

# Collaborative Trial Analyses of ABRF-91AAA

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## I. Introduction

Collaborative trials for assessment of the reliability of amino acid analyses have been done since the early fifties. The first 30 years of trials have been summarized and discussed by Williams (1), and more recently the ABRF series of trials (2-5) have provided information on various aspects of amino acid analyses. As has been noted previously (1), amino acid analysis is not as simple and routine as the wide use of the technique may suggest. Consequently, a large measure of self-evaluation by facilities providing this service is very necessary, and continuing education in the techniques and alternatives remains essential. The objective of the ABRF, in this instance, is then to provide a mechanism for self-evaluation and improvement of amino acid analytical capabilities of core facilities, by coordinated analyses of standard samples.

The ABRF studies to date have examined the recovery, accuracy and precision of amino acid analysis using either synthetic peptides, purified proteins, or a protein hydrolysate. These studies have in part concentrated on the sensitivity of analysis, with hydrolyses and analyses being performed in the 0.5-5 µg range. Laboratories have also been asked to follow well defined protocols to simplify data reduction and subsequent comparisons. The accuracy of analyses has generally been in the 80-90 % range. The present study was an attempt at investigating the quality of analyses

under conditions where laboratories might feel more comfortable, and where ample supply of sample was available. Three general aspects were explored. The first two were a realistic assessment of the accuracy of absolute quantitation and of the accuracy of relative yields of residues obtainable at present. The third aspect was the analysis of the problem amino acids tryptophan and cystine/cysteine where we were aiming at discovering which methods are used, and how well they performed.

## II. Methods and Materials

### A. Sample Preparation

Samples were generously prepared by Dr. Sam Margolis, National Institute of Standards and Technology, Gaithersburg, Maryland. Glassware and ampules were cleaned in a pyrolyzing oven to minimize contamination. Bovine serum albumin (7% solution, Standard reference Material 927a) was diluted by absorbance to a concentration of 0.75 mg per ml and dispensed into cleaned ampules using a Micromedic dispensing pump (1 ml) set at 20% volume. The samples were then lyophilized and sealed under an atmosphere of air. The samples were sent by regular mail to ABRF facility directors. The samples were distributed without identification of the protein as BSA. A questionnaire asked for a general strategy of analysis, a single answer of how many nmoles of each amino acid residue was present in the vial (a ballpark figure of 0.1 mg was given as guide) and a number of questions relating to the methodology and calibration.

The results were received by the committee after the identifying cover sheets were removed by an independent collaborator, Dr. B. Holmquist, to insure anonymity.

### B. Data Reduction

The data was coded into personal computer-based spreadsheets, and analyzed as follows. The amount of protein predicted by each reported residue was found by dividing by the known number per molecule for that residue. Individual amounts were compared with the average value for a given analysis and any that deviated by more than 15% were discarded; a mean amount was calculated from the remaining "relevant residues". Residues/mol were then determined by dividing the nanomoles of amino acid by this mean. Recoveries were calculated from the mean and the molecular weight of BSA and compared to the expected 0.15 mg to obtain a relative recovery (accuracy of quantitation).

The accuracy of each residue compared to the true value is expressed here as %Error. The overall accuracy of the composition is expressed as the Average %Error. Cystine/cystein and tryptophan errors were not included in the average percent error calculation.

Equations:

$$\%Error = 100 \times (\text{experimental} - \text{true residue value}) / \text{true}$$

