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Poster Presentation

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Amino Acid Analysis of Peptides and Proteins Using a Novel Activated Carbamate as a Pre-Column Derivatizing Reagent

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I. Introduction

Today many standard protein chemistry protocols such as Edman degradation and peptide mapping can be accomplished with low or even sub-picomole sample amounts through the use of modern liquid chromatography (1). Amino acid analysis has been assisted in this drive for higher sensitivity by the use of an array of pre-column derivatization reagents that yield easily detected labels. Fluorescent and chemiluminescent tags like orthophthalaldehyde (OPA) and fluorenyl methyl chloroformate (FMOC-Cl) provide the most sensitive tags, but the UV absorbing Edman reagent phenylisothiocyanate (PITC) remains the most widely used (2,3) tag for compositional analysis of peptide and protein hydrolyzates, perhaps because of its familiarity to protein chemists. Despite the wide choice in labelling chemistry, the desire for improved high sensitivity amino acid analysis continues to spur much active research into new reagents. In addition, many existing reagents exhibit less than ideal characteristics such as insufficient derivative stability, the formation of multiple products from a single amino acid, large interfering peaks from excess reagent or poor reproducibility or response linearity. We have recently described (4) the synthesis of a novel activated carbamate, 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC, patent pending), with unique spectroscopic properties which provides a pre-column derivatization method for the very accurate amino acid analysis of hydrolyzed peptides and proteins. A rapid reaction with amino acids forms highly stable asymmetric urea compounds with good fluorescence characteristics. A dramatic shift in fluorescence emission maximum for the amino acid adducts compared to the major reagent peak allows the direct injection of the sample reaction mixture with no interference from excess reagent. We herein report the application of AQC to the analysis of protein and peptide hydrolyzate samples, with an emphasis on the accuracy and reproducibility of the compositional data.

II. Materials and Methods

A. *Chemicals:*

Di(N-succinimidyl) carbonate (DSC) was purchased from Fluka Chemical Co. (Ronkonkoma, NY.); sodium acetate trihydrate (HPLC grade) and disodium ethylenediamine tetraacetic acid were from Baker Chemical Co. (Phillipsburg, PA.); triethylamine and 6-aminoquinoline (AMQ) were purchased from Aldrich Chemical Co. (Milwaukee, WI.). Amino acid standards were from Pierce Chemical Co. (Rockford, IL.); peptides and ß-Lactoglobulin A were obtained from Sigma Chemical Co. (St. Louis, MO.). Bovine serum albumin (BSA) was supplied by the Amino Acid Subcommittee of the Association of Biomolecular Resource Facilities.

Synthesis of AQC: Details of the synthesis are given in Reference 4. Briefly, DSC (3g, 12mmol) and AMQ (1.5g, 10mmol) were dissolved in dry acetonitrile, and the AMQ was added dropwise to the carbonate solution. After 30 minutes the reaction mixture was concentrated by rotary evaporation to about half its volume and, after cooling, the resulting crystals were filtered and washed with acetonitrile to yield 1.95g (66% of crude product). The product was recrystallized from acetonitrile as off-white crystals.

B. *Amino Acid Derivatization:*

Purified AQC was dissolved in dry acetonitrile at a concentration of 10mM. Standard solutions of amino acids were made from dilutions of commercial standard mixtures. Aliquots of standards in 5-50µl of water or dilute HCl were pipetted into glass tubes (6 x 50 or 12 x 75mm) and 0.2M borate buffer, pH 8.8 was added to bring the volume up to 40-400µl. Derivatization was carried out by adding one volume of AQC solution to four volumes of buffered sample followed by heating at 50°C for 10 minutes.

C. *Fluorescence Properties of AQC and Related Compounds:*

The AQC derivative of Ala (AQC-Ala) was synthesized by adding 1ml of 1M Ala in borate buffer to 1ml of a 10mM AQC solution in acetonitrile. Fluorescence spectra of AQC, AMQ and AQC-Ala were recorded on a Shimadzu RF-5000 fluorescence spectrophotometer. Stock solutions, 3mM, were diluted in acetonitrile to give a final concentration of 0.3mM.

