INFANT FORMULA AND ADULT NUTRITIONALS

Total Amino Acids by UHPLC-UV in Infant Formulas and Adult Nutritionals, First Action 2018.06

GREG JAUDZEMS and JOSEPH GUTHRIE
Nestlé Quality Assurance Center, 6625 Eiterman Rd, Dublin, OH 43017
SABINE LAHRICHI and CHRISTOPHE FUERER
Nestlé Research Center, Nestec Ltd., Vers-chez-les-Blanc, 1000 Lausanne 26, Switzerland

Background: **An acid hydrolysis UHPLC-UV method was evaluated for the determination of total amino acids in infant formula and adult/pediatric nutritional formula.** ***Objective:*** **It was assessed for compliance against AOAC *Standard Method Performance Requirements* (SMPR®) established by the AOAC INTERNATIONAL Stakeholder Panel for Infant Formula and Adult Nutritionals (SPIFAN).** ***Methods:*** **A single-laboratory-validation (SLV) study was conducted as a first step in the process to validate the method. In this SLV, 17 SPIFAN matrices representing a range of infant formula and adult nutritional products were evaluated for their amino acid content.** ***Results:*** **The analytical range was found to be within the needs for all products, some may require a dilution. Evaluation of trueness performed on SRM 1849a (Infant/Adult Nutritional Formula) showed all compounds met the SMPR theoretical value, with exceptions for THR and TYR. These may have a bias for the NIST data depending on hydrolysis used in the determination of the NIST CoA.** ***Conclusions:*** **Based on the results of this SLV, this method met the Standard Method Performance Requirements and was approved as a First Action method by the AOAC Expert Review Panel on Infant Formula and Adult Nutritionals on August 28, 2018.**

For the purposes of this method, the term "amino acids" (AA) refers (primarily) to L-α-amino acids of the general structure (except proline): specifically, the twenty common proteinogenic amino acids. Amino acids may be present either in their free forms or bound, as part of the protein/peptide component.

Although cysteine fits the general structure for α-amino acids, its disulfide form, cystine, is obviously included in the working definition of amino acids. The two are equivalent from a nutrition point of view and regulations treat them as such. Because of method overlap, taurine (2-amino-ethanesulfonic acid) is also included in this analyte category, despite not fitting the basic definition

Nutritional significance - α-amino acids primarily reflect protein quality, since in nutritional products the protein is a vehicle for delivering amino acid substrates for protein synthesis. Free amino acids substitute for protein in elemental formulas (Table 1 – Amino acid classification).

Total AA analysis requires hydrolysis and analysis steps. Each step introduces its own complications. (*1*) Complex collection of molecular structures. (*2*) No common structural features compatible with direct, sensitive, detection. (*3*) Asn and Gln are converted to Asp and Glu during acid hydrolysis, resulting in Asx and Glx determination. (*4*) As of this publication, no single method can release all of the amino acids from protein - Cys and Trp are destroyed during acid hydrolysis.

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Corresponding author's e-mail: greg.jaudzems@us.nestle.com
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Total Amino Acids
in Infant Formulas and Adult Nutritionals
UHPLC-UV
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Quantitative determination of total amino acids using 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (ACQ) derivatization followed by UHPLC separation and UV detection.

This method allows the determination, in one single analysis, of the following amino acids: alanine, arginine, aspartic acid (combined with asparagine), cystine (dimer of cysteine, combined with cysteine), glutamic acid (combined with glutamine), glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, taurine, threonine, tyrosine, and valine.

This method is not suitable for the determination of tryptophan.

[Applicable to infant and adult/pediatric nutritional formulas and other matrices such as infant cereals and pet foods.]

Caution: Refer to Material Safety Data Sheets prior to use of chemicals. Use appropriate personal protective equipment when performing testing. Because of the use of chemical solvents, acids and reagents, sample preparation should be conducted under a fume hood and appropriate safety precautions should be taken.

