Preface

Amino acid analysis is a technique that has become commonplace in biotechnology, biomedical, and food analysis laboratories. This book describes a variety of amino acid analysis techniques and how each technique can be used to answer specific biological questions.

The first two chapters in *Amino Acid Analysis Protocols* introduce the concepts, basic theory, and practice of amino acid analysis. The following chapters give detailed instructions on various methods and their applications.

As highlighted, there are many different approaches to amino acid analysis, but in all cases the results depend heavily on the quality of the sample. Therefore a new way to desalt samples prior to hydrolysis is covered as an introductory chapter (Chapter 3), and most authors have devoted a section to sample preparation, especially to the collection and storage of bodily fluids.

Some of the amino acid analysis methods described in this book are based on HPLC separation and analysis after precolumn derivatization. The precolumn derivatization techniques described use (a) 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) (Chapters 4 and 8); (b) 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (Marfey's reagent), which allows separation and analysis of enantiomeric amino acids (Chapter 5); (c) *O*-phthalaldehyde (OPA) (Chapters 6 and 10); (d) butylisothiocyanate (BITC) and benzylisothiocyante (BZITC) (Chapter 11); (e) phenylisothiocyanate (PITC) (Chapters 12 and 13); (f) ammonium-7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate (SBD-F) (Chapter 17); and (g) 9-fluorenylmethyl-chloroformate (FMOC-Cl) (Chapter 10).

Techniques have been described in which gas chromatography is used to separate and analyze (a) amino acids after *N*(*O,S*)-isoBOC methyl ester derivatization (Chapter 9); (b) *N*-isoBOC methyl esters of *O*-phosphoamino acids (Chapter 14); and (c) *N*(*S*)-isopropoxycarbonyl methyl esters derivatives of sulfur amino acids, glutathione, and other related aminothiols such as CysGly (Chapter 15). New techniques based on capillary electrophoresis separation (Chapter 16), high-performance anion-exchange chromatography (Chapter 7), and mass spectrometry of isotopically labeled proteins (Chapter 18) are also presented.

The applications of amino acid analysis are extremely varied and the technique remains the best means of accurate protein quantitation. Examples given in *Amino Acid Analysis Protocols* include the use of amino acid analysis for identification of picomolar amounts of protein on PVDF membranes (Chapter 8). The measurements of amino acids in bodily fluids and tissues such as urine (Chapters 9, 12, 14, 15), blood (Chapters 9, 10, 12, 14, 15, 17), seminal plasma (Chapter 6), or skeletal muscle tissue (Chapter 16), and measurement in the presence of high lipid content, such as in porcine lung (Chapter 13), are useful to help to identify diseases associated with changes in amino acid metabolism. Amino acid analysis, for example, is important to the study of such disorders as maple syrup urine disease (accumulation of branched-chain L-amino acids), phenylketonuria (high concentrations of phenylalanine), atherosclerosis (elevated levels of homocysteine), and galactosemia (often high concentrations of methonine). Amino acid and glucose analysis in fermentation broths of cell cultures (Chapter 7) enables the development of a feeding strategy that maintains the correct levels of nutrients. This is important since the use of such systems to make recombinant products is increasing. A method to determine the amino acid composition of foods (Chapter 11) is also included.

In addition to the standard methods used to separate the 20 commonly occurring amino acids, the analysis of unusual and modified amino acids is also addressed. Specifically, the analysis of homocysteine for monitoring the development of atherosclerosis (Chapter 17); hydroxyproline, a major amino acid found in collagen (Chapter 16); phosphoamino acids, which are difficult because they are acid labile (Chapter 14); aminothiols, such as cysteinylglycine and cystathionine (Chapter 15); and glycated lysine, implicated in diabetic complications and Alzheimer's disease (Chapter 18).

Overall *Amino Acid Analysis Protocols* presents an up-to-date, detailed methodology reference for a broad range of current techniques being used for amino acid analysis.

Catherine Cooper
Nicolle Packer
Keith Williams

2

Amino Acid Analysis, Using Postcolumn Ninhydrin Detection, in a Biotechnology Laboratory

**Frank D. Macchi, Felicity J. Shen, Rodney G. Keck,
and Reed J. Harris**

1. Introduction

Although lacking the speed and sensitivity of more widely heralded techniques such as mass spectrometry, amino acid analysis remains an indispensable tool in a complete biotechnology laboratory responsible for the analysis of protein pharmaceuticals.

Moore and Stein developed the first automated amino acid analyzer, combining cation–exchange chromatographic separation of amino acids with postcolumn ninhydrin detection ***(1)***. Commercial instruments based on this design were introduced in the early 1960s, though many manufacturers have abandoned this technology in favor of precolumn amino acid derivatization with separations based on reversed-phase chromatography ***(2–4)*** (*see* **Note 1**).

In our product development role, we still rely on amino acid analysis to generate key quantitative and qualitative data. Amino acid analysis after acid hydrolysis remains the best method for absolute protein/peptide quantitation, limited in accuracy and precision only by sample handling. We produced an Excel macro to process these data; the macro transfers and converts the amino acid molar quantities into useful values such as composition (residues per mol) and concentration. In addition, we employ several specialized amino acid analysis applications to monitor structural aspects of some of our recombinant products.

De novo biosynthesis of leucine in bacteria will lead to a minor amount of norleucine (Nle) production ***(5)***, particularly if recombinant proteins are produced in fermentations that have been depleted of leucine ***(6)***. The side-chain of Nle ($-CH_2-CH_2-CH_2-CH_3$) is similar enough to methionine ($-CH_2-CH_2-S-CH_3$) that some of the $tRNA^{Met}$ will be acylated by Nle, leading to incorporation of Nle at

From: *Methods in Molecular Biology*, vol. 159: *Amino Acid Analysis Protocols*
Edited by: C. Cooper, N. Packer, and K. Williams © Humana Press Inc., Totowa, NJ

Met positions ***(6,7)***. When this occurs, Nle may be incorporated at a low level at every Met position, and amino acid analysis is often the only method able to detect this substitution.

Hydroxylysine (Hyl) is a common modification of lysine residues found at -*Lys*-Gly- positions in collagens and collagen-like domains of modular proteins ***(8)***. This modification is also found at certain solvent-accessible -*Lys*-Gly- sites in noncollagenous proteins, usually at substoichiometric levels ***(9)***. Amino acid analysis is a useful screening technique for the identification of Hyl-containing recombinant proteins produced by mammalian cells.

The analysis of recombinant proteins using carboxypeptidases may still be required to assign the C-terminus when the polypeptide chain is extensively modified, thus ruling out making a C-terminal assignment based solely on mass and N-terminal analyses, or in cases where the C-terminal peptide cannot be assigned in a peptide map. When carboxypeptidase analyses are needed, a modified amino acid analysis program is needed to resolve Gln and Asn (which are not found in acid hydrolysates) from other amino acids.

Assignment of Asn-linked glycosylation sites is greatly facilitated by prior knowledge of the -*Asn*-Xaa-Thr/Ser/Cys- consensus sequence sites ***(10)***, and specific endoglycosidases, such as peptide:N-glycosidase F can be employed to quantitatively release all known types of Asn-linked oligosaccharides ***(11)***. O-linked sites are harder to assign, as these are found in less-stringent sequence motifs ***(12–14)***, and there is no universal endoglycosidase for O-glycans except for endo-α-*N*-acetylgalactosaminidase, which can only release the disaccharide Gal($\beta 1 \rightarrow 3$)GalNAc. In addition, O-glycosylation is often substoichiometric.

In mammalian cell products, at least two N-acetylglucosamine (GlcNAc) residues are found in Asn-linked oligosaccharides, whereas N-acetylgalactosamine (GalNAc) is found at the reducing terminus of the most common (mucin-type) O-linked oligosaccharides. A cation–exchange-based amino acid analyzer can easily be modified for the analysis of the amino sugars glucosamine ($GlcNH_2$) and galactosamine ($GalNH_2$) from acid hydrolysis of GlcNAc and GalNAc, respectively, allowing confirmation of the presence of most oligosaccharide types. In glycoproteins, HPLC fractions from peptide digests can be screened using amino sugar analysis to identify glycopeptides for further analysis.

Regulated biotechnology products are usually tested for identity using HPLC maps after peptide digestion ***(15,16)***. A key aspect of the digestion step for most proteins is obtaining complete reduction of all disulfide bonds, followed by complete alkylation of cysteines without the introduction of artifacts (e.g., methionine S-alkylation) ***(17)***. Amino acid analysis can be used to monitor cysteine alkylation levels for reduced proteins, such as are obtained after alkylation with iodoacetic acid, iodoacetamide or 4-vinylpyridine.