D. *Chromatography:*

Two HPLC systems were used routinely. System I consisted of (2) 510 Pumps, 712 WISP™ Injector, 470 Scanning Fluorescence Detector, Temperature Control Module and 860 Networking Computer. System II consisted of a 625 LC System, 717 Autosampler, 470 Scanning Fluorescence Detector, Column Heater Module and 860 Networking Computer (all Waters™ components, Millipore Corp., Milford, MA.) All separations were achieved using Waters Nova-Pak™ C18, 4µm, 3.9mm x 150mm columns (Millipore), maintained at 37°C, using multistep, linear gradient elution conditions at 1.0ml/min. Eluent A was 140mM sodium acetate, 17mM triethylamine, pH 5.05 with 1mg/L disodium EDTA. Eluent B was 60%(v/v) acetonitrile in Milli-Q® water (Millipore) in system I. System II used a ternary solvent system with eluent B acetonitrile and eluent C Milli-Q water.

E. *Preparation of Samples:*

Proteins and peptides (25-10,000ng) were hydrolyzed with gaseous HCl as previously described (5). After removing the excess HCl under vacuum, the amino acids were resolubilized with 20µl of 10-20mM HCl, the volume brought to 80µl with 0.2M borate buffer, pH 8.8, and the derivatives formed via the addition of 20µl of 10mM AQC in acetonitrile followed by heating at 50°C for 10 minutes. In one study, 50µl of salt (0.1M) or detergent solution (0.2% w/v) was added to the sample and dried prior to hydrolysis.

F. *Data Analysis:*

Compositional accuracy was calculated according to the method described in Reference 3:

$$\% \text{ Error} = 100 \times (\text{Experimental} - \text{True Residue Value})/\text{True Value}$$

$$\text{Average } \% \text{ Error} = \Sigma(\text{Absolute } \% \text{ Error})/16$$

III. Summary of Results

- AQC derivatization of amino acids is a novel method for protein compositional analysis that provides enhanced capabilites.
- Derivatization is rapid, reproducible, and quantitative with linear response (Figure 1 and Table I).
- Unique fluorescence properties permit direct sample injection (Figure 2).
- Highly fluorescent derivatives allow for sub-picomole detection (Figure 3 and Table I).
- Derivatives are stable for at least 1 week at 25°C.
- Protein compositional analysis by AQC derivatization is:
  - highly reproducible (Figure 4)
  - very accurate, even at sub-microgram levels (Figure 5 and Table IV)
  - permissable in the presence of salts and detergents (Table V).

IV. References

1. Young, P., Astephen, N.A., and Wheat, T.W. (1992) LC/GC, 10, 26-32
2. Cohen, S.A. and Strydom, D.J. (1988) Anal. Biochem. 174, 1-16
3. Strydom, D.J., Tarr, G.E., Pan, Y-C. E. and Paxton, R.J. (1992) in Techniques in Protein Chemistry III (R.H. Angeletti, ed.), Academic Press, San Diego, pp 261-274
4. Cohen, S.A. and Michaud, D.M. (1992) Anal. Biochem., submitted
5. Bidlingmeyer, B.A., Cohen, S.A., and Tarvin, T.L. (1984) J. Chromatogr. 336, 93-104
6. West, K.A/ and Crabb, J.W. (1990) in Current Research in Protein Chemistry: Techniques, Structure and Function, J.J. Villafranca, ed., Academic Press, Inc., pp 37-47

Table I: Reproducibility, Linearity, and Calculated Detection Limits

Amino Acid	r^2	Detection Limit (fmol)
Asp	0.9999	311
Ser	0.9996	237
Glu	0.9996	315
Gly	0.9995	293
His	0.9994	188
Arg	0.9995	119
Thr	0.9993	162
Ala	0.9992	155
Pro	0.9998	278
Cys	0.9996	794
Tyr	0.9995	93
Val	0.9996	57
Met	0.9996	74
Lys	0.9997	112
Ile	0.9998	47
Leu	0.9994	48
Phe	0.9999	38

Amino Acid	Area %CV	Retention Time %CV
Asp	0.82	0.56
Ser	0.58	0.48
Glu	1.32	0.50
Gly	1.36	0.42
His	1.60	0.40
Arg	1.17	0.23
Thr	1.05	0.21
Ale	0.76	0.18
Pro	0.47	0.15
Cys	1.34	0.08
Tyr	0.99	0.08
Val	0.59	0.08
Met	0.79	0.08
Lys	0.54	0.06
Ile	0.37	0.06
Leu	0.38	0.07
Phe	0.49	0.07
Average %CV	0.86	0.22

