Poster Presentation

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A Comparison of Analytical and Preparative Reverse Phase Packing Materials for the Purification of Peptides

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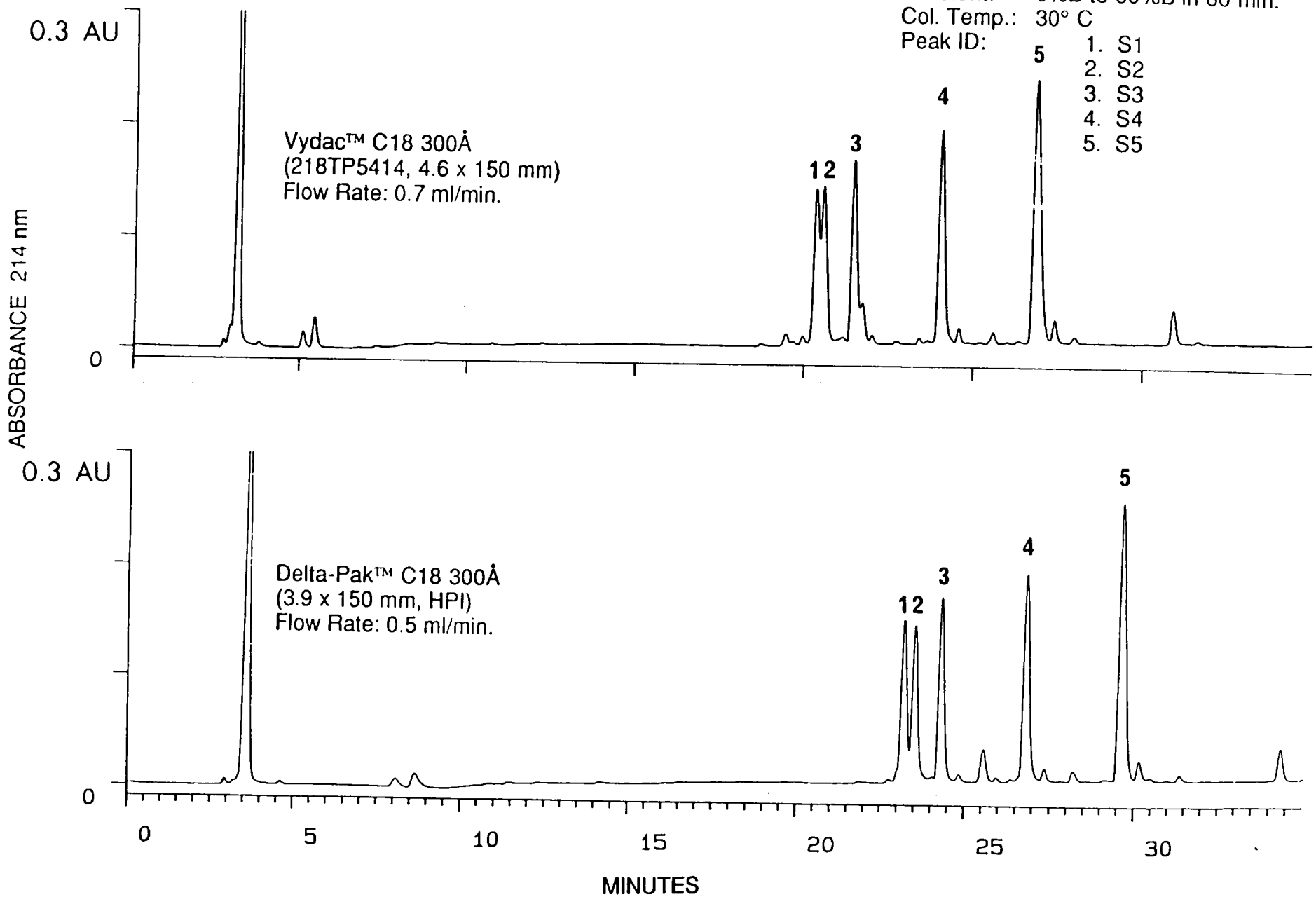
High-performance liquid chromatographic packing materials, representing various chromatographic modes of separation, provide the most powerful separation tools for the analysis and purification of complex mixtures of peptides. One of these modes (reverse phase) separates peptides by their hydrophobic retention on the surface of a bonded-silica packing material and desorption with eluents of increasing organic content. Packing materials utilized in this mode generally use pore enlarged silicas (~300 Å) bonded with alkyl-silanes of different alkyl chain lengths (C_4, C_8 or C_{18}). In this work, two 5 micron C_{18} analytical materials (Delta-Pak™ and Vydac™) and three 15-20 micron C_{18} preparative materials (Delta-Pak™, Bondapak®, and Vydac™) have been evaluated with the Pierce Peptide Retention Standard. The availability of the 5 micron Delta-Pak C_{18} in polymeric HPI (High Pressure Inert) column hardware and the preparative 15-20 micron Delta-Pak and Bondapak materials in inert polymeric PrepPak® cartridge columns allow for the use of HCl as well as TFA as polar mobile phase modifiers. The use of HCl allows for selectivity changes, enhanced sensitivity below 200 nm and the elimination of TFA in the final product. An example of scale-up purification of a synthetic peptide using HCl as the polar modifier from the 5 micron Delta-Pak C_{18} HPI column (3.9 x 150 mm) to a 15 micron Delta-Pak C_{18} PrepPak cartridge column (25 mm X 100 mm) will be described.

