Prep '90

Poster Presentation

Strategy for the Large Scale Preparative Isolation of a Synthetic Peptide Fragment Using High Efficiency 5 μm Reversed Phase Packings

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ABSTRACT

Peptides synthesized using solid-phase techniques and automated instrumentation must be purified to remove failed reaction sequences. Purity requirements for peptides used for diagnostic and therapeutic purposes have necessitated the use of high efficiency chromatographic packings for peptide purification. The use of 15 micron (μm) reversed phase packings for peptide purification has been demonstrated previously. Higher efficiency 5 μm packings in large columns have not been widely employed due, in part, to the limited ability of preparative HPLC instrumentation to deliver elevated flow rates at high back pressure.

A synthetic peptide fragment of human calcium activated neutral protease is being investigated for the treatment of Alzheimer's Disease. In order to obtain sufficient yield and purity, both 5 and 15 μm reversed phase packings with new preparative instrumentation were employed in the purification of the peptide fragment using optimized water/acetonitrile/trifluoroacetic acid gradients.

Introduction

Automated instrumentation for solid-phase peptide synthesis has has widely expanded the ability for most laboratories to produce biologically active peptides. However, for the synthetic peptide to be useful in a biological application, the peptide must be rigorously purified. The product of a synthetic protocol is often obtained in a complex mixture of peptide fragments including deletions and abbreviated sequences that may closely resemble the desired peptide.

Typically, peptides are separated by reversed phase high performance liquid chromatography (HPLC) using a gradient of increasing acetonitrile concentration in the presence of trifluoroacetic acid (TFA). Modifications in flow rate and gradient slope are used to enhance resolution of the desired peptide from contaminating materials. For small amounts of peptides, ranging from picomoles to low nanomoles, analytical columns (~3.9 mm i.d.) packed with 5µm-sized particles provide excellent resolution. For larger amounts, separations are routinely performed on preparative scale columns (>3.9mm i.d.) packed with 15 µm particles that provide higher capacity. While larger particles are more economical, some loss of efficiency and resolution can be expected when compared to 5 µm supports. Generally, it has not be practical to use higher efficiency 5 µm packings in large scale isolations because most existing instruments cannot deliver higher flow rates at elevated operating pressures associated with small particles. The introduction of a new preparative liquid chromatograph, the Delta Prep™ 4000, capable of operating at flow rates up to 150 ml/min with system pressures as high as 4000 psi has facilitated the investigation of 5 µm packing materials in larger columns.

In the present study, the utility of 5 µm and 15 µm packing materials in the preparative isolation of a peptide from a complex mixture was examined.

Materials and Methods

A 21-residue peptide fragment from human calcium activated protease (H_2N-AGIAAKLAKDREAAEGLGSHC-COOH) was synthesized by a continuous flow, automated solid phase 9-fluorenylmethyloxycarbonyl (FMOC) protocol on a model 9050 peptide synthesizer (MilliGen/Biosearch). FMOC-amino acid precursors and reagents were supplied by MilliGen/Biosearch. Following cleavage and deprotection, the synthetic product was lyophilized and stored at -20°C until analyzed.

Chromatography was performed on the Delta Prep™ 4000 (Waters Chromatography Division) equipped with a tunable ultraviolet (TUV) wavelength detector (model 484, Waters). Both small- and large-scale separations could be monitored with the dual flow function cell of the 484 detector. Several analytical and preparative scale columns packed with silica-based C18 reversed phase were used in the purification. Particle sizes were either 5 μm or 15 μm with 300 Å pores. All supports had low carbon load (<10%) and were fully end-capped.

Scaling Operating Parameters

Sample Load: $$Load_{large\text{-}scale} = Load_{small\text{-}scale} \frac{(D_{large\text{-}scale})^2 L_{large\text{-}scale}}{(D_{small\text{-}scale})^2 L_{small\text{-}scale}}$$

Where: D = Internal diameter of column
L = Length of column

Flow Rate: $$F_{large\text{-}scale} = F_{small\text{-}scale} \frac{(D_{large\text{-}scale})^2}{(D_{small\text{-}scale})^2}$$

Where: F = Flow rate (ml/min)
D = Diameter of column (cm)

Gradient Duration: $$GD_{large\text{-}scale} = GD_{small\text{-}scale} \frac{V_{large\text{-}scale}\ F_{small\text{-}scale}}{V_{small\text{-}scale}\ F_{large\text{-}scale}}$$

Where: V = Volume of column (ml)
GD = Gradient duration (min)
F = Flow rate (ml/min)

Gradient Delay: $$T = \frac{SV_{small\text{-}scale}}{F_{small\text{-}scale}} - \frac{SV_{large\text{-}scale}}{F_{large\text{-}scale}}$$

Where: T = Time delay (min)
SV = System volume (ml)
F = Flow rate (ml/min)