Table II:
Comparison of AQC to Other Reagents

	Dabsyl Chloride	PITC	FMOC-Cl	OPA/Analogs	OPA/FMOC Hybrid	**AQC**
Detection Mode	Vis @ 413nm	UV @ 254nm	Fluorescence	Fluorescence	Fluorescence	**Fluorescence**
Reagent Interference / Removal	Yes	Yes Volatile	Yes Extraction	No	No	**No**
Secondary Amino Acids	Yes	Yes	Yes	No	Yes	**Yes**
Stable Derivatives	Yes	Yes	His deriv.?	No (BME thiol) Yes (MPA thiol, analogs/CN^-)	Yes	**Yes**
Multiple Peaks	No	No	Yes	No (most) Yes/MPA Thiol	Yes/Lys with MPA thiol	**No**
Femtomole Levels	Barely	No	Yes	Yes	Yes	**Yes**
Salt/Detergent Interference	Not Reported	Some	Yes	?	?	**Minimal**

Table III: Protein Compositional Analysis

	ß-Lactoglobulin			Bovine Serum Albumin			
	Expected	Calculated	Calculated	Expected	Calculated	Calculated	Calculated
Asp	16	17.6	17.1	54	53.7	59.5	58.2
Ser	7	6.5	6.3	28	29.6	26.9	24.7
Glu	25	26.8	26.7	79	73.7	85.4	83.8
Gly	3	3.4	3.0	16	22.9	16.8	15.8
His	2	2.0	1.9	17	13.7	17.7	16.6
Arg	3	3.2	3.1	23	23.0	23.6	23.5
Thr	8	7.9	7.9	34	31.4	33.5	33.0
Ala	14	14.4	14.5	46	56.1	47.9	47.4
Pro	8	7.7	8.1	28	28.2	25.4	27.8
Tyr	4	3.6	3.7	19	16.2	17.7	17.8
Val	10	9.8	10.1	36	34.5	33.9	35.9
Met	4	3.5	3.8	4	4.6	4.1	4.1
Lys	15	15.5	16.0	59	52.0	61.9	60.7
Ile	10	9.5	9.6	14	14.3	13.4	13.8
Leu	22	21.8	22.7	61	54.8	57.8	61.5
Phe	4	4.0	4.0	27	25.8	26.3	27.6
Sample Amount (ng)		316	1801		30	185	1305
Average % Error		5.6	4.1		10.5	4.9	3.4

Table IV: Summary of Compositional Error Data

	ß-Lactoglobulin				Serum Albumin		
n	11	5	12	2	2	2	7
Range of Sample Amount (ng)[a]	90-389	587-790	1038-1915	9400-9770	30-33	175-185	1305-1932
Average Sample Amount (ng)	212	684	1665	9587	31	180	1739
Range of Average Errors	5.5-17.3	4.9-14.6	4.1-12.2	4.3-4.7	10.5-25.9	4.9-6.8	3.4-7.7
Average of Average Errors	10.7	8.6	6.4	4.5	18.2	5.8	5.5

a) Amounts are calculated from the amino acid recovery data.

Table V
Effect of Salts and Detergents

Summary of % error data, n=3 for each sample

	Control	SDS	Sodium Chloride	Sodium Acetate	Sodium Phosphate
Asp	6.7	9.1	10.2	15.0	2.3
Ser	11.7	20.3	12.1	30.4	43.4
Glu	10.6	13.0	13.4	11.3	4.8
Gly	9.2	17.3	20.9	22.7	40.4
His	1.3	2.6	3.5	5.9	3.5
Arg	3.6	6.5	5.6	3.9	17.1
Thr	7.4	10.7	8.4	31.9	24.2
Ala	1.9	5.0	1.4	3.6	0.6
Pro	2.3	5.0	2.5	3.1	2.7
Tyr	1.2	10.2	2.3	3.8	1.2
Val	3.0	1.3	3.2	1.0	0.8
Met	4.5	13.3	4.3	12.7	18.5
Lys	2.8	3.1	3.3	4.2	1.0
Ile	6.2	4.7	6.8	4.6	2.5
Leu	1.4	1.9	1.5	0.8	1.0
Phe	2.5	1.9	1.4	6.9	3.6
Average of Average Errors	4.8	7.9	6.3	10.1	10.5
Average Amount Hydrolyzed	1390	1133	1189	564	845

Fifty µL of salt (0.1M) or detergent (0.2%w/v) were added to the sample prior to hydrolysis, except for the phosphate solution which only used 10µL.