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



Figure 1 summarizes the yields that were attained by all the sites, relative to the 150 µg theoretical value. Although some sites achieved close to the theoretical yield, most did not. Discarding 18 sites as outliers (differing more than 2 standard deviations from the mean), the remaining 40 sites had an average yield of 91. ± 8.1%. This could signify an error in the “theoretical” yield, which is based among others on optical density measurements and amino acid analyses, or a pervasive bias. Such a bias could be due to pipetting errors (plastic-tipped dispensers), derivatizing inefficiencies or manipulative losses or to incorrect calibration, due perhaps even to incorrect standards. This study cannot address this question. The use of an internal standard may alleviate some of these potential problems. Sixteen sites used an internal standard Nle (13 sites) being the predominant choice, with 2 sites choosing a-aminobutyric acid and one hydroxyproline. Since only one of these analyses differed by more than two standard deviations from the mean, the overall result was demonstrably better than without internal standards. Conversely, many of the sites not using internal standards achieved good quantitation. The outliers in the latter group were however much more egregious. Although these data say that accuracy in absolute quantitation may not be good, the precision at 6% is better than in past collaborative trials.

The average of the absolute percent error of each residue in each sample, in comparison to the value obtained by sequence, is summarized in Figure 2. As in previous studies, the range extends from very good, 2. % for site 41, to very bad, over 25% for site 10. The average for all sites of 9.47% error is similar to that observed for high level hydrolyses (9% error) in the 1989 study (3) and better than the 13.45% error in the 1990 study (5). For the 21 sites with >10% average errors, the problem can be attributed in many cases to one or two bad residues, with

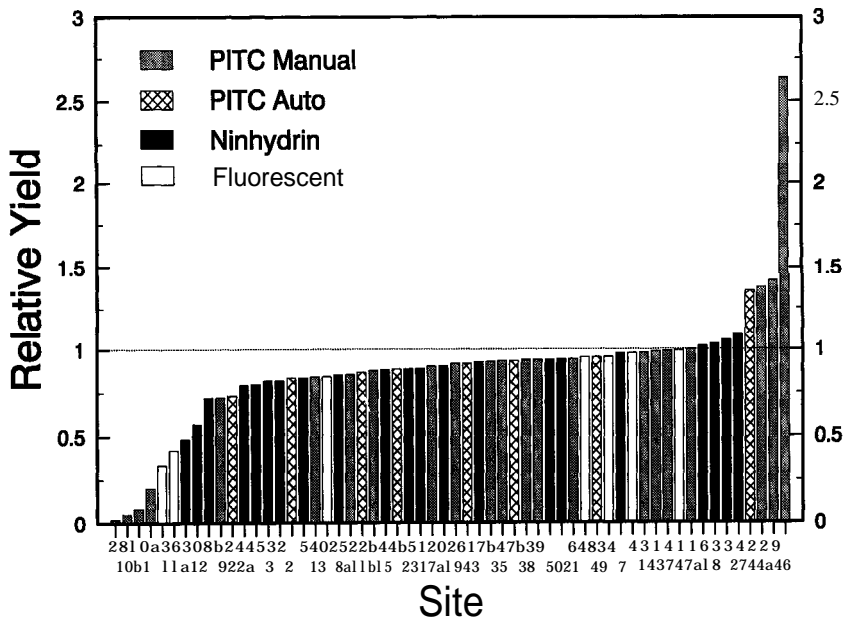
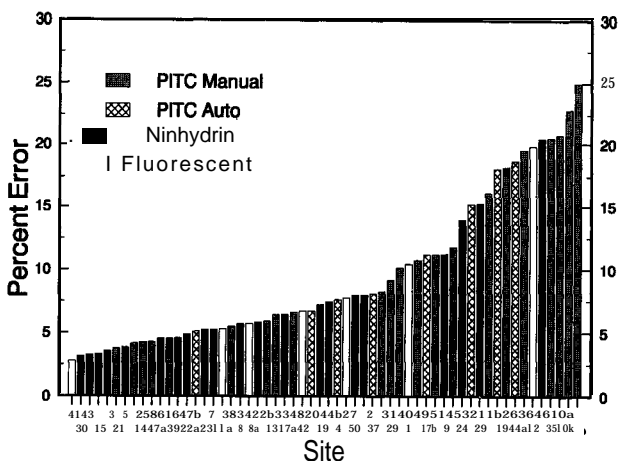


