

## A COLLABORATIVE AMINO ACID ANALYSIS STUDY FROM THE ASSOCIATION OF BIOMOLECULAR RESOURCE FACILITIES

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### I. INTRODUCTION

Amino acid analysis remains one of the more complex and difficult analytical methods in protein chemistry (1). In a continuing effort to enhance the performance of amino acid analysis technology in core facilities, the Association of Biomolecular Resource Facilities (ABRF) sponsored a 1989 amino acid analysis study. The ABRF amino acid analysis subcommittee, composed of the present authors, designed the study, prepared the samples and analyzed the results. The purpose of the study was to appraise the following three parameters relative to the amount of sample hydrolyzed and/or analyzed: the accuracy and precision of amino acid analysis method used in core facilities; the hydrolysis conditions used

in core facilities; and the ability of core facilities to quantify cysteine. To address these parameters with a minimum of analyses, the study was limited to two unidentified test samples, one hydrolyzed and the other not hydrolyzed. The prehydrolyzed sample provided each core facility with the same mixture of free amino acids generated from a protein rather than a standard calibration mixture. A protein was selected rather than a peptide (2) sample.  $\beta$ -lactoglobulin A chain was chosen because it is a nontoxic, readily available protein that contains cysteine. The preliminary results of the 1989 ABRF amino acid analysis study are presented here; the protein sequencing performance study is presented elsewhere in this volume (see Speicher *et al.*).

## II. MATERIALS AND METHODS

### A. Samples

Approximately 40  $\mu$ g of HPLC purified  $\beta$ -lactoglobulin A chain was provided as sample ABRF-89AAA-1 (AAA-1). Participants were requested to hydrolyze and analyze in triplicate two different amounts of AAA-1, either 5  $\mu$ g and 0.5  $\mu$ g or 0.5  $\mu$ g and 0.2  $\mu$ g depending upon the sensitivity of their instrumentation. In addition, participants were requested to determine the cysteine content of AAA-1 using a method of their choice.

Approximately 20  $\mu$ g of a hydrolysate of pyridylethyl  $\beta$ -lactoglobulin A chain was provided as sample ABRF-89 AAA-2 (AAA-2). Participants were requested to analyze in triplicate two different amounts of the prehydrolyzed AAA-2 sample, either 4.0  $\mu$ g and 0.4  $\mu$ g or 0.4  $\mu$ g and 0.1  $\mu$ g and to quantify pyridylethyl cysteine. A standard (Sigma) of pyridylethyl cysteine (PEC) was also supplied.

### B. Sample Preparation and Distribution

The AAA-1 and AAA-2 samples were prepared as follows.  $\beta$ -lactoglobulin A chain (Sigma) was pyridylethylated in the presence of 6 M guanidine hydrochloride (3) and both the modified and unmodified proteins purified by RP-HPLC using aqueous trifluoroacetic acid/acetonitrile solvents at the W.Alton Jones Cell Science Center, Lake Placid, NY. Sample AAA-1 was quantified and packaged for shipment at the University of California, Davis. Sample AAA-2 was quantified, hydrolyzed and packaged for shipment at Sterling Drug, Great Valley, PA. All AAA-2 samples were vapor phase hydrolyzed together in 6 x 50 mm borosilicate tubes within an evacuated dessicator using 6N HCl containing 1% phenol at 110°C for 24 h. Samples AAA-1, AAA-2, standard PEC and data sheets were distributed to participating core facilities by the Wisconsin Survey Research Laboratory, Madison, WI. Participants returned data to the Wisconsin Survey Research

Laboratory, identifying marks were removed and anonymous results in molar amounts were forwarded to the subcommittee for data reduction and interpretation.

### C. Calculations

Compositions in residues per molecule were calculated based upon the amount of protein analyzed. The amount of protein analyzed was determined by (i) dividing the amount of each individual residue by the known residue value, (ii) calculating a mean amount analyzed, (iii) discarding all individual values that deviated more than 15% from the mean, and (iv) recalculating the mean amount analyzed from the remaining relevant residues. The composition was calculated by dividing the amount of each amino acid by the relevant mean amount of protein.