A. Principle

Proteins are hydrolyzed in 6 M HCl for 24 h at 110°C in presence of phenol, 3-3'-Dithiodipropionic acid (DDP) and norvaline. Phenol (0.1%) is added to prevent halogenation of tyrosine. Norvaline is added as an internal standard. DDP is added to convert cystine and cysteine to S-2-carboxyethylthiocysteine (XCys) as described by Barkholt &

Table 1. Amino acid classification

Non-Essential	Essential (Regulated)	Conditionally essential
Alanine	Histidine	Arginine
Asparagine	Isoleucine	Cysteine
Aspartic acid	Leucine	Glutamine
Glutamic acid	Lysine	Glycine
	Methionine	Proline
	Phenylalanine	Serine
	Threonine	Taurine
	Tryptophan[a]	Tyrosine
	Valine	

[a]Tryptophan is not included in this method.

Jensen (1), and the resulting derivative can be separated from other amino acids for quantification.

After neutralization, amino acids and converted cysteine (XCys) are derivatized with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC). Derivatized amino acids are separated using reversed phase UHPLC with UV detection (Fluorescence detection is also an option.) at 260 nm.

During acid hydrolysis, glutamine (Gln) and asparagine (Asn) are converted to glutamic acid (Glu) and aspartic acid (Asp), respectively. Thus, Glu values represent the combined values of Glu and Gln, and Asp values represent the combined values of Asp and Asn. Cys2 values represent the combined values of cysteine and cystine since both are converted to XCys by DDP.

B. Apparatus

(a) *UHPLC system*.—This study was performed on an ACQUITY UPLC™ Quaternary solvent manager system (Waters Corp., Milford, MA; 2). An ACQUITY UPLC™ H-Class system was successfully used for the initial validation of the method. Alternative equipment may be used for this method, but will require adapting the separation gradient to ensure separation of all compounds.

(b) *Chromatography column.*—ACQUITY UPLC™ BEH C18 Column, 130 Å, 1.7 µm, 2.1 mm × 150 mm (Waters 186002353).

(c) *Micropipettes*.— Adjustable (10, 20, 200, and 1000 µL) and tips.

(d) *Vortex mixer.*

(e) *Analytical balance with a precision of 0.1 mg.*

(f) *Heating block*.—(55°C) ±2°C.

(g) *Laboratory oven*.—(110°C) ±2°C.

(h) *Syringe filter*.—Millipore 0.45 µm PVDF Millex®-HV (e.g., Millipore SLHV013NL).

(i) *Syringes*.—2 mL.

(j) *Borosilicate glass tubes*.—10 mL (e.g., Pyrex) with screw cap.

(k) *Microtubes*.—1.5 mL and 2 mL.

(l) V*ial with screw cap*.—4 mL.

(m) *Glass screw neck total recovery vial*.—12 × 32 mm (Waters 186000384C or equivalent).

C. Reagents

Remarks: Commercial references are only a guideline. Use equivalent chemicals or materials when listed items are not locally available. Before using chemicals, refer to the safety data sheets and ensure that the safety precautions are applied.

(a) *AccQ•Tag™ Ultra Derivatization kit*.—Waters 186003886.—*(1) Alternative derivatizing buffer*—Sodium tetraborate, decahydrate (e.g., J.T. Baker 3570-01).

(2) Alternative tagging reagent.—6-Aminoquinolyl-N-hydroxysuccinimidyl carbamate (e.g., Biosynth J-100019).

(b) *AccQ•Tag™ Ultra Eluent A concentrate*.—Waters 186003838.

(c) *AccQ•Tag™ Ultra Eluent B.*—Waters 186003839.—*(1) Alternative to AccQ•Tag™ Ultra Eluent B*.—w/w.

(2) Acetonitrile, gradient grade for LC.—e.g., Merck 1.00030 (>99.9%) 98%.

(3) Formic acid.—e.g., Sigma Aldrich 33015 2%.

(d) *Phenol*.—e.g., Sigma Aldrich P5566.

(e) *3,3'-Dithiodipropionic acid*.—e.g., Sigma Aldrich 109010.