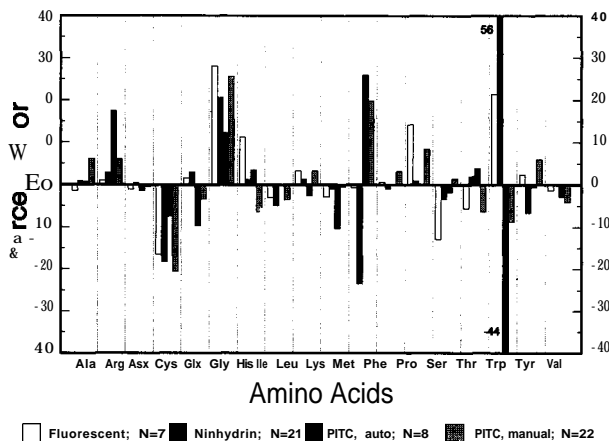
Figure 1. Yields of analyses, relative to the 150 µg dispensed to the sites.



**Figure 2.** The average of the absolute percent error of each residue in each sample. These values were calculated relative to the sequence values.

methionine and glycine being the most common. By deleting the worst residue in the average % error calculation, the average % error of 12 of these sites decreases to 10% or less. Among the methods, the classical ninhydrin methodology generally afforded better data than the pre-column PITC methods, although all methods had individual analyses in the low % error range. This perhaps reflects the larger experience base of the classical methodology.

The relative errors for each residue are summarized in Figure 3. Note that no samples were removed from the set. The general trend is that tryptophan, cystine, and methionine remain problematic in many laboratories. A discussion of the various problems highlighted by these comparisons is as follows.



**Figure 3.** Relative errors for each amino acid residue. No samples were removed from the set.

**Tryptophan** Most laboratories do not analyze for tryptophan, probably due to these difficulties. Trp is the least abundant residue in BSA at 2 residues per 582, which of course compounded the problems experienced. The raw values and methodology for tryptophan estimation are summarized in Table II. The methanesulfonic acid based method of hydrolysis accounted for half of the reported Trp analyses (8 sites), with only 3 of them within 20% of the sequence value. The excessively high value (162% or 220% error) found e.g. by one of the PITC and one of the OPA based analyses, is probably due to a chromatographic artifact, while difficulties in integrating small amounts of Trp in addition to hydrolytic losses probably underlie the low values found in other analyses. Three sites reported a total lack of tryptophan. No method can be statistically selected as superior, although the thioglycolic acid method (2 successful analyses out of two) may need some attention.

**Table II. Analyses of Tryptophan in ABRF-9IAAA**

Hydrolysis Medium	Analysis Method	Percent Error of Individual Sites		
Methane Sulfonic Acid	Ninhydrin	<b>-100.0</b>	-35.2	
	ptc	-10.7	-69.8	6.7
	fluor	<b>162.2</b>	<b>-100.0</b>	<b>3.6</b>
Thioglycolic acid		<b>-3.1</b>	<b>-13.1</b>	
Dodecane thiol		9.3	<b>-100.0</b>	
HCl	As dihydro tryptophan	<b>41.8</b>		
Mercaptoethane sulfonic acid		72.8	26.0	
3%phenol		<b>220.3</b>		

**Cvstine/Cysteine** was determined by 44/58 sites and raw error values are summarized in Table III. Oxidative preparation of samples remains the most popular, 23 samples being analyzed after such treatment. Direct analyses of HCl hydrolysates were done in 12 cases, and 4 sites used induced disulfide exchange during hydrolysis to form tractable derivatives. A further 4 sites reduced and alkylated the protein and then did a "normal" analysis. Disulfide-exchange and prior alkylation were the most successful methods.

If 25% error is tolerated, three quarters of the samples remain. The "direct" analyses fall into two clear groups, those with more than 45% losses and those with less than 18% error, perhaps pointing to the general utility, but also to considerable uncontrolled problems. The oxidative methods, presumably popular due to their historical use, average 18% low, with a range from +6.8 to -63.1%. Some of the

higher values were obtained by use of standard proteins (sites 25 and 39) to correct for both chemical and hydrolytic losses, but site 31 still had only a 50% yield.