Figure 1: Derivatization Chemistry

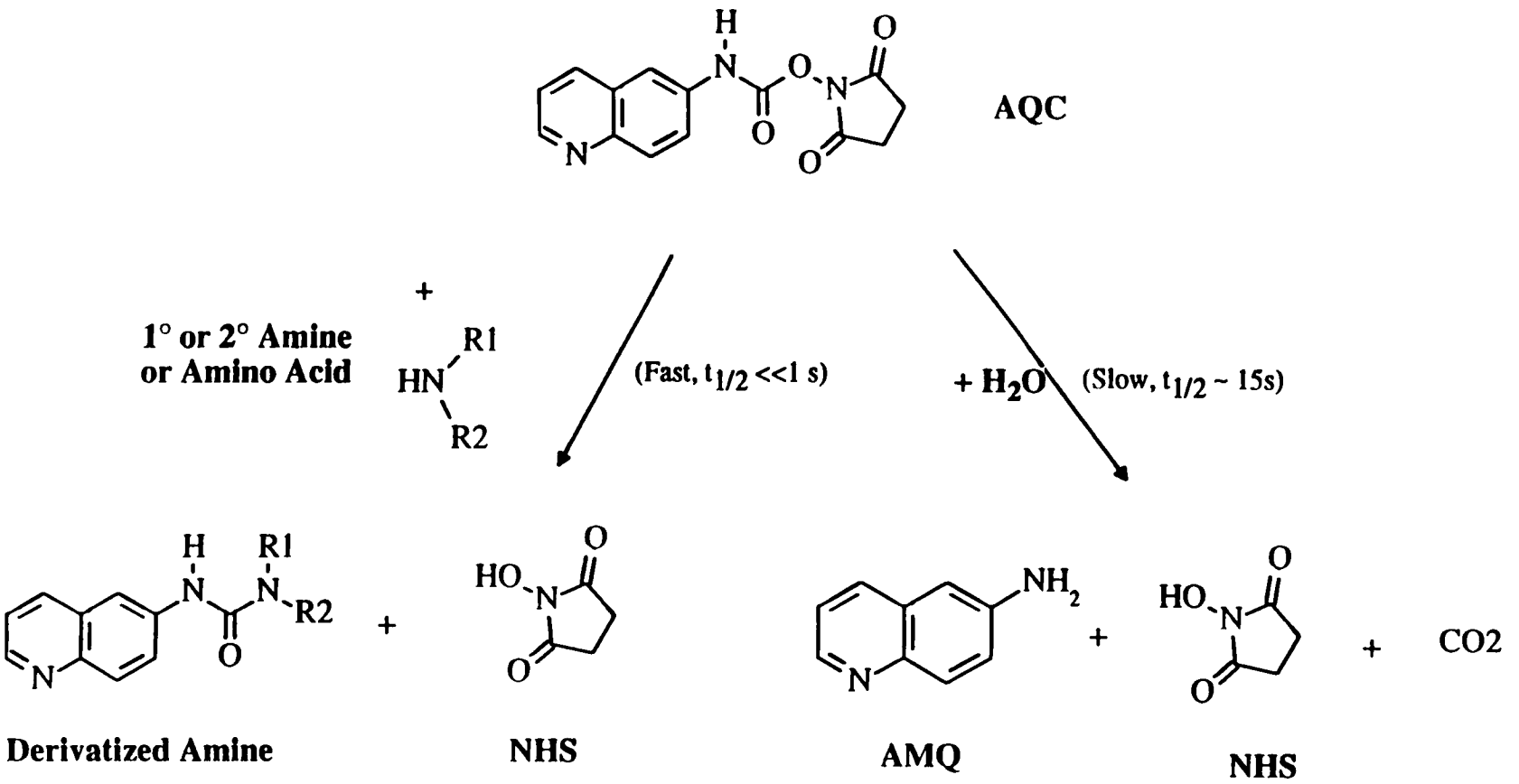