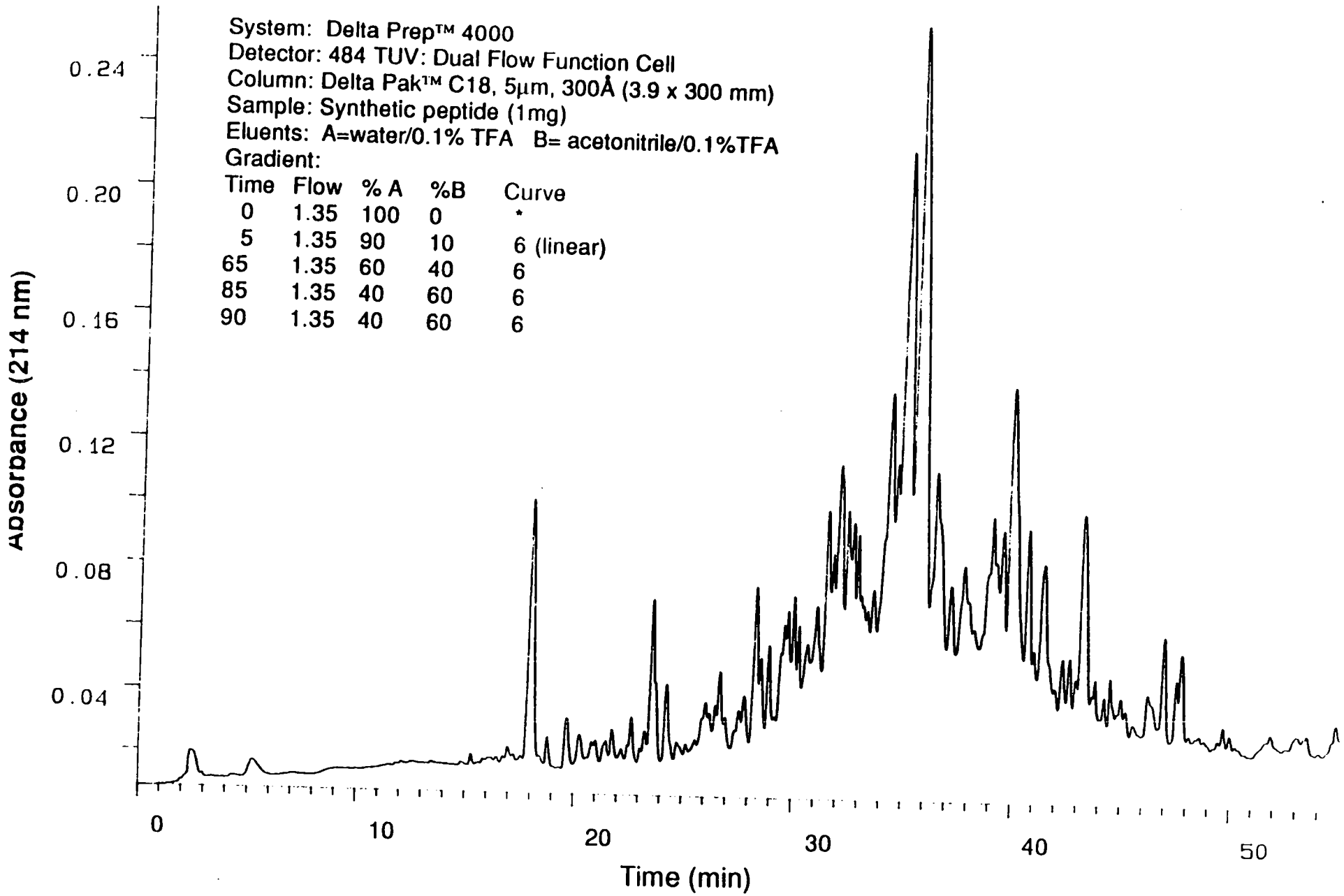
System: Delta Prep™ 4000
Detector: 484 TUV: Dual Flow Function Cell
Column: Delta Pak™ C18, 5µm, 300Å (3.9 x 300 mm)
Sample: Synthetic peptide (1mg)
Eluents: A=water/0.1% TFA B= acetonitrile/0.1%TFA
Gradient:
Time Flow % A %B Curve
0 1.35 100 0 *
5 1.35 90 10 6 (linear)
65 1.35 60 40 6
85 1.35 40 60 6
90 1.35 40 60 6
Absorbance (214 nm)
0.24
0.20
0.16
0.12
0.08
0.04
0
10
20
30
40
50
Time (min)

Figure 1 Small-scale Separation of 1mg Synthetic Peptide on 5 μm Reversed Phase Support. The reaction mixture was separated on a small (3.9 x 300 mm) high resolution 5 μm C18 column. An optimized gradient was developed to maximize the resolution of the peptide eluting at 35.4 min. Due to the complexity of this mixture, a multi-step approach to the purification was developed.

