AOAC Official Method 999.13
Lysine, Methionine, and Threonine in Feed Grade Amino Acids and Premixes

First Action 1999

[Method is applicable to the determination of the free nonprotein-bound amino acids (lysine, methionine, and threonine) in feed grade amino acid trade products or in premixes with more than 10% individual amino acid content.]

See Table **999.13** for the results of the interlaboratory study supporting acceptance of the method.

A. Principle

The amino acid trade products or premix are dissolved in 0.1M HCl and diluted with sodium citrate buffer. Norleucine internal standard solution is added and the amino acids are separated by an amino acid analyzer or by HPLC using a cation exchange resin and sodium citrate buffer eluent solutions. The amino acids are measured colorimetrically following post-column reaction with ninhydrin, or by fluorescence detection after post-column reaction with *o*-phthaldialdehyde (OPA).

B. Apparatus

(**a**) *Amino acid analyzer or HPLC*.—With cation exchange resin (polystyrene sulfonate in Na^+ form), ninhydrin or OPA post-column derivatization, and data system for integration of peaks. *See also* **D**(**f**) and (**g**) and the note in **E**.

(**b**) *Bottles*.—500 mL wide-mouth, polyethylene or glass.

(**c**) *Dilutor*.—Optional for volumetric dilution. (*Note*: If ninhydrin reaction is used, use volume dilution ratio of about 1:20. Use higher ratio for OPA derivatization. Check the relative standard deviation [RSD] of dilution regularly with a balance; the RSD must be <1%).

(**d**) *Glassware*.—Glass beakers, 1000 mL; volumetric flasks, 50, 100, 500, 1000 and 5000 mL; graduated cylinders, 100 and 1000 mL; graduated pipets, 5 and 10 mL.

(**e**) *Magnetic stirring plate*.

(**f**) *Membrane filter units*.—Cellulose acetate, 0.22 μm.

(**g**) *pH meter*.—Calibrated with buffers of pH 2.0, 4.0, and 7.0.

(**h**) *Vacuum desiccator*.—With phosphorus pentoxide for water absorption.

(**i**) *Analytical balance*.—Accurate to 0.1 mg.

C. Reagents

(*Note*: The amino acids can be either of racemic or L-enantiomeric form.)

(**a**) *Lysine·HCl*.—Crystals, purity 99+%.

(**b**) *Methionine*.—Crystals, purity 99+%.

(**c**) *Norleucine*.—Crystals, purity 98+%.

(**d**) *Threonine*.—Crystals, purity 99+%.

(**e**) *Thiodiglycol*.—Liquid, purity 98+%.

D. Preparation of Solutions

(**a**) *Sodium hydroxide solution*.—30%. Weigh 300.0 g NaOH in tared 1 L beaker. Slowly dissolve pellets in ca 600 mL water with cooling. Transfer quantitatively to a 1 L volumetric flask, dilute to mark with water, and mix thoroughly.

(**b**) *Sodium citrate buffer, pH 2.20*.—Weigh 19.6 g trisodium citrate dihydrate in a 1 L beaker and dissolve in ca 800 mL water. While stirring, add 5 mL thiodiglycol, **C**(**e**), 1 g phenol crystals and 16 mL concentrated HCl. Adjust pH to 2.20 with some drops of HCl or NaOH solution. Transfer solution quantitatively into 1 L volumetric flask and dilute to volume with water. (*Note*: Use this buffer for all extract dilution or amino acid stock solutions for calibration to obtain the test solutions for the analyzer or HPLC. May be purchased commercially.)

(**c**) *HCl solutions*.—(*1*) *1M HCl*.—To 800 mL water in a 1 L volumetric flask, add 83 mL concentrated HCl from a graduated cylinder. Cool to room temperature, dilute to volume with water, and mix thoroughly. (*2*) *0.1M HCl*.—Pour 500 mL 1M HCl, using a volumetric flask, into a 5 L volumetric flask. Dilute to volume with water and mix thoroughly.