Compositional accuracy was calculated as average % Error for all amino acids measured excluding Trp and Cys. All other values were included and not corrected for partial destruction, incomplete hydrolysis or contamination. Percent Error per amino acid was calculated after rounding the measured value to the nearest integer.

$$\% \text{ Error} = 100 \times \frac{|\text{known value} - \text{measured integer value}|}{\text{known value}}$$

$$\text{Average \% Error} = \frac{\% \text{ Error for 16 amino acids}}{16}$$

Precision of residue values obtained from the multiple analyses was measured as percent standard deviation (%SD). Compositional precision was calculated by averaging the % SD values for all the amino acids measured, excluding Trp and Cys.

### D. Data Reduction

The University of Washington Biostatistics Service Group adapted the Statistical Package for Social Sciences (SPSS) software to perform the designated calculations for composition and precision, entered all raw data and made these calculations by computer. Final data manipulation for compositional accuracy and summary data were performed on a desktop computer by L.E. using Microsoft Multiplan.

## E. Survey

Participants in the study were also requested to answer a brief survey concerning amino acid analysis methods and applications. Responses to the survey questions were compiled by D.A.

## III RESULTS AND DISCUSSION

### A. Instrumentation and Methods

A total of 36 core facilities responded with amino acid analysis data generated from 43 instruments during June through August, 1989. A summary of the instrumentation and methods used by the respondents is presented in Table I. Essentially half the participants utilized postcolumn derivatization and the other half precolumn derivatization methods. The majority of results in the study were produced with either PTC (20 responses) or ninhydrin (19 responses) based amino acid analysis systems.

Table I  
Summary of Instrumentation and Methods  
1989ABRF Amino Acid Analysis Study

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#### Instrumentation

15	Beckman Models 6300/7300
10	Waters HPLC/PICO TAG
7	Applied Biosystems Model 420/130
3	Beckman HPLC
3	Beckman Model 119/121
1	Dionex Model D500
1	Hewlett Packard Model 1090
1	St Johns Associates HPLC
1	Varian HPLC
1	Homemade HPLC

#### Methods

20	PTC, Phenyl thiocarbonyl
19	Ninhydrin
2	OPA, o-Phthaldialdehyde
1	Fluorim, Fluorescamine
1	FMOC, 9-Fluoromethylchloroformate
22	Total Postcolumn derivatization
21	Total Precolumn derivatization

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## B. Accuracy and Precision

The average amino acid analysis results obtained from 228 analyses of sample AAA-1 and 187 analyses of sample AAA-2 are presented in Tables II and III, respectively. The compositional data is tabulated based upon the approximate amount of protein hydrolyzed for AAA-1 and analyzed for AAA-2. Hydrolysis/analysis amounts in this report are grouped according to the requested levels and the participant's quantity identification labels. Several laboratories provided data from three load levels. The results are further categorized according to methodology, allowing comparison of the major chemistries, namely PTC and ninhydrin. The time constraints for this report prohibited compilation of recovery data, evaluation of all methodologies, identification of exemplary performance and other statistical considerations. Furthermore, the accuracy and precision data has not yet been separated within a laboratory from a comparison of those between laboratories. This will be done in a more comprehensive presentation elsewhere.

Similar performance of ninhydrin and PTC methods was demonstrated with intermediate and greater amounts of sample. For example, amino acid compositions of AAA-1 (Table II) at the high level from ninhydrin analyzers exhibited about 9% average error compared with 11% average error from PTC analyzers. In the intermediate range ninhydrin data showed about 17% error versus about 16% average error in the PTC data. Interestingly, at the lower range of protein hydrolyzed, the accuracy of the PTC results remained at about 16% average error. Compositions of the prehydrolyzed AAA-2 sample (Table III) determined by ninhydrin and PTC analyzers also exhibited similar levels of accuracy. Surprisingly, slightly greater error was found in the data from prehydrolyzed AAA-2 compared to AAA-1. This could be due to the hydrolysis work up procedure and/or to extended storage and shipping. It should be emphasized that the AAA-2 data was compared to the known  $\beta$ -lactoglobulin composition and a different accuracy value would be obtained if the data were compared to the actual composition of the mixture.

