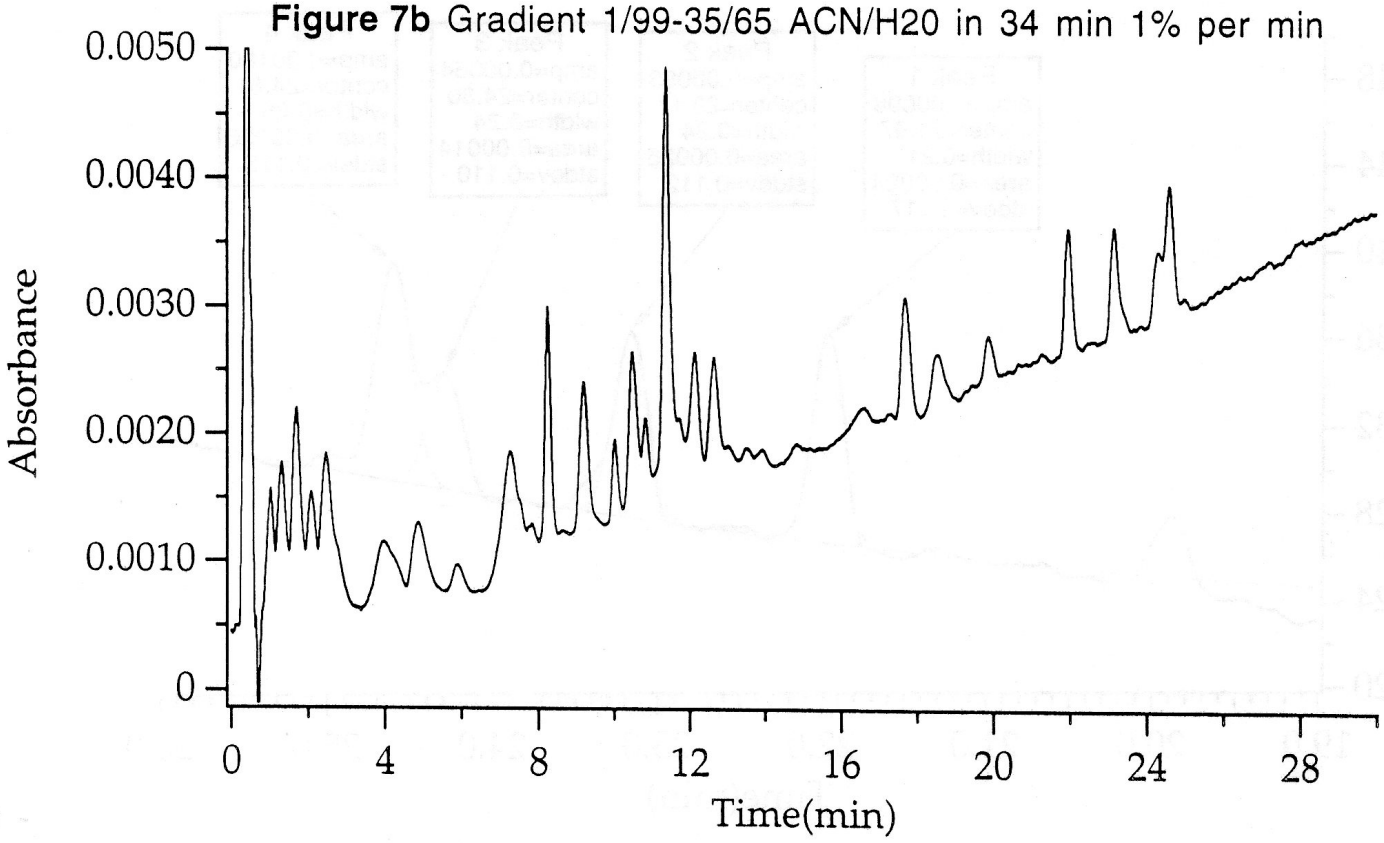


Figure 7b Gradient 1/99-35/65 ACN/H20 in 34 min 