AMINO ACID ANALYSIS OF PROTEIN HYDROLYSATES USING UPLC® AMINO ACID ANALYSIS SOLUTION

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INTRODUCTION

Amino acid analysis is used in the protein structure laboratory to provide two kinds of information. The total quantity of amino acids is a direct measure of the amount of protein in a sample. Additionally, the measurement of the proportions of amino acids provides information to confirm the identity and to detect modifications. Both applications require robust, accurate, and sensitive measurements that identify and quantitate the amino acids.

An amino acid analysis system solution has been developed for these applications. It includes a defined combination of instrument, chemistry, and methods. The standard configuration for this solution includes a tunable UV detector, but additional detector options provide flexibility to meet the needs for other assays in the laboratory.

The quantitative properties of the method were evaluated with the tunable UV (TUV), photo diode array (PDA), and fluorescence (FLR) detectors. Sensitivity, linearity and reproducibility were compared. Factors influencing quantitative derivatization were systematically optimized. Acid hydrolysates of bovine serum albumin were used as the model protein to test the accuracy of the analysis for measurement of composition and total protein concentration.

METHODS

Chromatographic Conditions

Standard UPLC® Amino Acid Analysis Solution instrument and chemistry configurations were used for all experiments. No changes were made to mobile phases, column or derivatization chemistry, or operating conditions.

Sample Preparation and Evaluation

Linearity and Reproducibility—A 2.5µmol/mL Hydrolysate standard was serially diluted to give concentrations of 10, 50, 100, 250, and 500 pmol/µL. The amount injected corresponded to 1, 5, 10, 25, and 50 pmol on column. Six replicate 50 pmol injections were made to test reproducibility.

Protein Hydrolysate—Acid-hydrolyzed bovine serum albumin was prepared in an independent laboratory as part of a collaborative study and supplied at an estimated concentration of 1.0 mg/mL in 0.1 M HCl, sealed under argon in glass ampoules. Samples were diluted 1:11 with 0.1 M HCl.

- 10 µL diluted sample
- 10 µL 0.1 N NaOH
- 60 µL AccQ·Tag™ Ultra Borate Buffer
- 20 µL AccQ·Tag™ Ultra Reagent

The protein hydrolysate analysis was repeated over five separate days, with two columns and a total of five mobile phase preparations. Independent sample dilutions were prepared on five days. On each day, the sample was derivatized five separate times. Each derivatized sample was then injected in triplicate. These 75 analyses are described below.

Detector Options

Figure 1A 50 pmole Standard with TUV Detection

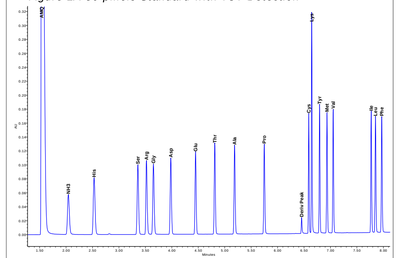


Figure 1B 50 pmole Standard with PDA Detection

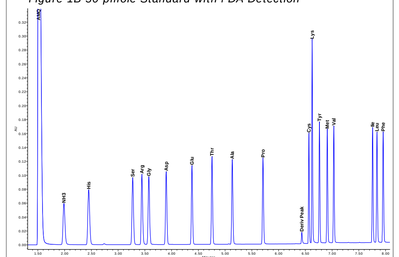


Figure 1C 50 pmole Standard with FLR Detection

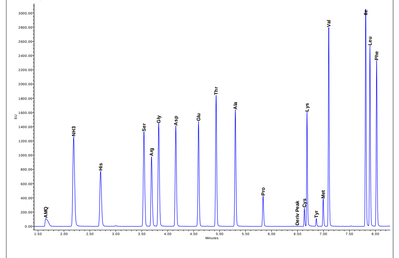
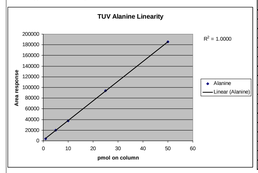


Figure 1. Chromatographic comparison of detector options with 50 pmole Hydrolysate Standard. Figure 1A (TUV) and 1B (PDA) show similar chromatographic profiles, while Figure 1C (FLR) is characterized by different peak sizes because of differences in fluorescence yields among the amino acids.

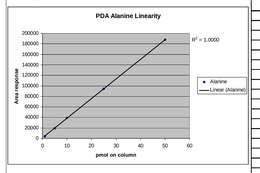
Quantitative Properties

Figure 2A Quantitation with TUV



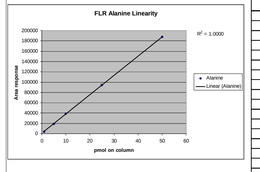
TUV	50 pmol RT Std Dev (min)	50 pmol Area % RSD	Correlation Coefficient 1 - 50 pmol
His	0.013	0.18	1.000
Ser	0.006	0.17	0.999
Arg	0.004	0.19	1.000
Gly	0.004	0.19	1.000
Asp	0.003	0.19	1.000
Glu	0.002	0.18	1.000
Thr	0.001	0.21	1.000
Ala	0.001	0.17	1.000
Pro	0.001	0.16	1.000
Cys	0.001	0.19	1.000
Lys	0.001	0.25	1.000
Tyr	0.001	0.23	1.000
Met	0.001	0.21	1.000
Val	0.001	0.21	1.000
Ile	0.001	0.22	1.000
Leu	0.001	0.19	1.000
Phe	0.001	0.25	1.000