2. Materials

2.1. Equipment

1. 1-mL hydrolysis ampoules (Bellco, Vineland, NJ; part number 4019-00001) (*see* **Note 2**).
2. Savant SpeedVac.
3. Oxygen/methane flame.
4. Glass knife (Bethlehem Apparatus Co., Hellertown, PA).
5. 1/4" ID × 5/8" OD Tygon tubing.
6. Model 6300 analyzers (Beckman Instruments, now Beckman Coulter, Fullerton CA). The sum of the 440 nm and 570 nm absorbances is converted to digital format using a PE Model 900 A/D converter, and the data are collected by a PE Turbochrom Model 4.1 data system (*see* **Notes 3–5**).
7. Lithium-exchange column (Beckman part number 338075, 4.6 × 200 mm).

2.2. Reagents and Solutions

1. Constant boiling (6 *N*) HCl ampoules are obtained from Pierce (Rockland, IL) (*see* **Note 6**).
2. Mobile phase buffers purchased from Beckman Instruments include sodium citrate buffers Na-D, Na-E, Na-F, Na-R, and Na-S; lithium citrate buffers include Li-A, Li-B, Li-C, Li-D, Li-R, and Li-S.
3. Ninhydrin kits (Nin-Rx) are also purchased from Beckman; these must be mixed thoroughly before use (usually 2 h at room temperature), and care must be taken to avoid skin discoloration because of contact with ninhydrin-containing materials.
4. Dialysis may be used to desalt samples into dilute acetic acid prepared from deionized water (Milli-Q, Millipore) and Mallinckrodt U.S.P. grade glacial acetic acid.
5. Amino acid standards: are diluted from the stock Beckman standard (part number 338088) with Na-S buffer to final concentration of 40 nmol/mL or 20 nmol/mL (*see* **Note 7**).
6. 2 *N* glacial acetic acid.

3. Methods

3.1. Sample Preparation

Proteins should be desalted to obtain optimal compositional data. Dialysis against 0.1% acetic acid removes salts while keeping proteins in solution, but quantitative data will often require direct hydrolysis (i.e., without dilution or sample losses introduced during dialysis). When proteins must be analyzed without desalting, neutral buffers such as 50 m*M* Tris can be used without compromising the results. Excipients to avoid include urea (which generates abundant ammonia during hydrolysis), sugars (which caramelize during hydrolysis), and detergents such as the polysorbate and Triton types that can

Table 1
Standard Amino Acid Analysis

Time (min)	Event	Conditions
0.0	Sample injection	Na-E buffer, 48°C
8.5	Start temp. gradient	48°C to 60°C in 8 min
24.5	Buffer change	Na-E to Na-F
41.0	Buffer change	Na-F to Na-D
78.0	Reagent pump	Ninhydrin to water
79.0	Buffer change	Na-D to Na-R
80.0	Buffer change	Na-R to Na-E
82.5	Temperature change	60°C to 48°C
84.0	Reagent pump	Water to ninhydrin
97.0	Recyle (start next run)	

Buffer pump: 16 mL/h.
Reagent pump: 8 mL/h.

damage cation–exchange columns. Samples in enzyme-linked immunosorbent assays (ELISA)-type buffers should be avoided as they typically contain albumin or gelatins, whose amino acids cannot be distinguished after hydrolysis from the protein of interest. Peptides generally can be desalted by reverse phase (RP)-HPLC using volatile solvents such as 0.1% TFA in water/acetonitrile.

1. Place samples in hydrolysis ampoules (*see* **Note 8**), then dry under vacuum using a Savant SpeedVac.
2. Place approx 100 µL of 6 *N* HCl in the lower part of the ampoule (*see* **Note 9**). Freeze in a dry ice/ethanol bath, attached to a vacuum system via 1/4" ID × 5/8" OD Tygon tubing, then slowly thaw and evacuate to < 150 mtorr.
3. Use an oxygen/methane flame to seal the neck of the tube at the constriction.
4. Place the sealed ampoules in a 110°C oven for 24 h (*see* **Note 10**), then allow to cool before opening after scoring them with a glass knife.
5. Remove the acid by vacuum centrifugation, again using a Savant system, with a NaOH trap inserted between the centrifuge and cold trap.
6. After hydrolysis and acid removal, samples that contain 0.5–10 µg of protein, or 0.1–1 nmol of peptide fractions should be reconstituted with 60–200 µL of Na-S sample buffer (*see* **Note 11**).

3.2. Protein/Peptide Quantitation

1. Subject triplicate samples containing 0.5–10 µg of protein or 0.1–1 nmol of peptide to 24-h hydrolysis *in vacuo* as aforementioned.
2. Follow the standard operating conditions given in **Table 1** (*see* **Note 12**). A standard chromatogram containing 2 nmol of each component is shown in **Fig. 1**.

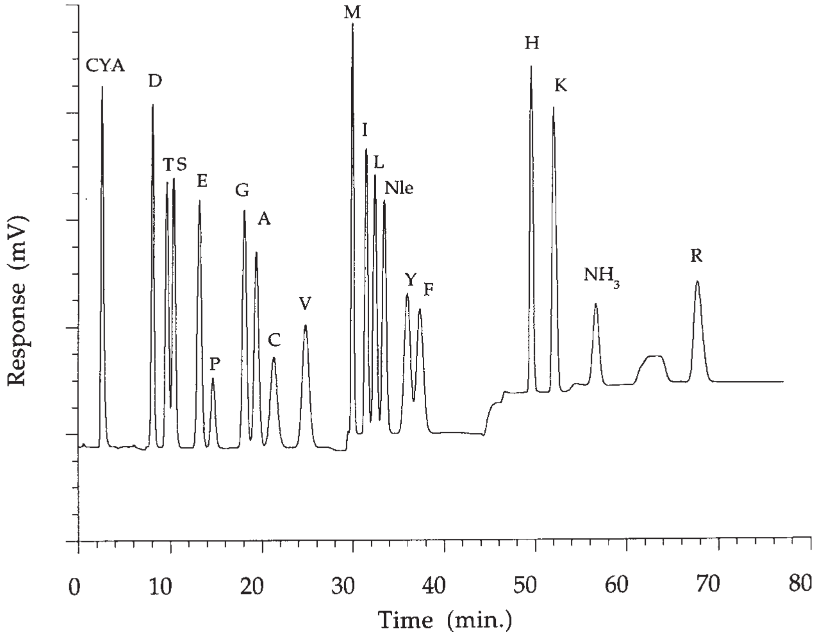


Fig. 1. Analysis of a standard amino acid mixture. The standard contains 2 nmol of each component except for NH_3. Operating parameters are given in **Table 1**.

3. Peak area data from the Turbochrom system are converted to nmol values by external standard calibration; internal standards are not necessary if a reliable autosampler is used.
4. The amino acid nmol values are also automatically converted to .tx0 files that can be imported into a custom Microsoft Excel program called the AAA MACRO (**Table 2**) for analysis using a PC-based computer.
5. The first step in running the AAA MACRO is to open a template, such as the example "protein.xls" given in **Table 3**. The residues per mol and molecular mass calculations must be modified and saved for each different protein/peptide; Asn and Asp are reported as Asx, whereas Gln, Glu and pyroglutamate are reported as Glx.
6. The macro asks for some background information (e.g., requestor's name, sample name, number of replicates), sample prep information (e.g., volumes of hydrolysate loaded vs reconstitution volume, original sample volume), then processes the data, providing a single-page report showing calculated compositions and concentration, as shown in **Fig. 2** (*see* **Note 13–15**).

3.3. Norleucine Incorporation

1. Detection of trace Nle levels in *Escherichia coli*-derived proteins require 24-h hydrolysis of 25–100 μg of protein (*see* **Note 16**).