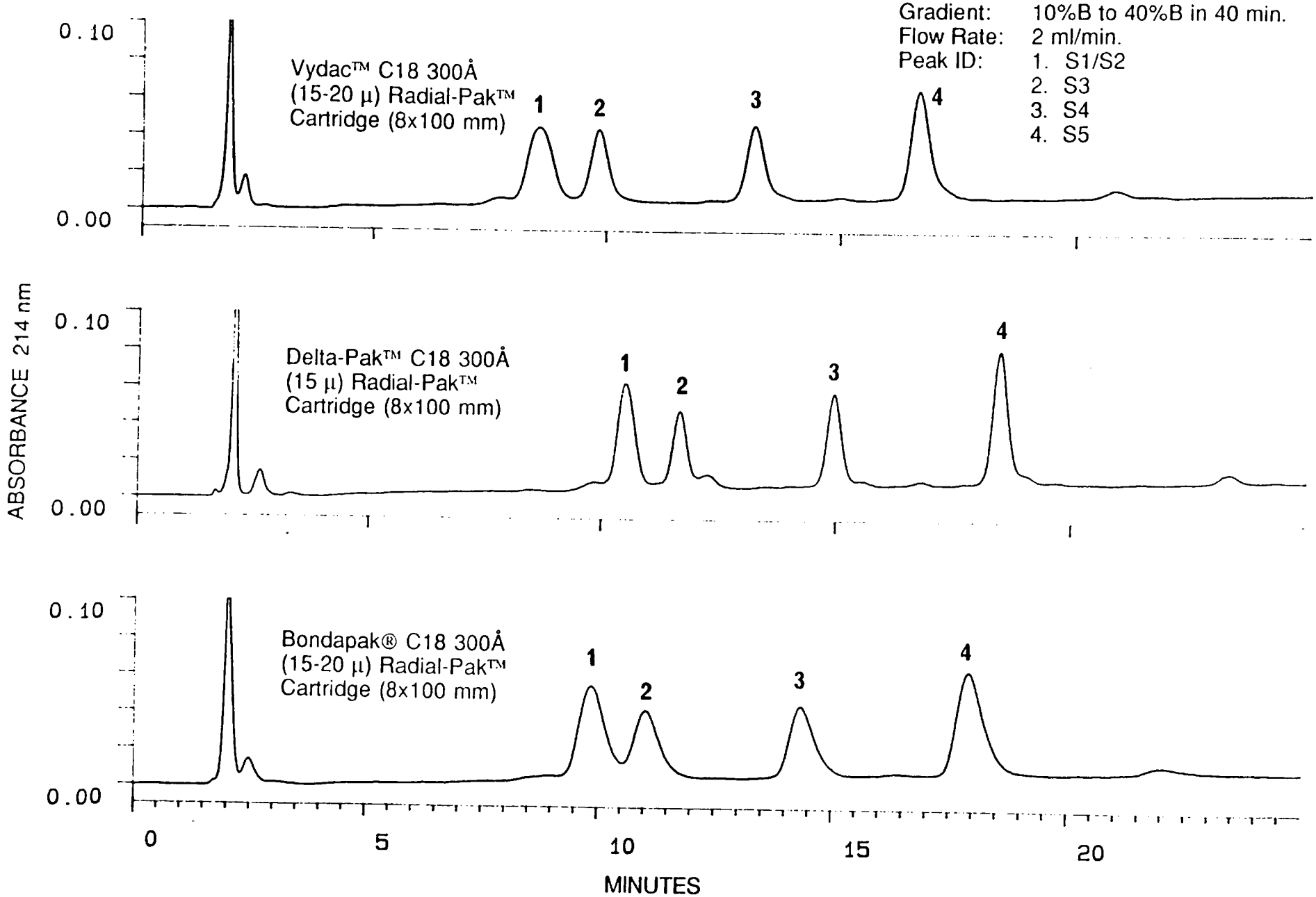
Comparison of Analytical Reverse Phase Packings - Peptide Retention Standard

Solvent A: Water/0.1% TFA
Solvent B: Acetonitrile/0.1% TFA
Gradient: 0%B to 60%B in 60 min.
Col. Temp.: 30° C
Peak ID:
1. S1
2. S2
3. S3
4. S4
5. S5