(**d**) *Norleucine internal standard stock solution*.—Weigh 0.32–0.34 g norleucine crystals [ca 2.5 mmol/L; **C**(**c**)] and transfer quantitatively with 1M HCl, (**c**)(*1*), into a 1 L volumetric flask. Dissolve crystals with about 100 mL 1M HCl, (**c**)(*1*) and dilute to volume with water. Store solution in a refrigerator for a maximum of 4 weeks.

(**e**) *Amino acids standard solutions*.—[*Notes*: (*1*) Standard solutions must contain only the amino acids being analyzed, that is, lysine, methionine, and threonine. The commercially available mixed standard solutions, containing, e.g., 18 amino acids, do not give optimal results. (*2*) The dilution and addition of internal standard must be carried out either with a dilutor/graduated pipets or by weighing.] Before first use, dry crystalline amino acids in vacuum desiccator for 2 days over phosphorus pentoxide, and then store in vacuum desiccator.

(*1*) *Amino acids stock solution (each amino acid concentration is approximately 2.5 mmol/L)*.—Weigh 0.45–0.47 g lysine·HCl, **C**(**a**), 0.36–0.38 g methionine, **C**(**b**), and 0.29–0.31 g threonine, **C**(**d**), and transfer quantitatively with 1M HCl, (**c**)(*1*), into a 1 L volumetric flask and dissolve in 100 mL 1M HCl, (**c**)(*1*). Dilute to volume with water. Store in refrigerator for a maximum of 4 weeks.

(*2*) *Amino acids calibration solution (using balance)*.—Weigh 2.5 mL (W_{aa}) amino acids stock solution, (*1*), and 2.5 mL ($W_{n\text{-}c}$) norleucine solution, (**d**), into a 50 mL volumetric flask. Dilute to volume with sodium citrate buffer, (**b**). Store solution in refrigerator for a maximum of 1 week.

(*3*) *Amino acids calibration solution (using dilutor/pipets)*.—Dilute equal volumes of amino acids stock solution, (*1*), and norleucine solution, (**d**), with sodium citrate buffer, (**b**), using 1:20 ratio, (e.g., 50 μL amino acids stock solution and 50 μL norleucine solution are diluted by the dilutor to 1.000 mL with sodium citrate buffer, (**b**), or 5.0 mL both solutions are pipetted in 100 mL volumetric flask and diluted to volume with buffer). Store solution in refrigerator for a maximum of 1 week. (*Note*: The dilution of the solutions can be adjusted to the sensitivity of the analyzer and post-column derivatization used.)

(**f**) *Elution buffers for cation exchanger column*.—Purchase from the manufacturer of the amino acid analyzer or HPLC or prepare them according to supplier's recommendations. Typically 3–5 aqueous buffer solutions are used containing sodium citrates or carbonates and small amounts of special additives and preservatives. During elution of the amino acids, the concentration of Na^+ and the pH are increased.

(**g**) *Ninhydrin or OPA reagent*.—Purchase from the manufacturer of the amino acid analyzer or HPLC or prepare it according to supplier's recommendations.