Table 2
Amino Acid Analysis Data Conversion Macro

Commands	
Macro 4(a)	
=ACTIVATE("MACRO4A.XLM")	
=HIDE()	
=\TC4\DATA	
=SELECT(!B4)	
=INPUT("Requestor's Name?",2,"Name",""	Asks for Requestor's Name
=IF(A7=FALSE,HALT())	Halts macro if Cancel button is clicked
=FORMULA(A7)	Returns name to data worksheet cell B4
=SELECT(!B5)	
=INPUT("Requestor's Extension?",1,"Telephone extension","")	Asks for Requestor's Extension
=IF(A11=FALSE,HALT())	Halts macro if Cancel button is clicked
=FORMULA(A11)	Returns extension to data worksheet cell B5
=SELECT(!E4)	
=INPUT("Sample to be analyzed?",2,"Sample name", "")	Asks for protein to be analyzed
=IF(A15=FALSE,HALT())	Halts macro if Cancel button is clicked
=FORMULA(A15)	Returns protein's name to data worksheet cell E4
=SELECT(!E5)	
=INPUT("Requestor's Mail Stop?",1,"Mail Stop","")	Asks for Requestor's MailStop
=IF(A19=FALSE,HALT())	Halts macro if Cancel button is clicked
=FORMULA(A19)	Returns Requestor's MailStop to data worksheet cell E5
=SELECT(!B28)	
=INPUT("Molecular Mass of protein to be analyzed?",1, "Molecular Mass (g/mole)","")	Asks for MW
=IF(A23=FALSE,HALT())	Halts macro if Cancel button is clicked
=FORMULA(A23)	Returns MW to data worksheet cell B28
=SELECT(!F29)	
=INPUT("µL in Ampoule?",1,"Ampoule volume (µL)","")	Asks for amount sample put in ampoule
=IF(A27=FALSE,HALT())	
=FORMULA(A27)	
=SELECT(!F30)	
=INPUT("Sample reconstitution volume?",1,"Reconstituted volume (µL)","")	Asks for reconstitution volume of sample
=IF(A31=FALSE,HALT())	Halts macro if Cancel button is clicked
=FORMULA(A31)	
=SELECT(!F31)	

```
=INPUT("Dilution Factor?",1,"Dilution factor",""                              Asks if samples were diluted prior to analysis
=IF(A35=FALSE,HALT())                                                          Halts macro if Cancel button is clicked
=FORMULA(A35)
=SELECT(!B1)                                                                   Selects cell B1 on worksheet
=INPUT("How many replicates will you be analyzing today?",   1,"Number of replicates","")   Asks how many replicate samples will be processed
=IF(A39=FALSE,HALT())                                                          Halts macro if Cancel button is clicked
=FORMULA(A39)                                                                  Places users sample number in cell B2
=SET.NAME("counter1",1)                                                        Resets counter1
=SELECT(!B41)                                                                  Selects active cell to be B6
=DIRECTORY("\TC41\data")                                                       Selects disk in drive as AAA directory
=FILES("*.*")                                                                  Opens AAA disk
=OPEN?("*.*",0,FALSE,2)                                                        Top of loop and Select data file from listed files
=SELECT("R38C6:R57C6")                                                         Select data file to open
=COPY()                                                                        Copies AAA nanomole data
=CLOSE()                                                                       Closes data file
=ACTIVATE.NEXT()
=SELECT("RC[1]")
=PASTE()                                                                       Pastes nanomole values into worksheet
=SET.NAME("counter1",counter1+1)                                               Adds value of 1 to the counter1
=IF(counter1<=(B1),GOTO(A46))                                                  Checks to see what value = counter1 if >=1 then loops up to the
                                                                                 top of the loop, if 0 then proceeds downward
=SELECT(!C40)
=INPUT("What is the name of your first replicate?",2,   "Name of 1st replicate","")          Asks for name of first data file chosen
=IF(A56=FALSE,HALT())
=FORMULA(A56)
=SELECT(!D40)
=INPUT("What is the name of your second replicate?",2,   "Name of 2nd replicate","")         Asks for name of second data file chosen
=IF(A60=FALSE,HALT())
=FORMULA(A60)
=SELECT(!E40)
=INPUT("What is the name of your third replicate?",2,   "Name of 3rd replicate","")          Asks for name of third data file chosen
=IF(A64=FALSE,HALT())
=FORMULA(A64)
=SAVE.AS?(,1)                                                                  Asks if you want to save data
=IF(A67=FALSE,HALT())                                                          Halts macro if Cancel button is clicked
=ACTIVATE("MACRO4A.XLM")
=UNHIDE()
=ACTIVATE.NEXT()
=RETURN()                                                                      Halts the macro
```

Table 3
AAA Macro Template

A	B	C	D
AAA Macro	3		
			Protein Template
Name:		Researcher:	Protein Name:
Extension:	0		Mail Stop:
Amino Acid	Theoretical Composition	= (C66)	= (D66)
CyA	0	= C67	= D67
Asx	35	= C68	= D68
Thr	37	= C69	= D69
Ser	59	= C70	= D70
Glx	41	= C71	= D71
Pro + Cys SH	24	= C72	= D72
Gly	33	= C73	= D73
Ala	30	= C74	= D74
1/2 Cys-Cys	10	= C75	= D75
Val	35	= C76	= D76
Met	3	= C77	= D77
Ile	12	= C78	= D78
Leu	32	= C79	= D79
Nle	0	= C80	= D80
Tyr	22	= C81	= D81
Phe	13	= C82	= D82
His	7	= C83	= D83
Lys	27	= C84	= D84
Arg	12	= C86	= D86
Molecular Mass (g/mole)	47503.01		
	ul in Ampoule		
	smp recon(ul)		
	dilution factor		
column load (ul)	50		
	nMois Protein	= F62	= G62
	Protein Concentration mg/mL	= ([C33*B28*0.001*F30/B32]/F29)*F31	= ([D33*B28*0.001*F30/B32]/F29)*F31
Amino Acid	Theoretical Composition	data file #1	data file #2
CyA	= B7	0.01	0.011
Asx	= B8	5.132	5.07
Thr	= B9	5.146	5.086
Ser	= B10	7.692	7.58
Glx	= B11	5.875	5.8
Pro + CySH	= B12	4.415	4.227
Gly	= B13	4.831	4.76
Ala	= B14	4.545	4.474
1/2 Cys-Cys	= B15	1.263	1.375
Val	= B16	4.922	4.851
Met	= B17	0.39	0.365
Ile	= B18	1.68	1.662
Leu	= B19	4.68	4.812
Nle	= B20	0	0
Tyr	= B21	3.11	3.066
Phe	= B22	1.916	1.893
His	= B23	1.039	1.022
Lys	= B24	3.971	3.906
NH4	0	7.145	6.989
Arg	= B25	1.753	1.736
Total nMoles	= SUM(B42:B60)	= SUM(C41:C60)-C59	= SUM(D41:D60)-D59
Total nMoles/Total # residues		C61/B61	D61/B61

E	F	G	H	
				1
				2
				3
Protein X				**4**
0				**5**
= (E66)	Averages			**6**
= E67	= AVERAGE(C7:E7)			**7**
= E68	= AVERAGE(C8:E8)			**8**
= E69	= AVERAGE(C9:E9)			**9**
= E70	= AVERAGE(C10:E10)			**10**
= E71	= AVERAGE(C11:E11)			**11**
= E72	= AVERAGE(C12:E12)			**12**
= E73	= AVERAGE(C13:E13)			**13**
= E74	= AVERAGE(C14:E14)			**14**
= E75	= AVERAGE(C15:E15)			**15**
= E76	= AVERAGE(C16:E16)			**16**
= E77	= AVERAGE(C17:E17)			**17**
= E78	= AVERAGE(C18:E18)			**18**
= E79	= AVERAGE(C19:E19)			**19**
= E80	= AVERAGE(C20:E20)			**20**
= E81	= AVERAGE(C21:E21)			**21**
= E82	= AVERAGE(C22:E22)			**22**
= E83	= AVERAGE(C23:E23)			**23**
= E84	= AVERAGE(C24:E24)			**24**
= E86	= AVERAGE(C25:E25)			**25**
				26
				27
				28
	20			**29**
	150			**30**
	1			**31**
				32
= H62				**33**
= ([E33*B28*0.001*F30/B32]/F29)*F31	= AVERAGE(C25:E25)			**34**
data file #3	Ave nM cal	Ave nM cal	Ave nM cal	**40**
0.011				**41**
5.075	= C42/B42	= D42/B42	= E42/B42	**42**
5.091				**43**
7.586				**44**
5.801	= C45/B45	= D45/B45	= E45/B45	**45**
4.417				**46**
4.765				**47**
4.481	= C48/B48	= D48/B48	= E48/B48	**48**
1.182				**49**
4.86				**50**
0.401				**51**
1.653				**52**
4.797	= C53/B53	= D53/B53	= E53/B53	**53**
0				**54**
3.067				**55**
1.889	= C56/B56	= D56/B56	= E56/B56	**56**
1.02	= C57/B57	= D57/B57	= E57/B57	**57**
3.914	= C58/B58	= D58/B58	= E58/B58	**58**
7.148				**59**
1.75	= C60/B60	= D60/B60	= E60/B60	**60**
= SUM(E41:E60)-E59				**61**
= E61/B61	= AVERAGE (F42:F60)	= AVERAGE (G42:G60)	= AVERAGE (H42:H60)	**62**