(f) *Amino acid standard solution*.—Containing the following 17 amino acids at 2.5 µmol/mL each (except L-cystine at 1.25 µmol/mL): L-alanine, L-arginine, L- aspartic acid, L-cystine, L-glutamic acid, L-glycine, L-histidine, L-isoleucine, L-leucine, L-lysine, L-methionine, L-phenylalanine, L-proline, L serine, L-threonine, L-tyrosine and L-valine (e.g., Sigma AAS18).

(g) *L-cystine*.—e.g., Sigma Aldrich 30199.

(h) *Taurine*.—e.g., Sigma Aldrich 86329.

(i) *Norvaline*.—e.g., Sigma Aldrich N7627.

Table 2018.06A. Cystine concentration (final, after derivatization)

Solution	10 pmol/µL	5 pmol/µL	2.5 pmol/µL	1 pmol/µL	0.5 pmol/µL	0 pmol/µL
Cystine solution, µL	200[a]	100[a]	50[a]	200[b]	100[b]	0
Water, µL	900	1000	1050	900	1000	1100
1% DDP in 0.2 M NaOH, µL	600	600	600	600	600	600
0.2 M HCl, µL	600	600	600	600	600	600
10 mM Nva stock solution, µL	200	200	200	200	200	200
0.1% Phenol in 12 M HCl, µL	2500	2500	2500	2500	2500	2500

[a] 10 mM cystine stock solution.

[b] 1 mM cystine solution.

Table 2018.06B. Amino acid concentrations (each, final, after derivatization)

Solution	25 pmol/µL	10 pmol/µL	5 pmol/µL	1pmol/µL	0.5 pmol/µL	0 pmol/µL
Amino acid solution, µL	50[a]	100[b]	50[b]	100[c]	50[c]	0
Taurine solution, µL	50[d]	100[e]	50[e]	100[f]	50[f]	0
2.5 mM Nva in 0.1 M HCl, µL	20	20	20	20	20	20
0.1 M HCl, µL	380	280	380	280	380	480

[a] 2.5 mM AA stock solution.

[b] 0.5 mM AA solution.

[c] 0.05 mM AA solution.

[d] 2.5 mM Tau stock solution.

[e] 0.5 mM Tau solution.

[f] 0.05 mM Tau solution.

(j) *Sodium hydroxide pellets*.—Reagent grade (e.g., Merck 106498).

(k) *Sodium hydroxide solution.*—1 M (e.g., Merck 109137).

(l) *Sodium hydroxide solution (optional)*.—6 M (e.g., Merck 137032).

(m) *Hydrochloric acid fuming 37% (12 M) GR for analysis.*—e.g., Merck 100317.

(n) *Hydrochloric acid*.—1 M (e.g., Merck 109057).

(o) *Hydrochloric acid*.—0.1 M (e.g., Merck 109060).

(p) *Water*.—Laboratory grade Type 1.

D. Reagents and Standard Preparation

(a) *Sodium hydroxide (NaOH) solutions*.—6, 0.2, and 0.05 M.

(b) *Hydrochloric acid (HCl) solution*.—0.2 M.

(c) *1% DDP in 0.2 M NaOH.*

(d) *0.1% phenol in 12 M HCl.*

(e) *AccQ•Tag™ Ultra Derivatization kit*.—Prepare the reagents included in the kit following the manufacturer's instructions. *(1) AccQ•Tag™ Ultra Borate buffer (reagent 1)*.—Ready-to-use solution. *(i) Alternative reagent.*—5% (w/v) sodium tetraborate in water.

(2) AccQ•Tag™ Ultra reagent (vial 2A and 2B).—Reconstitute AccQ•Tag™ Ultra reagent (vial 2A) according to the manufacturer's instructions: *(i)* Preheat a heating block to 55°C.

(*ii*) Tap vial 2A lightly before opening to ensure all AccQ•Tag™ Ultra reagent powder is at the bottom of the vial.