1% per min



Expanded view of Tryptic digest of Myoglobin

Figure 8a 0.05% TFA mobile phase

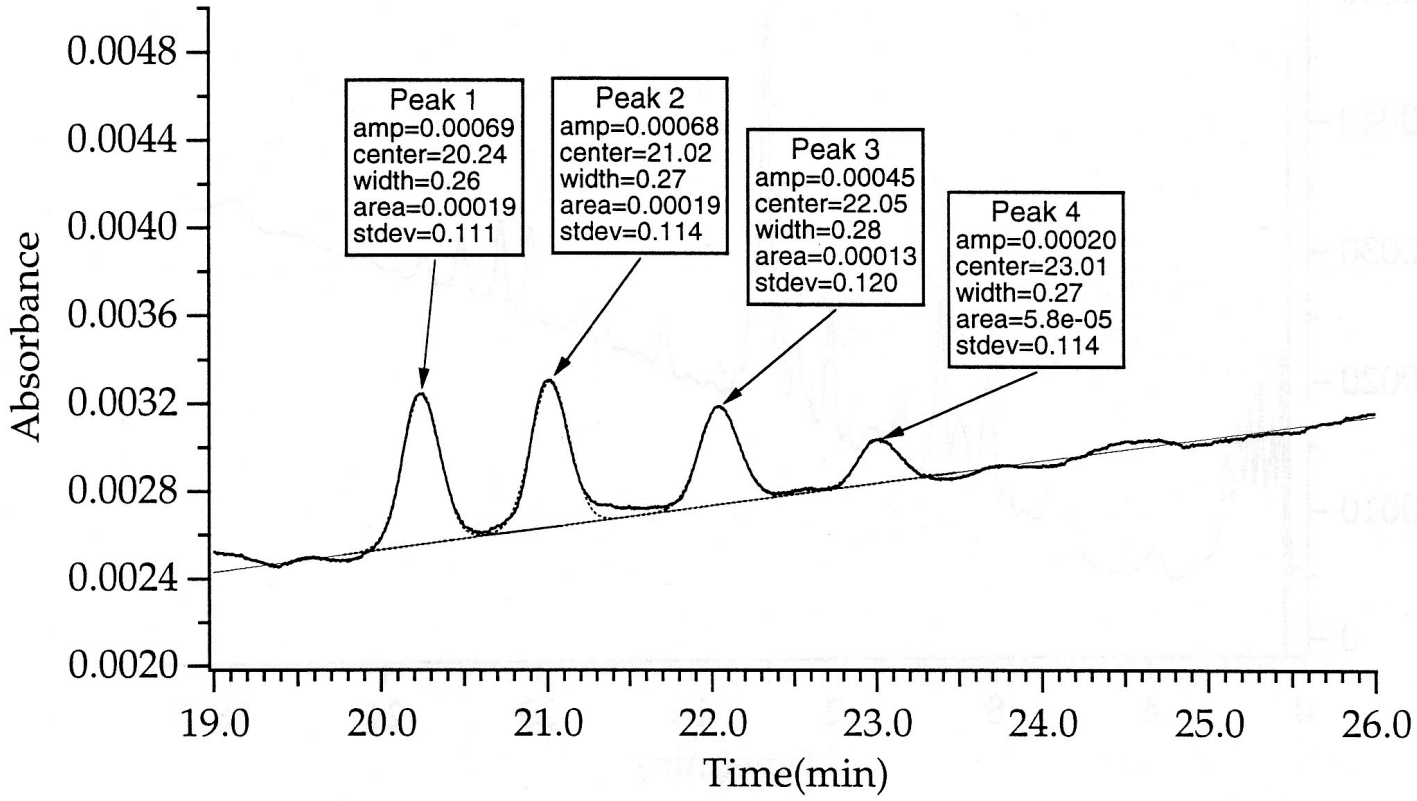
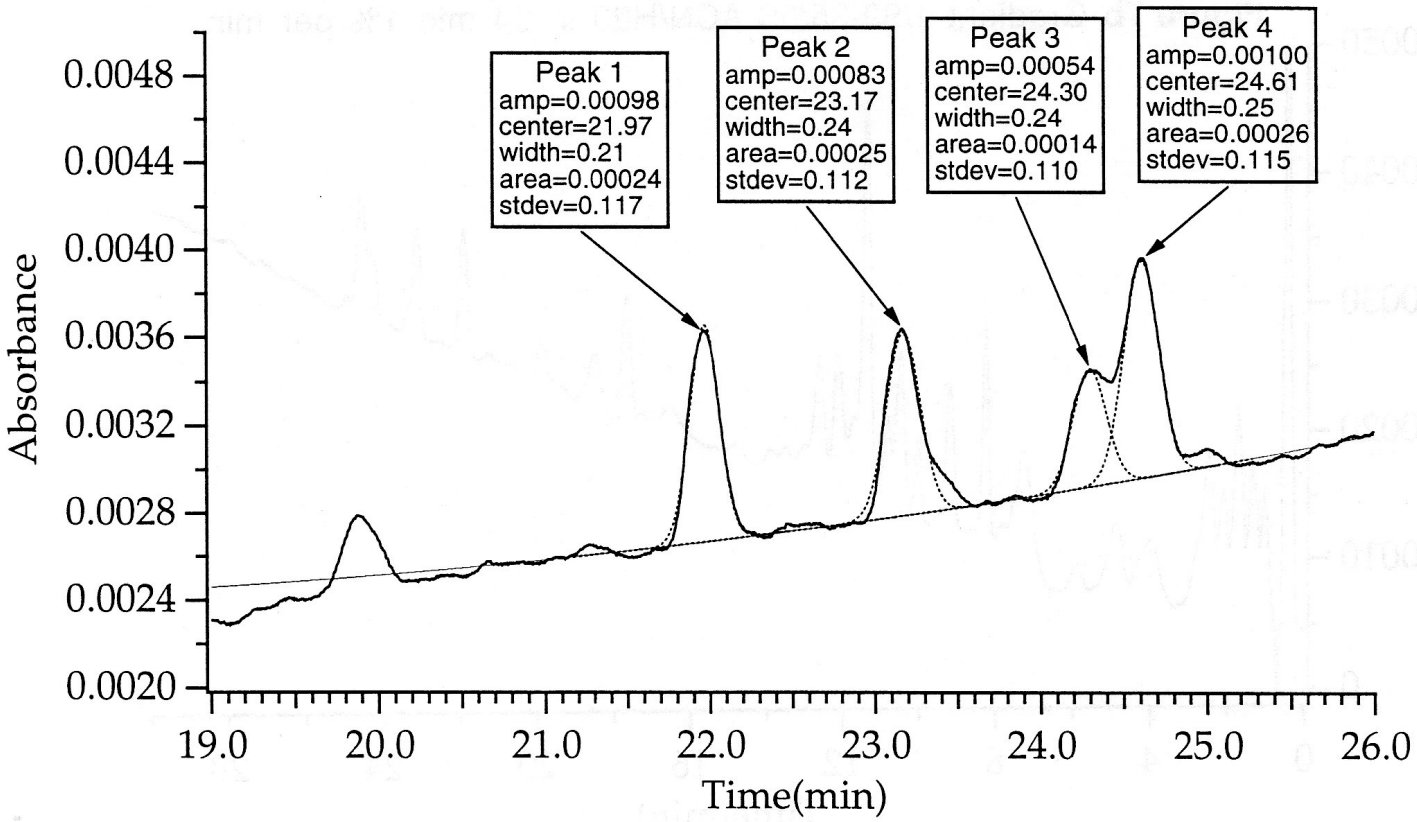