Although greater in error, the precision of the AAA-2 results (Table III) were superior to that of the AAA-1 results (Table II) within each load level regardless of the methodology. The greater variability (%SD) in AAA-1 results generated from individual hydrolysis (Table II) emphasizes the importance of the hydrolysis step. All participants used 6N HCl for hydrolysis. About 44% of the respondents performed a vapor phase hydrolysis at 105-115°C for 18-24h and 13% performed a vapor phase hydrolysis at 150-165°C for 0.75-1.5h. About 44% of the respondents performed a liquid phase hydrolysis at 105-115°C for 18-24 h. Most laboratories (72%) included phenol (0.05%-3.0%) in the HCl as a halogen scavenger. Five laboratories included  $\beta$ -mercaptoethanol (0.01%-0.05%) and one laboratory used 4% thioglycolic as reducing agents in the HCl.

Table II

## Average ABRF1989AAA1 Amino Acid Analysis Results

Amino Acid	Known Composition	Amount Hydrolyzed	All Responses			Ninhydrin		PTC		
			Low	Intermediate	High	Intermediate	High	Low	Intermediate	High
Ala	14		13.2	13.8	14.8	13.6	14.8	13.5	14.1	14.6
Arg	3		3.7	4.4	3.4	4.6	3.4	4.2	4.4	3.6
Asx	16		14.8	14.9	15.3	15.2	15.7	14.9	15.0	13.8
Cys	5									
Glx	25		23.2	23.4	23.2	23.8	23.8	23.2	23.6	21.5
Gly	3		6.0	5.2	4.1	5.7	4.1	5.6	5.1	4.3
His	2		2.0	2.1	2.2	2.1	2.2	1.9	1.9	2.0
Ile	10		7.9	8.1	8.4	8.4	8.7	8.2	8.1	8.2
Leu	22		19.5	19.6	21.2	20.6	21.5	19.9	19.4	21.6
Lys	15		14.6	13.8	14.7	13.8	14.1	15.4	13.7	14.7
Met	4		2.8	2.8	3.3	2.8	3.2	2.7	2.7	3.4
Phe	4		4.1	4.0	4.1	3.8	4.1	3.8	4.0	4.1
Pro	8		8.0	8.6	8.5	7.4	8.2	8.0	9.3	9.0
Ser	7		7.8	7.1	6.8	6.9	6.7	7.6	7.4	7.0
Thr	8		7.8	7.89	7.8	7.6	7.8	7.9	8.2	7.8
Tyr	4		4.0	3.8	3.5	3.6	3.6	3.7	3.6	3.3
Val	10		8.9	8.8	9.6	8.9	10.0	9.1	8.9	9.0
	sites		16	31	24	10	15	14	19	7
	n		57	103	68	31	45	42	59	18
	Average %SD		29.4	28.5	21.0	26.4	20.0	27.2	25.7	18.0
	Average% Error		15	15	9	17	9	16	16	11

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Average residue per molecule from n number of analyses. Methods and calculations are as described in the text. Amounts hydrolyzed are approximate; Low refers to  $-0.2 \mu\text{g}$ , Intermediate to  $-0.5 \mu\text{g}$  and High to  $-5 \mu\text{g}$ . Results for Cys are presented in Table IV