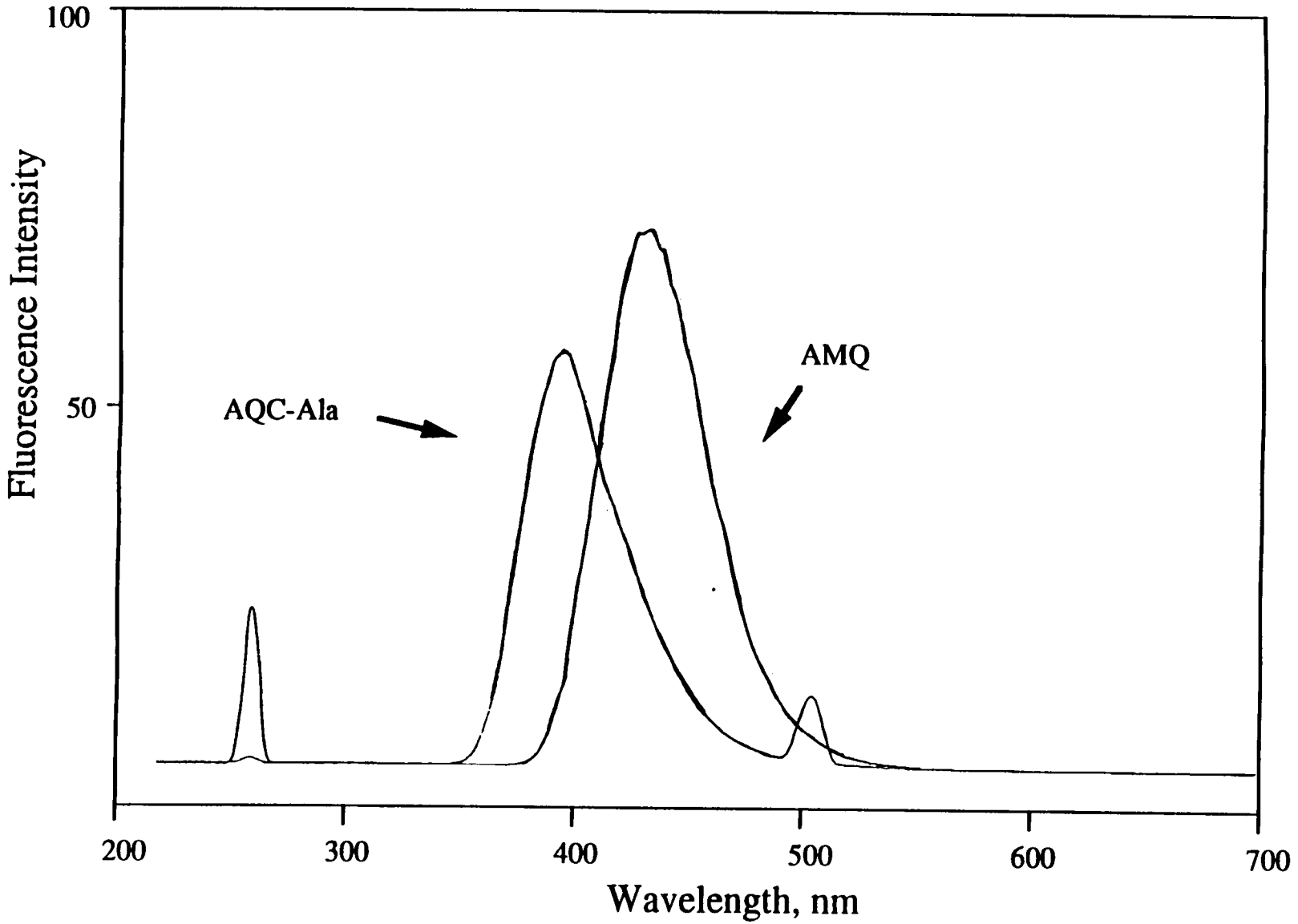