(*iii*) Rinse a clean micropipette by drawing and discarding 1 mL of AccQ•Tag™ Ultra reagent diluent from vial 2B (ready-to-use solution). Repeat 2 times.

(iv) Draw 1.0 mL from vial 2B and transfer it to the AccQ•Tag™ Ultra reagent powder in vial 2A. Cap the vial tightly.

(v) Vortex mix for approximately 10 s.

(vi) Heat vial 2A on top of the preheated heating block until the AccQ•Tag™ Ultra reagent powder is dissolved. Do not heat the reagent for longer than 10 min.

Once reconstituted, the AccQ•Tag™ Ultra reagent is approximately 10 mM. Store reconstituted AccQ•Tag™ Ultra reagent in a desiccator at room temperature for up to 1 week.

Caution: AccQ•Tag™ Ultra reagent reacts with atmospheric moisture. Seal the container tightly when not in use. Do not refrigerate. Do not use discolored reagent, especially if it is yellow or green.

(3) Alternative reagent.—Into a 4 mL vial, weigh out approximately 3.0 to 4.0 mg of 6-Aminoquinolyl-N-hydroxysuccinimidyl carbamate. Continue with step **D(e)***(2)(iii)* using LC-grade acetonitrile instead of the AccQ•Tag™ Ultra reagent diluent.

(f) *Norvaline (Nva) internal standards.—(1) 10 mM Nva stock solution*.—Weigh 117.16 mg Nva into a 100 mL volumetric flask and make up to 100 mL with 0.1 M HCl.

(2) 2.5 mM Nva solution.—Pipet 2.5 mL of 10 mM Nva stock solution into a 10 mL volumetric flask and make up to 10 mL with 0.1 M HCl.

Store both Nva solutions at –20°C for up to 6 months as 2 mL aliquots.

(g) *Cystine calibration standards.—(1) 10 mM cystine stock solution*.—Weigh 140 mg cystine into a 100 mL volumetric flask and make up to 100 mL with 0.05 M NaOH.

Store 10 mM cystine stock solution at –20°C for up to 3 months as 1 mL aliquots.

(2) 1 mM cystine solution.—Add 900 µL of 0.05 M NaOH to 100 µL of 10 mM cystine stock solution.

1 mM cystine solution is prepared freshly for each analysis.

(h) *Amino acid (AA) calibration standards (with exception of cystine and taurine).—(1) 2.5 mM AA stock solution*.—Amino acid standard solution is ready-to-use and contains 2.5 mM of each amino acid (although present in this solution, cystine is not used for quantification and is prepared separately, *see* section **D(g)***(1)* and **D(g)***(2)*.

Store 2.5 mM calibration standard stock solution at 20°C for up to 6 months as 250 µL aliquots. *(1) 0.5 mM AA solution*.—add 600 µL 0.1 M HCl to 150 µL 2.5 mM AA solution.

(2) 0.05 mM AA solution.—Add 900 µL 0.1 M HCl to 100 µL 0.5 mM AA solution.

0.5 mM and 0.05 mM AA solutions are prepared freshly for each analysis.

(i) *Taurine (Tau) calibration standards.—(1) 2.5 mM Tau stock solution*.—Weigh 31.29 mg Tau into a 100 mL volumetric flask and make up to 100 mL with water.

Store 2.5 mM Tau stock solution at –20°C for up to 6 months as 250 µL aliquots.

(2) 0.5 mM Tau solution.—Add 800 µL water to 200 µL 2.5 mM Tau stock solution.

(3) 0.05 mM Tau solution.—Add 900 µL water to 100 µL 0.5 mM Tau solution.

0.5 mM and 0.05 mM Tau solutions are prepared freshly for each analysis.

(j) *Chromatography solvents (mobile phases*).—*(1) Eluent A (Solvent A).*—Prepare Eluent A from AccQ•Tag™ Ultra Eluent A concentrate as follows:

(i) Measure 850 mL of water into a 1 L graduated cylinder.

(ii) In a separate graduated cylinder, measure 150 mL of AccQ•Tag™ Ultra Eluent A concentrate.