Table III

Average ABRF1989 AAA2 Amino Acid Analysis Results  
Prehydrolyzed Sample

Amino Acid	Known Composition	Amount Analyzed	Al 1 Responses			Ninhydrin		PTC		
			Low	Intermediate	High	Intermediate	High	Low	Intermediate	High
Ala	14		16.6	16.9	17.5	17.4	17.6	16.3	16.9	16.6
Arg	3		3.5	3.1	2.5	2.5	2.4	4.0	3.3	2.7
Asx	16		20.8	17.8	19.1	18.6	18.9	22.0	17.9	19.2
Cys	5		5.6	5.7	5.5	4.6	5.4	5.8	5.9	5.7
Glx	25		27.5	26.3	26.9	27.1	26.8	27.7	26.6	26.7
Gly	3		4.2	4.6	4.1	5.2	4.2	4.3	4.3	4.3
His	2		1.5	1.8	2.0	1.8	1.7	1.3	1.5	1.7
Ile	10		6.8	7.1	7.2	7.0	7.2	6.7	7.4	7.7
Leu	22		19.9	21.0	22.1	22.1	22.5	18.9	21.1	21.6
Lys	15		12.0	13.5	13.1	14.4	14.6	11.8	13.3	9.3
Met	4		3.1	3.7	4.3	3.7	4.1	3.0	3.7	4.2
Phe	4		3.9	3.9	3.8	3.6	3.7	3.4	4.0	4.1
Pro	8		11.2	10.1	10.1	9.7	10.3	11.5	10.3	9.4
Ser	7		7.9	7.4	7.3	7.6	7.3	8.1	7.4	7.2
Thr	8		7.9	7.4	7.2	7.4	7.2	8.4	7.5	7.2
Tyr	4		3.4	3.2	3.0	3.1	3.0	3.0	3.2	3.2
Val	10		7.3	7.6	8.2	7.9	8.1	7.0	7.6	8.4
	sites		15	29	21	9	14	12	17	5
	n		37	94	56	27	37	24	57	14
	Average %SD		25.7	21.3	16	14.9	11.4	26.4	15.9	10.2
	Average % Error		2.0	1.5	1.5	1.8	1.6	2.5	1.6	1.6

Average residues per molecule from n number of analyses. Methods and calculations are as described in the text. Amounts analyzed are approximate; Low refers to  $\sim 0.1 \mu\text{g}$ , Intermediate to  $\sim 0.4 \mu\text{g}$  and High to  $\sim 4 \mu\text{g}$ .