**Methionine:** Large variations in methionine quantitation were found. The biggest factor is most probably the low level of methionine in BSA - only 4 residues. Figure 3 shows methionine to be analyzed in the most biased fashion by the two major methods. Ninhydrin, when in error, consistently underestimates Met, with values as low as a total lack; no site overestimated it. This could imply either a consistent hydrolysis problem (oxidation) or an integration problem on a small peak. The PITC methodology frequently overestimates Met, being up to 3 fold high in 2 cases. Low values were also seen, which is consistent with a hydrolysis problem as seen with ninhydrin. The high values are most probably due to erroneous identification of a common "reagent" peak. Overall, the error rate in Met correlates well with the average error rate of all residues, with only 2 of the best 28 and 7 of the remaining 30 analyses having a higher than 20% error in Met. There was no correlation with the additive used for hydrolysis.

**Table III. Analyses of Cystine/Cysteine in ABRF-91AAA**

Derivative	Analysis Method	N	average	std dev	range
performic acid	ninhydrin	8	-16.8	14.7	-36.5 - 4.2
	ptc	6	-14.6	17.0	-49.1 - 6.6
	fluor	2	-35.4	6.7	-42.1 - -28.7
alkylated		4	5.8	14.9	-7.4 - 29.4
None	ninhydrin	3	-40.5	40.6	-90.3 - 13.8
	ptc manual	7	-36.3	29.3	-81.1 - -3.6
	ptcauto	2	-17.4	2.1	-19.5 - -15.3
hcl/dithiopropionic acid		3	4.5	10.2	-3.7 - 18.9
dms0/HCl		6	-18.8	25.1	-63.1 - 6.8
dithiodigly col		1	-0.5	-	
' abdf titration		1	-4.3	-	
periodate		1	-12	-	
<b>Total</b>		<b>44</b>			
<b>Summary</b>					
Oxidized		23	-18.2	18.7	-63.1 - 6.8
Alkylated		4	5.8	14.9	-7.4 - 29.4
Disulfide exchange		4	3.2	9.1	-3.7 - 18.9
Direct		12	-34.2	31.9	-90.3 - 13.8

**Aspartic acid:** The worst problems in Asp analyses were seen with the PITC methodology, where 3/8 automated analyses had negative errors larger than -10%, and 5/22 of the manual sites had similar errors. Two of these sites had large positive errors. These negative errors are probably due to derivatization or solubilization problems caused by leachates from the glassware used for hydrolysis. This may sometimes be alleviated by appropriate chemical procedures (7).

**Glutamic acid:** In the past it has been suggested that Glu values may be overestimated due to erroneous calibration with standards in which partial formation of pyroglutamic acid has taken place. More Glu values are low (or high) than Asp values are, but in the majority of cases they parallel errors in Asp. A few very large errors cause the large deviations seen in Figure 3. The slightly positive average error seen for ninhydrin may reflect the expected calibration errors but are inconsequential in the context of the generally high errors seen in this study as a whole.

**Histidine:** The fluorescent methods overestimate His, while the manual PITC methods underestimate it. Thus, 4 of the 7 fluorescent sites overestimated His by at least 2 residues out of 17. Contrariwise 6 of the 22 manual PITC sites underestimated His by at least 2 residues, with 2 of those yielding only half the theoretical value. Calibration values were similarly low (see below, Section D). As noted before, this may be due to chemical problems in the derivatization, and/or chromatographic problems. The ninhydrin sites fared better with only one underestimating (and that at 40%) and two overestimating.