(iii) Add the concentrate to the water and mix thoroughly.

Note: Eluent A concentrate, once opened, must be stored tightly capped at around 4°C. Dilute Eluent A is stable for 1 week at room temperature.

(2) Eluent B (Solvent B).—AccQ•Tag™ Eluent B is supplied as a working solution; no additional preparation is required. Eluent B, once opened, must be stored tightly capped at around 4°C for no longer than 1 month.

Alternative Eluent B.—Use HPLC-grade Acetonitrile supplemented with 2% (w/w) formic acid.

(k) *Wash solvents*.—*(1)* The weak needle wash solvent is 5% (v/v) acetonitrile in water.

(2) The strong needle wash solvent is 95% (v/v) acetonitrile in water.

(3) The seal wash solvent is 50% (v/v) acetonitrile in water.

E. Sample Analysis

(a) *Sample preparation*.—Reconstitute powders by adding 25 g powder to 200 g water and mix thoroughly. Weigh out 220±20 mg reconstituted powders or ready-to-feed liquids into a 10 mL glass tube with screw cap. Report the sample mass to 0.1 mg. Complete to 1100 mg with water.

To each tube, add:

(1) 600 µL DDP solution 1% DDP in 0.2 M NaOH.

(2) 600 µL 0.2 M HCl.

(3) 200 µL 10 mM Nva stock solution (10 pmol/µL final concentration after derivatization).

(4) 2500 µL phenol/HCl solution (0.1 % phenol in 12 M HCl).

Note: Phenol/HCl solution has to be added under the hood.

Sparge the tube a minimum of ~5 s with a stream of nitrogen to displace oxygen.

Close tubes with screw caps and vortex.

Note: Make sure the caps are perfectly clean (i.e. devoid of any particle) to ensure tightness and avoid evaporation during hydrolysis.

(b) *Cystine calibration standards preparation*.—Table **2018.06A** describes how to prepare calibration standards for converted cystine at (0 to 10) pmol/µL and Nva at 10 pmol/µL (all are final concentrations after derivatization).

Note: phenol/HCl solution has to be added under the hood.

Sparge the tube a minimum of ~5 s with a stream of nitrogen to displace oxygen.

Close tubes with screw caps and vortex.

Note: Make sure the caps are perfectly clean (i.e. devoid of any particle) to ensure tightness and avoid evaporation during hydrolysis.

(c) *Hydrolysis (of samples and cystine standards)*.—Place tubes in an oven at 110 °C ± 2 °C for 24 h ± 0.5 h.

(d) *Neutralization and dilution (of samples and cystine standards)*.—Take the tubes out of the oven. Allow hydrolysates to cool down and particles to settle down prior to taking an aliquot. When transferring aliquots, pipet about 1 cm below the top of the liquid. Perform neutralization under the hood.

Transfer 0.2 mL of each hydrolysate (samples and converted cystine standards) into a 1.5 mL microtube, add 0.2 mL of 6 M NaOH and then 0.4 mL of 0.1 M HCl. Mix well and filter through a 0.45µm membrane filter into another 1.5 mL microtube.

(e) *Amino acids calibration standards preparation–not needed to be acid hydrolyzed.*—Table **2018.06B** shows how to prepare 0.5 mL calibration standards at (0 to 25) pmol/µL and Nva at 10 pmol/µL (all are final concentration after derivatization). If quantification of taurine is not needed, replace the taurine solution by water.

The amino acid solutions are stable for 1 week when stored at 4 °C (± 2 °C).

(f) *Derivatization (of samples, cystine standards, and amino acids standards)*.—Derivatization converts free amino acids into highly stable derivatives. Standards and samples are derivatized following the manufacturer's instruction as described: *(1)* Preheat a heating block to 55°C.

(2) With a micropipette, add 70 µL of AccQ•Tag™ Ultra Borate buffer (reagent 1, *see* **D(e)***(1)* to a clean 12 × 32 mm glass screw neck total recovery vial.