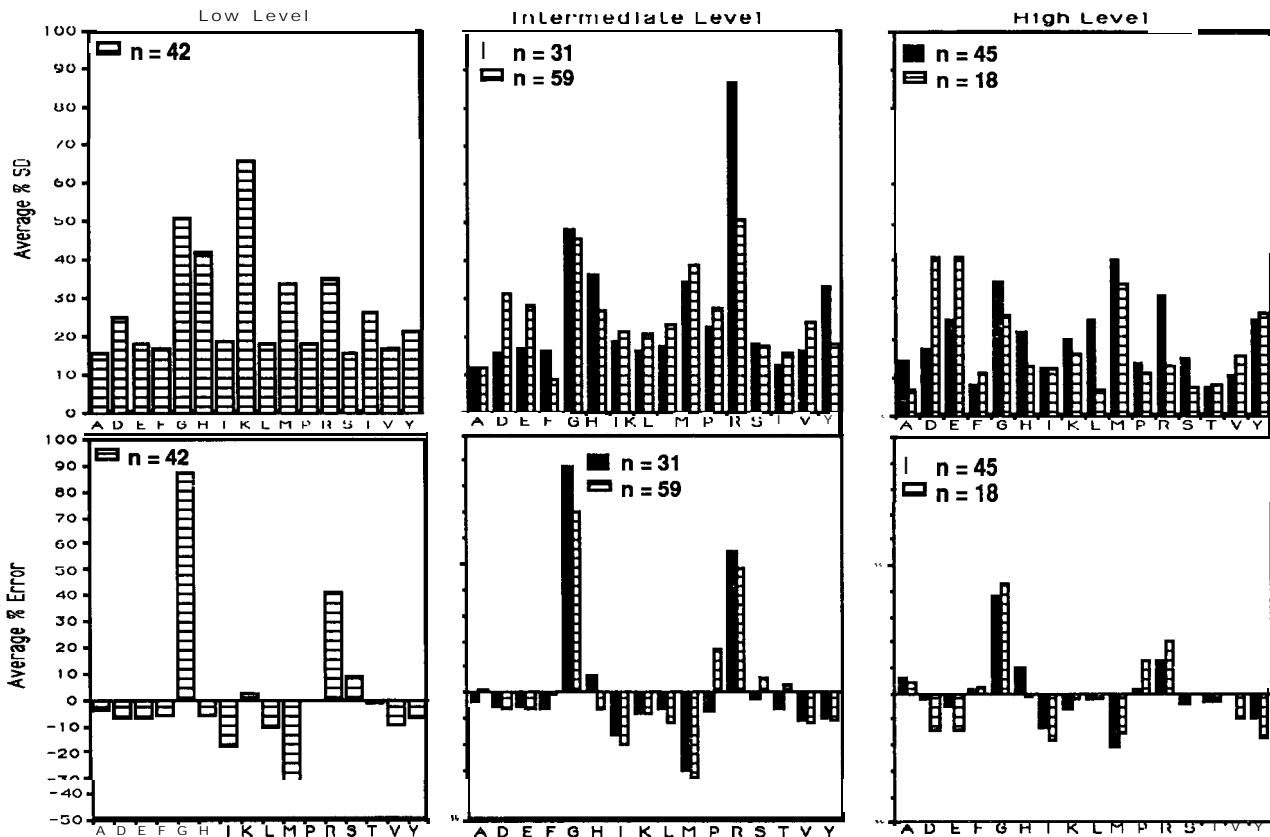


Figure 1. Average Error and precision of each amino acid from analysis of AAA1. |, ninhydrin; ▨, PTC; % SD, percent standard deviation. Amounts analyzed are approximate; Low level refers to  $\sim 0.2 \mu\text{g}$ , Intermediate to  $\sim 0.5 \mu\text{g}$  and High to  $\sim 5 \mu\text{g}$ .