Comparison of Preparative Reverse Phase Packings - Peptide Retention Standard

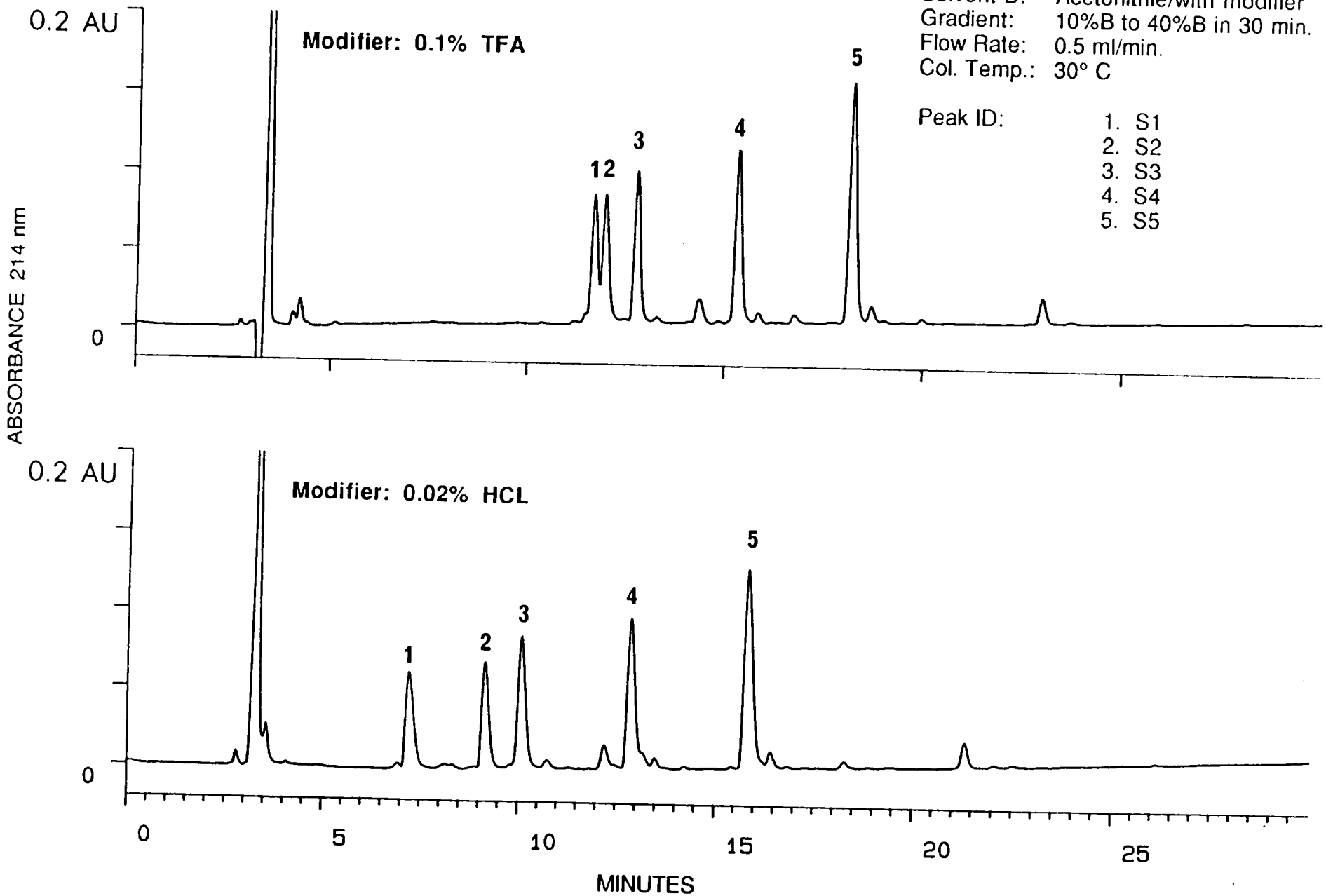
Solvent A: Water/0.1% TFA
Solvent B: Acetonitrile/0.1% TFA
Gradient: 10%B to 40%B in 40 min.
Flow Rate: 2 ml/min.
Peak ID:
1. S1/S2
2. S3
3. S4
4. S5



Comparison of Mobile Phase Modifiers - Peptide Retention Standard

Column: Delta-Pak™ C18 300Å, 5μ (3.9 x 150 mm, HPI)
Solvent A: Water/with modifier
Solvent B: Acetonitrile/with modifier
Gradient: 10%B to 40%B in 30 min.
Flow Rate: 0.5 ml/min.
Col. Temp.: 30° C

Peak ID:
1. S1
2. S2
3. S3
4. S4
5. S5



Comparison of Mobile Phase Modifiers - Peptide Retention Standard

Column: Delta-Pak™ C18 300Å, 5µ (3.9 x 150 mm, HPI)
Solvent A: Water/with modifier
Solvent B: Acetonitrile/with modifier
Gradient: 10%B to 40%B in 30 min.
Flow Rate: 0.5 ml/min.
Col. Temp.: 30° C