(continued)

Table 3 *(continued)*

A	B	C	D
Amino Acid	Theoretical Composition	= C40	= D40
CyA	= B7	= IF(C41/[F$62]=0, " " ,[C41/F$62])	= IF(D41/[G$62]=0, " " ,[D41/G$62])
Asx	= B8	= IF(C42/[F$62]=0, " " ,[C42/F$62])	= IF(D42/[G$62]=0, " " ,[D42/G$62])
Thr	= B9	= IF(C43/[F$62]=0, " " ,[C43/F$62])	= IF(D43/[G$62]=0, " " ,[D43/G$62])
Ser	= B10	= IF(C44/[F$62]=0, " " ,[C44/F$62])	= IF(D44/[G$62]=0, " " ,[D44/G$62])
Glx	= B11	= IF(C45/[F$62]=0, " " ,[C45/F$62])	= IF(D45/[G$62]=0, " " ,[D45/G$62])
Pro + CySH	= B12	= IF(C46/[F$62]=0, " " ,[C46/F$62])	= IF(D46/[G$62]=0, " " ,[D46/G$62])
Gly	= B13	= IF(C47/[F$62]=0, " " ,[C47/F$62])	= IF(D47/[G$62]=0, " " ,[D47/G$62])
Ala	= B14	= IF(C48/[F$62]=0, " " ,[C48/F$62])	= IF(D48/[G$62]=0, " " ,[D48/G$62])
1/2 Cys-Cys	= B15	= IF(C49/[F$62]=0, " " ,[C49/F$62])	= IF(D49/[G$62]=0, " " ,[D49/G$62])
Val	= B16	= IF(C50/[F$62]=0, " " ,[C50/F$62])	= IF(D50/[G$62]=0, " " ,[D50/G$62])
Met	= B17	= IF(C51/[F$62]=0, " " ,[C51/F$62])	= IF(D51/[G$62]=0, " " ,[D51/G$62])
Ile	= B18	= IF(C52/[F$62]=0, " " ,[C52/F$62])	= IF(D52/[G$62]=0, " " ,[D52/G$62])
Leu	= B19	= IF(C53/[F$62]=0, " " ,[C53/F$62])	= IF(D53/[G$62]=0, " " ,[D53/G$62])
Nle	= B20	= IF(C54/[F$62]=0, " " ,[C54/F$62])	= IF(D54/[G$62]=0, " " ,[D54/G$62])
Tyr	= B21	= IF(C55/[F$62]=0, " " ,[C55/F$62])	= IF(D55/[G$62]=0, " " ,[D55/G$62])
Phe	= B22	= IF(C56/[F$62]=0, " " ,[C56/F$62])	= IF(D56/[G$62]=0, " " ,[D56/G$62])
His	= B23	= IF(C57/[F$62]=0, " " ,[C57/F$62])	= IF(D57/[G$62]=0, " " ,[D57/G$62])
Lys	= B24	= IF(C58/[F$62]=0, " " ,[C58/F$62])	= IF(D58/[G$62]=0, " " ,[D58/G$62])
NH4	0	= IF(C59/[F$62]=0, " " ,[C59/F$62])	= IF(D59/[G$62]=0, " " ,[D59/G$62])
Arg	= B25	= IF(C60/[F$62]=0, " " ,[C60/F$62])	= IF(D60/[G$62]=0, " " ,[D60/G$62])

2. After removal of the acid, reconstitute the samples with Li-S buffer, then analyze using lithium citrate buffers with a lithium-exchange column.
3. Use the analysis conditions given in **Table 4**. If needed, the separation between Nle and Tyr (which elutes after Nle) can be increased by lowering the column temperature.
4. Set the detector to the most sensitive scale (0.1 AUFS) (*see* **Note 17**). A chromatogram is given in **Fig. 3**.

3.4. Hydroxylysine Analysis

1. Hydrolyze samples containing 50–100 µg of protein (*see* **Note 18**) for 24 h as described in **Subheading 3.1**.
2. Remove the acid, then reconstitute the samples with Li-S buffer, and analyze using the modified program given in **Table 5** (*see* **Note 19**). The standard chromatogram is given in **Fig. 4** (*see* **Note 20**).

3.5. Carboxypeptidase Analysis

Applications involving single or combinations of carboxypeptidases to assign C-terminal protein sequences have been adequately described elsewhere ***(18)***.

1. Add norleucine to samples prior to the addition of carboxypeptidases at equimolar ratios (e.g., 10 nmol Nle for a sample containing 10 nmol of polypeptide).
2. Take aliquots at various time-points and place in Eppendorf tubes containing an equal volume of 2 *N* glacial acetic acid.
3. Heat for 2 min at 100°C on a boiling water bath to halt the digestion and precipitate the protein.

E	
= E40	**66**
= IF(E41/[H$62]=0, " " ,[E41/H$62])	**67**
= IF(E42/[H$62]=0, " " ,[E42/H$62])	**68**
= IF(E43/[H$62]=0, " " ,[E43/H$62])	**69**
= IF(E44/[H$62]=0, " " ,[E44/H$62])	**70**
= IF(E45/[H$62]=0, " " ,[E45/H$62])	**71**
= IF(E46/[H$62]=0, " " ,[E46/H$62])	**72**
= IF(E47/[H$62]=0, " " ,[E47/H$62])	**73**
= IF(E48/[H$62]=0, " " ,[E48/H$62])	**74**
= IF(E49/[H$62]=0, " " ,[E49/H$62])	**75**
= IF(E50/[H$62]=0, " " ,[E50/H$62])	**76**
= IF(E51/[H$62]=0, " " ,[E51/H$62])	**77**
= IF(E52/[H$62]=0, " " ,[E52/H$62])	**78**
= IF(E53/[H$62]=0, " " ,[E53/H$62])	**79**
= IF(E54/[H$62]=0, " " ,[E54/H$62])	**80**
= IF(E55/[H$62]=0, " " ,[E55/H$62])	**81**
= IF(E56/[H$62]=0, " " ,[E56/H$62])	**82**
= IF(E57/[H$62]=0, " " ,[E57/H$62])	**83**
= IF(E58/[H$62]=0, " " ,[E58/H$62])	**84**
= IF(E59/[H$62]=0, " " ,[E59/H$62])	**85**
= IF(E60/[H$62]=0, " " ,[E60/H$62])	**86**

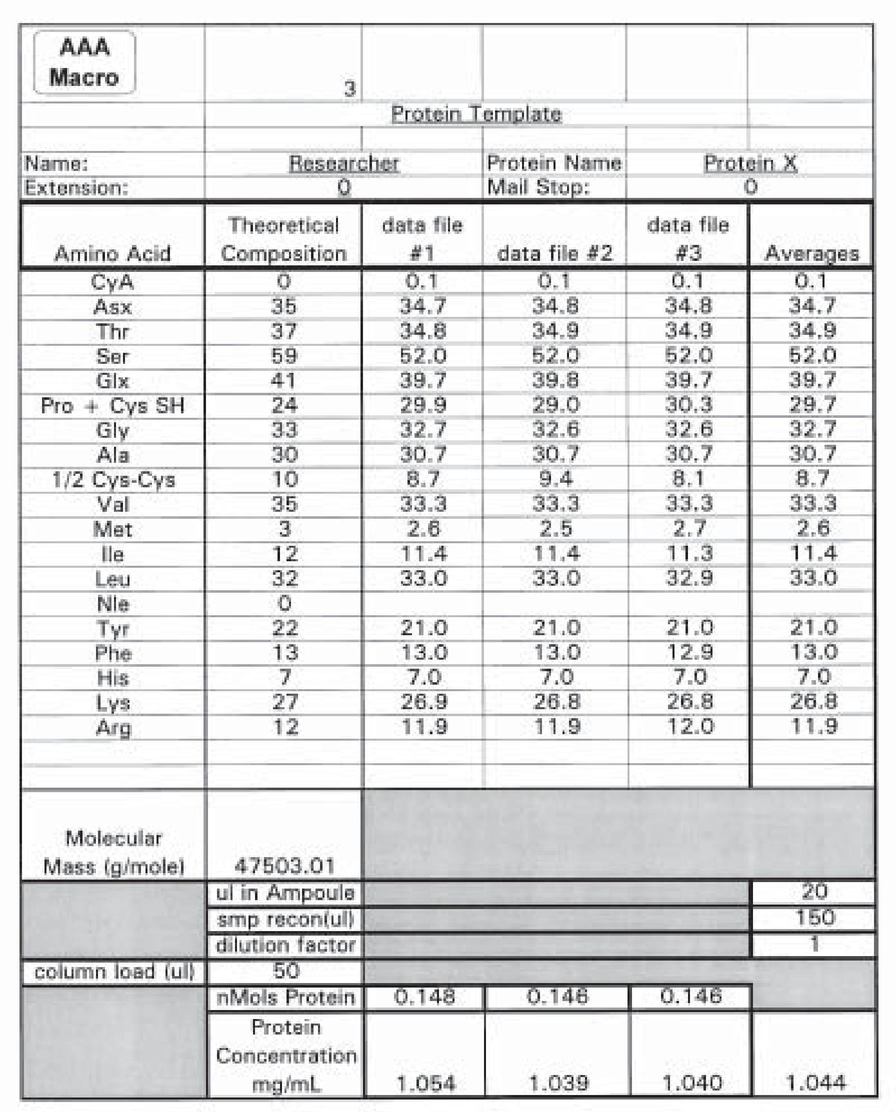
4. After cooling on wet ice, centrifuge the samples and transfer the supernatant to another Eppendorf tube.
5. Dry by rotary evaporation using a Savant SpeedVac.
6. Reconstitute the samples with Li-S buffer.
7. Follow the operating conditions given in **Table 6**. The initial 40-min segment of the chromatogram obtained using this modified program is shown in **Fig. 5**.