Table 999.13 Interlaboratory study results of the determination of amino acids

Sample	Amino acid	Expected value, %	No. of labs	Mean	s_r	RSD_r, %	s_R	RSD_R, %	$r = 2.8\ s_r$	$R = 2.8\ s_R$
No. 1 Biolys™	Lysine		17	45.89	0.39	0.84	1.06	2.31	1.08	2.96
No. 2 Premix	Methionine	27.00	16	26.55	0.41	1.56	0.49	1.85	1.16	1.37
	Threonine	15.00	16	14.85	0.19	1.29	0.35	2.33	0.54	0.97
No. 3 Premix	Lysine	20.80	17	20.56	0.27	1.33	0.51	2.49	0.77	1.44
	Methionine	11.00	17	11.26	0.14	1.24	0.21	1.84	0.39	0.58
	Threonine	8.00	17	8.22	0.05	0.65	0.16	1.90	0.15	0.44
No. 4 Premix	Lysine	16.80	17	16.63	0.22	1.29	0.39	2.32	0.60	1.08
	Methionine	14.00	17	14.21	0.13	0.93	0.27	1.92	0.37	0.76
	Threonine	11.00	17	11.19	0.09	0.82	0.25	2.22	0.26	0.70
No. 5 Premix	Lysine	12.80	17	12.58	0.09	0.74	0.23	1.86	0.26	0.65
	Methionine	32.00	17	32.04	0.16	0.50	0.83	2.59	0.45	2.32
	Threonine	22.00	17	22.10	0.18	0.81	0.43	1.94	0.50	1.20
No. 6 Premix	Lysine	12.29	17	12.30	0.21	1.68	0.26	2.13	0.58	0.73
	Methionine	30.72	17	30.59	0.32	1.06	0.76	2.50	0.90	2.14
	Threonine	21.12	17	21.25	0.25	1.18	0.40	1.87	0.70	1.11
No. 7 Premix	Lysine	10.40	17	10.27	0.12	1.21	0.19	1.81	0.35	0.52
	Methionine	9.00	17	9.10	0.08	0.92	0.13	1.48	0.23	0.38
	Threonine	14.00	17	14.03	0.10	0.70	0.29	2.07	0.27	0.81
No. 8 Premix	Lysine	10.24	17	10.12	0.12	1.21	0.15	1.50	0.34	0.42
	Methionine	8.86	17	8.91	0.10	1.17	0.17	1.89	0.29	0.47
	Threonine	13.79	17	13.83	0.19	1.35	0.31	2.25	0.52	0.87
No. 9 Premix	Lysine	24.00	17	23.48	0.26	1.13	0.50	2.15	0.74	1.41
	Methionine	19.00	17	19.09	0.19	0.99	0.33	1.73	0.53	0.93
	Threonine	15.00	17	15.09	0.15	1.00	0.30	1.99	0.42	0.84
No. 10 Premix	Lysine	23.28	17	22.85	0.19	0.84	0.41	1.78	0.54	1.14
	Methionine	18.43	17	18.65	0.13	0.68	0.29	1.53	0.36	0.80
	Threonine	14.55	17	14.65	0.14	0.96	0.31	2.15	0.40	0.88
No. 11 L-Lysine·HCl	Lysine	76.04	17	74.11	0.69	0.93	1.32	1.78	1.93	3.70
No. 12 DL-Methionine	Methionine	93.55	17	93.27	0.79	0.85	1.42	1.52	2.22	3.98
Number 13 L-Threonine	Threonine	95.95	17	95.46	1.11	1.17	2.07	2.16	3.12	5.78

E. Calibration

[*Note*: The chromatographic system must separate the amino acids from each other and from any other components which react with the reagent used (e.g., ammonia, amines, peptides, or aminosugars). The analyte amino acids must be 100% baseline separated from all other peaks that are eluted to avoid erroneous results caused by peak overlap. The chromatographic system should provide a linear response over the concentration range of the standard curve.]

For calculation, assume that the standard amino acids are 100% pure. Calculate the lysine content in lysine·HCl as follows:

$$\text{Lysine base} = 0.800 \times \text{lysine·HCl}$$

Calibrate the system as follows: Filter a suitable amount of the amino acid calibration solution, **D**(**e**)(*2*) or **D**(**e**)(*3*), through membrane filter units, **B**(**f**). Inject and analyze clear solution 5 times by ion exchange chromatography. The injection volume normally is 20–50 μL.

Determine the peak areas by the chromatographic data system and calculate the mean response factor (RF_{aa}) for each amino acid (*see* **H**, *Calculations*). The relative standard deviation (RSD_r) of RF_{Lys}, RF_{Met}, and RF_{Thr} over the 5 replicates of calibration is <1% for each analyte.

