

AAARG2003 Study: Quantitation of proteins by Amino Acid Analysis and Colorimetric Assays

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Introduction

The Amino Acid Analysis Research Group of the Association of Biomolecular Resource Facilities periodically provides member and non-member laboratories with test samples in order to assist them in maintaining and improving the quality of AAA and assess the reliability of AAA.

The AAA2003 study is a continuation of a previous study focusing on quantitation of proteins by amino acid analysis and dye-binding assays. Each participating laboratory received five separate solutions of pure proteins for the determination of their protein concentration by AAA. The concentration of each sample was also determined by the BCA or Bradford dye-binding assays. An additional standard sample was supplied for the calibration of the dye-binding assay. The amino acid analysis results were used by Research Group members to calculate the protein concentration in each sample and to compare these data with the relative precision and accuracy of the colorimetric assays. The goal of this study is to show whether colorimetric assays are a reliable method of quantitating unknowns when using a standard protein as reference.

Materials and Methods

Aprotinin (sample 1), beta-lactoglobulin (sample 2), fetuin (sample 3), hemoglobin (sample 4), and ubiquitin (sample 5) were purchased from Sigma; the standard sample (bovine serum albumin) for the calibration of the dye-binding assays was obtained from the National Bureau of Standards. All proteins were dissolved in water at a concentration of 2.5 mg/mL. The standard sample was supplied undiluted as the original 7% solution.

The data received from the participating laboratories were evaluated using a "best fit" procedure previously described [1] to calculate the experimentally determined protein concentration. By comparing with the theoretical compositions of the mature proteins, based on a 16 amino acid constellation excluding Trp and Cys, average %error were determined for each data set. Protein concentration determined by the dye-binding assays were tabulated as submitted by the participating laboratories.

1. Mahrenholz, A.M., et al., (1996) in *Techniques in Protein Chemistry VII*, Academic Press, San Diego, 323-330. Amino Acid Analysis - Recovery from PVDF membranes: ABRF-95AAA collaborative trial.

Table 1: Site identification key

| Site | Identification | Site | Identification | Site | Identification |
|------|----------------|------|----------------|------|----------------------|
| 1 | 715 | 10 | 20032 | 19 | 86340 |
| 2 | 10023 | 11 | 20090 | 20 | 51840 |
| 3 | 10258 | 12 | 21322 | 21 | 53025 |
| 4 | 12080 | 13 | 2C-OH | 22 | 53602 |
| 5 | 12345 | 14 | 32039 | 23 | 61001 |
| 6 | 13236 | 15 | 3383 | 24 | 88666 |
| 7 | 13445 | 16 | 34623 | 25 | CBTBE |
| 8 | 14220 | 17 | 40952 | 26 | MAXY1 |
| 9 | 15021 | 18 | 43072 | 27 | ORION |
| | | | | 28 | Kalibration advanced |