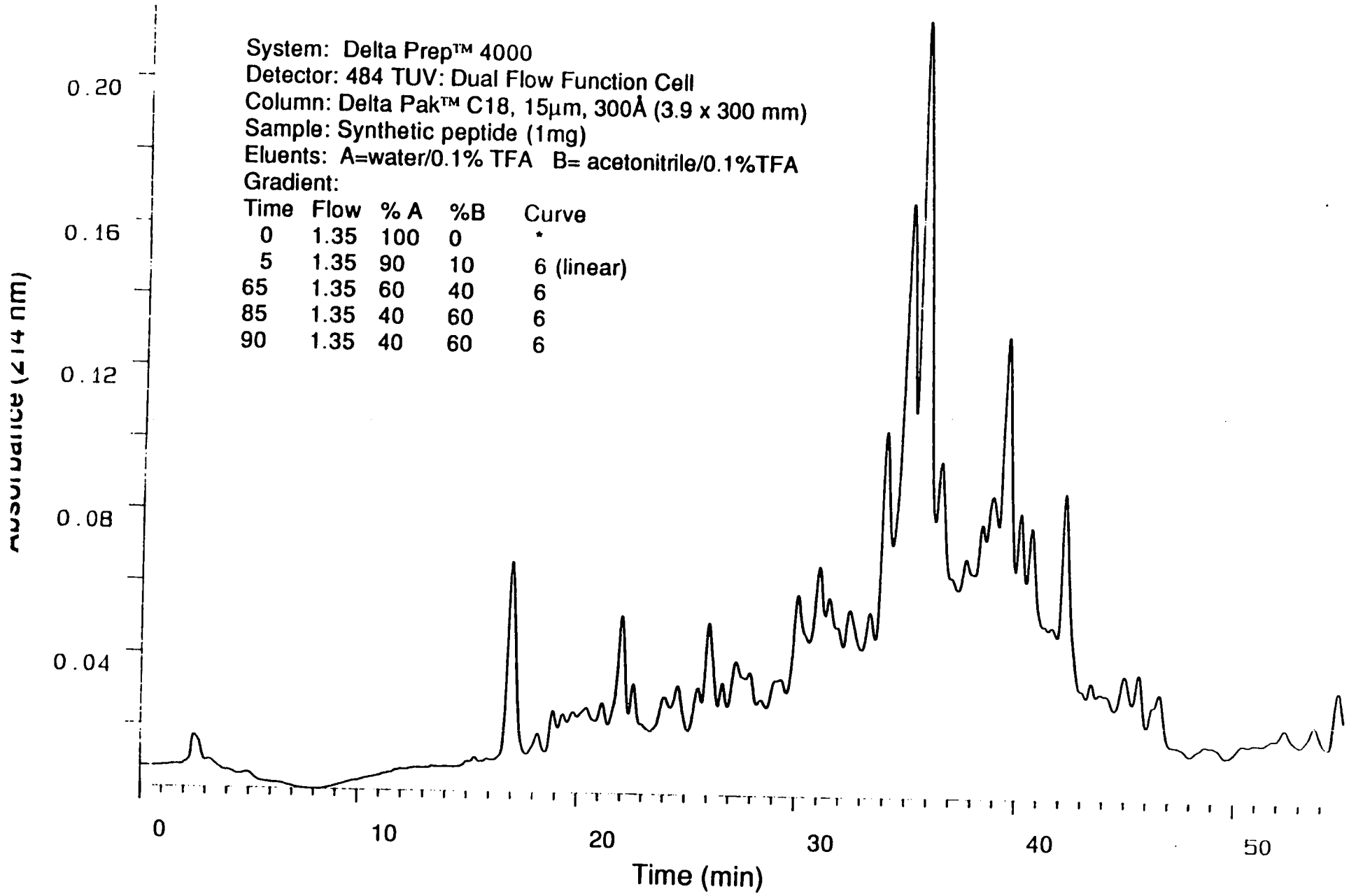
System: Delta Prep™ 4000
Detector: 484 TUV: Dual Flow Function Cell
Column: Delta Pak™ C18, 15µm, 300Å (3.9 x 300 mm)
Sample: Synthetic peptide (1mg)
Eluents: A=water/0.1% TFA B= acetonitrile/0.1%TFA
Gradient:
Time Flow % A %B Curve
0 1.35 100 0 *
5 1.35 90 10 6 (linear)
65 1.35 60 40 6
85 1.35 40 60 6
90 1.35 40 60 6
Absorbance (214 nm)
0.20
0.16
0.12
0.08
0.04
0
10
20
30
40
50
Time (min)

Figure 2 Small-scale Separation of 1 mg Synthetic Peptide on 15 μm Reversed Phase Support. The 21-mer was separated on a 15 μm column with the same gradient described in Figure 1. The 5 μm packing material (Figure 1) provided greater resolution of the smaller detail of the peptide contaminants than the 15 μm support. The overall elution profile was similar on both packings. The major component eluted at the same retention time and was separated from the contaminating peptides with a similar degree of resolution. Thus, this separation provided the framework for scale-up to larger column sizes packed with more economical 15 μm supports.

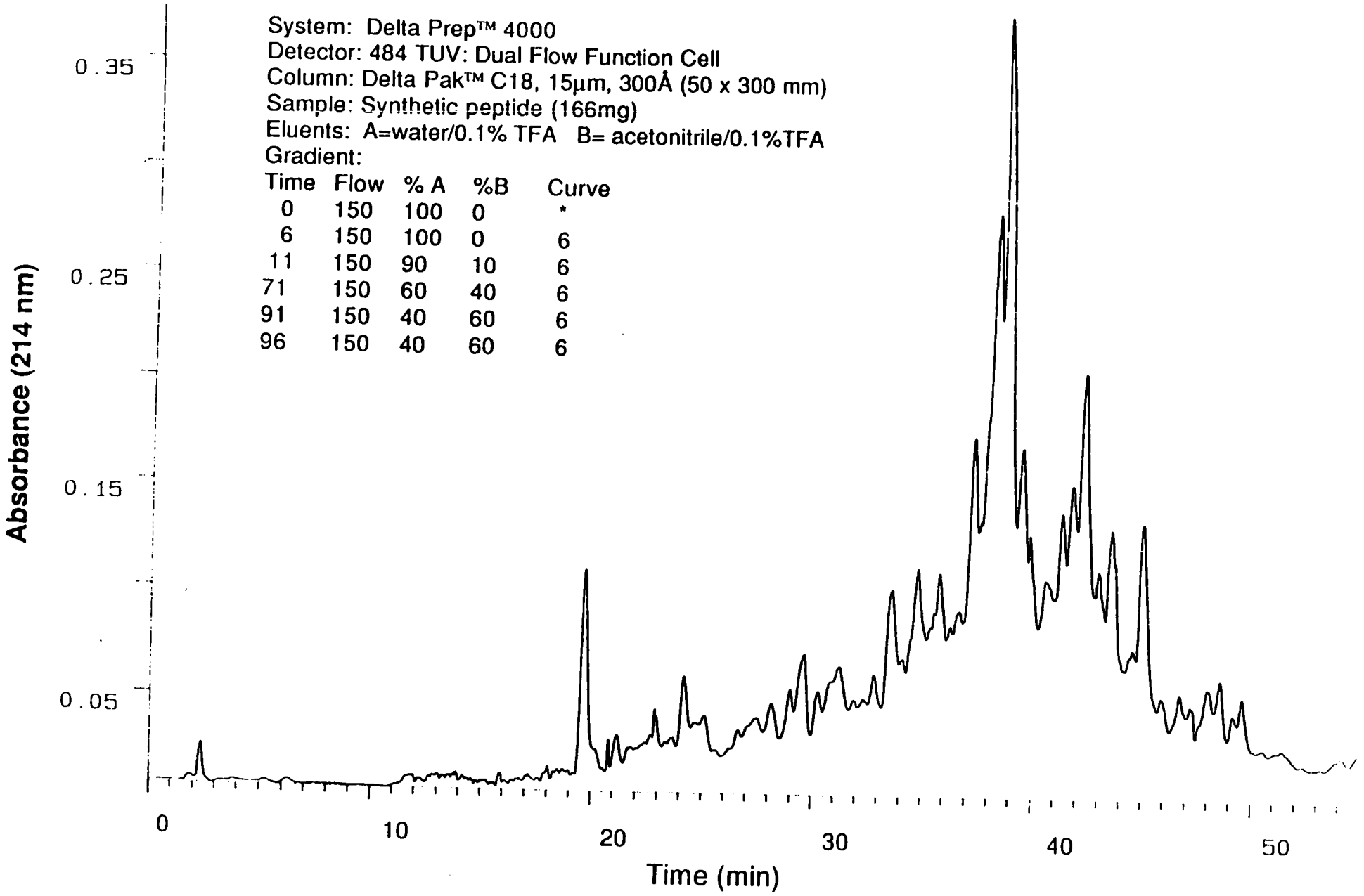
System: Delta Prep™ 4000
Detector: 484 TUV: Dual Flow Function Cell
Column: Delta Pak™ C18, 15µm, 300Å (50 x 300 mm)
Sample: Synthetic peptide (166mg)
Eluents: A=water/0.1% TFA B= acetonitrile/0.1%TFA
Gradient:
Time Flow % A %B Curve
0 150 100 0 *
6 150 100 0 6
11 150 90 10 6
71 150 60 40 6
91 150 40 60 6
96 150 40 60 6
Absorbance (214 nm)
0.35
0.25
0.15
0.05
0
10
20
30
40
50
Time (min)