3.6. Amino Sugar Analysis (see Note 21)

1. Divide samples containing 2–20 µg of protein or 0.5–5 nmol of peptide fractions into two identical aliquots.
2. Hydrolyze one aliquot for 24 h at 110°C as described in **Subheading 3.1.**
3. Hydrolyze the other aliquot for only 2 h at 110°C (*see* **Note 22**).
4. After removal of the acid, reconstitute the 2-h hydrolysates with Na-S buffer and analyze using a modified program given in **Table 7** (*see* **Note 23**).
5. Analyze the 24-h hydrolysates using the standard method (**Table 1**) (**Fig. 6**) for quantitation of the protein/peptide to permit molar GlcNAc and/or GalNAc determinations. Tryptophan standards should also be analyzed to ensure that Trp does not coelute with $GalNH_2$; if necessary, this resolution can be improved by lowering the column temperature (*see* **Note 24**).

3.7. Cysteine Alkylation Monitoring

1. Hydrolyze desalted samples containing 2–20 µg of S-carboxy-methylated or S-carboxyamidomethylated proteins for 24 h as described in **Subheading 3.1.**
2. After removal of the acid, reconstitute the samples with Na-S buffer, and analyze using the standard program (**Table 1**) (*see* **Note 25**). Representative chromato-



AAA Macro	3				
	Protein Template				
Name:	Researcher		Protein Name	Protein X	
Extension:	0		Mail Stop:	0	
Amino Acid	Theoretical Composition	data file #1	data file #2	data file #3	Averages
CyA	0	0.1	0.1	0.1	0.1
Asx	35	34.7	34.8	34.8	34.7
Thr	37	34.8	34.9	34.9	34.9
Ser	59	52.0	52.0	52.0	52.0
Glx	41	39.7	39.8	39.7	39.7
Pro + Cys SH	24	29.9	29.0	30.3	29.7
Gly	33	32.7	32.6	32.6	32.7
Ala	30	30.7	30.7	30.7	30.7
1/2 Cys-Cys	10	8.7	9.4	8.1	8.7
Val	35	33.3	33.3	33.3	33.3
Met	3	2.6	2.5	2.7	2.6
Ile	12	11.4	11.4	11.3	11.4
Leu	32	33.0	33.0	32.9	33.0
Nle	0				
Tyr	22	21.0	21.0	21.0	21.0
Phe	13	13.0	13.0	12.9	13.0
His	7	7.0	7.0	7.0	7.0
Lys	27	26.9	26.8	26.8	26.8
Arg	12	11.9	11.9	12.0	11.9
Molecular Mass (g/mole)	47503.01				
	ul in Ampoule				20
	smp recon(ul)				150
	dilution factor				1
column load (ul)	50				
	nMols Protein	0.148	0.146	0.146	
	Protein Concentration mg/mL	1.054	1.039	1.040	1.044

Fig. 2. Summary sheet using the AAA macro. Average compositions are given in the right-hand column, and the average mg/mL value is provided in the lower right hand box. CyA refers to cysteic acid, which is present when samples are oxidized intentionally.

grams for the standard mixture containing carboxymethylcysteine (CMCys) and for an *S*-carboxymethylated recombinant antibody sample are given in **Fig. 7A,B**, respectively.

3. Monitor CMCys and half-cystine residue/mol values to determine the extent of cysteine alkylation (*see* **Notes 26** and **27**).

Table 4
Norleucine Analysis

Time (min)	Event	Conditions
0.0	Sample injection	Li-A buffer, 38°C
44.0	Buffer change	Li-A to Li-D
73.0	Buffer change	Li-D to Li-R
74.0	Reagent pump	Ninhydrin to water
77.0	Buffer change	Li-R to Li-A
80.0	Reagent pump	Water to ninhydrin
90.0	Recyle (start next run)	

Buffer pump: 20 mL/h.
Reagent pump: 10 mL/h.

4. Notes

1. Precolumn derivatization with RP-HPLC separation is used for amino acid analysis; a popular version is the Waters AccQTag system ***(21)***. These precolumn methods may not be suitable for detection of trace levels of minor amino acids (the needle-in-a-haystack problem) because the peak resolutions are diminished when the sample loads are increased, whereas resolution is maintained with higher loads using the cation–exchange systems. In addition, precolumn accuracy may be limited if derivatization is incomplete, a problem that does not occur with postcolumn derivatization systems. Similarly, cation–exchange systems are more tolerant of salts and residual HCl than the precolumn systems.
2. Hydrolysis ampoules are wrapped in heavy duty foil and pyrolyzed by heating for 24 h at 400°C in a muffle furnace before use.
3. Production of the Beckman 6300 analyzers described in this chapter has been halted, but a similar system can be fashioned using components offered by Pickering Labs (Mountain View, CA) ***(19)***. Pickering also supplies amino acid analysis buffers, reagents, and columns for the Beckman 6300, but care must be taken not to combine Pickering's Trione ninhydrin reagent with mobile phases that contain alcohols (such as Beckman's Na-A and Na-B) as this combination may clog the analyzer's reactor.
4. Hitachi also offers a cation–exchange amino acid analysis instrument.
5. Dionex has recently introduced an anion–exchange system that detects underivatized amino acids using pulsed amperometric detection ***(20)*** (*see also* Chapter 7, this volume), but we have no experience with this system.
6. A wash bottle containing 1 *M* sodium bicarbonate is kept nearby wherever HCl ampoules are opened to neutralize spills.
7. The prepared standards should be stored refrigerated in aliquots using screw-top Eppendorf tubes equipped with a rubber gasket to prevent evaporation. Tryptophan tends to degrade over time in acid conditions, so fresh Trp standards should be prepared when needed; commercial preparations containing Trp may not be reliable.

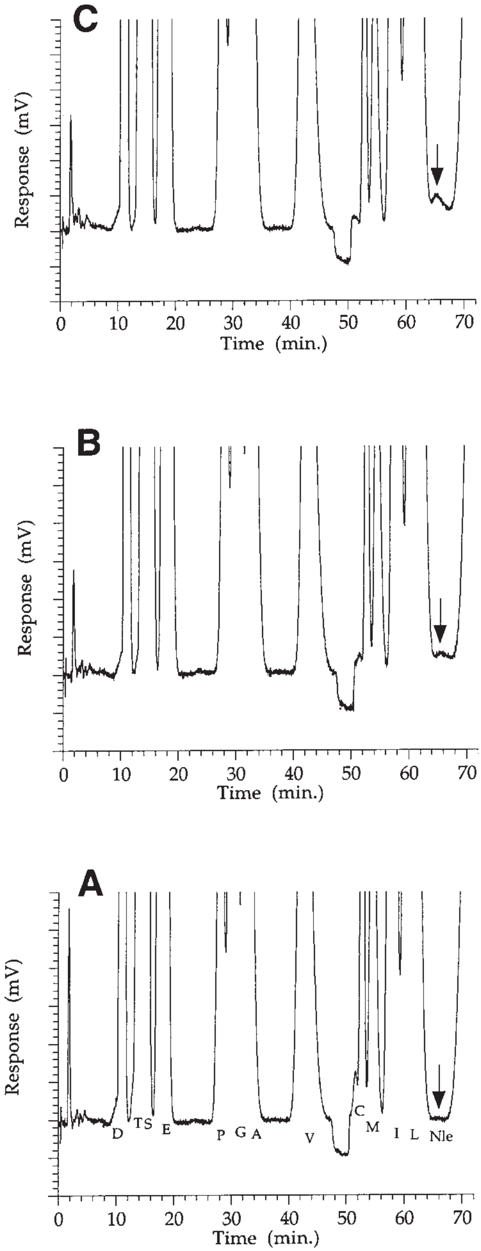


Fig. 3. Analysis for norleucine incorporation at Met positions. Aliquots from 40 µg of a recombinant protein are given, with the arrow indicating the Nle peak after additions of (**A**) 0 pmol Nle (**B**) 200 pmol Nle, or (**C**) 400 pmol Nle. Operating parameters are given in **Table 4**.