Peak ID:
1. S1
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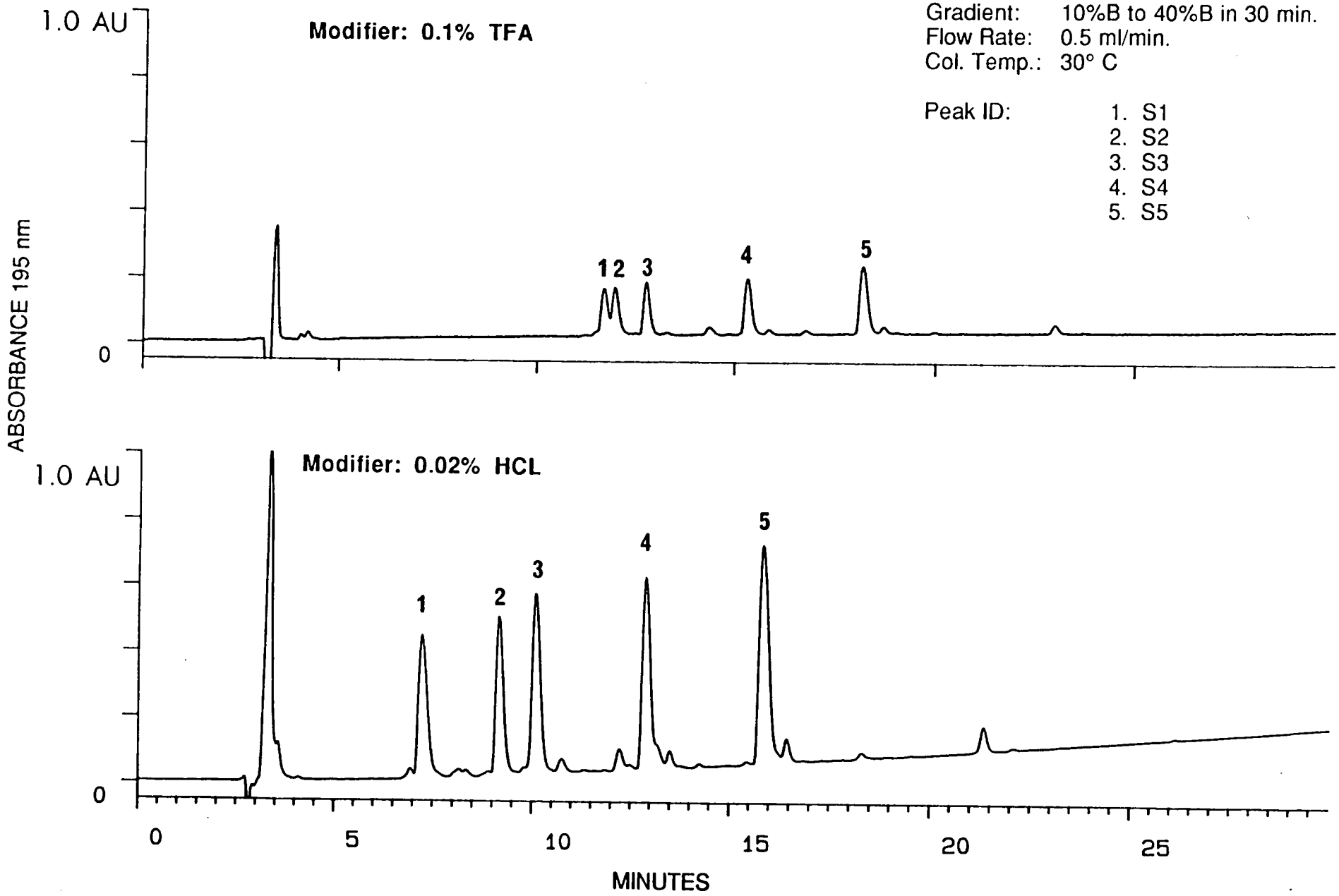
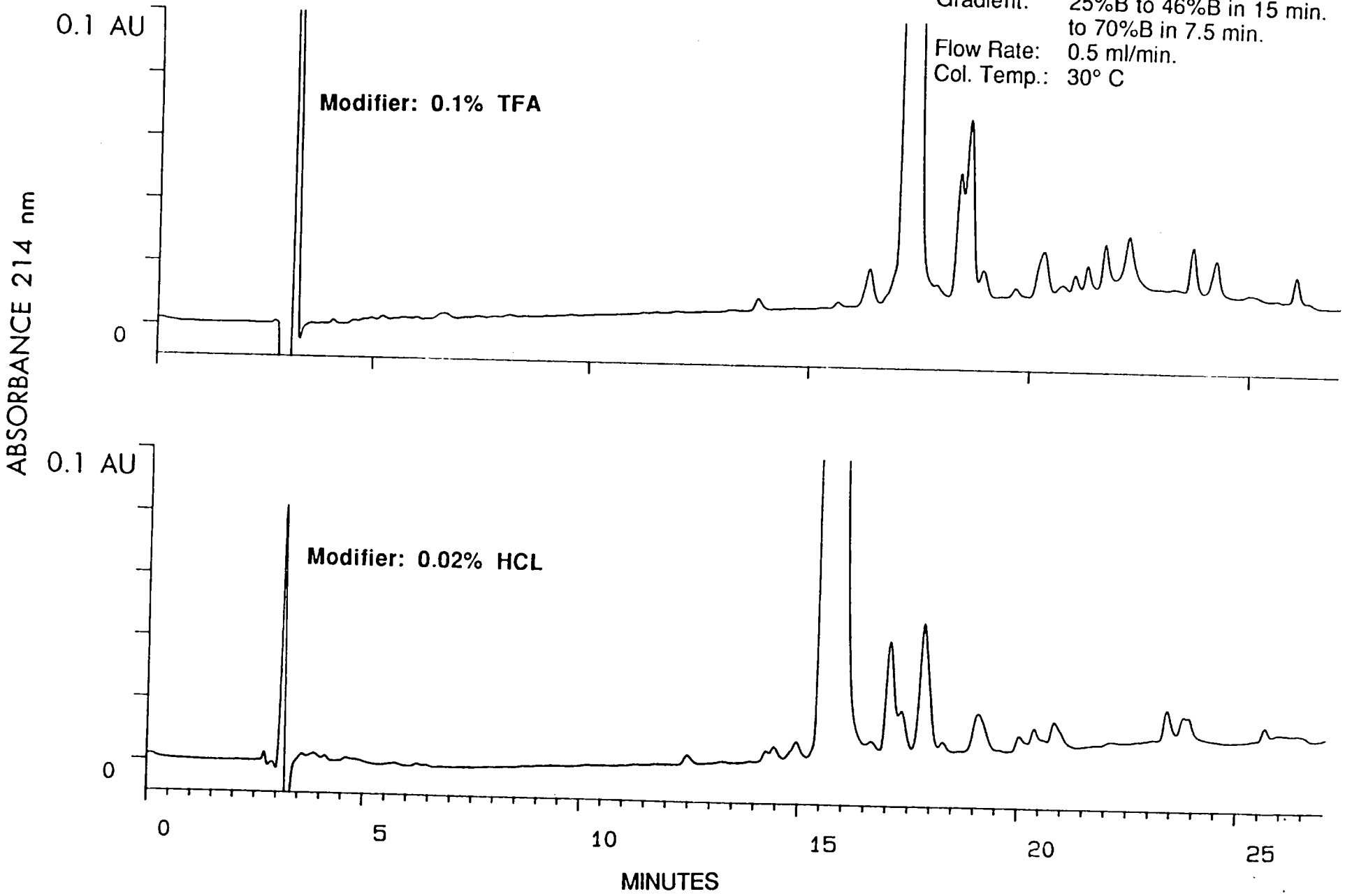
3. S3
4. S4
5. S5



