Complex Carbohydrate Society '89

Poster Presentation

Characterization of Glycoproteins by HPLC -- Peptide Mapping and Analysis of Site Specific Glycosylation

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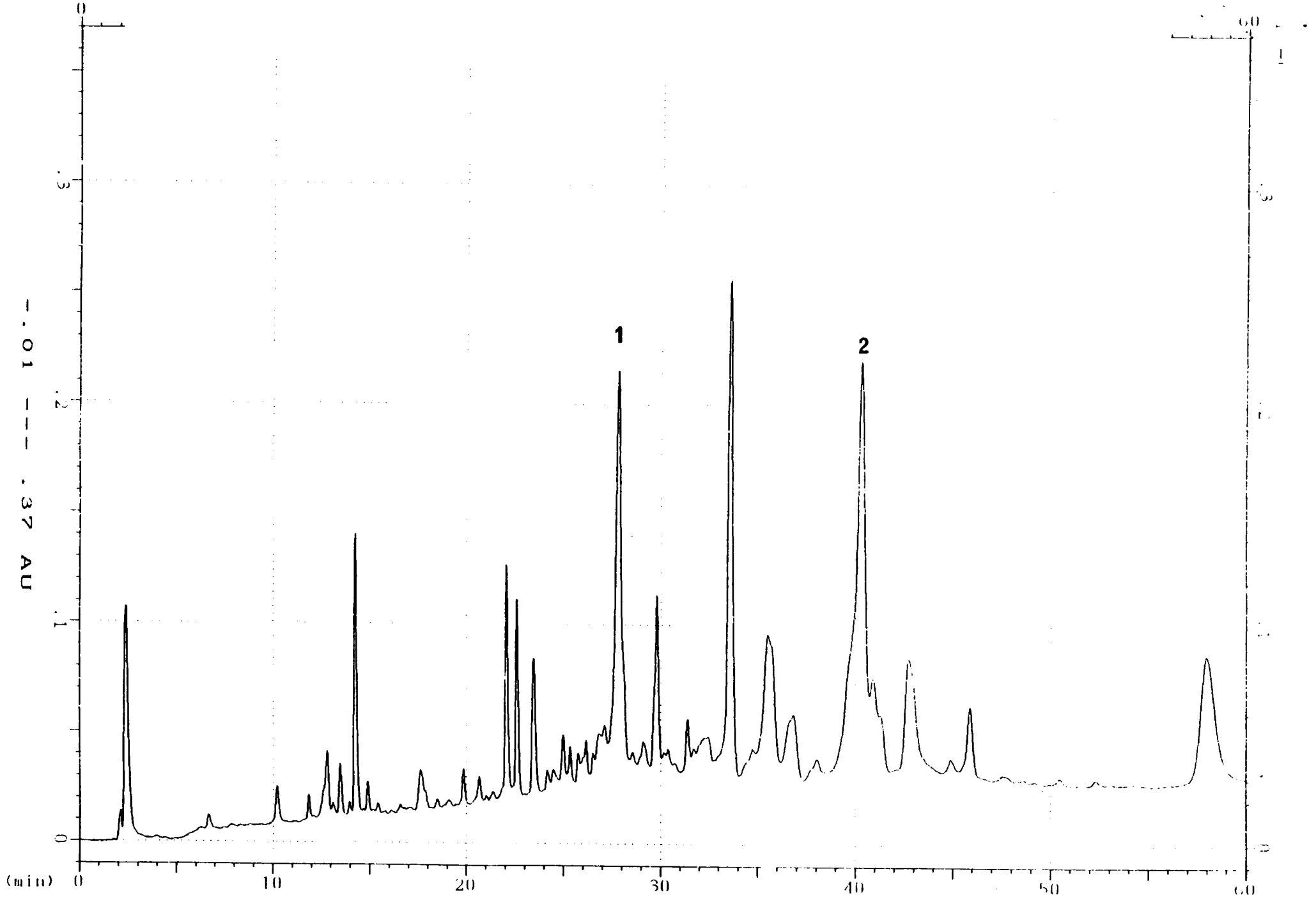
INTRODUCTION:

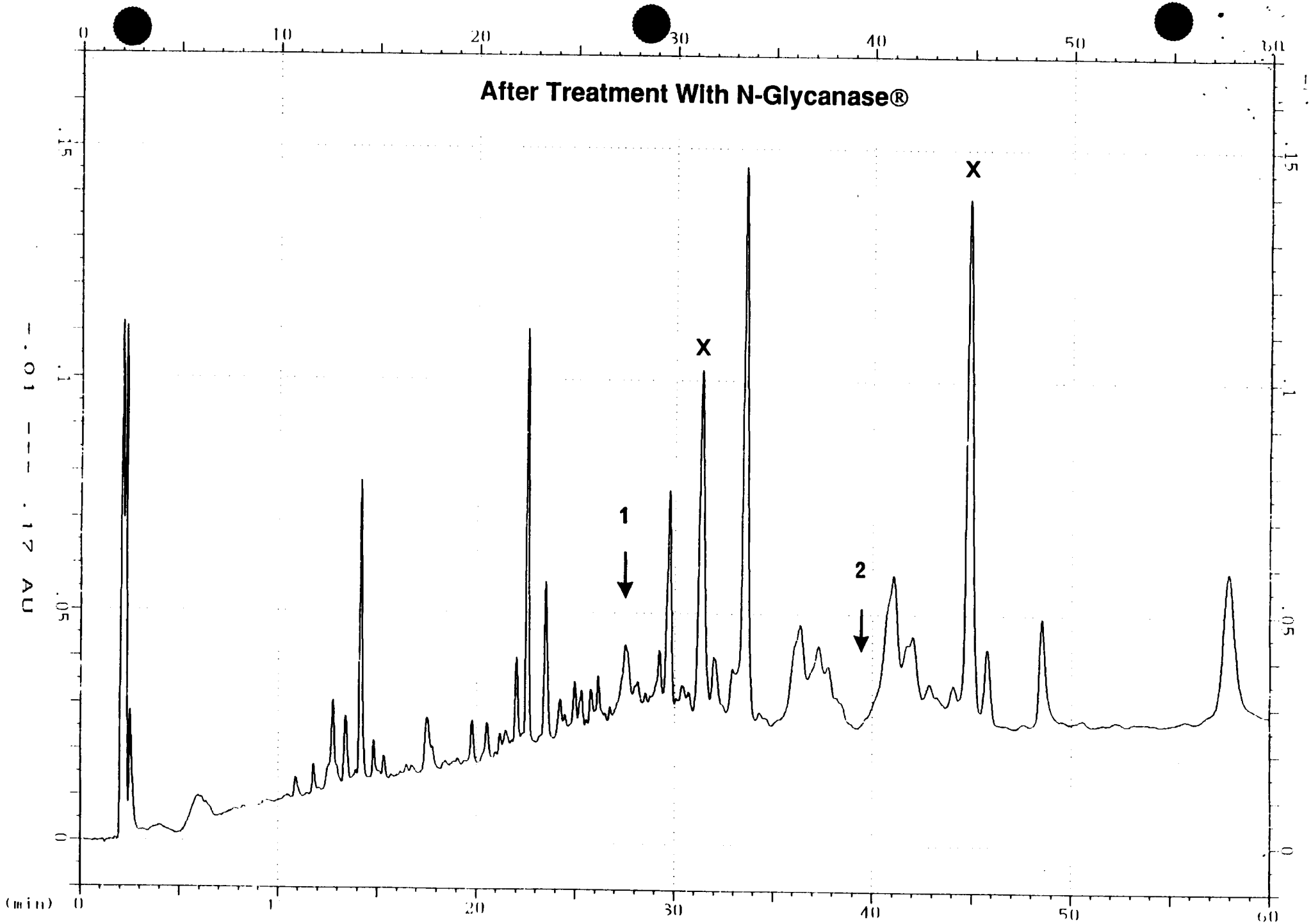
The need to characterize glycoproteins which are produce by recombinant techniques requires that several methodologies be used to ensure batch to batch reproducibility. To verify that proper glycosylation has occurred on the product, structure elucidation of the oligosaccharide by NMR and MS techniques must be carried out. Prior to structure elucidation, other methodologies which utilize HPLC techniques, such as peptide mapping, amino acid analysis and carbohydrate profiling, must be employed to substantiate the characterization of the product. In this paper, fetuin is used as a representative glycoprotein. After reduction and alkylation, fetuin was digested with trypsin and a peptide map was generated on a Waters Peptide Analyser. Treatment of the digest with N-glycanase, a specific amidase which hydrolyses N-linked oligosaccharides, followed by analysis on the Peptide Analyser, revealed which peaks contained the carbohydrates bound to asparigine by demonstrating a relative shift in retention time. Two major fractions from the original digest were collected after scale-up chromatograpy and amino acid composition was determined for each fraction using Pico-Tag procedure. Each fraction was then digested with N-glycanase and analysis on the Glyco-Pak DEAE provided a profile of the carbohydrates attached to the glycopeptides. This procedure provides, at least in part, methods which could be used in quality control laboratories to assure the analysis of site-specific glycosylation.

ABSTRACT:

Once purified to homogeneity, recombinantly produced glycoproteins are characterized by a number of methodologies including peptide mapping, amino acid analysis, sequencing and carbohydrate profiling, in order to ensure batch to batch reproducibility. Two of these methodologies, namely peptide mapping and profiling of site-specific glycosylation, have been performed using new column chemistries and instrumentation. A tryptic digest of fetuin, a representative glycoprotein, was analysed by HPLC before and after treatment with N-Glycanase using the Waters Peptide Analyser. Carbohydrate containing peptides were preparatively isolated by HPLC and treated with N-Glycanase to release the N-linked oligosaccharides. Following removal, the oligosaccharides were fractionated according to the degree of sialylation using a new stable, polymeric column chemistry, Glyco-Pak DEAE. This fractionation provides a profile of the oligosaccharides attached to a specific glycopeptide.