Table 5
Hydroxylysine Analysis

Time (min)	Event	Conditions
0.0	Sample injection	Li-A buffer, 38°C
12.0	Temperature change	38°C to 50°C over 8 min
42.0	Buffer change	Li-A to Li-B
60.0	Temperature change	50°C to 71°C over 8 min
70.0	Buffer change	Li-B to Li-C
110.0	Reagent pump	Ninhydrin to water
111.0	Buffer change	Li-C to Li-R
114.0	Buffer change	Li-R to Li-A
125.0	Reagent pump	water to ninhydrin
135.0	Recyle (start next run)	

Buffer pump: 20 mL/h.
Reagent pump: 10 mL/h.

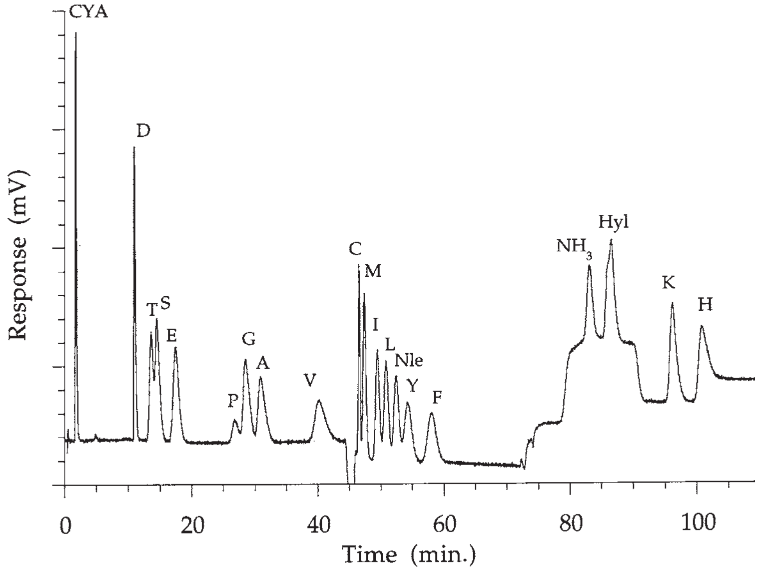


Fig. 4. Analysis for hydroxylysine. A standard mixture containing 1 nmol of each component was loaded. Hyl appears as a poorly-resolved peak pair. Operating parameters are given in **Table 5**.

8. Protein/peptide quantitation can be compromised by multiple sample transfers. When accuracy is essential, samples should be transferred directly from the primary container to the hydrolysis ampoule.

Table 6
Analysis of Carboxypeptidase Supernatants

Time (min)	Event	Conditions
0.0	Sample injection	Li-A buffer, 38°C
12.0	Temperature change	38°C to 50°C over 8 min
43.0	Buffer change	Li-A to Li-B
60.0	Temperature change	50°C to 73°C over 8 min
60.0	Buffer change	Li-B to Li-C
130.0	Reagent pump	Ninhydrin to water
132.0	Buffer change	Li-C to Li-R
134.0	Buffer change	Li-R to Li-A
140.0	Temperature change	73°C to 38°C
140.0	Reagent pump	Water to ninhydrin
155.0	Recyle (start next run)	

Buffer pump: 20 mL/h.
Reagent pump: 10 mL/h.

Table 7
Amino Sugar Analysis

Time (min)	Event	Conditions
0.0	Sample injection	Na-F buffer, 66°C
55.0	Reagent pump	Ninhydrin to water
57.0	Buffer change	Na-F to Na-R
58.5	Buffer change	Na-R to Na-F
60.0	Reagent pump	Water to ninhydrin
74.0	Recyle (start next run)	

Buffer pump: 20 mL/h.
Reagent pump: 10 mL/h.

9. Alternative hydrolysis systems have been proposed, including the Waters PicoTag batch hydrolysis system, in which the 6 *N* HCl is placed outside the sample tubes in a chamber that can be evacuated, closed, and heated. Phenol must also be added to prevent destruction of tyrosine. This system has the advantage that direct contact with the acid is avoided, eliminating a potential source of contamination, but in our experience the poor hydrolysis of Ile-Ile, Ile-Val, and Val-Val bonds with vapor–phase hydrolysis makes this technique unsuitable.
10. Hydrolysis at 155°C for 60 min has also been proposed, but we seldom use this procedure because Thr and Ser values are greatly reduced. Also, because we typically batch samples together, a 24-h hydrolysis is often more convenient from an operational standpoint.
11. A Perkin Elmer Model 200 autosampler has replaced the original coil system, with a fixed 50-µL volume used for standards and samples.

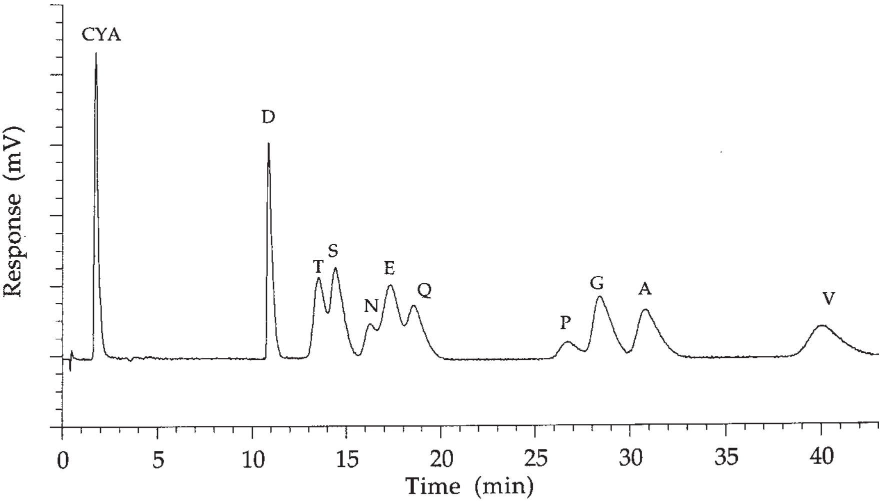


Fig. 5. Analysis of carboxypeptidase digestion samples (expanded view). A standard mixture containing 1 nmol of each component was loaded. For clarity, only the early region of the chromatogram is provided to show the elution positions of Asn and Gln; the complete chromatogram is essentially the same as **Fig. 8**. Operating parameters are given in **Table 6**.

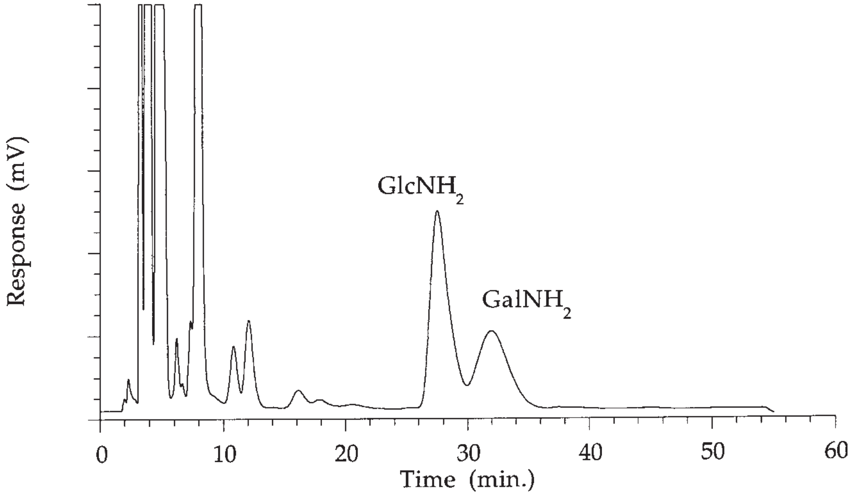


Fig. 6. Amino sugar analysis. A hydrolysate from 1 nmol of a recombinant glycoprotein was loaded. Operating parameters are given in **Table 7**.

