Method of analysis for Amino acids

- **Reagents, Solvents, and Materials**

Sodium phosphate monobasic monohydrate, Sodium hydroxide, Boric acid, Acetonitrile (LC grade), and Methanol (LC grade), OPA reagent, Borate buffer was prepared by adjusting 0.4 N boric acid to pH 10.2 with NaOH. Constant-boiling HCl. Chromatographic-grade water

Disposable glass test tubes (50 × 6 mm) and hydrolysis reaction vials (25 × 120 mm) with Mininert valves ,Amber wide-opening vials, glass conical inserts with polymer feet, and screw caps .

- **Amino Acid Standard Solutions**

Amino acid standard samples were prepared by mixing 95 µL of the 250 pmol/µL amino acid standard mixture with 5 µL of 10 mM norvaline and analyzed directly by RP-HPLC, within 24 h from preparation. Solutions for linearity study were prepared in duplicate by diluting the 1 nmol/µL amino acid standard solution, and contained 20, 50, 130, 250, or 500 pmol/µL of amino acid standard mixture together with 0.5 mM norvaline.

- **Protein Samples**

Glass test tubes (50 × 6 mm) were marked with incisions and soaked in a detergent solution for at least 12 h. They were rinsed thoroughly in Milli-Q water and dried in an oven at 80°C. Protein samples (7–75 µg) were transferred into the glass test tubes and spiked with 0.5 mM norvaline. They were quickly spun in a low-velocity centrifuge, then frozen and dried in a lyophilizer. Samples were then transferred into the reaction vial containing 0.5 mL of constant-boiling HCl on the bottom. Up to 12 test tubes could be accommodated in a reaction vial. The reaction vial was tightly closed and transferred into a pre-heated oven at 110°C for 18 h. The reaction vial was cooled at room temperature, then carefully opened under an aspirated hood. The test tubes were centrifuged and dried again in the lyophilizer to remove any liquid traces (condensed vapors). The dried residues were dissolved in 100 µL of 0.1 N HCl and transferred into the HPLC glass insert vials.

- **Analytical Procedure**

After derivatization, an amount equivalent to 2.5 µL of each sample was injected on column, , at 40°C, with detection at λ = 338 nm. Mobile phase A was 40 mM NaH_2PO_4, adjusted to pH 7.8 with NaOH, while mobile phase B was acetonitrile/methanol/ water (45/45/10 v/v/v). The separation was obtained at a flow rate of 2 mL/min that allowed for

1.9 min at 0% B followed by a 16.3-min step that raised eluent B to 53%. Then washing at 100% B and equilibration at 0% B was performed in a total analysis time of 26 min.

➢ RESULTS AND DISCUSSION

Acid hydrolysis is a crucial step that considerably influences amino-acid recovery. In fact, during acid hydrolysis, tryptophan and cysteine are destroyed and serine and threonine are also partially lost, while methionine can undergo oxidation. Moreover, some amino acids such as glycine and serine are common contaminants; therefore, their quantification needs careful subtraction of average responses in blank runs, which, in the case of glycine, is also complicated by the fact that this residue is known to give rise to multiple derivatives after OPA reaction. Therefore, the validation parameters were estimated using the following seven best-recovered amino acids: Asx (Asn+Asp), Glx (Glu+Gln), Arg, Ala, Phe, Leu, and Lys.In order to fully assess the method's performance, both a standard amino acid mixture and a reference protein (e.g., BSA) should be assayed along with the product. The standard amino acid mixture enables the verification of the HPLC method's performance, including derivatization, while the reference protein samples assess the completeness of the hydrolysis step. In addition, L-norvaline, which is added as the internal standard, provides a control for sample-to-sample variability.

➢ Specificity

Specificity was documented by comparing retention times obtained in the standard amino acid mixture (five samples) with those obtained from the reference protein samples (three samples). Results are reported. The minimal difference between retention times (<0.1%) allows confident, highly specific, peak identification. Usually, a difference within ±3% is considered acceptable between retention times of the same amino acids present in the standard mixture and in the hydrolyzed sample.

➢ Linearity and Range

Linearity was studied in the range from 20 to 500 pmol/µL of standard amino acids and from 1.5 to 15 pmol/µL of rFab, corresponding approximately to 20–200% of the test concentration. Five concentration points were assayed in duplicate. Both the standard amino acid mixture and the test product showed good linearity in the tested range. The area response obeyed the equation y = mx + C, where the intercept C was zero within 95% confidence limits and the square correlation coefficient (R2) was always greater then 0.985 (Table 33).