Figure 2B Quantitation with PDA



PDA	50 pmol RT Std Dev (min)	50 pmol Area % RSD	Correlation Coefficient 1 - 50 pmol
His	0.021	0.31	1.000
Ser	0.010	0.26	1.000
Arg	0.008	0.27	1.000
Gly	0.007	0.32	1.000
Asp	0.005	0.33	1.000
Glu	0.003	0.32	1.000
Thr	0.002	0.26	1.000
Ala	0.001	0.24	1.000
Pro	0.001	0.27	1.000
Cys	0.001	0.24	1.000
Lys	0.001	0.13	1.000
Tyr	0.001	0.25	1.000
Met	0.001	0.25	1.000
Val	0.001	0.25	1.000
Ile	0.001	0.22	1.000
Leu	0.001	0.20	1.000
Phe	0.001	0.15	1.000

Figure 2C Quantitation with FLR



FLR	50 pmol RT Std Dev (min)	50 pmol Area % RSD	Correlation Coefficient 1 - 50 pmol
His	0.013	0.32	1.000
Ser	0.008	0.33	1.000
Arg	0.006	0.32	1.000
Gly	0.006	0.35	1.000
Asp	0.005	0.34	0.999
Glu	0.004	0.34	0.999
Thr	0.003	0.38	1.000
Ala	0.003	0.27	1.000
Pro	0.002	0.37	1.000
Cys	0.001	0.33	0.999
Lys	0.001	0.34	0.998
Tyr	0.001	0.37	0.999
Met	0.001	0.36	0.999
Val	0.001	0.36	0.999
Ile	0.001	0.40	0.999
Leu	0.001	0.33	0.999
Phe	0.001	0.35	0.999

Figure 2. Reproducibility and linearity data for TUV (2A), PDA (2B), and FLR (2C) detector options, with Alanine plotted for each. Each plot is accompanied by a table for all amino acids that show the standard deviation of retention time and % RSD of area for replicate injections of 50 pmoles.

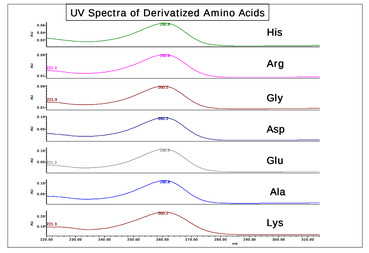


Figure 3. UV spectra for selected amino acid peaks at spectral resolution setting of 1.2 nm width on PDA detector. All the amino acids have the same absorbance maximum at 260 nm and same spectral shape. The PDA does not provide peak identification or peak purity assessment in this application.

Controlling Derivatization

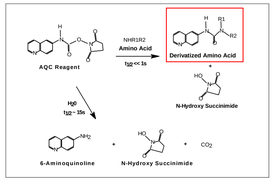


Figure 4. Reaction Mechanism for the derivatization of amino acids and subsequent hydrolysis of excess AQC Reagent

Requirements for optimal derivatization

- Optimal reaction is between pH 8 and pH 10
  - Standard derivatization cocktail includes 70 µL of borate buffer at pH 8 to neutralize sample acidity
  - If sample is equivalent to 0.1 M HCl, no neutralization is required
  - For higher concentrations of acid, replace part of the borate buffer volume with a volume of NaOH equal to the sample volume. The concentration of NaOH should match the sample acid concentration
- Ensure a 5x reagent excess
  - Derivatization cocktail contains ~250 nmoles of reagent
  - A standard derivatization cocktail is used for 1 µL injection from 100 µL total volume
  - Sample should not exceed 50 nmoles of amino acids
  - An injection could contain at most 500 pmoles divided among the many peaks
  - Useful guidelines
    - 1 pmole on column or 100 pmoles total of least abundant amino acid
    - 1—3 µg total protein in derivatization cocktail

Experimental approach to unknown samples

- Test two level of neutralization with sodium hydroxide
- Test two concentration levels
- Evaluate
  - Total peak area
  - Ratio of Ala/Phe
  - The amount of mono-derivatized Lys can also indicate the level of excess reagent in sample

Table 1. Evaluating quality of derivatization of BSA Hydrolysate. Asp, Glu, Ala, and Lys are most sensitive to incomplete derivatization, while Phe is least affected. The areas shown are corrected for dilution. Peak areas are not significantly larger with additional neutralization. Dilution of the sample to increase reagent excess does not increase yield. The Ala/Phe ratio is constant across all conditions. These observations confirm complete derivatization of the sample.