**Tyrosine:** Tyr have 20 tyrosyl residues (8), as opposed to the 19 residues we have assumed for these calculations (9). The marked negative bias in the ninhydrin-based analyses of Tyr may therefore be 50% larger than shown in Figure 3 and is typically associated with hydrolysis conditions. The magnitude of these errors precludes their being only due to integration errors of the usually broad Tyr peaks. The positive bias of the PITC-based analyses may similarly be decreased to a small positive value. Since hydrolysis conditions should be independent of analysis method, this positive bias in the PTC analyses is probably due to "reagent" peaks which elute in close proximity to Tyr in most chromatography systems.

**Lysine:** Manual PITC methodology occasionally lead to low yields of Lys (4/22 worse than -10% error), while the automated sites showed markedly negatively biases (4/8 worse than -10% error). This would suggest a pervasive problem which may be due to solubility properties of the PTC-amino acids or the quality of the PITC.

**Arginine:** of the 8 auto-PITC sites could analyze Arg with less than 10% error, mostly positively biased. A small number of the manual PITC sites likewise were positively biased. Such bias has been seen in unpublished studies to be due, among others, to contaminated methanol used during derivatization.

**Proline:** 2 of the 3 sites which did analysis of Pro by a fluorescent derivative had large positive errors, emphasizing that extra care is still needed for analysis of secondary amino acids. Ninhydrin sites had few problems with Pro, in contrast to

conventional wisdom. A strong correlation of overall error with more than 10% error in Pro, is seen with the PITC methods, where 7 of the 8 erroneous Pro values fall in the 12 analyses with the worst average percent error. Proline reacts very easily with PITC to form the most stable PTC-derivative, so that a bad proline value may be taken as a predictor of erroneous analyses in general. Standards with markedly variant relative color yields and especially high Pro color yields will most readily alert operators to this condition in a set of samples.

Methionine large errors were seen for glycine, probably due to adventitious contamination, some perhaps already in the original samples (about 5%). There are however some samples which are very much in error - 5 are higher than 50% in error, ranging to 200%.

To summarize the error-level of the whole set of analyses, they can be categorized in three sets; < 5% average error (n=14), 5-10% average error (n=23), and > 10% error (n = 21). The notable differences between the best sites and the average sites was in the ability to accurately quantitate methionine and histidine. The worst sites, on the other hand, had great difficulty in analyzing methionine and possibly had contamination problems as evidenced by high glycine values.

### *C. The Influence of Hydrolysis Conditions*

Hydrolysis conditions were varied, although nearly all of the 58 (effective) sites partaking in the study used 6N HCl as the digestion acid of choice. One site used 2: 1 HCl:trifluoroacetic acid, and one site used this in addition to 6 N HCl. Gas-phase versus liquid-phase hydrolyses are equally popular with 26 sites doing the former and 24 the latter. No difference is obvious in the quality of the analyses done by these two procedures. Interestingly, no participant used micro-wave assisted hydrolysis.

The early work in this field strongly suggested the use of a good vacuum during hydrolyses (e.g. 10), and many sites seem to still adhere to this. Thus 13 sites used the traditional <0.1 mm Hg pressure, while 15 sites used between 0.1 and 1 mm Hg. A further 9 sites used higher pressures, 12 did not report their vacuum conditions and one site used no vacuum. The 8 automated hydrolyses made use of vacuum as required by that instrument. No correlation was seen of quality of analysis and vacuum conditions.

The commonly used 110°C as hydrolysis temperature is still the most popular with 38 sites using temperatures ranging from 106° to 120°, and hydrolyses times from 18 hrs to 96 hrs. Temperatures of 147° to 170° were used by 12 sites for manual hydrolyses and by the 8 automated sites. In the latter cases hydrolyses times were from 30 min to 3 hrs. The higher temperatures lead to both excellent and very bad analyses, pointing perhaps again to the importance of operator experience, rather than specific techniques. The use of an inert gas to assist in displacing oxygen from hydrolysis tubes was seen at 41 sites; 29 used Nitrogen, 11 Argon and one Helium. No difference in analysis quality correlated with this.