Figure 3 Large-scale Separation of 166 mg Synthetic Peptide on 15 µm Reversed Phase Support. Using the sample load and optimized gradient developed for the small-scale separations, the separation was scaled-up to a 50 x 300 mm column. The peptide mixture (166 mg) was solubilized in 0.1% TFA (100 ml) and applied to the column at a flow rate of 10 ml/min. Following sample application, initial conditions were held for 6-min to duplicate the effect of the system delay volume seen in the small-scale isolations. In the above chromatogram, the overall resolution of the separation was retained. Several fractions enriched in the peak-of-interest were collected, chromatographed individually (data not shown), and pooled (120 ml) for further purification.

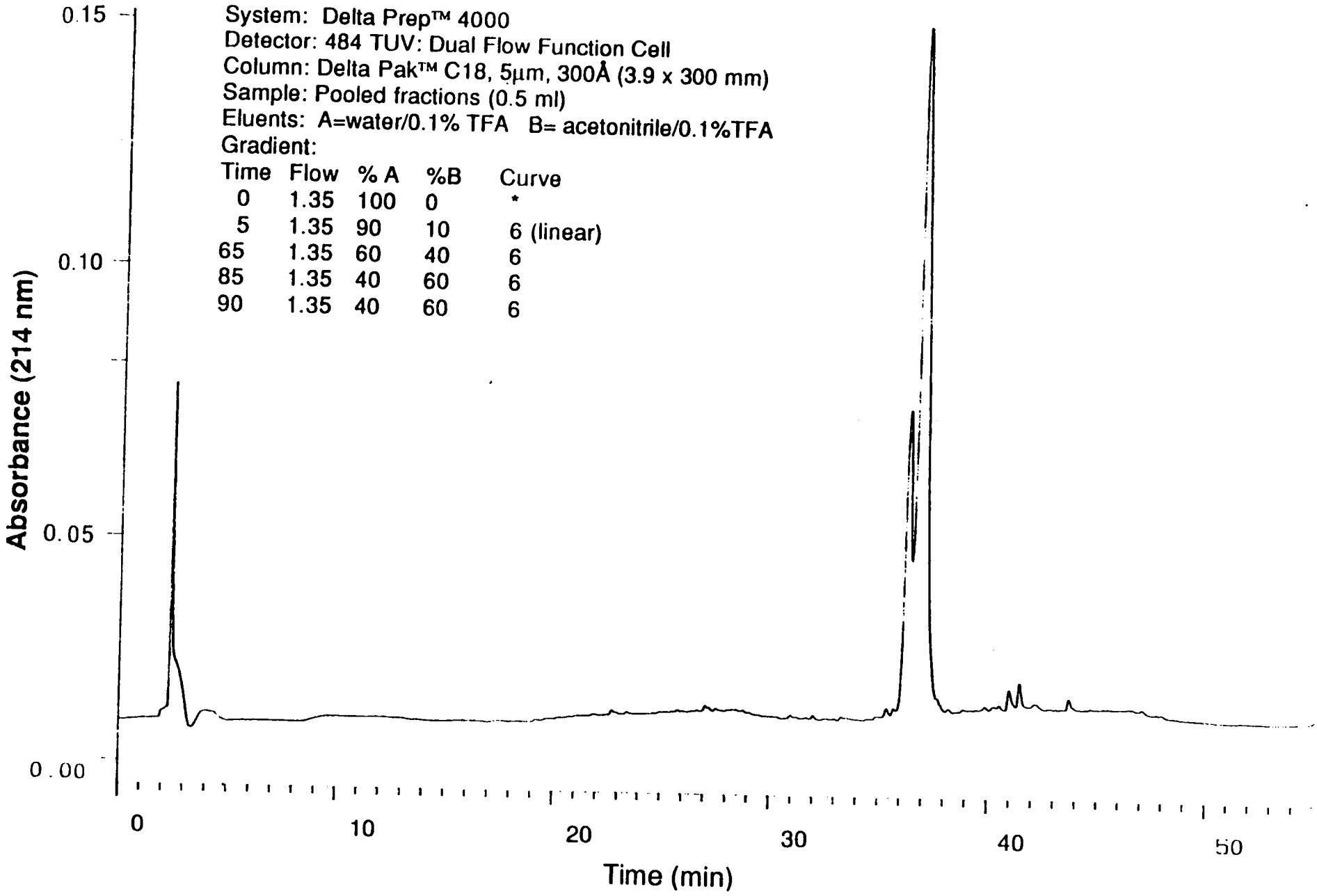
System: Delta Prep™ 4000
Detector: 484 TUV: Dual Flow Function Cell
Column: Delta Pak™ C18, 5µm, 300Å (3.9 x 300 mm)
Sample: Pooled fractions (0.5 ml)
Eluents: A=water/0.1% TFA B= acetonitrile/0.1%TFA
Gradient:
Time Flow % A %B Curve
0 1.35 100 0 *
5 1.35 90 10 6 (linear)
65 1.35 60 40 6
85 1.35 40 60 6
90 1.35 40 60 6
0.15
0.10
0.05
0.00
Absorbance (214 nm)
0
10
20
30
40
50
Time (min)