Amino Acid	22x Dilution	22x Dilution w/1x Neutralization	22x Dilution w/2x Neutralization	10x Dilution	50x Dilution
Asp	285890	275352	285142	279360	292750
Glu	419716	403876	419364	408600	436900
Ala	244024	234278	241010	237960	249950
Lys	469590	440264	449416	459790	471400
Phe	148126	141438	147246	143930	153250
Ala/Phe ratio	1.647	1.656	1.637	1.653	1.631

Sample Analysis

Figure 5A Analysis of Hydrolyzed BSA with TUV Detection

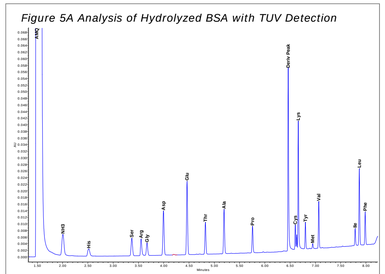


Figure 5B Analysis of Hydrolyzed BSA with FLR Detection

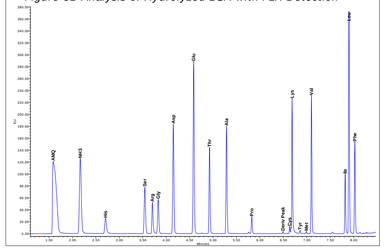


Figure 5. Analysis of Bovine Serum Albumin (BSA) Hydrolysate with TUV (5A) and FLR (5B) detection, approximately 9 ng on column. While the responses vary for the amino acids between the detectors, quantitation is consistent, as shown in Tables 2 and 3.

Table 2. Comparison of calculated and expected composition derived from known sequence of BSA for both UV and fluorescence detection. Results are the mean ± standard deviation for 75 replicate determinations.

Amino Acid	Expected Residues	*Observed Residues TUV	FLR
His	17	15.36 ± 0.19	15.73 ± 0.16
Ser	28	26.00 ± 0.08	25.90 ± 0.41
Arg	23	22.37 ± 0.08	22.39 ± 0.20
Gly	16	17.68 ± 0.20	16.65 ± 0.42
Asp	54	55.47 ± 0.21	55.18 ± 0.32
Glu	79	80.68 ± 0.20	80.27 ± 0.44
Thr	33	31.92 ± 0.06	32.01 ± 0.07
Ala	47	47.51 ± 0.15	47.40 ± 0.16
Pro	28	28.35 ± 0.14	28.92 ± 0.13
Lys	59	57.78 ± 0.38	57.83 ± 0.99
Tyr	20	20.19 ± 0.08	20.67 ± 0.34
Met	4	4.16 ± 0.15	4.04 ± 0.05
Val	36	35.67 ± 0.13	35.38 ± 0.13
Ile	14	13.15 ± 0.16	13.44 ± 0.16
Leu	61	63.13 ± 0.28	63.18 ± 0.28
Phe	27	26.57 ± 0.13	27.00 ± 0.33

*** Average of 75 data points (25 derivatizations, each injected in triplicate)**

Table 3. Determination of the protein concentration of a sample. The amino acid amounts are expressed as the residue molecular weights, and the sum of these amounts is equal to the weight of the protein. The BSA hydrolysate was derivatized five times on each of five separate days, and each derivatization injected in triplicate. The values reported in Table 3 are the means of the triplicate injections. The apparent variability in the concentration measurement is approximately 7%. Detailed examination shows that much of the variance of the data can be attributed to pipetting errors. The TUV replicates from Day 5 are much lower for all derivatization preparations as compared to the other four days. This is consistent with a volumetric error in the initial dilution on that day. With the FLR detector, the first derivatization on Day 5 is much higher than any other derivatization on any day. This is consistent with a pipetting error in that derivatization cocktail. Addition of an internal standard to the sample to be hydrolyzed will improve the reliability of the final analytical result.

Sample ID	Total µg AA/mL Hydrolysate TUV	FLR
BSA 1-1	699.64	664.27
BSA 1-2	694.09	656.91
BSA 1-3	697.88	665.76
BSA 1-4	698.21	655.55
BSA 1-5	695.43	655.40
BSA 2-1	716.66	747.45
BSA 2-2	717.87	741.41
BSA 2-3	715.65	746.38
BSA 2-4	709.75	748.39
BSA 2-5	707.93	733.88
BSA 3-1	714.93	721.30
BSA 3-2	711.50	721.43
BSA 3-3	708.46	710.33
BSA 3-4	708.14	725.01
BSA 3-5	708.10	681.68
BSA 4-1	674.16	730.93
BSA 4-2	678.43	729.37
BSA 4-3	683.07	721.29
BSA 4-4	678.62	732.51
BSA 4-5	678.94	740.78
BSA 5-1	591.40	927.97
BSA 5-2	604.30	743.11
BSA 5-3	571.78	740.98
BSA 5-4	571.57	742.96
BSA 5-5	599.18	726.54
Mean	677.43	724.46
Std Dev	47.98	53.21
% RSD	7.08	7.34

CONCLUSION AND SUMMARY

- **The UPLC® Amino Acid Analysis Solution can be used to accurately measure amino acid composition and to derive the protein concentration of a sample.**
- **The system can include different detectors and the same final result is obtained with all detectors.**
- **Optimal sample derivatization is critical for accurate quantitation of amino acids.**
- **Consistent results over a large number of replicates confirms robustness of the analytical solution.**

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