Extrapolation of the analytical values for Ser/Thr and Ile/Val determined from multiple hydrolysis times, were no more successful in yielding "correct" values, than values taken from single time period hydrolyses.

The only hydrolysis conditions that seemed to correlate weakly with quality, was the use of additives, such as phenol. The use of mercaptans or disulfide-containing reagents coincided with none of the lowest ranking 22 analyses, while only four phenol-treated samples fell in the lowest 15. It is not clear whether the automated analyses used additives. Additives are not however absolutely necessary, as demonstrated by sites 7, 25, 38 and 47.

## D. Calibration

Accurate amino acid analyses require accurate calibration, an obvious fact that tends to be discounted. In the case of precolumn analysis with PITC, the accuracy of calibration can be judged from the extinction coefficients of the PTC-amino acids in the analytical solvent at the analytical wavelength. These constants are known (11).

**Table IV**

	CALIBRATION % ERROR					BSA % ERROR			
	Raw		Absolute			Raw		Absolute	
	Ave	Ave	H-non	>1-1	Berr	Ave	Ave	H-non	>1-1
Ala	0.4	3.6	1.5	1.0	-0.4	2.9	5.6	6.2	-4.3
Arg	5.4	6.7	0.3	4.9	3.7	3.0	7.3	5.2	-4.1
Asx	2.4	5.8	-3.5	-1.3	-1.8	-0.5	5.5	-0.6	2.3
Glx	-2.0	3.5	3.0	-0.0	1.8	-2.9	6.4	6.1	-2.4
GIY	-3.4	4.4	-4.3	-1.6	-1.4	13.3	14.2	-2.3	-6.0
His	-11.2	11.2	1.8	3.3	-6.6	-7.3	10.8	-3.7	-3.2
Ile	1.0	5.4	1.6	2.0	2.0	-4.8	7.9	3.6	-4.2
Leu	1.6	3.8	0.5	-0.7	-0.3	0.7	6.1	6.0	-8.0
Lys	-0.5	3.4	-3.0	-2.1	0.5	-0.4	7.5	2.0	-8.9
Met	-2.3	9.2	7.8	-0.4	1.6	7.1	16.5	8.5	-0.5
Phe	3.0	4.6	-2.1	0.3	-0.4	0.7	5.6	3.8	-7.2
Pro	-3.0	4.9	0.9	0.7	0.7	5.5	7.4	4.9	-3.8
Ser	-8.5	10.0	8.7	-3.4	3.5	-3.8	10.9	0.7	-7.1
<b>Thr</b>	7.4	9.2	-2.6	-1.1	4.6	-7.4	10.7	5.8	-4.3
Tyr	-1.1	7.2	4.8	-0.9	1.1	1.2	6.3	5.5	-4.8
Val	-1.3	4.1	1.5	-1.3	-2.5	-3.2	5.6	2.7	-0.8
av-WC	-0.8	6.1	1.1	-0.0	0.4	0.2	8.4	3.4	-4.2

Calibration "errors" for PTC-amino acid analyses were computed from areas per nmol for each site's standard minus the extinctions at 254 nm, with data sets normalized to the non-problematic amino acids E A P L & F. Column 1 shows the crude ave % errors, with negative values meaning lower recovery values than expected, while column 2 shows the average absolute % errors. Sites (n= 15) were divided three different ways into roughly equal bins; columns 3-5 show the results of subtracting in each pair the errors of one from the other. H-non: hydrolyzed standards - non-hydrolyzed. > 1-1: calibration using the average of >one standard - only one. Berr: sites reporting <5 % ave error (ignoring W & C) in their analysis of BSA - those with >6 % ave error. The BSA columns have a similar meaning. One calibration error each for G & Y and one BSA error were excluded from the calculation because of extreme values.

Some of the results are shown in Table IV for the 15 sites submitting calibration data based on areas at 254 nm.