Figure 8b 0.05% acetic acid mobile phase



Tryptic digest of Cytochrome C

Figure 9a Gradient 5/95-35/65 ACN/H20 in 30 min 1% per min
0.05% TFA pH=2.2

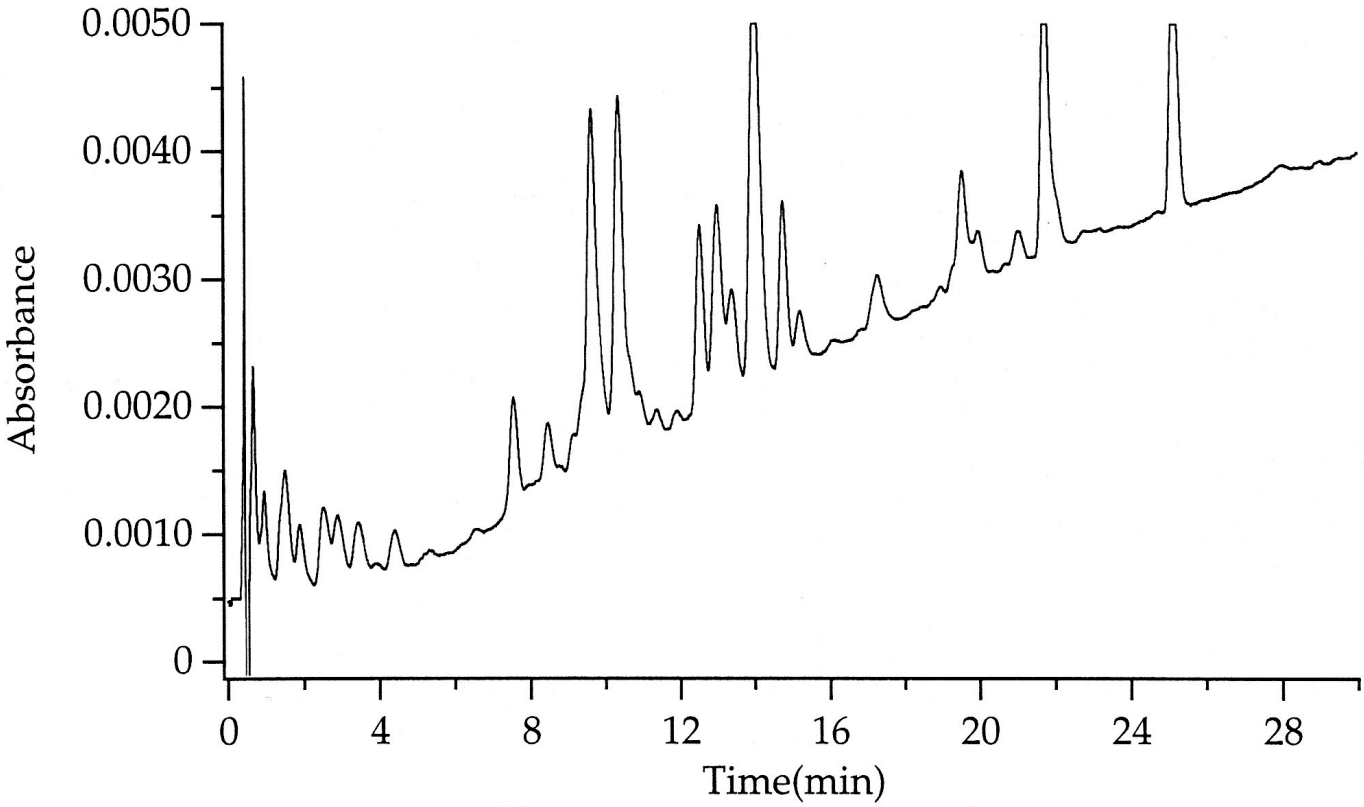


Figure 9b Gradient 1/99-35/65 ACN/H20 in 34 min 1% per min
0.05% Acetic Acid pH=3.1