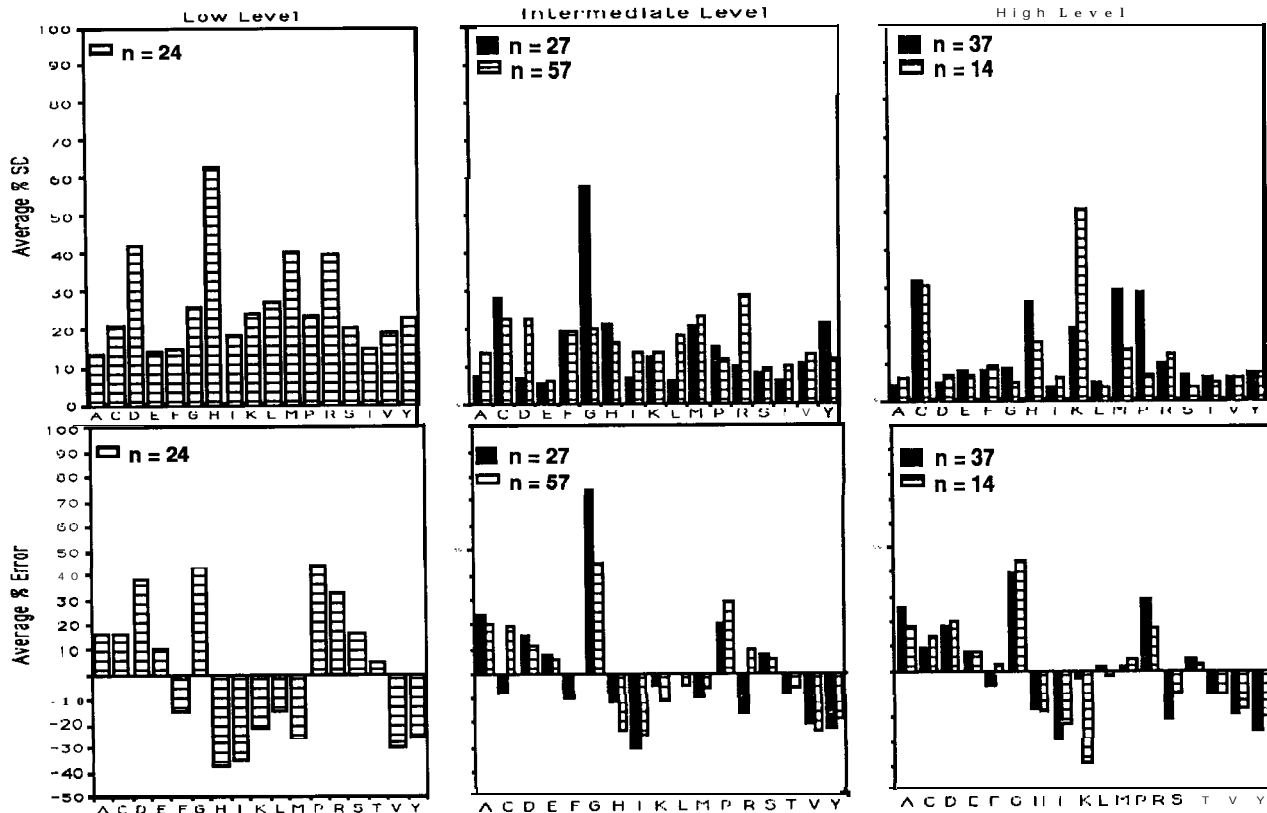


Figure 2. Average Error and precision of each amino acid from analysis of AAA2. |, ninhydrin; ▨, PTC; % SD, percent standard deviation. Amounts analyzed are approximate; Low level refers to  $\sim 0.1 \mu\text{g}$ , Intermediate to  $\sim 0.4 \mu\text{g}$  and High to  $\sim 4 \mu\text{g}$ .

The average error and precision found per amino acid in samples AAA-1 and AAA-2 are compared at three levels in Figures 1 and 2, respectively. Overall, Gly, Met and Arg contributed significantly to the error in both the PTC and ninhydrin data. For Gly, error is due in part to contamination; oxidation contributes to the error associated with Met. For Arg, inflated values in PTC analyses can result from chromatographic co-elution with both methionine sulfoxide and dehydroalanine. With regard to error in AAA-2, Pro also was a problem for ninhydrin analyses and Lys a problem for PTC analyses at the high levels. At the low level, the majority of the residues in AAA-2 exhibited 20% error. The more variable residues with regard to %SD in AAA-1 and AAA-2 included Gly, Met, Arg and His in both the ninhydrin and PTC data. The low abundance of Gly and His in  $\beta$ -lactoglobulin (3 and 2 residues, respectively) also contributes to the predominant error and variability values associated with these residues. Tyr and Pro also vary considerably in the ninhydrin data and Asp, Glu and Lys in the PTC results.

### C. Cysteine Quantification

Average results from cysteine analysis are presented in Table IV. Pyridylethyl cysteine was quantified in the prehydrolyzed AAA-2 sample by 20 laboratories which returned a total of 29 ninhydrin, 2 OPA and 78 PTC analyses. Ninhydrin analyzers using the lithium acetate buffer system were able to resolve PEC and, relative to the overall data, obtained very accurate results (8-9% average error) at the intermediate and high levels. However, for ninhydrin systems using a sodium acetate buffer in the chromatography, PEC usually co-eluted with ammonia or Lys. PTC amino acid analysis systems reported PEC values for AAA-2 exhibiting on the average about 16% error at the low level. Cysteine analysis of sample AAA-1 was performed by 16 core facilities reporting 44 analyses. Two laboratories quantified carboxymethyl cysteine following alkylation with iodoacetic acid, thirteen laboratories quantified cysteic acid following performic acid oxidation and one laboratory reported a combination of these two methods. Overall, the cysteic acid analysis of AAA-1 exhibited about 17% average error from 39 analyses. The error associated with the CMC data was higher but the total number of analysis was much fewer ( $n = 5$ ).