Figure 4 Small-scale Purification of Pooled Fractions on 5 µm Support. In order to enhance the resolution of the peptide from closely-eluting contaminants, the next step in the purification was performed on a high-resolution 5 µm support using the same gradient as Figures 1 and 2. The Pooled Fractions from the preparative isolation (Figure 3) contained a significant contaminant seen as a shoulder on the leading edge of the peak-of-interest.

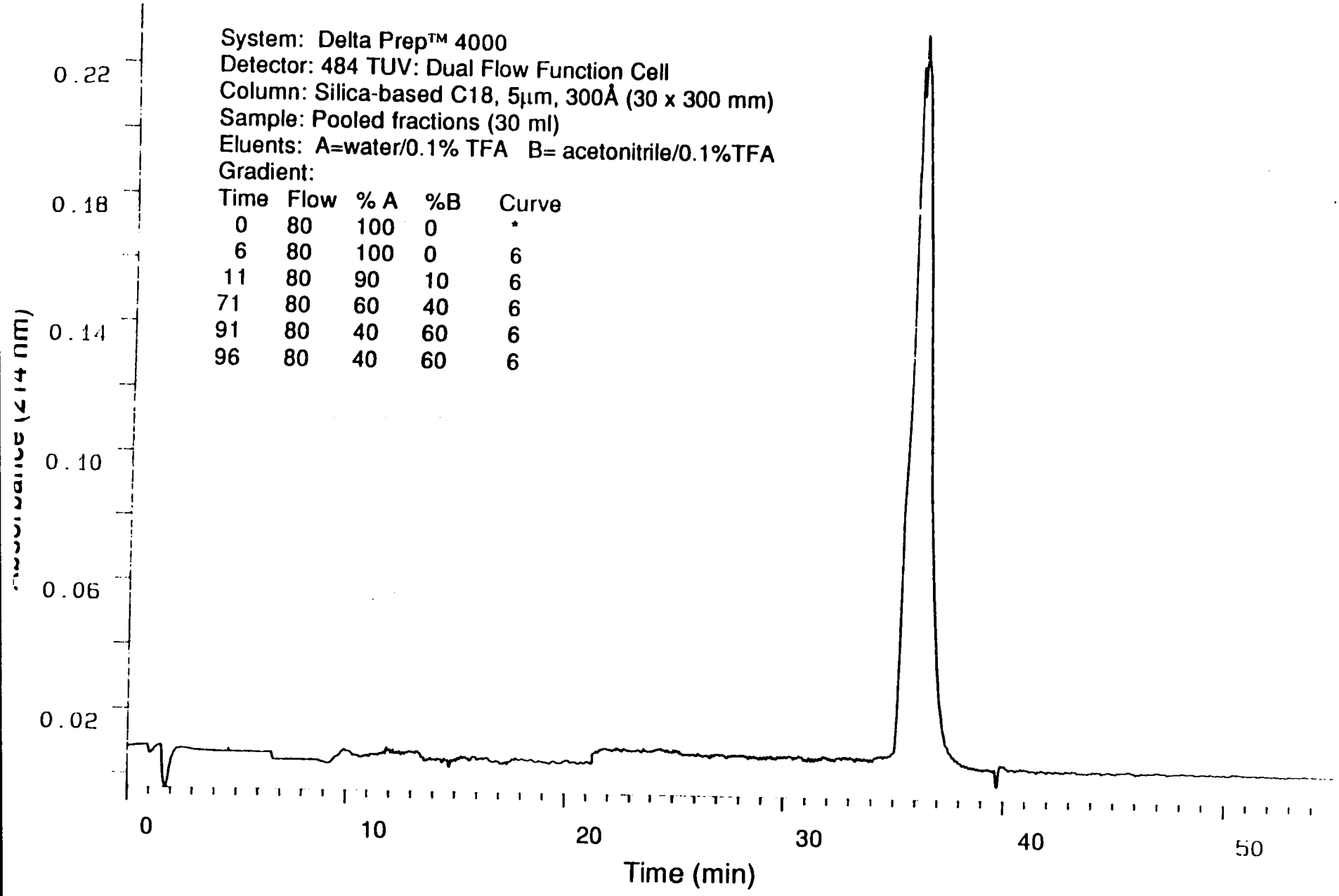
System: Delta Prep™ 4000
Detector: 484 TUV: Dual Flow Function Cell
Column: Silica-based C18, 5µm, 300Å (30 x 300 mm)
Sample: Pooled fractions (30 ml)
Eluents: A=water/0.1% TFA B= acetonitrile/0.1%TFA
Gradient:
Time Flow % A %B Curve
0 80 100 0 *
6 80 100 0 6
11 80 90 10 6
71 80 60 40 6
91 80 40 60 6
96 80 40 60 6
0.22
0.18
0.14
0.10
0.06
0.02
0
10
20
30
40
50
Time (min)