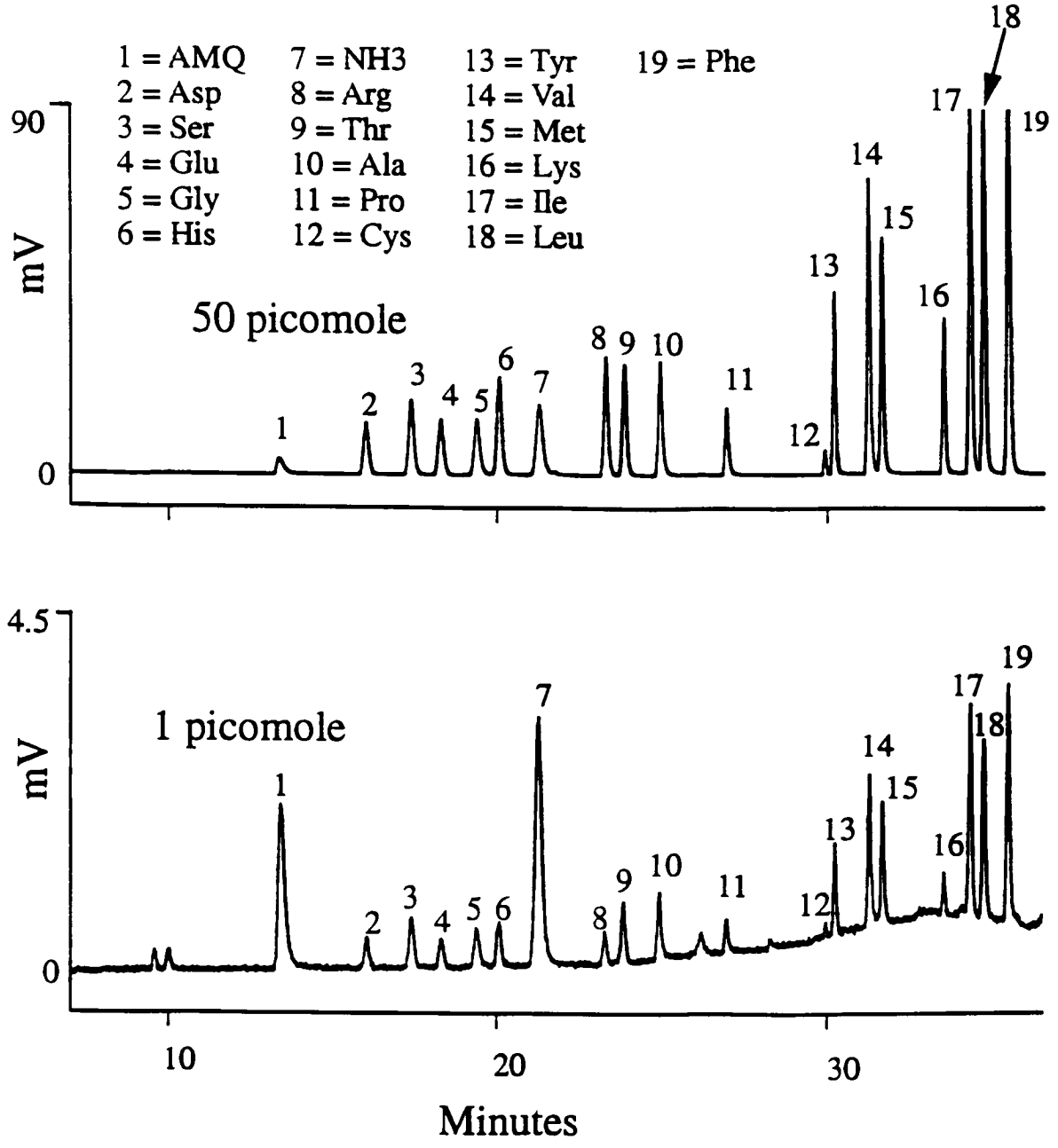
Figure 2

Emission Spectra for AQC-Ala and AMQ



Favorable fluorescence emission properties allow for the detection of derivatized amino acids at 395nm with minimal interference from the reagent hydrolysis product AMQ.