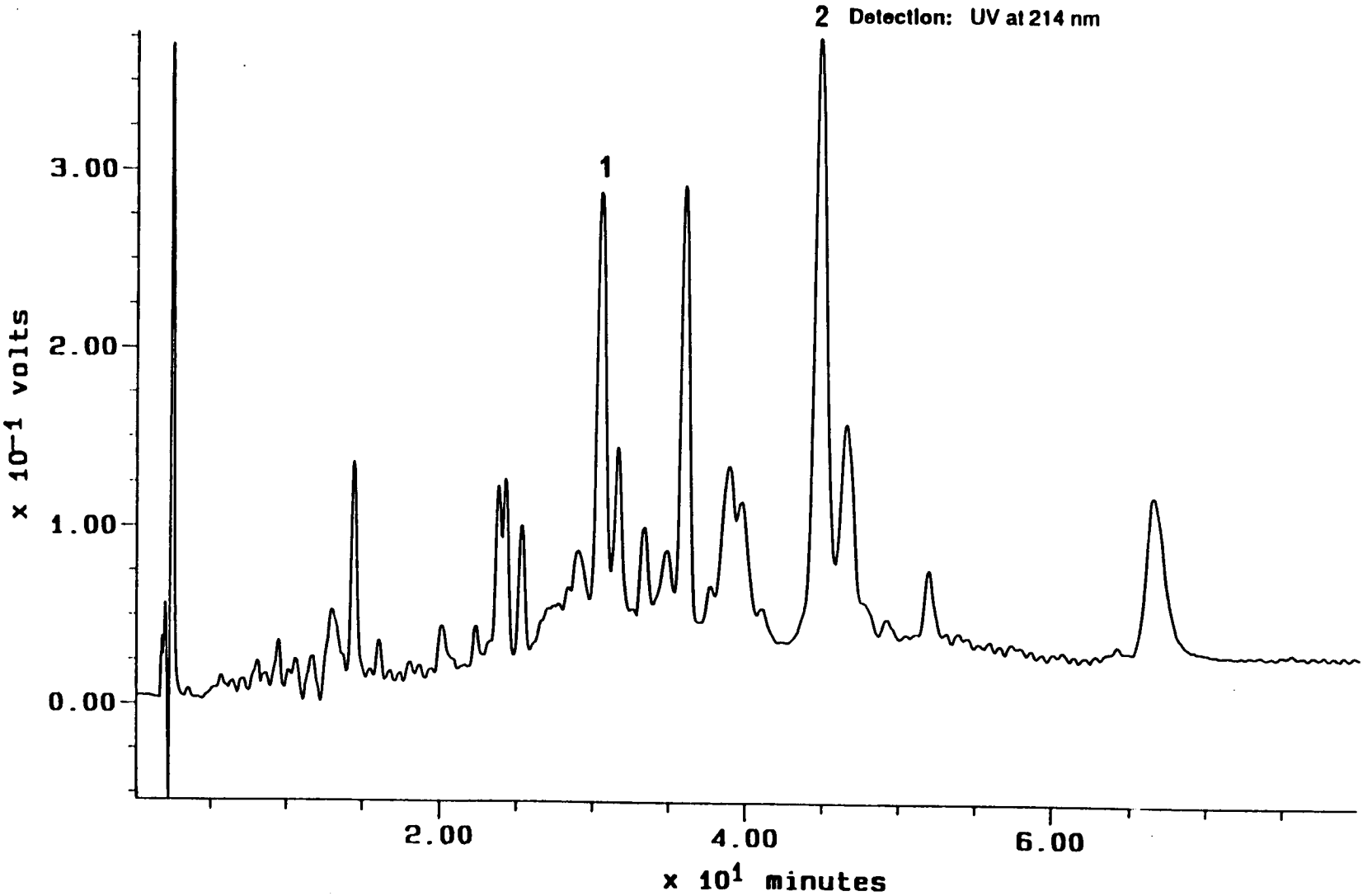
HIGH RESOLUTION PEPTIDE MAP USING 5u 300Å
DELTA PAK C18 3.9mmx150mm




After Treatment With N-Glycanase®
X
X
1
2
-.01 --- .17 AU
(min)
0
10
20
30
40
50
60
.15
.1
.05
0