Figure 5 Large-scale Purification of Pooled Fractions on 5 µm Support. The Pooled Fractions were purified on the preparative-scale with minimal loss of resolution. Fractions were collected and screened for purity by reversed phase HPLC.

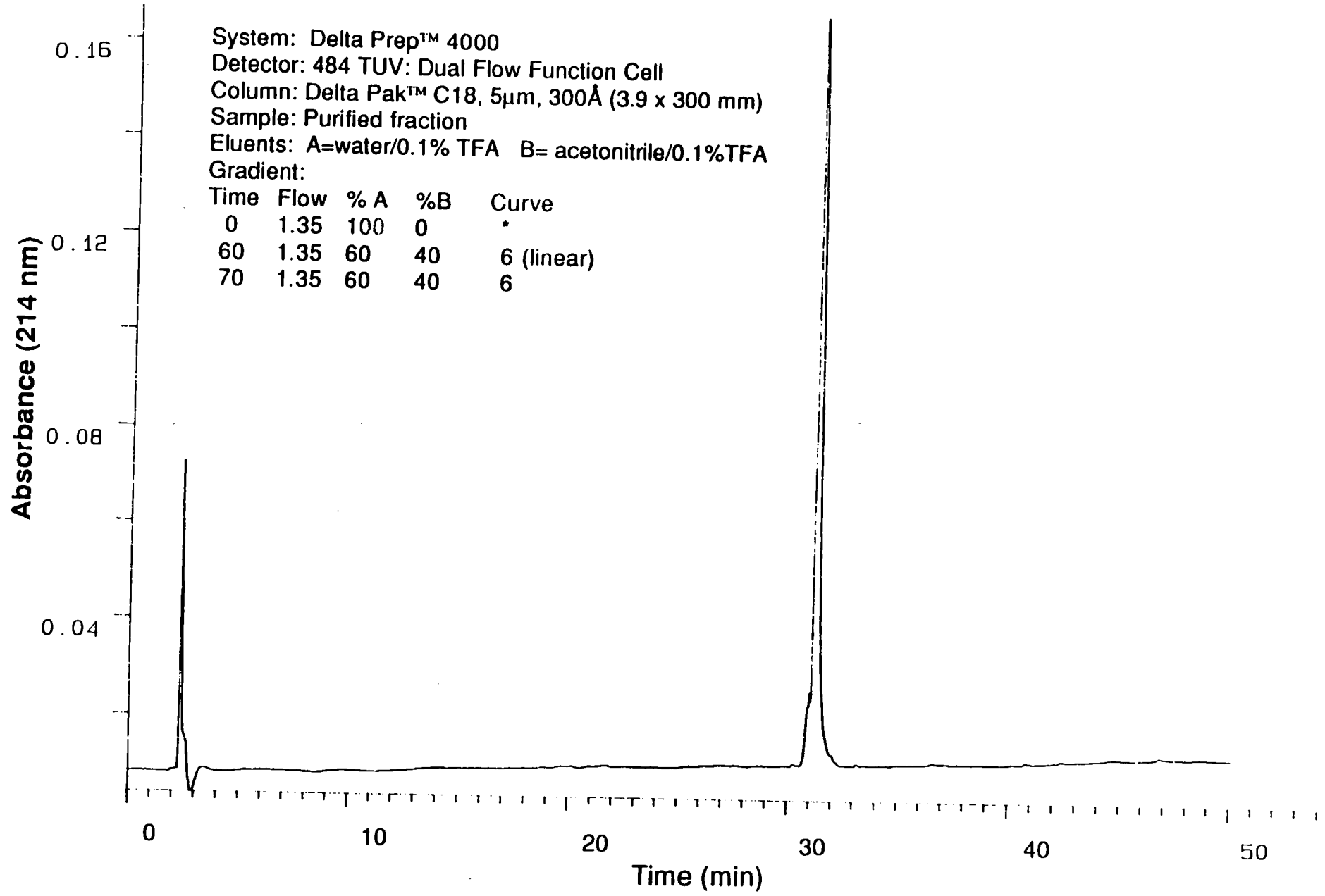
System: Delta Prep™ 4000
Detector: 484 TUV: Dual Flow Function Cell
Column: Delta Pak™ C18, 5µm, 300Å (3.9 x 300 mm)
Sample: Purified fraction
Eluents: A=water/0.1% TFA B= acetonitrile/0.1%TFA
Gradient:
Time Flow % A %B Curve
0 1.35 100 0 *
60 1.35 60 40 6 (linear)
70 1.35 60 40 6
0.16
0.12
0.08
0.04
Absorbance (214 nm)
0
10
20
30
40
50
Time (min)

Figure 6 Chromatography of the Purified Fraction from the 5 µm large-scale purification. Each fraction from the 5 µm preparative isolation was screened for purity using reversed phase HPLC on 5 µm columns. Chromatography of the most pure fraction is shown above.