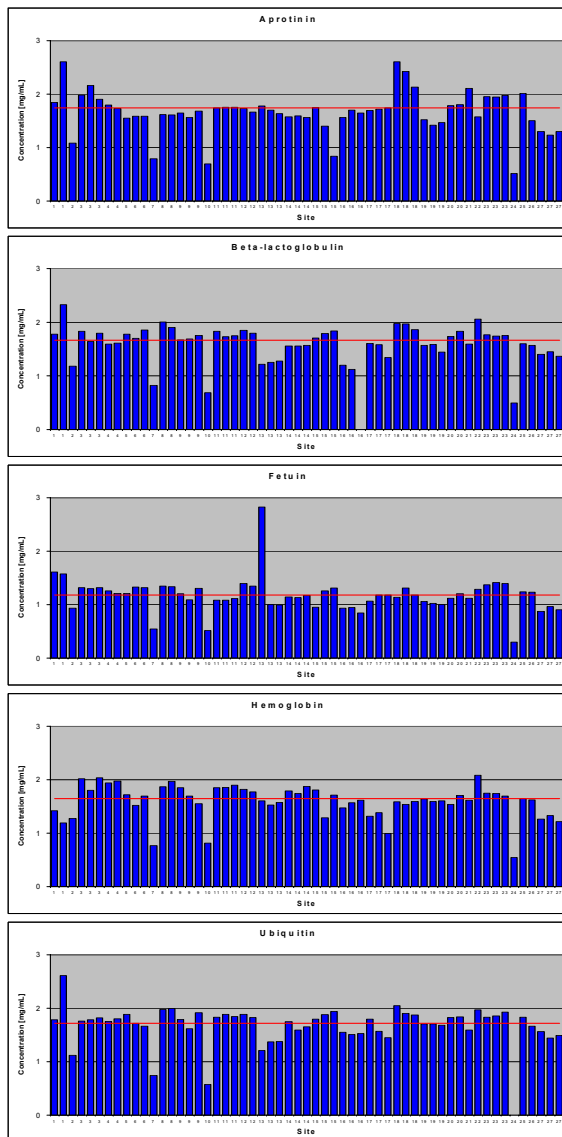


Figure 1: Protein concentration determined by AAA. The protein concentration (blue bars) for each data set was calculated as indicated in the Methods section. Mean concentrations (red line) are: 1.74 ± 0.28 for aprotinin, 1.67 ± 0.23 for beta-lactoglobulin, 1.18 ± 0.17 for fetuin, 1.64 ± 0.23 for hemoglobin and 1.72 ± 0.20 for ubiquitin. The site identification key is shown in table 1.

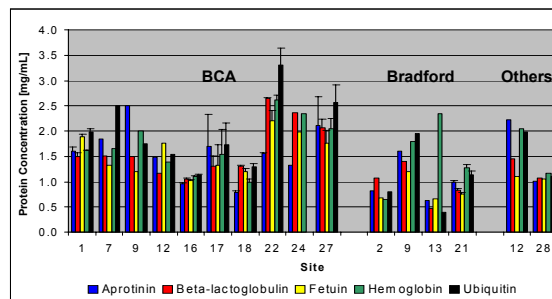


Figure 2: Protein concentration determined by dye-binding assay. Others: Lowry assay (site 12), home made assay (site 28). Data from sites 2, 24, 13, and 28 are single measurements. All other sites performed multiple analysis (BCA assay: Site 1: n=10, site 7: n=7, site 9: n=2, site 12: n=2-3, site 16: n=9, site 17: n=3, site 18: n=3, site 22: n=2, site 27: n=9-15. Bradford assay: Site 9: n=3, site 21: n=4. Others: site 12: n=2). Data were reported as mean values but some of the laboratories have not indicated the standard deviation of their multiple measurements.

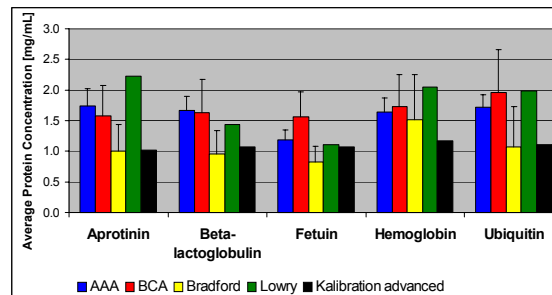


Figure 3: Comparison of protein concentrations determined by AAA and dye-binding assays. For the AAA values data from sites 7, 10, and 24 for all samples were excluded. For aprotinin also the values from site 2 and for fetuin the value from site 13 were excluded. For AAA: n=53-54, for BCA: n=10, for Bradford: n=4, for Lowry and Kalibration advanced: n=1. Average protein masses are: 6518 (aprotinin), 18281 (beta-lactoglobulin), 36353 (fetuin), $2 \times 15954 + 2 \times 15044$ (hemoglobin), and 8565 (ubiquitin).