F. Homogenization and Extraction of Samples

Grind or crush test samples in mortar to pass a 0.25 mm sieve and mix thoroughly. Ensure that no coarse particles are present. Extract each test sample in duplicate. Proceed with (**a**) or (**b**) below.

(**a**) *Extraction using dilution by weighing*.—[*Note*: This procedure saves 60% of the 0.1M HCl, **D**(**c**)(*2*), and permits several dilutions from one extract.]

For pure amino acids.—Weigh 2.5 mmol (0.45–0.47 g) lysine·HCl, 0.36–0.38 g methionine, or 0.29–0.31 g threonine into weighed 500 mL labeled bottles. Add approximately 400 mL 0.1M HCl, **D**(**c**)(*2*), and dissolve while stirring with a magnetic stirrer for 30 min. Determine total weight of extraction solution (W_{ex}) and weigh an aliquot of 1 mL by use of a graduated pipet into 50 mL volumetric flask (W_a). Weigh additionally 2.5 mL norleucine solution, **D**(**d**), into that flask ($W_{n\text{-}ts}$), fill to the mark with citrate buffer, **D**(**b**), and mix well. Proceed to **G**.

For premixes or nonpure amino acids trade products.—Calculate approximate test portion weight [$W_{tp\ (premix)}$], g, based on the amino acid with lowest expected content as follows:

$$W_{tp\ (premix)} = \frac{\times}{}$$

$W_{tp\ (pure\ aa)}$ = test portion amount (2.5 mmol) used for pure amino acid, g; C_{exp} = expected content of aa, %.

Weigh in the calculated test portion amount into weighed 500 mL labeled bottles. [*Example*: A premix with 20% content of DL-methionine, **C**(**b**), requires test portion amount of 1.8–1.9 g.] Add approximately 400 mL 0.1M HCl, **D**(**c**)(*2*), and extract while stirring with a magnetic stirrer for 30 min. Determine total weight of extraction solution (W_{ex}) and weigh an aliquot of 1 mL by using a graduated pipet into 50 mL volumetric flask (W_{ali}). Weigh additionally 2.5 mL norleucine solution, **D**(**d**), into that flask ($W_{n\text{-}ts}$), fill to volume with citrate buffer, **D**(**b**), and mix well. Proceed to **G**. (*Note*: If the premix contains other amino acids with higher expected contents, dilutions using less than 1 mL aliquot of extract plus 2.5 mL norleucine may be prepared.)

(**b**) *Extraction using volumetric dilution.—For pure amino acids test samples*.—Weigh 2.5 mmol, that is 0.45–0.47 g, lysine·HCl, **C**(**a**), 0.36–0.38 g methionine, **C**(**b**), or 0.29–0.31 g threonine samples, **C**(**d**), transfer quantitatively with 0.1M HCl, **D**(**c**)(*2*), into 1 L volumetric flask, and dissolve in about 900 mL 0.1M HCl, **D**(**c**)(*2*), while stirring with a magnetic stirrer for 30 min. Fill to volume with 0.1M HCl, **D**(**c**)(*2*), and mix well. Dilute and add norleucine solution, **D**(**d**), following identical procedure as in the preparation of the calibration solution, **D**(**e**)(*3*). Proceed to **G**.

For premixes or nonpure amino acids trade products.—Calculate approximate test portion amounts [$W_{tp\ (premix)}$] based on each expected amino acid content as described in (**a**). Use for extraction an average test portion amount to achieve that all amino acid peak areas are in the linear range of the calibration. Weigh in this average amount and transfer quantitatively with 0.1M HCl, **D**(**c**)(*2*), into 1 L volumetric flask, extract in about 900 mL 0.1M HCl, **D**(**c**)(*2*), while stirring with a magnetic stirrer for 30 min. Fill to volume with 0.1M HCl, **D**(**c**)(*2*), and mix well. Dilute and add norleucine solution, **D**(**d**), following identical procedure as in the preparation of the calibration solution, **D**(**e**)(*3*). [*Note*: If amino acid contents differ strongly in the premix, make more than one extraction using different calculated amounts $W_{tp\ (premix)}$ and determine amino acid contents from the optimal extract.]