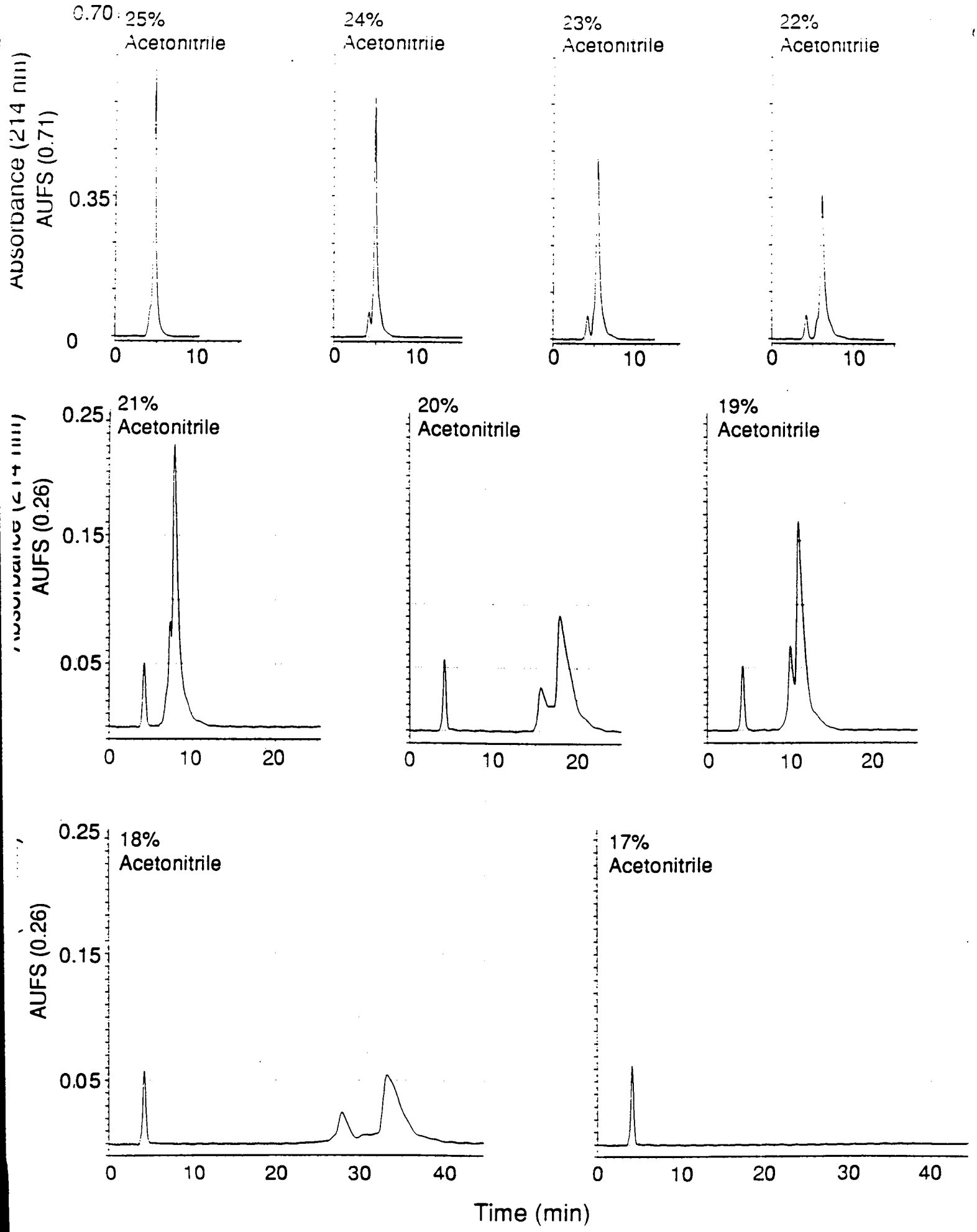
0.70
25%
Acetonitrile
24%
Acetonitrile
23%
Acetonitrile
22%
Acetonitrile
Absorbance (214 nm)
AUFS (0.71)
0.35
0
0
10
0
10
0
10
0
10
0.25
21%
Acetonitrile
20%
Acetonitrile
19%
Acetonitrile
AUFS (0.26)
0.15
0.05
0
10
20
0
10
20
0
10
20
0.25
18%
Acetonitrile
17%
Acetonitrile
AUFS (0.26)
0.15
0.05
0
10
20
30
40
0
10
20
30
40
Time (min)

Figure 7 Alternate Separation of Pooled Fractions. Gradient elution is most commonly used to purify peptides, but may not provide the best resolution in every case. The utility of isocratic chromatography was tested with the Pooled Fractions from the 15 µm preparative isolation. In the elution profile in Figure 4, the peak-of-interest eluted at approximately 25% acetonitrile. When run isocratically at 25% acetonitrile, the material eluted at the void volume of the column. A series of isocratic separations where the acetonitrile concentration was reduced in 1% increments was performed using an automated, low-dispersion system (Waters 625 with model 712 autosampler and 991 photodiode array detector). Subsequent eluent compositions were generated using the Auto-Blend™ method where the proportions of water and acetonitrile were generated from eluents A and B. As expected, with decreasing organic concentration, the peptides were retained longer by the reversed phase support and eluted in a larger volume. However, resolution increased dramatically, with optimal resolution at 18% acetonitrile. The isocratic separation was highly susceptible to subtle changes in acetonitrile as seen by the markedly different retentions obtained in the range of 19-17% acetonitrile.