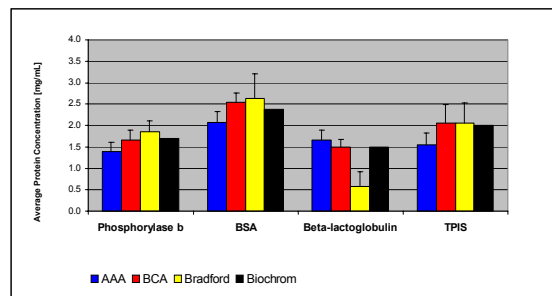


Figure 4: AAA2001 study: Comparison of protein concentrations determined by AAA and dye-binding assays. For AAA: n=18, for BCA: n=5, for Bradford: n=6, for Biochrom: n=1. Average protein masses are: 97158 (phosphorylase b), 66433 (BSA), 18281 (beta-lactoglobulin), and 26625 (TPIS).

Results

Data were returned from 28 laboratories for the AAA2003 Study. Amino acid analysis was performed by 27 laboratories, of which only 10 laboratories determined the protein concentration by the BCA assay, 4 by the Bradford assay, and 1 by a Lowry assay. One laboratory reported results obtained by a homemade dye-binding assay.

The average accuracy (%error) of beta-lactoglobulin, fetuin, hemoglobin, and ubiquitin range between 8.8 to 11.7% and are in the same range as those reported for comparable samples in previous studies [2, 3]. With an average %error of 17.7% the accuracy for aprotinin is significantly lower than in the other samples. The higher values are due to the exceptional high average errors of serine ($51.9 \pm 21.7\%$) and valine ($45.7 \pm 48.6\%$). In all data sets their absolute values are slightly higher than expected. Mature aprotinin has only one serine and one valine residue and thus only a small deviation from the theoretical values yields a relatively large %error value. However, the constantly increased amounts of these two amino acid residues may indicate the presence of an impurity in this protein preparation.

For the calculation of the average protein concentration (fig. 1) data from sites 7, 10, and 24 were excluded. Additionally, data from site 2 were excluded for aprotinin and beta-lactoglobulin, and from site 13 for fetuin. The average protein concentrations determined by AAA were 1.67 and 1.64 mg/ml for beta-lactoglobulin and hemoglobin and 1.74 and 1.72 mg/ml for aprotinin and ubiquitin. Assuming the weight recorded on the Sigma bottle was accurate and assuming small pipetting errors on our part in preparing the samples the recoveries for aprotinin, beta-lactoglobulin, hemoglobin, and ubiquitin were 69%, 66%, 70%, and 69%, respectively. This accuracy is typical, and is attributed to the water and residual salt content of the protein preparation. With an average of 1.18 mg/ml (47% protein recovery) the concentration of fetuin was significantly lower and additionally reflects the carbohydrate content of this protein.

We hoped to eliminate the large variations of the dye-binding assays observed in the AAA2001 study by supplying the participating laboratories with a calibration solution. However, the protein concentrations of the same sample determined by the BCA or Bradford assays still show large variations between different laboratories (fig. 2) and there is no difference between the two studies.

The comparison of the average data of the AAA2001 study (fig. 4) with the present study (fig. 3) shows that the concentrations determined by the BCA assay are comparable to those obtained by AAA while the concentrations determined by the Bradford assay are more variable. Lower values are obtained with proteins of low mass (aprotinin, ubiquitin) but the results also indicate that other yet unknown features of a protein may lead to lower concentration values (fetuin, beta-lactoglobulin).

2. Strydom, D.J., Andersen, T.T., Apostol, I., Fox, J.W., Paxton, R.J. and Crabb, J.W. 1993. Cysteine and tryptophan amino acid analysis of ABRF92-AAA. In *Techniques in Protein Chemistry IV*. (R. Hogue-Angeletti, ed.) pp. 279-288. Academic Press, San Diego.

3. Schegg, K.M., Denslow, N.D., Andersen, T.T., Bao, Y.A., Cohen, S.A., Mahrenholz, A.M. and Mann, K. 1997. Quantitation and identification of proteins by amino acid analysis. In *Techniques in Protein Chemistry VIII*. (D.R. Marshak, ed.) pp. 207-216. Academic Press, San Diego.

Conclusion

The BCA colorimetric method gave protein concentrations for all proteins that were consistent with the values obtained by amino acid analysis. The Bradford assay yielded variable values depending on the nature of the proteins, but firm conclusions are difficult for the Bradford assay because of the limited data sets.