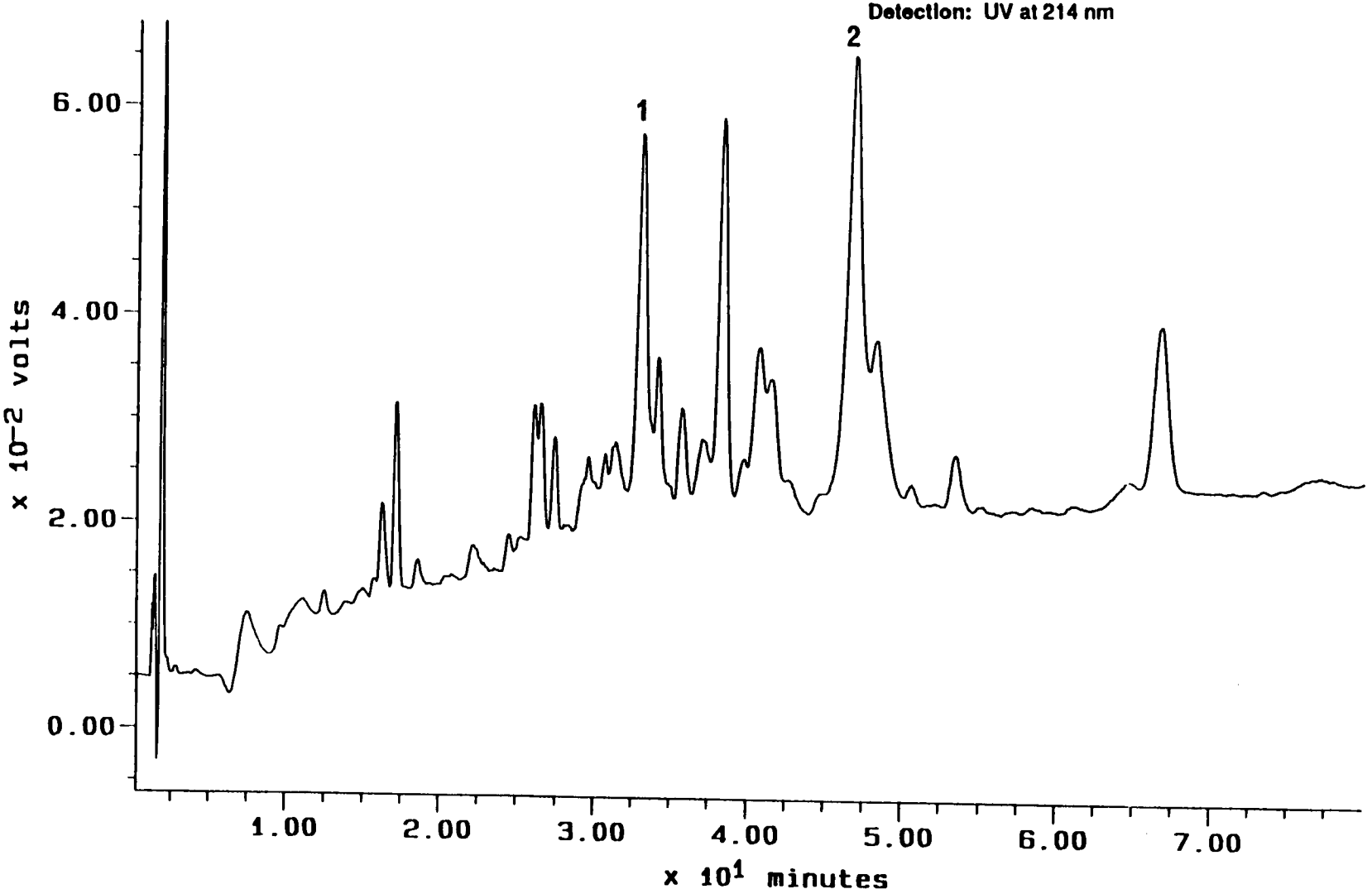
[redacted]CALE-UP OF PEPTIDE MAP USING [redacted]5u 300Å DELTA PAK C18 25x10 CARTRIDGE COLUMN

HCM 25 x 10
Column: Delta-Pak C_{18}, 15 micron, 300A with [redacted]d column
Sample: Reduced and alkylated fetuin tryptic digest
Injection: 500 ul
Mobile Phase: Solvent A: Water with .1% TFA
Solvent B: Acetonitrile with .1% TFA
Gradient Conditions: 0-29.2 min., 0-25% B
29.2-122.5 min., 25-60% B
122.5-134.1min., hold at 60% B
134.1-135.5 min., 60-0% B
Flow Rate: 19.5 ml/min.
Detection: UV at 214 nm