Figure 3:
Chromatography of Standards

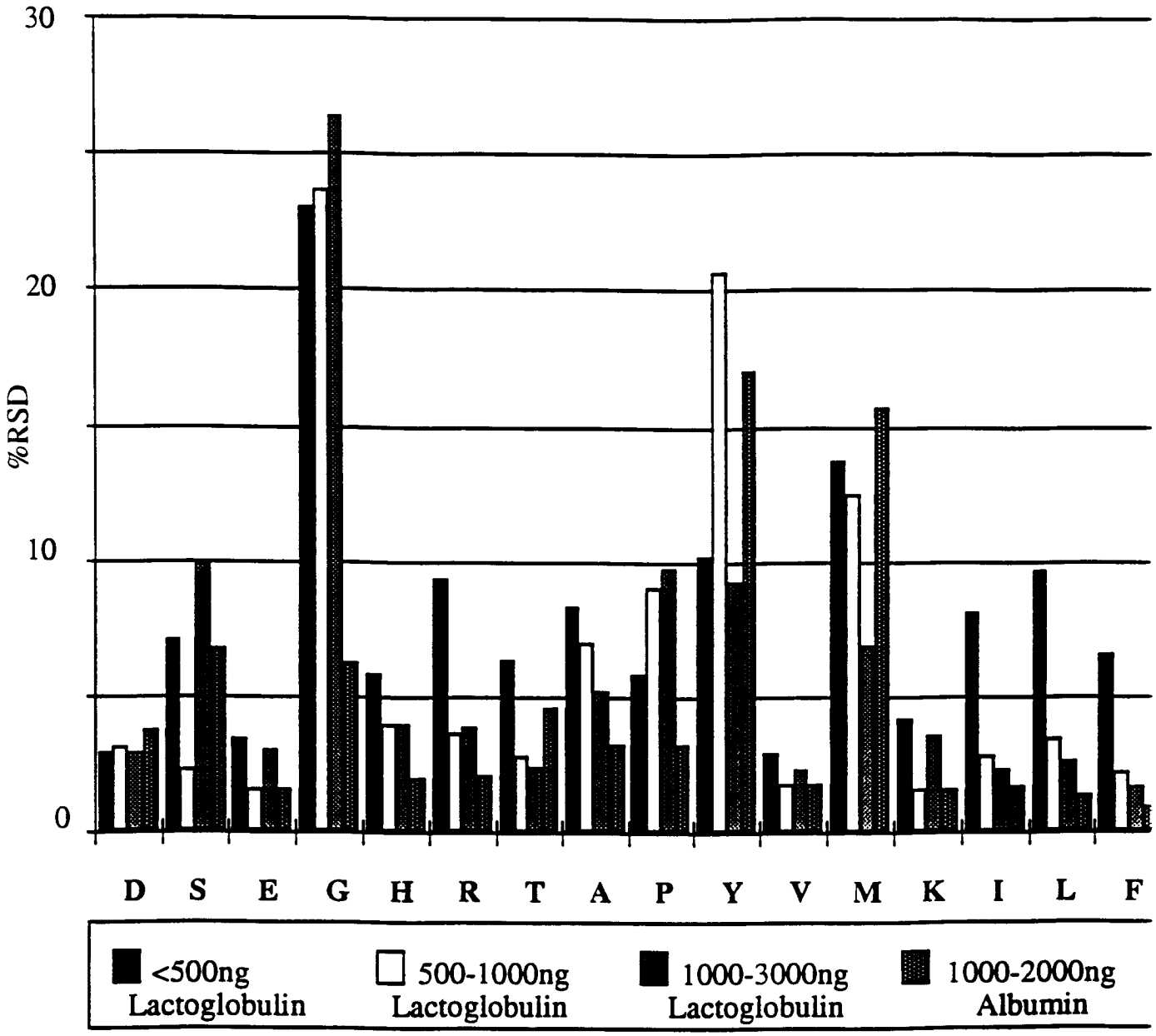


Eluent A : 140mm NaOAc,17mm TEA,pH = 5.04
Eluent B : 60% Acetonitrile
Column Temperature = 37°

Optimized chromatographic conditions provide for near- or baseline components. Fluorescence detection gives extremely quiet baselines and in conjunction with high quantum yields permits 50-350 femtomole detection limits (see Table I).

Figure 4

Reproducibility of Compositional Data



The data set described in the methods section was used to generate reproducibility data for compositional analysis. Each analysis was treated separately according to Reference 1. Virtually all values are less than 10% and most analyses with 500ng have %RSD less than 5% except for the hydrolytically unstable Tyr and Met, and the Gly values for lactoglobulin which are easily distorted by even small levels of contamination.