Comparison of Mobile Phase Modifiers - Crude Synthetic Peptide

Column: Delta-Pak™ C18 300Å, 5μ (3.9 x 150 mm, HPI)
Sample: Neurotensin (Synthesis #2)
Solvent A: Water/with modifier
Solvent B: Acetonitrile/with modifier
Gradient: 25%B to 46%B in 15 min. to 70%B in 7.5 min.
Flow Rate: 0.5 ml/min.
Col. Temp.: 30° C



The flow rate and sample load for the preparative column can be calculated according to the following equations. (In this example a sample loading study on the 3.9 x 150 mm was not done.)

$$F_p = F_a \times \frac{V_p}{V_a}$$

F_p=flow rate of the preparative column=?

F_a=flow rate of the analytical column=0.5 ml/min

V_p=preparative column void volume=32.66 ml

V_a=analytical column void volume=1.559 ml

$$Fp = 0.5 \times \frac{32.66}{1.559}$$

$$Fp = 10.5 \text{ ml/min}$$

$$Load_p = Load_a \times \frac{V_p}{V_a}$$

$Load_p$=sample load of the preparative column

$Load_a$=flow rate of the analytical column

V_p=preparative column void volume

V_a=analytical column void volume

Gradient Duration:

When the flow rate is scaled in proportion to the column volumes, the gradient time profile of the analytical and preparative columns remains the same with the exception for the need of a gradient delay for the preparative column.