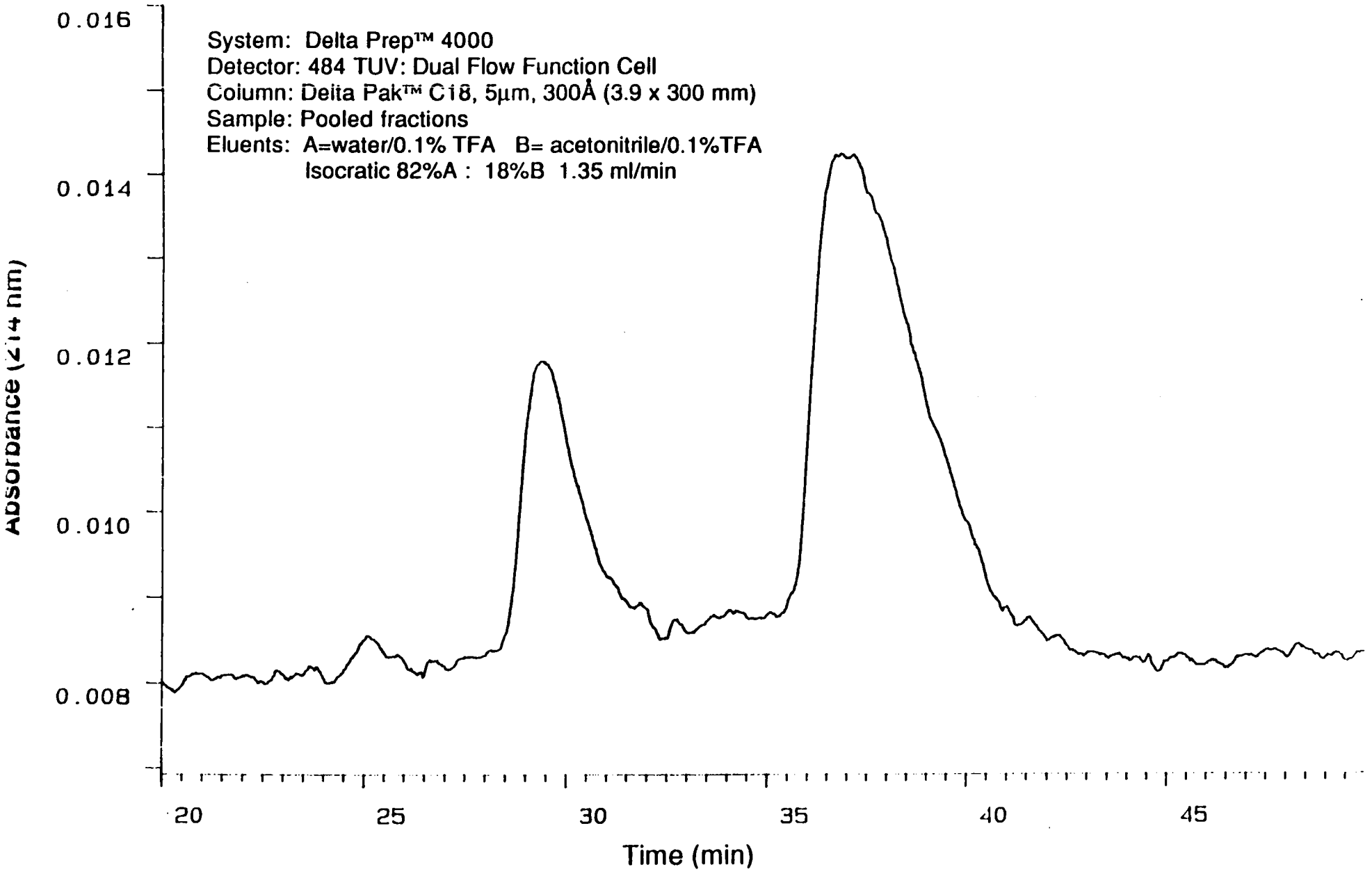
System: Delta Prep™ 4000
Detector: 484 TUV: Dual Flow Function Cell
Column: Delta Pak™ C18, 5µm, 300Å (3.9 x 300 mm)
Sample: Pooled fractions
Eluents: A=water/0.1% TFA B= acetonitrile/0.1%TFA
Isocratic 82%A : 18%B 1.35 ml/min
0.016
0.014
0.012
0.010
0.008
Absorbance (214 nm)
20
25
30
35
40
45
Time (min)

Figure 8 Isocratic Separation of Pooled Fractions on the Delta Prep 4000. The isocratic separation of the Pooled Fractions at 18% acetonitrile (Figure 5) was transferred to the Delta Prep 4000. Again, using the using the Auto-Blend method, the isocratic elution of the Pooled Fractions was resolved similarly. Thus, it was possible to generate fragile isocratic separations on the Delta Prep using Auto-Blend and stock eluents

Conclusions

1. The reaction products from peptide synthesis contain several similar peptide sequences that closely resemble the desired product. One or more purification steps are required to ensure homogeneity of the peptide product. The most useful method is by reversed phase HPLC.

2. Preparative HPLC using 5 µm supports may provide better resolution of a complex sample. However, for large preparative isolations, it may be advantageous to trade the enhanced resolution obtained on 5 µm materials for the economy of 15 µm packing materials. This is especially true for the first step in a multi-step purification protocol. A 15 µm support could be used to reduce the sample mass while removing many of the diverse reaction products that could conceivably erode the efficiency of the 5 µm support.

3. The higher efficiency 5 µm supports may be used later in the purification as a final step to remove very closely-eluting contaminants.

4. The Delta Prep 4000 provides a convenient and accurate approach to scaling a separation from an analytical-scale column to the preparative-scale column. When the same type of particle is used, a small-scale separation can be scaled to large-scale by adjusting sample load, flow rate, and gradient duration to obtain essentially the same elution profile.

5. Isocratic elution can provide excellent resolution of closely-eluting contaminants. Using the Auto-Blend method and stock eluents of water/0.1% TFA and acetonitrile/0.1% TFA an isocratic purification can be easily optimized.