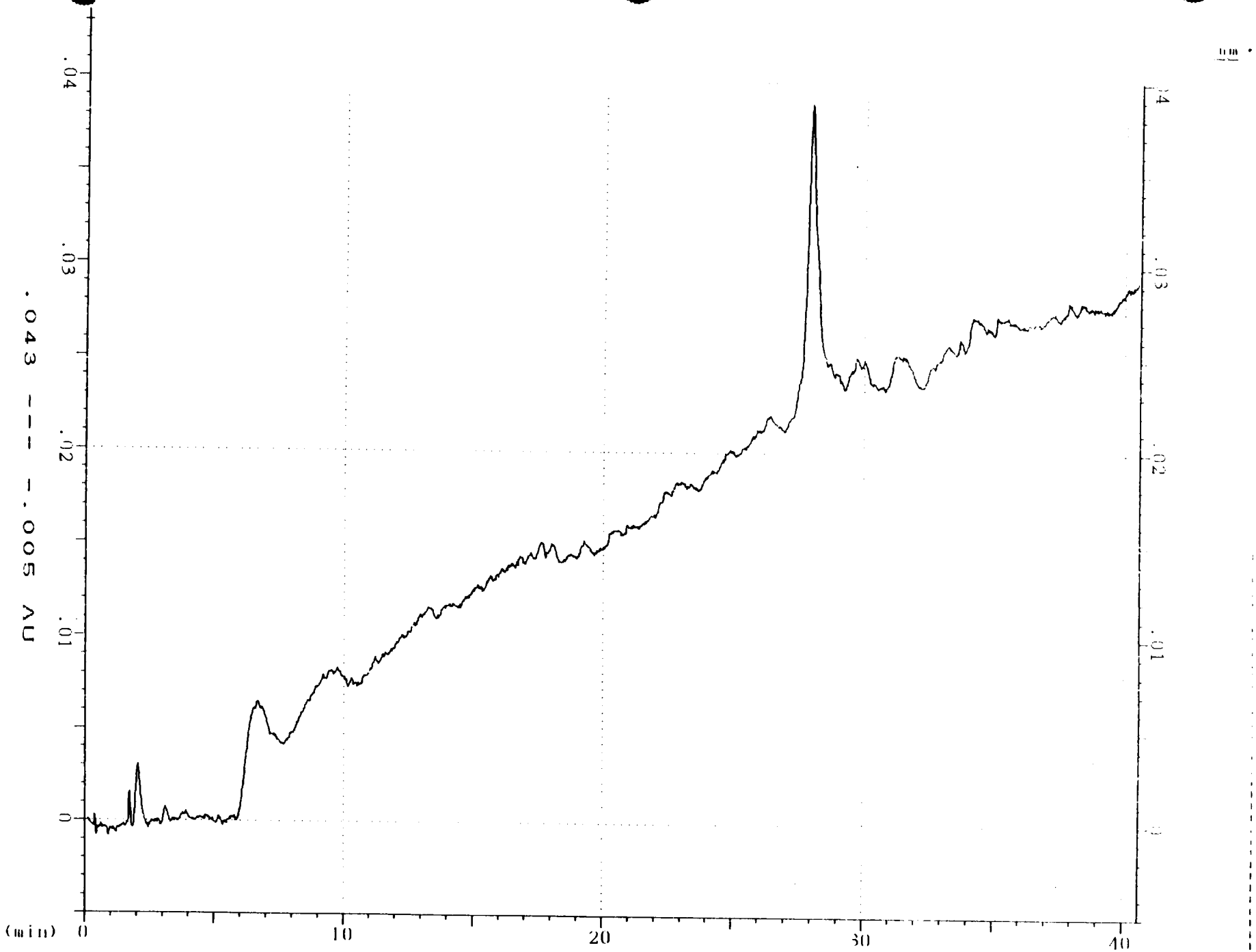


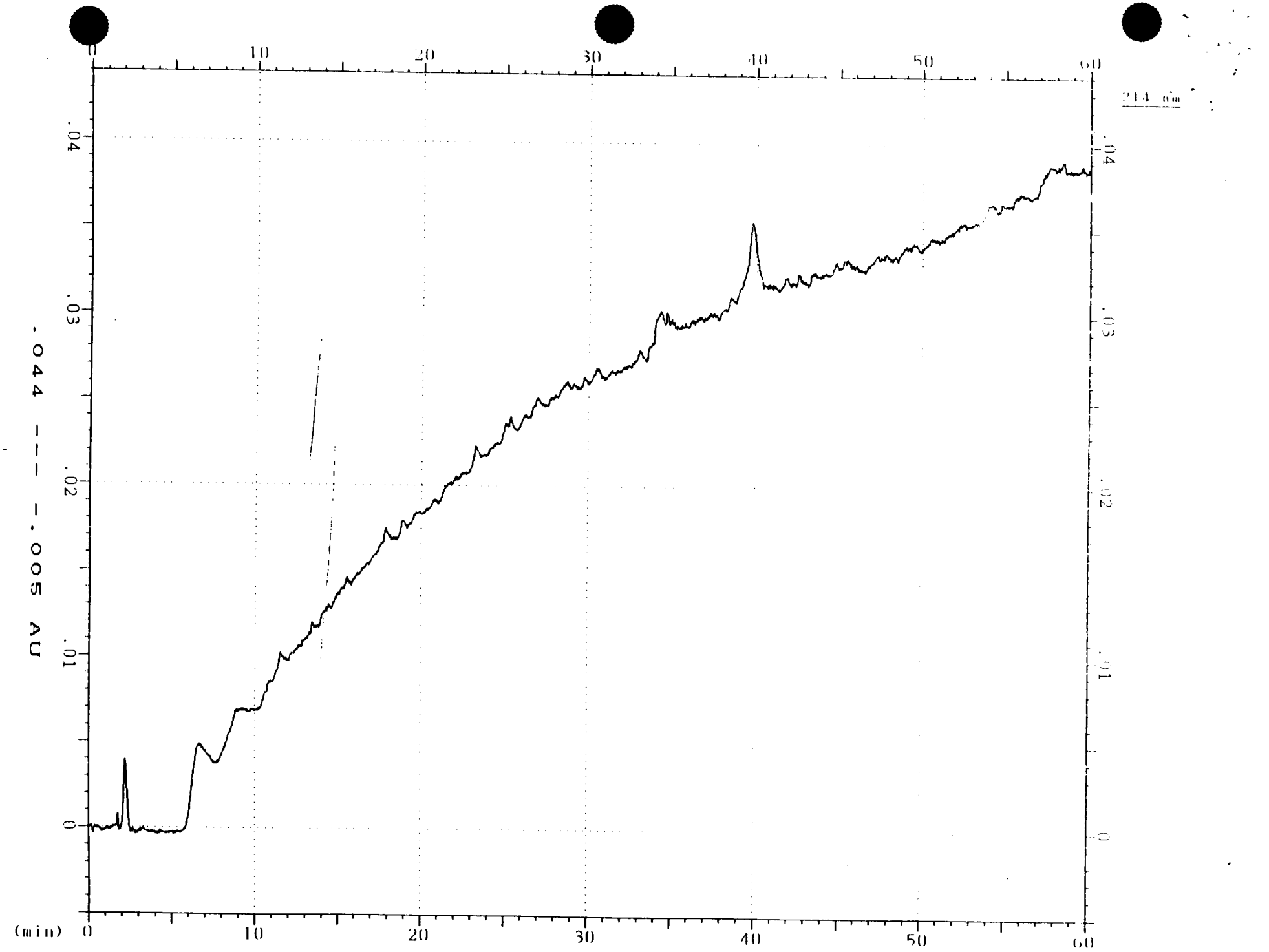
OPTIMIZATION OF PEPTIDE MAP USING 15u 00Å DELTA PAK C18 8x10 CARTRIDGE COLUMN

HCM 8 x 10
Column: Delta-Pak C_{18}, 15 micron, 300A
Sample: Reduced and alkylated fetuin tryptic digest
Injection: 10 ul
Mobile Phase: Solvent A: Water with .1% TFA
Solvent B: Acetonitrile with .1% TFA
Gradient Conditions: 0-29.2 min., 0-25% B
29.2-122.5 min., 25-60% B
122.5-134.1min., hold at 60% B
134.1-135.5 min., 60-0% B
Flow Rate: 2.0 ml/min.
Detection: UV at 214 nm