The His calibration error was the largest and was recovered consistently low (note equal magnitude of “raw” and absolute % errors). Moreover, it was the strongest predictor of problems in the analysis of BSA: the large negative number in column 5 indicates that the sites with better analyses did markedly better calibrating His. The calibration error is sufficiently great to account for the large error in His values for BSA (column 7), while similarly high errors for Gly, Met, Ser & Thr are better explained as contamination and hydrolysis losses.

The expectation that sites which hydrolyzed their standard(s) would show a high “error” for Ser was fulfilled (column 3), as Ser is partially destroyed by hydrolysis. Did they therefore do better determining Ser in BSA? Column 8 shows they did not. Perhaps contaminating Ser offset the error necessarily experienced by the sites which did not compensate for that loss. Thr is also partially destroyed by hydrolysis, but is rarely a contaminant, so some other process must be inflating its apparent recovery: note that Met is high in column 3, meaning it is lost on hydrolysis. The most likely product is Met sulfoxide, which is known to coelute with Thr in many PTC separations. The high error for Thr in column 8 shows that this “compensation” does not work; however, sites with the better analyses (column 5) did show lowered recovery of Thr (& Ser), suggesting a beneficial compensation in some cases. But in general, the values in columns 3 & 8 suggest a disadvantage in hydrolyzing one’s standard.

The benefits of averaging calibration standards are far more clear: all but one of the values in column 9 are negative, indicating lower errors in the BSA analysis. The calibration is distinctly more accurate as well, if one ignores the aberrant values for Arg & His (column 4). But the general impression of this data is noise: the absolute “raw” averages (columns 1 & 6) are often much less than the average absolute values (columns 2 & 7), indicating the cancellation of fluctuations about a mean; and the average difference is small between hydrolyzing versus not, averaging many vs one, or even doing in general a good analysis of BSA vs doing a poor one.

Recommendations, especially to those doing PTC analyses: average several calibration standards and pay attention to response factors that are far from the usual or a priori expected values, especially for His. Unusual values imply problems in sampling (contamination), chemistry (incomplete reaction, loss), chromatography (coeluting garbage peak, baseline irregularity), or integration.

### *E. The "Best" Analyses*

The quality of many of the analyses reported in this trial was good and Table V reports an example of a few such analyses. These represent the “best” analyses, including Trp and Cys, as acquired by each of the different types of method.

Table V. Examples of Best Total Analyses of ABRF-9IAAA by Each Method!

Site #	41	6	33	2	39	47a	Seqb
	Fluor	Ninhydrin		Auto	PITC		
Ala	46.2	47.4		46.3	47.2	45.9	46
Arg	22.9	23.0	22.2	26.0	23.8	26.4	23
Asx	53.3	55.7	52.8	59.0	47.6	57.5	54
CyS	34.4	30.0	22.2	37.4	37.3	33.7	35
Glx	82.4	84.0	78.8	81.8	81.2	80.2	79
Gly	17.2	19.4	16.8	20.8	17.7	15.8	16
His	16.5	16.9	17.6	15.3	16.4	17.2	17
Ile	12.6	11.8	13.8	11.8	13.7	12.1	14
Leu	62.5	60.2	61.6	61.6	62.9	61.4	61
Lys	57.8	60.6	58.3	56.4	58.7	59.5	59
Met	3.9	3.5	2.3	4.6	3.8	4.1	4
Phe	27.2	26.6	26.4	26.3	27.5	26.5	27
Pro	29.2	29.9	29.8	27.6	29.5	28.6	28
Ser	28.2	27.1	21.8	25.9	26.3	27.4	28
Thr	33.4	34.4	35.2	31.1	32.8	35.4	34
Trp	2.1	1.3	1.9	2.2	2.1	1.7	2
Tyr	19.3	19.7	18.0	19.0	20.2	18.3	19
Val	35.8	33.5	35.6	33.5	34.9	32.0	36

<sup>a</sup>Values are given as residues per mol of protein.