(3) Add 10 µL of calibration standard [**E(e)**], neutralized sample solution [**E(d)**], or neutralized converted cystine standard [**E(d)**] to the vial.

(4) Vortex mix briefly.

(5) Add 20 µL of reconstituted AccQ•Tag™ Ultra reagent [**D(e)***(2)*] to the sample vial.

(6) Mix the solution immediately by pipetting up and down several times. Cap and mix by vortex immediately for several seconds and tap the vial to ensure no bubble is trapped.

(7) Let stand for 1 min at room temperature.

(8) Heat the vial in a heating block for 10 min at 55°C (±1°C).

(g) *UHPLC separation*.—*(1)* Prime solvent lines for 5 min.

(2) Prime wash/sample syringes for 4 cycles.

(3) Allow the chromatographic system to stabilise before injecting standards and samples. Make sure the system pressure and initial conditions are stable before performing injections (around 9000 psi).

(4) Before starting a series of analyses, inject two blanks (water) to condition the column.

(5) Inject 1 µL of each derivatized calibration standards, and then inject 1 µL of derivatized sample solutions. Perform single injections. Add a blank injection (water) at the end of each calibration series.

(6) Perform UHPLC under the conditions in Table **2018.06C**.

(7) Operating conditions may vary depending on the apparatus. Follow the supplier's instructions.

(h) *Peak identification and integration*.—Identify the amino acids peaks in the sample solution by comparison with the retention times of the corresponding peaks obtained in the calibration standards. If a peak has not been integrated correctly, call the recorded data and reintegrate.

To verify the system is stable inject a mid level standard a minimum of three times (5× for USP requirements) and ensure response and retention times have a RSD% < 2.

Check that peaks are separated with a good resolution (baseline

Table 2018.06C. UHPLC conditions

Column temp, °C	50			
UV detector, nm	260			
Injection vol., µL	1			
Flow rate, mL/min	0.4			
Mobile phase A	Eluent A [**C(b)**]			
Mobile phase B	Eluent B [**C(c)**]			
Elution gradient	Time, min	A, %	B, %	Curve
	0.00	99.9	0.1	
	5.50	99.9	0.1	2
	15.22	90.9	9.1	7
	20.47	78.8	21.2	6
	21.26	40.4	59.6	6
	21.29	10	90	6
	22.84	10	90	6
	26.00	99.9	0.1	6
	32.00	99.9	0.1	6
	0.00	99.9	0.1	

separation). If this is not the case, adapt the chromatographic conditions (gradient, temperature, tubing length…) accordingly.

Note: To check that the derivatization reagent was present in sufficient amount (excess), verify that the derivatization peak is present in the chromatogram.

Verify that excess derivatization reagent was present for the sample. The response of the excess reagent is present in the chromatography as the large AMQ peak first to elute. The response should be equal to that of the 25 pmol/µL standard or the reaction and sample should be flagged and discarded. The derivatization peak at ~17 min prior to Lysine can be ignored.

(i) *Calculation and expression of results.—(1) Calibration curve*.—Establish the calibration curve from the six different calibration standards for each amino acid and converted cystine at the beginning of each series of analyses by plotting the response (peak area ratio of analyte vs. internal standard, multiplied by the concentration of the internal standard, see below) against analyte concentration.

$$response = \frac{A_s}{A_{is}} \times C_{is}$$

Force the linear regression through zero.

Check the linearity of the calibration (the correlation coefficient R^2 must be above 0.99).

(2) Amino acid calculation.—Calculate the amount of individual amino acids present in the sample in pmol/µL from the calibration curve using the following equation:

$$C_s = \frac{A_s \times C_{is}}{A_{is} \times S}$$

Note: C_s is determined automatically when Empower is used to create the calibration curves.

where C_s = Concentration of individual amino acid in the test sample solution in pmol/µL; A_s = Peak area of individual amino acid in the test sample solution; C_{is} = Concentration of internal standard injected in pmol/µL; A_{is} = Peak area of internal standard chromatogram; S = Slope of the calibration curve (all curves are forced through zero, equation: y=ax).