➢ Accuracy

Accuracy is defined as the agreement between the found value and the true, independently determined, concentration value. It was studied on BSA samples prepared from a calibrated standard, which is normally used in protein determination assays. The accuracy of the method was evaluated considering two different parameters: the absolute percent error and the recovery. The absolute percent error (%ABS) was calculated for each well-recovered amino acid. It was always less then 7%, and the average percent error was less then 2.5% (Table 44).). Based on the found amino acid concentrations and the known composition of BSA, the percent recovery of BSA was calculated. Considering each single amino acid, the recovery was found within the range 97–108%, while the average recovery, considering all the amino acids, was 102% (Table 55),), which is largely within the 90–110% range that is considered acceptable.5

TABLE 4

Absolute Percent Error (%ABS) of Found to Theoretical (Nt) Residue Numbers for the BSA Molecule

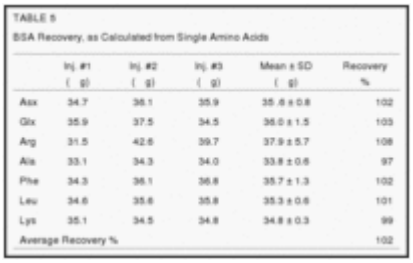

TABLE 5

BSA Recovery, as Calculated from Single Amino Acids

	Inj. #1 (g)	Inj. #2 (g)	Inj. #3 (g)	Mean ± SD (g)	Recovery %
Asx	34.7	36.1	35.9	35 .6 ± 0.8	102
Glx	35.9	37.5	34.5	36.0 ± 1.5	103
Arg	31.5	42.6	39.7	37.9 ± 5.7	108
Ala	33.1	34.3	34.0	33.8 ± 0.6	97
Phe	34.3	36.1	36.8	35.7 ± 1.3	102
Leu	34.6	35.6	35.8	35.3 ± 0.6	101
Lys	35.1	34.5	34.8	34.8 ± 0.3	99
Average Recovery %					102

TABLE 5

BSA Recovery, as Calculated from Single Amino Acids

Precision

Precision was measured as repeatability and intermediate precision. Reproducibility, which refers to the use of the analytical procedure in different laboratories, was beyond the scope of the present study.

Repeatability was studied on six injections (and derivatization) of the same BSA sample. The mean accuracy of each well-recovered amino acid was calculated. The percentage coefficient of variation (%CV) for each well-recovered amino acid was less than 2.5% (Table 66),), compared with an acceptance range of 5%.

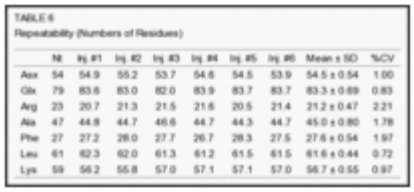

TABLE 6
Repeatability (Numbers of Residues)

	Nt	Inj. #1	Inj. #2	Inj. #3	Inj. #4	Inj. #5	Inj. #6	Mean ± SD	%CV
Asx	54	54.9	55.2	53.7	54.6	54.5	53.9	54.5 ± 0.54	1.00
Glx	79	83.6	83.0	82.0	83.9	83.7	83.7	83.3 ± 0.69	0.83
Arg	23	20.7	21.3	21.5	21.6	20.5	21.4	21.2 ± 0.47	2.21
Ala	47	44.8	44.7	46.6	44.7	44.3	44.7	45.0 ± 0.80	1.78
Phe	27	27.2	28.0	27.7	26.7	28.3	27.5	27.6 ± 0.54	1.97
Leu	61	62.3	62.0	61.3	61.2	61.5	61.5	61.6 ± 0.44	0.72
Lys	59	56.2	55.8	57.0	57.1	57.1	57.0	56.7 ± 0.55	0.97

TABLE 6

Repeatability (Numbers of Residues)

Intermediate precision was studied by running the whole method on three different days. Each day, three equivalent BSA samples were prepared, hydrolyzed, derivatized, and injected. The %CV for each well-recovered amino acid was generally not greater than 2.5%, with only one exception for arginine at 8%, as reported in Table 77.

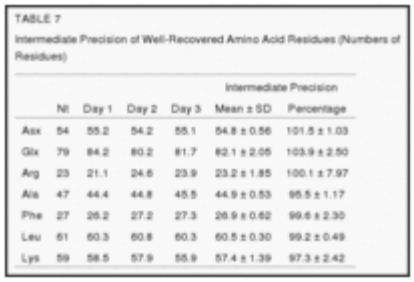

TABLE 7
Intermediate Precision of Well-Recovered Amino Acid Residues (Numbers of Residues)

		Intermediate Precision				
	Nt	Day 1	Day 2	Day 3	Mean ± SD	Percentage
Asx	54	55.2	54.2	55.1	54.8 ± 0.56	101.5 ± 1.03
Glx	79	84.2	80.2	81.7	82.1 ± 2.05	103.9 ± 2.50
Arg	23	21.1	24.6	23.9	23.2 ± 1.85	100.1 ± 7.97
Ala	47	44.4	44.8	45.5	44.9 ± 0.53	95.5 ± 1.17
Phe	27	26.2	27.2	27.3	26.9 ± 0.62	99.6 ± 2.30
Leu	61	60.3	60.8	60.3	60.5 ± 0.30	99.2 ± 0.49
Lys	59	58.5	57.9	55.9	57.4 ± 1.39	97.3 ± 2.42

TABLE 7

Intermediate Precision of Well-Recovered Amino Acid Residues (Numbers of Residues)

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