Preparative Gradient Delay:

The gradient may need some additional adjustment due to the fact that there is a delay volume between the point of mixing of the gradient and the top of the column. This delay volume results in the fact that the separation is isocratic until the gradient delay volume is purged. The effect is more pronounced on a small volume analytical column than on a large volume preparative column and this short isocratic section may need to be simulated on the preparative column.

$$t_d = \frac{D_a}{F_p} \times \left(\frac{V_p}{V_a} - 1\right)$$

t_d=gradient delay for the preparative instrument

D_a=analytical instrument gradient delay volume=0.5835 ml

F_p=flow rate of the preparative column=10.5 ml/min

V_p=preparative column void volume=32.66 ml

V_a=analytical column void volume=1.559 ml

$$t_d = \frac{0.5835}{10.5} \times \left(\frac{32.66}{1.559} - 1\right)$$

t_d=1.11 min

Analytical Versus Preparative Gradient Table:

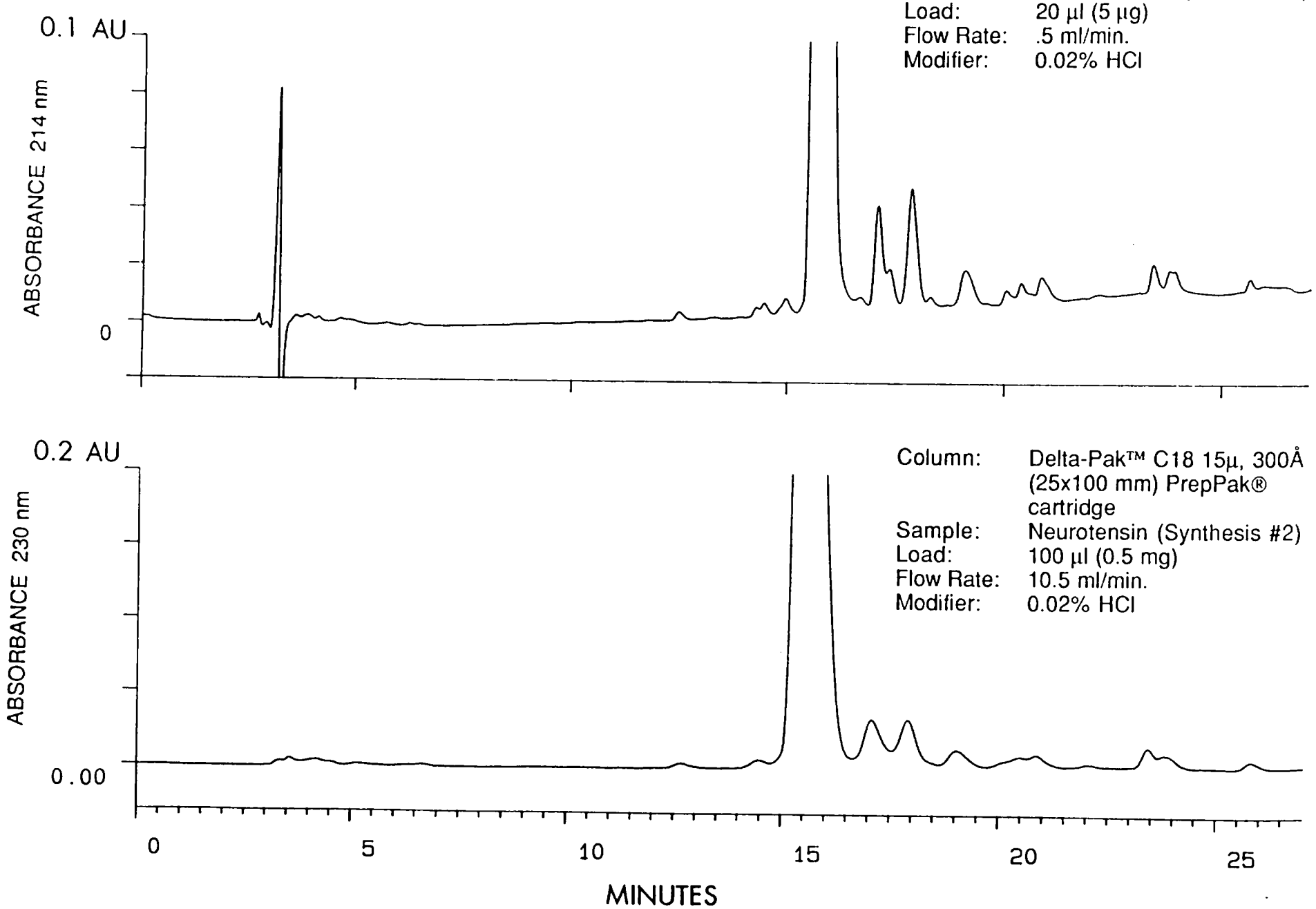
Analytical:

Time	Flow rate	%A	%B	Curve
Initial	0.5	75	25	*
15.0	0.5	54	46	6
22.5	0.5	30	70	6
30.0	0.5	75	25	11

Preparative:

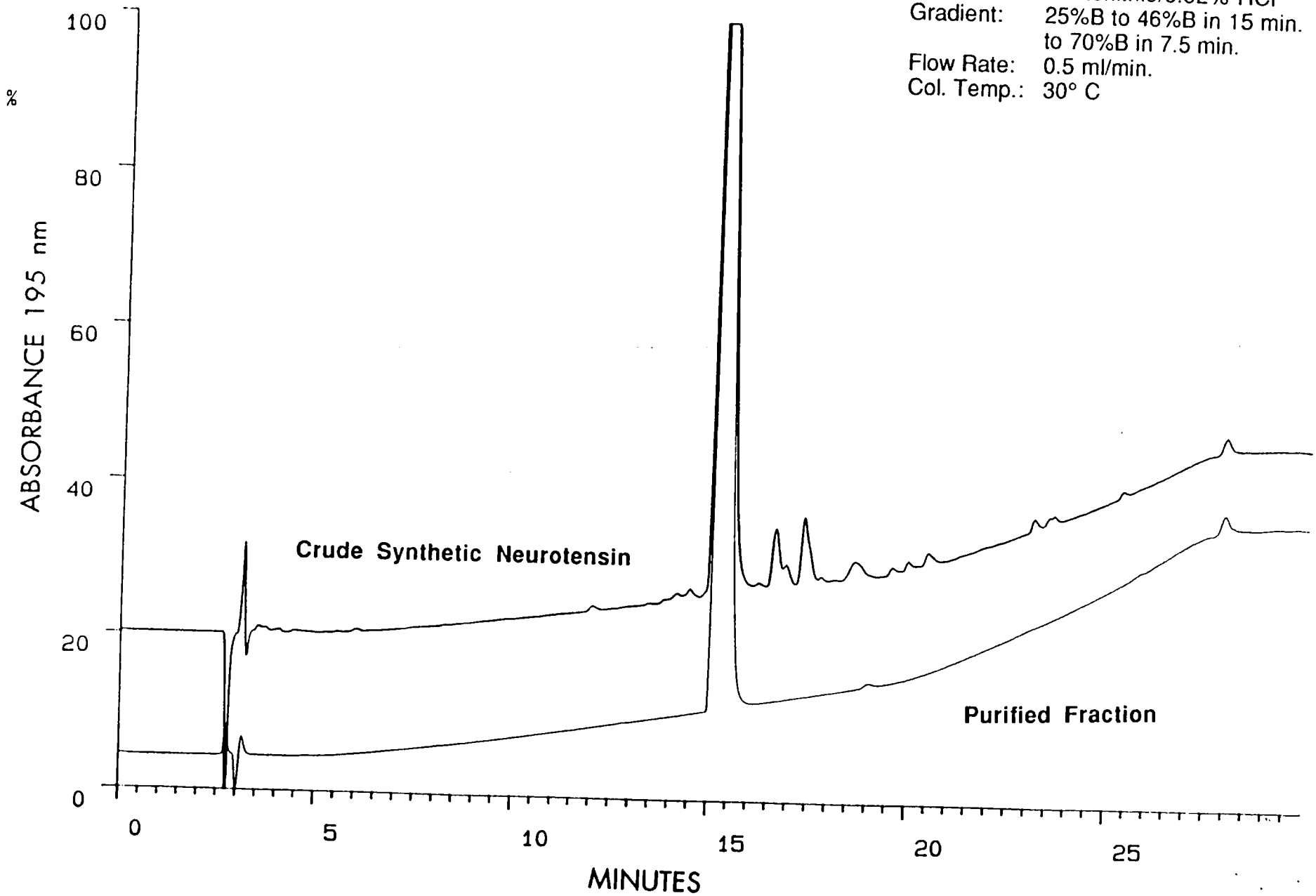
Time	Flow rate	%A	%B	Curve
Initial	10.5	75	25	*
1.11	10.5	75	25	6
16.11	10.5	54	46	6
23.61	10.5	30	70	6
31.11	10.5	75	25	11