Table 2018.06D. Molecular Weights *(MW_{AA})* of Amino Acids (g/mol)

Aspartic acid	133.11
Threonine	119.12
Serine	105.09
Glutamic acid	147.13
Proline	115.13
Glycine	75.07
Alanine	89.10
Cystine	240.30
Valine	117.15
Methionine	149.21
Isoleucine	131.18
Leucine	131.18
Tyrosine	181.19
Phenylalanine	165.19
Lysine	146.19
Histidine	155.16
Arginine	174.20
Taurine	125.15

Calculate the mass fraction, w, of each amino acid, in milligram per 100 grams of product, using the following equation:

$$w = \frac{C_s \times MW_{AA} \times V_s \times d_1 \times d_2}{m_S \times 10}$$

where MW_{AA} = Molecular weight of individual amino acids in g/mol (*see* Table **2018.06D**); V_s = Volume of hydrolysis solution in mL (typically 5 mL); d_1 = Dilution factor in the neutralization step (4); d_2 = Dilution factor in the derivatization step (10); m_s = mass of the test portion in mg; 10 = Combined factor to convert pg to mg (10^{-9}), mL to µL (10^3) and mg to 100 g ($1/10^{-5}$)

Note: Software (Empower) can be configured to calculate the mass fraction default entries are sample weight, dilution of 0.02, plus any required reconstitution factor multiplier (9 for 25 g powder to 200 g water)

Results and Discussion

Standard Method Performance Requirements (SMPR)

According to their concentration in reconstituted powders or ready-to-feed liquids, analytes are categorized into three groups: 0.5–5.0, 5.0–150, and 150–2500 mg/100 g reconstituted product. Table 2 from AOAC SMPR 2014.013 (3; below) indicates the different requirements for each group.

Note: The requirements listed in Table 2 correspond to the

Table 2. Method performance requirements[a]

Parameter		Minimum acceptable criteria
Analytical range 0.4–2500[b]		0.5–2500[b]
Limit of quantitation (LOQ)		≤0.5[b]
Recovery	0.5–5.0	±12%
	5.0–150	±10%
	150–2500	±7%
Repeatability (RSD_r)	0.5–5.0	≤7%
	5.0–150	≤5%
	150–2500	≤3%
Reproducibility (RSD_R)	0.5–5.0	≤11%
	5.0–150	≤8%
	150–2500	≤5%

[a] Concentrations apply to: (a) "ready-to-feed" liquids "as is"; (b) reconstituted powders (25 g into 200 g water); and (c) liquid concentrates diluted 1:1 by weight using water.

[b] mg/100 g Reconstituted final product.

updated version approved by the stakeholder panel during the August 2018 annual meeting.

Linearity

For each analyte, three standard curves with six to ten calibration levels between 0.25 and 50 pmol/µL were injected (1 µL). The concentration of the internal standard norvaline (Nva) was 10 pmol/µL.

The calibration range (0.25 to 50 pmol/µL) corresponds to concentrations between 2.5 and 500 mg/100 g reconstituted final product [*see* section **E(i)** for conversion details].

Note: Concentrations lower than 2.5 mg /100 g reconstituted final product (as listed in the SMPR) were not tested. However, all analytes in all samples tested were above this value (lowest value was 3.5 for taurine in sample 9). Sample dilution can be done if values fall above standard curves.

From the correlation coefficient R^2 of the regression lines (> 0.99), the standard deviation of the residuals, and the graphs of the residuals (Table 3 and Figure 1, we conclude that the calibration curves are linear between (0.25 and 10) pmol/µL for XCys and (0.25 and 50) pmol/µL for all other amino acids.

Limit of Quantification

The scope of this method implies working at effective concentrations higher than the limit of quantification (LOQ) stipulated in the SMPR. Therefore, the LOQ for the different amino acids were set to the lowest point of their linearity ranges (*see* **F(b)**). This is equivalent to 0.25 pmol per injection (1 µL) for all amino acids studied.