12. In the tables, the "reagent pump" event refers to changing the solution added postcolumn from a ninhydrin-containing reagent to water (or vice versa); this is done to avoid having NaOH (Na-R) or LiOH (Li-R) mix with the ninhydrin reagent.

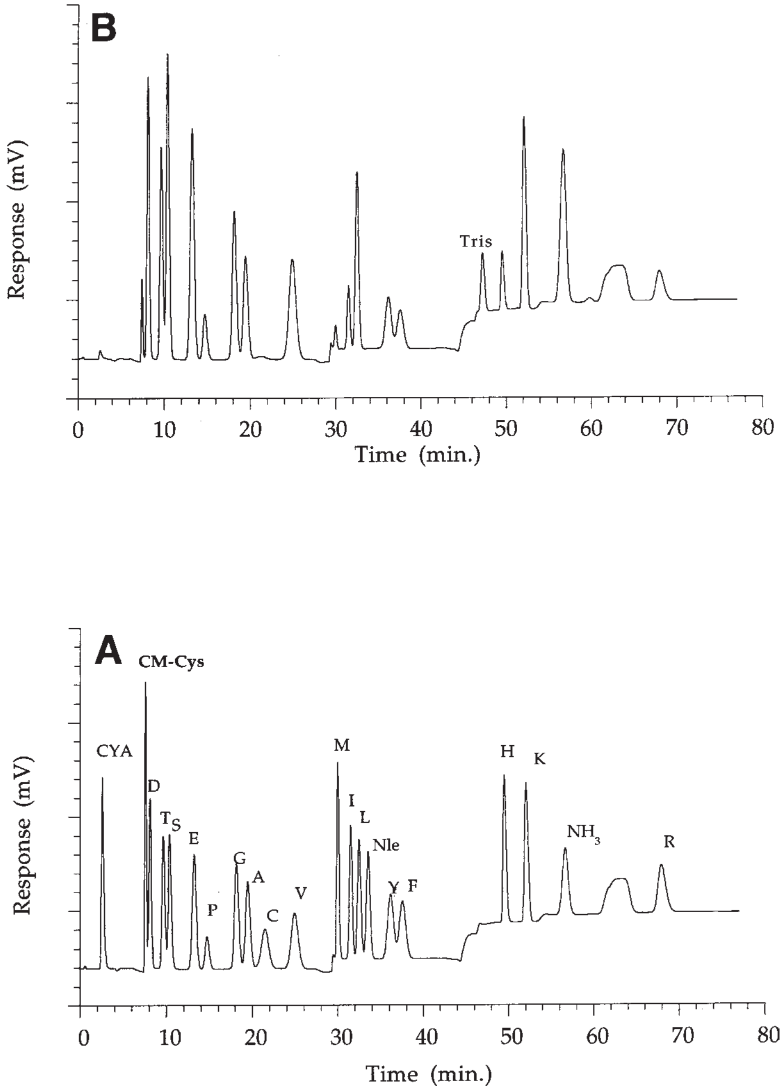


Fig. 7. Carboxymethylcysteine analysis. **(A)** Standard mixture containing 2 nmol of each component. **(B)** Analysis of 5 µg of a recombinant antibody after reduction and S-carboxymethylation. Operating parameters are given in **Table 1**. The peak eluting at approx 47 min is Tris buffer.

13. Key amino acids that typically provide quantitative recoveries (e.g., Asx, Glx, Ala, Leu, Phe, His, Lys, Arg) are used to determine the total nmol of protein or peptide (in the example provided for data file #1 in **Table 3**, 5.132 nmol of Asx are divided by 35 residues of Asx expected per mol of protein to produce a nmol

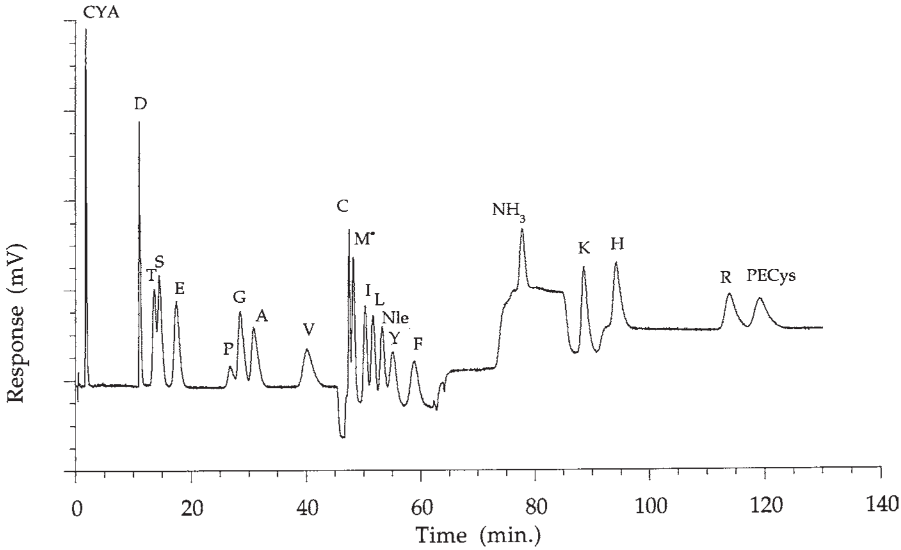


Fig. 8. Pyridylethylcysteine analysis. Analysis of a standard mixture containing 1 nmol of each component. Operating parameters are given in **Table 6**.

protein value); use of these selected amino acids avoids the low recoveries experienced for the acid-labile amino acids, especially Trp, Cys, Thr, and Ser *(1)*.

14. The amino acid nmol values are divided by the total protein/peptide nmol value to produce residues/mol values that are averaged in the right-hand column (in the example provided in **Table 3** and **Fig. 2**, the nmol values in rows 42–60 of columns C, D, and E are divided by the average protein value of 0.148, 0.146, and 0.146, respectively, then averaged). The nmol protein/peptide values in **Fig. 2** are also multiplied by the molecular mass to provide the total µg injected on the analyzer, which is then converted to a µg/µL value (same as a mg/mL value) by dividing by the volume loaded in the ampoule (e.g., 20 µL in **Fig. 2**), then correcting for the reconstitution volume and µL injected (e.g., multiplied by 150/50 in **Fig. 2**); these concentrations are then averaged as shown in the lower right-hand box of **Fig. 2**.
15. When the protein/peptide composition is not known, or if the sample contains a mixture of proteins, then quantitation is performed by summing the nanograms contributed by each amino acid residue. For example, instead of calibrating to a 2 nmol standard value for alanine, the data system calibrates to 142 ng (equivalent to 2 nmol of alanine using the residue mass). The values for all amino acids are summed. This method generates values that are usually 5–10% lower than their true quantity because of poor recoveries of acid-labile amino acids, but in our experience these values are likely to be more sensitive and reliable than colorimetric methods.