1 AND 2 USING 5u 300 DELTA PAK C18 3.9mmx150mm




214 nm
.044 --- -.005 AU
(min)
0
10
20
30
40
50
60
.04
.03
.02
.01
0

AMINO ACID COMPOSITION OF FETUIN GLYCOPEPTIDES

Amino Acid	Pico-Tag Analysis Peptide 1	Peptide 2	126-141	Reported[1] 54-85	142-169
ASP	2.8(3)	2.9(3)	5.1(5)#	5.4(6)#	2.3(2)
GLU	0.9(1)	2.9(3)	-	3.3(3)	3.8(4)
SER	1.6(1)	1.8(2)	1.4(1)	0.9(1)	2.2(2)
GLY	0.5(?)	1.2(1)	-	1.2(1)	1.1(1)
HIS	-	0.5(1)	-	1.0(1)	1.0(1)
ARG	0.7(1)	1.0(1)	1.0(1)	2.0(2)	1.1(1)
THR	-	2.7(3)	-	4.1(4)	1.2(1)
ALA	1.2(1)	2.0(2)	1.7(1)	1.3(1)	3.6(3)
PRO	2.5(3)	3.4(3)	3.0(3)	3.1(3)	0.8(1)
TYR	-	-	-	0.8(1)	0.8(1)
VAL	-	2.6(3)	-	2.7(3)	3.5(5)
MET	-	-	-	-	-
CYS	-	-	-	-	-
ILE	-	1.0(1)	-	2.0(2)	1.0(1)
LEU	2.7(3)	1.9(2)	3.6(4)	2.7(3)	3.0(3)
PHE	-	-	-	-	1.1(1)
LYS	0.8(1)	-	0.6(1)	-	-