Scale-Up from an Analytical Column to a Preparative Cartridge



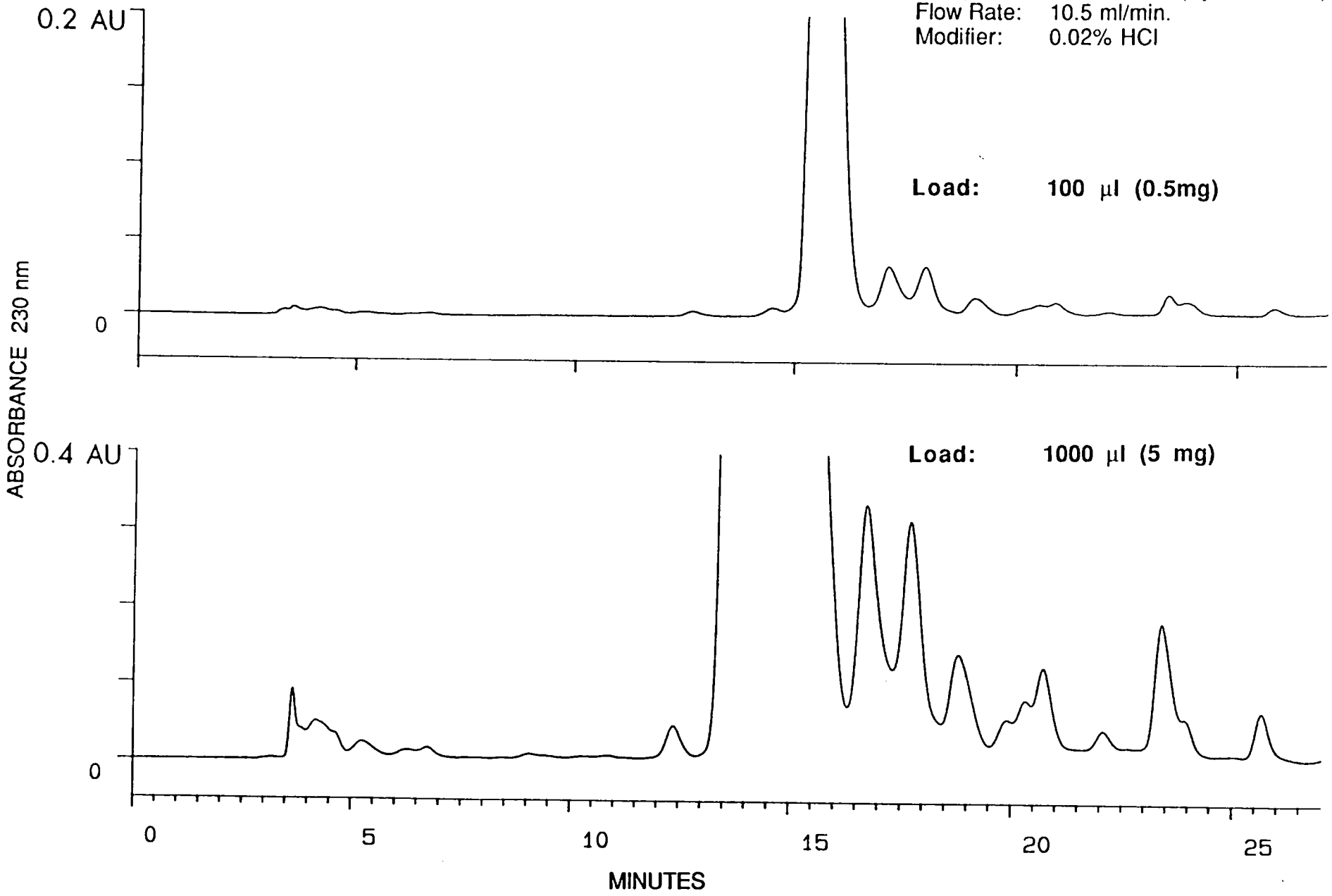
Analysis of Purified Fraction From Preparative Chromatography

Column: Delta-Pak™ C18 300Å, 5μ (3.9 x 150 mm, HPI)
Solvent A: Water/0.02% HCl
Solvent B: Acetonitrile/0.02% HCl
Gradient: 25%B to 46%B in 15 min. to 70%B in 7.5 min.
Flow Rate: 0.5 ml/min.
Col. Temp.: 30° C



Sample Load Comparison on a Preparative Cartridge

Column: Delta-Pak™ C18 15µ, 300Å (25x100 mm) PrepPak® cartridge
Sample: Neurotensin (Synthesis #2)
Flow Rate: 10.5 ml/min.
Modifier: 0.02% HCl



In order to scale-up from the 3.9 x 150 mm HPI column to the preparative 25 x 100 mm PrepPak cartridge it is important to realize that both the column diameter and column length, as well as the packing particle size are changing. It is therefore easiest to determine the flow rate and the sample load of the preparative column in proportion to the column volumes. This will result in identical retention times for both the analytical and preparative column; but a decrease in resolution will be observed for the preparative column due to the 15 micron packing material.

Flow Rate and Sample Load:

Before the chromatography can be scaled-up from the 3.9 mm ID analytical column to the 25 mm ID preparative column the actual column void volumes and instrument gradient delays need to be determined. The column void volumes are obtained with acetone and the instrument gradient delay is obtained by removing the column, spiking eluent B with acetone and running a gradient from 100%A to 100%B (curve 11).

625 Analytical System:

V_a=3.9 x 150 mm HPI column void volume=1.559 ml

D_a=analytical instrument gradient delay=0.5835 ml

600 Preparative System:

V_p=25 x 100 mm PrepPak cartridge void volume = 32.66 ml

D_p=preparative instrument gradient delay=6.6462 ml

Preparative Purification of a Synthetic Peptide-Neurotensin

Scale-Up by Column Volume

1. Using HCl as a modifier

2. Scaling-up from a 3.9 mm ID HPI column to a 25 mm ID PrepPak cartridge

   a. Different column length (15 cm for the HPI column versus 10 cm for the PrepPak cartridge)

   b. Same Delta-Pak™ C18, 300Å chemistry but different particle size (5 micron for the HPI column versus 15 micron for the PrepPak cartridge)

3. Using two different HPLC sysytems for the separations (625 LC System for the analytical and 600 Multi Solvent Delivery System for the preparative)