If analysis is not done immediately after preparation, store solutions, (**a**) or (**b**), at <5°C for a maximum of 3 days.

G. Determination

Filter a suitable amount of the test solution, **F**(**a**) or **F**(**b**), through membrane filter units, **B**(**f**), into autosampler vials and inject into analyzer. The injection volume normally is 20–50 μL. Analyze calibration solution, **D**(**e**)(*2*) or **D**(**e**)(*3*), after every 4 test solutions. Calculate the amino acid recoveries (*see Calculations*) and compare the amino acid contents with the expected values. If the results fall outside of the 99–101% range recovery, repeat calibration and check the chromatographic system for errors.

H. Calculations

Determine the area of the amino acid peaks in calibration solutions and test extracts by integration and calculate the amino acid content in percent of the test portion as described in (**a**) or (**b**) below.

(**a**) *Calculation if dilution by weighing is applied*.—Calculate the response factor (RF) for each analyzed amino acid as follows:

$$RF_{aa} = \frac{\times}{\times}$$

where $P_{aa–c}$ = peak area of amino acid in calibration solution; $P_{n–c}$ = peak area of norleucine in calibration solution; W_{aa} = weight of 2.5 mL amino acid stock solution (g); $W_{n–c}$ = weight of 2.5 mL norleucine stock solution (g).

Calculate the amino acid content of the test sample as follows:

$$\text{Amino acid, \%} = \frac{_{t} \times \quad \times \quad \times \quad _{t} \times}{_{t} \times \quad \times \quad \times}$$

where $P_{aa–ts}$ = peak area of amino acid in test solution; $P_{n–ts}$ = peak area of norleucine in test solution; RF_{aa} = response factor of amino acid; C_{aa} = concentration of amino acid in stock solution (g/1000 g),

assuming 1 L solution weighs 1000 g; W_{n-ts} = weight of 2.5 mL norleucine stock solution in test solution (g); W_{ex} = weight of total extract solution (g); W_{ali} = weight of extract aliquot used (g); W_{tp} = weight of test portion (g).

(**b**) *Calculation if volumetric dilution is applied*.—Calculate the response factor for each analyzed amino acid as follows:

$$RF_{aa} = \text{——}$$

where P_{aa-c} = peak area of amino acid in calibration solution; P_{n-c} = peak area of norleucine in calibration solution.

Calculate the amino acid content of the test sample as follows:

$$\text{Amino acid, \%} = \frac{_{t} \times \quad \times}{_{t} \times} \times 100$$

where P_{aa-ts} = peak area of amino acid in test solution; P_{n-ts} = peak area of norleucine in test solution; RF_{aa} = response factor of amino acid; C_{aa} = concentration of amino acid in stock solution (g/L); W_{tp} = weight of test portion (expressed in g/L).

[*Notes*: (*1*) Results of this calculation are expressed on the product "as is." For the calculation of purity according to a specification, that is, amino acid content in dry product, determine the dry matter content of the product. The purity is directly obtained, if the product is dried prior to analysis. (*2*) Lysine results may be expressed as lysine base or as lysine·HCl using the transformation: Lysine·HCl = 1.25 × lysine base. Indicate how the lysine results were calculated.]

I. Reporting Results

Perform the analysis in duplicate. Calculate the relative spread, $\%D = |x_1 - x_2| \times 200/(x_1 + x_2)$. Report the mean value if %D is ≤1.5%.

If the spread is greater, perform analysis from a third extraction and report mean of the 3 determinations. If RSD is >3%, reanalyze the test solutions. If the RSD of the results of each of the first 2 test solutions is confirmed (RSD ≤1.5%), the test sample is not homogeneous enough. Rehomogenize the test sample. If the results again are not confirmed, the error is due to the chromatography. Check the analyzer and repeat all affected measurements.

Round results to 2 decimal places.

Reference: *J. AOAC Int.* **83**, 771(2000).

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