<sup>b</sup>from ref. 9.

### F. Comparison with previous analyses of BSA

Table VI summarizes a few earlier analyses of BSA (12-15) together with the current analyses. The analyses of 1946 (microbiological) were good by 1991 standards, with a better average error than most of the present analyses. The chromatographic analyses of 1949, 1964 and 1970, all done before the sequence was known, are better in average error than nearly all of this year's analyses. Although the quantities used in those studies were considerably larger than the present, these data demonstrate an ideal for present methodology which is surely inherently attainable. Operator experience is part of the answer, but if the chromatography of those analyses are compared to current chromatograms, the better resolution of the earlier analyses are apparent, suggesting that some improvement may be attained through improved resolution of the fast present-day separations. The existence of many baseline disturbances in the fast analyses likewise compare unfavorably with the steady

baselines of analyses at the  $> 100$  nmol level. Site 41, using OPA postcolumn methodology, had the lowest average %error in this trial and compares very favorably with the older analyses. The average of all the analyses and of the best 14 analyses, both are very near the true analysis, suggesting that the large errors are mostly random and therefore average out. The higher average error values for Cys and Gly in the pooled data of all 58 sites, but not in the selected group, signifies specific problems with those residues which could be alleviated with proper technique.

**Table VI. Comparison of Analyses of ABRF-9IAAA with Literature Values**

	Percent Error						
	all 1991	top 14 1991	site #41 1991	1946a	1949b	1964c	1970d
<b>Ala</b>	1.1	0.3	0.4	nd	4.1	0.0	-1.4
<b>Arg</b>	4.5	2.6	-0.6	2.1	0.4	-2.7	3.2
<b>Asx</b>	-1.5	-0.6	-1.4	-3.2	3.6	0.4	2.4
<b>Cys</b>	-18.1	-8.2	-1.7	0.6	4.5	2.0	3.5
<b>Glx</b>	-2.4	1.8	4.3	-5.1	-3.1	-1.9	4.5
<b>Gly</b>	20.6	7.9	7.3	0.3	3.1	-0.3	-3.9
<b>His</b>	-1.2	-0.3	-2.7	-4.4	3.5	-1.0	0.8
<b>Ile</b>	-4.7	-5.2	-10.3	3.2	-3.2	-0.1	1.7
<b>Leu</b>	0.4	0.5	2.5	11.8	4.5	0.7	-2.4
<b>Lys</b>	-3.5	0.3	-2.0	-5.6	1.4	4.5	0.7
<b>Met</b>	0.2	-2.5	-2.5	-12.2	-7.8	-5.8	-4.6
<b>Phe</b>	-0.3	0.6	0.6	-9.3	0.7	-1.3	-2.5
<b>Pro</b>	3.1	2.9	4.4	13.2	0.3	6.7	2.5
<b>Ser</b>	-3.5	-3.5	0.8	-0.8	-1.8	-7.2	-3.4
<b>Thr</b>	-2.9	-2.3	-1.9	4.7	-1.8	0.7	-2.3
<b>Tip</b>	6.9	9.5	3.6	-9.2	-2.4	1.5	3.8
<b>Tyr</b>	-1.3	-0.3	1.8	4.5	0.3	6.4	5.4
<b>Val</b>	-3.1	-1.0	-0.7	0.9	-4.3	1.1	-1.1
<b>abs av</b>	3.4	2.0	2.8	5.4	2.7	2.5	2.7

ref. 12    bref. 13    'ref. 14    <sup>d</sup>ref. 15

## Acknowledgements

This work was partially supported by NSF grant DIR **9003100** to John Crabb (W.Alton Jones Cell Science Center) on behalf of the ABRF. We would like to thank the anonymous ABRF member facilities that participated in the study, Dr. Sam Margolis for his interest in the study and for preparing the samples, and Dr. Barton Holmquist for receiving and handling the voluminous results and enabling the maintenance of anonymity of the collaborating facilities.

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