1. Jour. Biol. Chem. 263, 111-117, (1988)

- (Cm-Cys) coelution with Asp

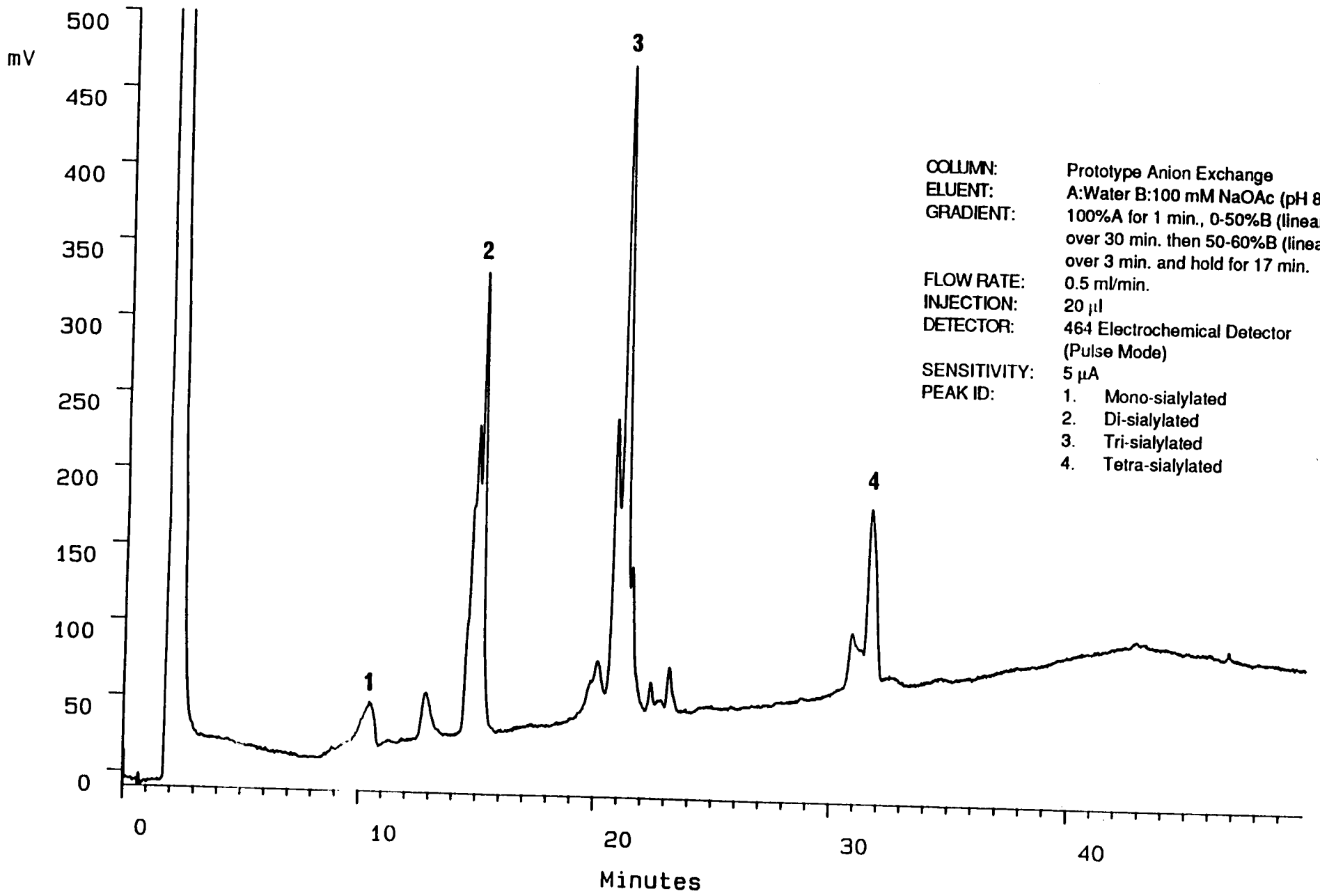
138 (K)LCPDCPLLAPLN*DSR

81 RPTGEVYDIEIDTLETTCHVLDPTPLAN*CSVR

158 VVHAVEVALATFNAESN*GSYLQLVEI[PR]

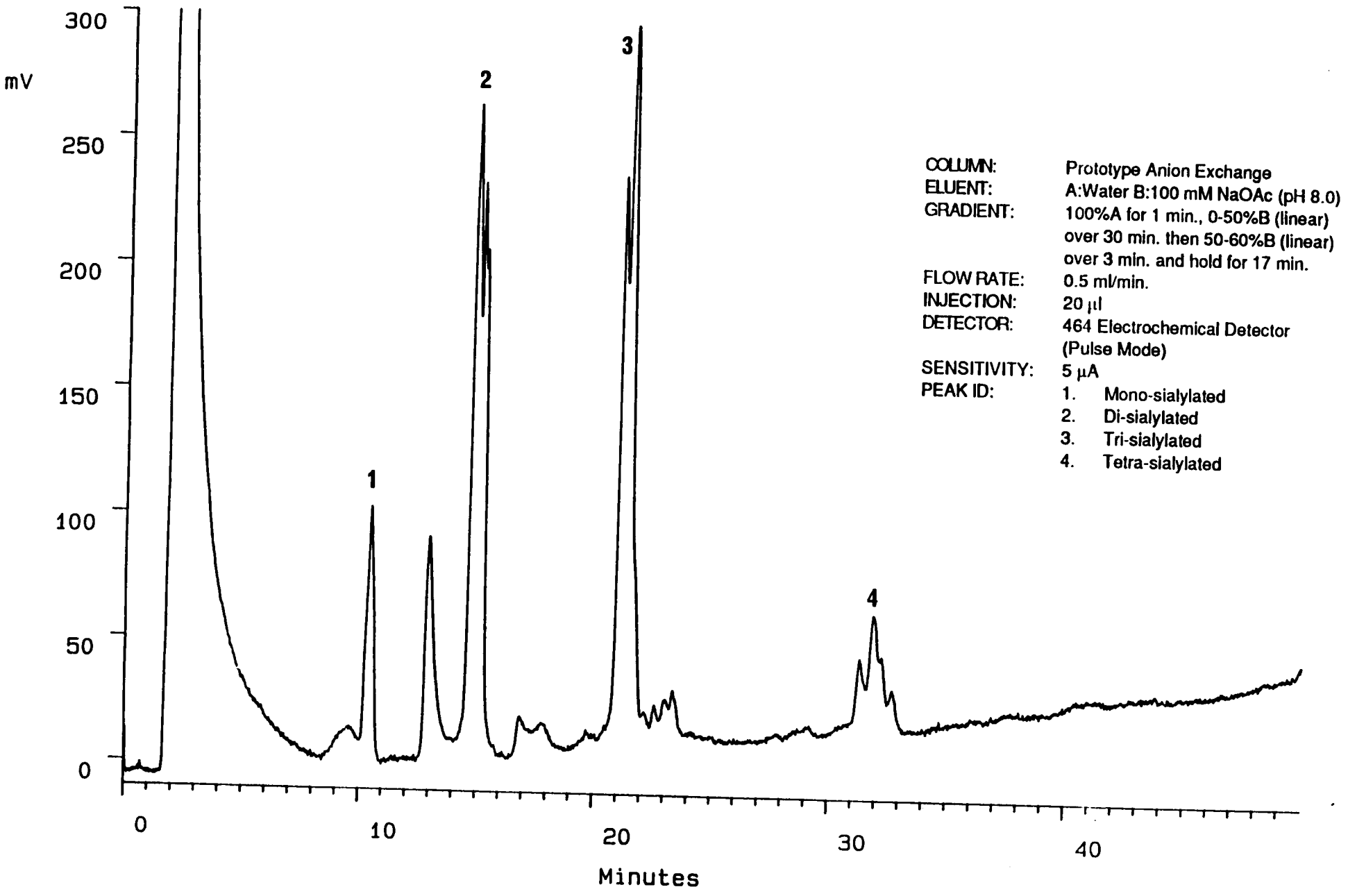
N-Linked Oligosaccharides Derived From Fetuin