16. Norleucine quantitation is difficult at levels below 1% Nle-for-Met replacement. Nle added at several levels to samples can establish the lowest level of quantitation, which for us is typically about 75 pmol. In our experience, the incorporation of Nle occurs proportionately at every Met position; therefore, the mol Nle per mol protein value can be divided by the number of methionines to provide percent Nle-for-Met replacement values. Nle replacement can sometimes be observed by electrospray mass spectrometry of intact proteins (18 Dalton lower mass), provided that no other sources of heterogeneity are present (V. Ling, unpublished data).
17. Most amino acids will be present in great abundance, saturating the detector, but the glycine and proline peaks are usually still on-scale, and thus can be used for mol Nle per mol protein quantitation. Chromatograms for 40 µg hydrolysates of a recombinant protein spiked with 0, 200, or 400 pmol Nle are given in **Fig. 3A**, **B**, and **C**, respectively.
18. Sensitive detection of hydroxylysine (Hyl) is difficult because of the fact that most noncollagenous molecules are at most partially modified at just one -Lys-Gly- positio n, thus the overall percentage of modified Lys is very low.
19. In the standard amino acid analysis method (**Table 1**), Hyl coelutes with histidine, so it would not be observed in an intact protein that contains His, and it might be misinterpreted in a peptide fraction. The long delay for the second buffer change is needed to increase the resolution of Hyl from ammonia. Hyl appears as a partially resolved doublet peak due to racemization of the δ carbon during hydrolysis; therefore, the peak areas are summed.
20. Once it has been determined that Hyl is present, peptide maps may be used to assign the site provided the investigator is aware that Hyl–Gly bonds are fairly resistant to trypsin and endoproteinase Lys-C digestion *(22)*.
21. Amino sugar analysis does not provide complete monosaccharide determinations of the types obtained by techniques such as HPAEC-PAD or GC-MS, but it does have the advantages that no additional equipment is required, and the results are routinely quantitative. This approach is most useful when assaying proteins for the mucin-type O-linked oligosaccharides that contain GalNAc at their reducing termini. O-linked structures that lack GalNAc are rare, but have been found in EGF-like domains of several glycoproteins *(23)*. Some N-linked structures contain GalNAc, particularly in proteins from human embryonic kidney (293) cells *(24)* or melanoma cells *(25)*, but these N-linked structures can be released using PNGaseF to allow discrimination between N-linked and O-linked GalNAc residues.
22. After 2 h of hydrolysis, GlcNAc is hydrolyzed to $GlcNH_2$, whereas GalNAc is hydrolyzed to $GalNH_2$, and both are released quantitatively.
23. This program starts with the second buffer used in the standard analysis, so most amino acids elute near the beginning of the chromatogram. A chromatogram from 1 nmol of a hydrolysate of a recombinant glycoprotein containing both N-linked and O-linked sites is given in **Fig. 6**.

24. Proteins that are highly glycosylated will have some residual amino sugars that will appear as a broad peak that elutes in the Ile-Leu-Nle region of the standard chromatogram. Increasing the hydrolysis time to 72 h will eliminate this peak.
25. Methionine residues can also be unintentionally S-alkylated, but this can be detected by the presence of trace levels of homoserine, a hydrolysis product of S-carboxymethylmethionine that elutes between Ser and Glx.
26. Samples that have been alkylated using 4-vinylpyridine need to be analyzed using the lithium citrate program that is used for the carboxypeptidase digestion samples (**Table 6**). Pyridylethylcysteine (PECys) is very basic, and elutes after Arg (**Fig. 8**).
27. When monitoring Cys alkylation conditions, attention should be paid to methionine recoveries, as the conditions (such as trace metals or residual O_2) that affect Met recoveries will also affect CMCys recoveries. In addition, methionine sulfoxide can coelute with CMCys; therefore, samples should be analyzed promptly after acid removal.

References

1. Moore, S. and Stein, W. H. (1958) Chromatographic determination of amino acids by the use of automatic recording equipment. *Methods Enzymol.* **6,** 819–831.
2. Schuster, R. (1988) Determination of amino acids in biological, pharmaceutical, plant and food samples by automated precolumn derivatization and high performance liquid chromatography. *J. Chromatog.* **431,** 217–284.
3. Heinrickson, R. L. and Meredith, S. C. (1983) Amino acid analysis by reverse-phase high-performance liquid chromatography: precolumn derivatization with phenylisothiocyanate. *Anal. Biochem.* **136,** 65–74.
4. van Wandlen, C. and Cohen, S. A. (1997) Using quaternary high-performance liquid chromatography eluent systems for separating 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate-derivatized amino acid mixtures. *J. Chromatog. A* **763,** 11–22.
5. Kisumi, M., Sugiura, M., and Chibata, I. (1976) Biosynthesis of norvaline, norleucine and homoisoleucine in Serratia marcescens. *J. Biochem.* **80,** 333–339.
6. Tsai, L. B., Lu, H. S., Kenney, W. C., Curless, C. C., Klein, M. L., Lai, P.-H., et al. (1988) Control of misincorporation of de novo synthesized norleucine into recombinant interleukin–2 in *E. coli*. *Biochem. Biophys. Res. Commun.* **156,** 733–739.
7. Bogosian, G., Violand, B. N., Dorward-King, E. J., Workman, W. E., Jung, P. E., and Kane, J. F. (1989) Biosynthesis and incorporation into protein of norleucine by Escherichia coli. *J. Biol. Chem.* **264,** 531–539.
8. Kivirikko, K. I., Myllyla, R., and Pihlajaniemi, T. (1992) Hydroxylation of proline and lysine residues in collagens and other animal and plant proteins, in *Posttranslational Modifications of Proteins* (Harding, J. J. and Crabbe, M. J., eds.), CRC, Boca Raton, FL, pp. 1–51.
9. Molony, M. S., Wu, S.-L., Keyt, L., and Harris, R. J. (1995) The unexpected presence of hydroxylysine in non-collagenous proteins, in *Techniques in Protein Chemistry VI* (Crabbe, J., ed.), Academic, San Diego, CA, pp. 91–98.

10. Kornfeld, R. and Kornfeld, S. (1985) Assembly of asparagine-linked oligosaccharides. *Annu. Rev. Biochem.* **54,** 631–664.
11. Tarentino, A. L., Gomez, C. M., and Plummer, T. H. (1985) Deglycosylation of asparagine-linked glycans by peptide: N-glycosidase F. *Biochemistry* **24,** 4665–4671.
12. O'Connell, B., Tabak, L. A., and Ramasubbu, N. (1991) The influence of flanking sequences on O-glycosylation. *Biochem. Biophys. Res. Commun.* **180,** 1024–1030.
13. Wilson, I. B. H., Gavel, Y., and von Heijne, G. (1991) Amino acid distributions around O-linked glycosylation sites. *Biochem. J.* **275,** 528–534.
14. Pisano, A., Packer, N. H., Redmond, J. W., Williams, K. L., and Gooley, A. A. (1994) Characterization of O-linked glycosylation motifs in the glycopeptide domain of bovine κ-casein. *Glycobiology* **4,** 837–844.
15. Garnick, R. L., Solli, N. J., and Papa, P. A. (1988) The role of quality control in biotechnology: an analytical perspective. *Anal. Chem.* **60,** 2546–2557.
16. Lundell, N. and Schreitmüller, T. (1999) Sample preparation for peptide mapping — a pharmaceutical quality-control perspective. *Anal. Biochem.* **266,** 31–47.
17. Jones, M. D., Merewether, L. A., Clogston, C. L., and Lu, H. S. (1994) Peptide map analysis of recombinant human granulocyte stimulating factor: elimination of methionine modification and nonspecific cleavages. *Anal. Biochem.* **216,** 135–146.
18. Allen, G. (1989) Determination of the carboxy-terminal residue, in *Sequencing of Proteins and Peptides*, Elsevier, Amsterdam and New York, pp. 67–71.
19. Grunau, J. A. and Swaider, J. M. (1992) Chromatography of 99 amino acids and other ninhydrin-reactive compounds in the Pickering lithium gradient system. *J. Chromatog.* **594,** 165–171.
20. Clarke, A. P., Jandik, P., Rocklin, R. D., Liu, Y., and Avdalovic, N. (1999) An integrated amperometry waveform for the direct, sensitive detection of amino acids and amino sugars following anion-exchange chromatography. *Anal. Chem.* **71,** 2774–2781.
21. Strydom, D. J. (1996) Amino acid analysis using various carbamate reagents for precolumn derivatization, in *Techniques in Protein Chemistry VII* (Marshak, D. R., ed.), Academic, San Diego, CA, pp. 331–339.
22. Molony, M. S., Quan, C., Mulkerrin, M. G., and Harris, R. J. (1998) Hydroxylation of Lys residues reduces their susceptibility to digestion by trypsin and lysyl endopeptidase. *Anal. Biochem.* **258,** 136–137.
23. Harris, R. J. and Spellman, M. W. (1993) O-Linked fucose and other post-translational modifications unique to EGF modules. *Glycobiol.* **3,** 219–224
24. Yan, S. B., Chao, Y. B., and van Halbeek, H. (1993) Novel Asn-linked oligosaccharides terminating in GalNAcβ(1→4)[Fucα(1→3)]GlcNAcβ(1→•) are present in recombinant human Protein C expressed in human kidney 293 cells. *Glycobiology* **3,** 597–608.
25. Chan, A. L., Morris, H. R., Panico, M., Eteinne, A. T., Rogers, M. E., Gaffney, P., et al. (1991) A novel sialylated N-acetylgalactosamine-containing oligosaccharide is the major complex-type structure present in Bowes melanoma tissue plasminogen activator. *Glycobiology* **1,** 173–185.