### D. Survey

A total of 34 laboratories responded to the amino acid analysis survey questionnaire. Essentially all core facilities that offer amino acid analysis as a routine service (85% of the responses) utilize the methodology for several applications within their facility. Overall, 90% of the responses indicated a need for 90% or greater accuracy (i.e., 10% error or less) in amino acid analysis applications and 36% of the responses

Table IV  
Average Results from Cysteine Analysis

Sample/Method	Al <sub>1</sub> Responses			Ninhydrin		PTC		
	Low	Intermediate	High	Intermediate	High	Low	Intermediate	High
<u>1989 ABRFAAA1</u>								
Cysteic Acid								
n	9	15	15		13	9	15	2
%SD	27.5	18.0	29.4		33.3	27.5	18.0	3.7
% Error	17	17	15		14	17	17	24
Carboxymethylcysteine								
n		3	2	3				
% SD		3.0	11.4	3.0				
%Error		21	26	21				
<u>1989 ABRF AAA2</u>								
Pyridylethylcysteine								
n	27	62	30	11	18	21	45	12
%SD	25.2	26.9	30.7	28.4	32.0	21.1	22.7	30.1
%Error	12	14	11	8	9	16	19	14

indicated a need for 95% or greater accuracy. The most prevalent applications of amino acid analysis included compositional analysis of unknown peptides and proteins (94%), confirmation of the identity of known sample (85%) and quantification for sequence analysis (78%). About 62% of responding facilities use an internal standard in amino acid analysis. Eighty-six percent of the facilities using any internal standard use norleucine. Amino acid analysis data is reported in several formats; however, the most common practice (82% of the responses) is to report molar amounts (e.g. picomoles or nanomoles) followed by residues per molecule (50% of the responses) and mole percent (35% of the responses). Eighty-eight percent of the responses indicated that the ABRF amino acid analysis test samples were useful for their facilities.

#### IV. SUMMARY AND CONCLUSIONS

A significant segment of the Association of Biomolecular Resource Facilities participated in the 1989 amino acid analysis study. Approximately 110 core facilities received the AAA samples and 36 laboratories returned data. In contrast, several collaborative amino acid analysis trials by others have been composed of 11 laboratories or less (1). The majority of the laboratories in the present study employed either postcolumn, ninhydrin based amino acid analysis systems (47%) or precolumn PTC based instrumentation (44%). Overall results demonstrate similar performance of the ninhydrin and PTC instrumentation above the low load level. Accuracy was better with larger amounts of the AAA-1 sample; average errors were near 10% at the high range and about 15-17% at the intermediate range. Precision within these load levels was also similar for both methods. Notably the average data allow identification of  $\beta$ -lactoglobulin but do not discriminate between the A and B chains which differ by 4 residues (i.e., Ala, Gly, Asx and Val). The results from prehydrolyzed AAA-2 exhibited less variability but more error than the AAA-1 data, and illustrate the contribution of the hydrolysis procedure. PTC methodology was employed by 80% or more of the laboratories that analyzed AAA-1 at the low level; average error was about 15% and variability was about 29% SD at both low and intermediate levels.

Fifty-six percent of the responding facilities quantified pyridylethyl cysteine in AAA-2 with about 13% average error. Less than half (44%) of the responding laboratories measured cysteine in unmodified sample AAA-1. Of the laboratories that analyzed cysteine in AAA-1, about 80% carried out performic acid oxidation and quantified cysteic acid with about 17% average error.

Amino acid analysis is a quantitative yet statistical type of measurement and multiple analyses are usually necessary to obtain reliable data. However, experimental samples are often not available in amounts sufficient for multiple analyses and background contributions commonly

skew high sensitivity results. The present results provide a useful perspective of current amino acid analysis technology using ninhydrin and PTC methods. Results from the 1988 ABRF amino acid analysis test peptide (2) are consistent with these performance levels. In order to attain the high quality performance sought by the majority of the survey respondents (i.e. less than 10% error), better approaches and methodologies for hydrolysis and amino acid analysis must be found that can routinely deliver the accuracy that is required.

## V. ACKNOWLEDGEMENTS

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