N-Glycanase® Released From Peptide 1

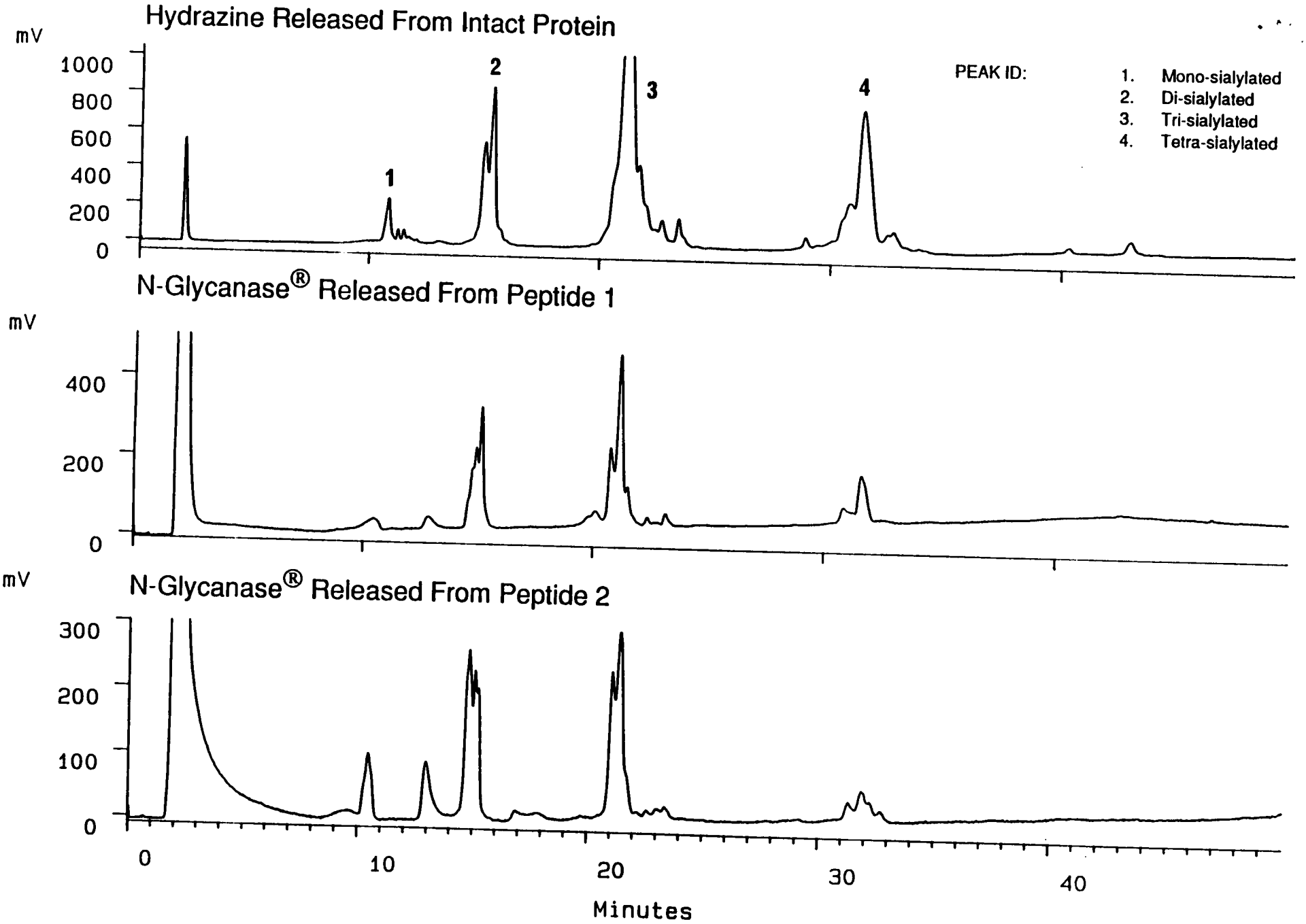


N-Linked Oligosaccharides Derived From Fetuin

N-Glycanase® Released From Peptide 2



COLUMN:	Prototype Anion Exchange
ELUENT:	A:Water B:100 mM NaOAc (pH 8.0)
GRADIENT:	100%A for 1 min., 0-50%B (linear) over 30 min. then 50-60%B (linear) over 3 min. and hold for 17 min.
FLOW RATE:	0.5 ml/min.
INJECTION:	20 µl
DETECTOR:	464 Electrochemical Detector (Pulse Mode)
SENSITIVITY:	5 µA
PEAK ID:	1. Mono-sialylated 2. Di-sialylated 3. Tri-sialylated 4. Tetra-sialylated


Hydrazine Released From Intact Protein
mV
1000
800
600
400
200
0
1
2
3
4
PEAK ID:
1. Mono-sialylated
2. Di-sialylated
3. Tri-sialylated
4. Tetra-sialylated
N-Glycanase® Released From Peptide 1
mV
400
200
0
N-Glycanase® Released From Peptide 2
mV
300
200
100
0
0
10
20
30
40
Minutes

CONCLUSIONS:

Peptide mapping, amino acid analysis, and carbohydrate profiling are techniques used to aid in the characterization of glycoproteins. High resolution peptide mapping combined with specific endoglycosidases or amidases can serve as useful tools to identify N-linked containing glycopeptides. In addition, the judicious use of proteinases on a protein of known sequence will simplify the interpretation of compositional amino acid analysis. The release of oligosaccharides by enzymic or chemical means permits the profiling of the carbohydrates attached to glycopeptides. These methods do not, however, determine carbohydrate structure and should be used in conjunction with recognized techniques for structure elucidation such as NMR and MS to ensure that proper glycosylation has occurred on a recombinant product.