Using an average molecular weight of 110 g/mol and a sample mass of 200 mg (reconstituted powder), 0.25 pmol/µL (derivatized sample) corresponds to

Table 3. Calibration levels for all components. Converted cystine noted as XCys

		Concn range		Slope		Intercept			
Analyte	Unit	Min	Max	Central value	Slope=0? (Y/N)	Central value	Intercept=0? (Y/N)	Coefficient of determination R^2	Standard deviation of residuals
Ala		0.25	50	0.982	N	-0.043	Y	0.999	0.533
Arg	pmol/µL	0.50	50	0.948	N	-0.194	Y	0.999	0.569
Asp	pmol/µL	0.25	50	0.901	N	0.190	Y	0.998	0.637
XCys	pmol/µL	0.25	10[a]	1.834	N	0.049	Y	1.000	0.093
Glu	pmol/µL	0.25	50	0.916	N	0.087	Y	0.999	0.560
Gly	pmol/µL	0.50	50	0.927	N	-0.097	Y	0.999	0.548
His	pmol/µL	0.25	50	0.956	N	-0.176	Y	0.999	0.539
Ile	pmol/µL	0.25	50	1.005	N	-0.126	Y	0.999	0.546
Leu	pmol/µL	0.25	50	0.983	N	-0.145	Y	0.999	0.537
Lys	pmol/µL	0.25	50	1.597	N	-0.071	Y	0.999	0.951
Met	pmol/µL	0.25	50	0.979	N	-0.168	Y	0.999	0.541
Phe	pmol/µL	0.25	50	0.993	N	-0.143	Y	0.999	0.552
Pro	pmol/µL	0.25	50	0.897	N	-0.066	Y	0.999	0.565
Ser	pmol/µL	0.25	50	0.945	N	-0.100	Y	0.999	0.508
Tau	pmol/µL	0.25	50	0.931	N	-0.109	Y	0.999	0.587
Thr	pmol/µL	0.25	50	0.968	N	-0.104	Y	0.999	0.524
Tyr	pmol/µL	0.25	50	0.991	N	-0.145	Y	0.999	0.554
Val	pmol/µL	0.25	50	0.981	N	-0.267	Y	0.999	0.542

[a] Cystine follows a linear regression up to 10 pmol/µL. In the matrices used for the validation of this method, concentrations of cystine did not exceed 3 pmol/µL.

2.5 mg/100 g in the reconstituted sample according to the following three equations:

Equation 1.—Concentration of analyte in the hydrolysate (4 and 10 are the dilutions occurring during the neutralization and derivatization steps, respectively).

$$0.25\left[\frac{pmol}{\mu L}\right]\times 110\left[\frac{pg}{pmol}\right]\times 4\times 10\times 10^{3}\left[\frac{\mu L}{mL}\right]\times 10^{-9}\left[\frac{mg}{pg}\right]=0.0011\left[\frac{mg}{mL}\right] \quad (1)$$

Equation 2.—Amount of analyte in the sample

$$0.0011\left[\frac{mg}{mL}\right]\times 5[mL]=0.0055[mg]$$

Equation 3.—Amount of analyte in 100 g of reconstituted powder

$$\frac{0.0055[mg]}{220[mg_s]\times 10^{-5}\left[\frac{100g_s}{mg_s}\right]}=2.5\left[\frac{mg}{100g_s}\right]$$

Based on the actual results (Table 4), concentrations in the reconstituted samples are at least 25× higher than this value (except for taurine, which is around 4 mg/100 g reconstituted sample), indicating that 2.5 mg/100 g reconstituted sample could be considered as the LOQ for this study instead of the 0.5 mg/100 g reconstituted sample value stipulated in the SMPR. However, the SPIFAN ERP approved this deviation of the method performance compared to the SMPR. *See* Table 5 for each components actual LOQ.

Analytical Range

Following calculations similar to those described in section **F(c)**, the analytical range can be converted from pmol/µL (concentration of the derivatized sample) to

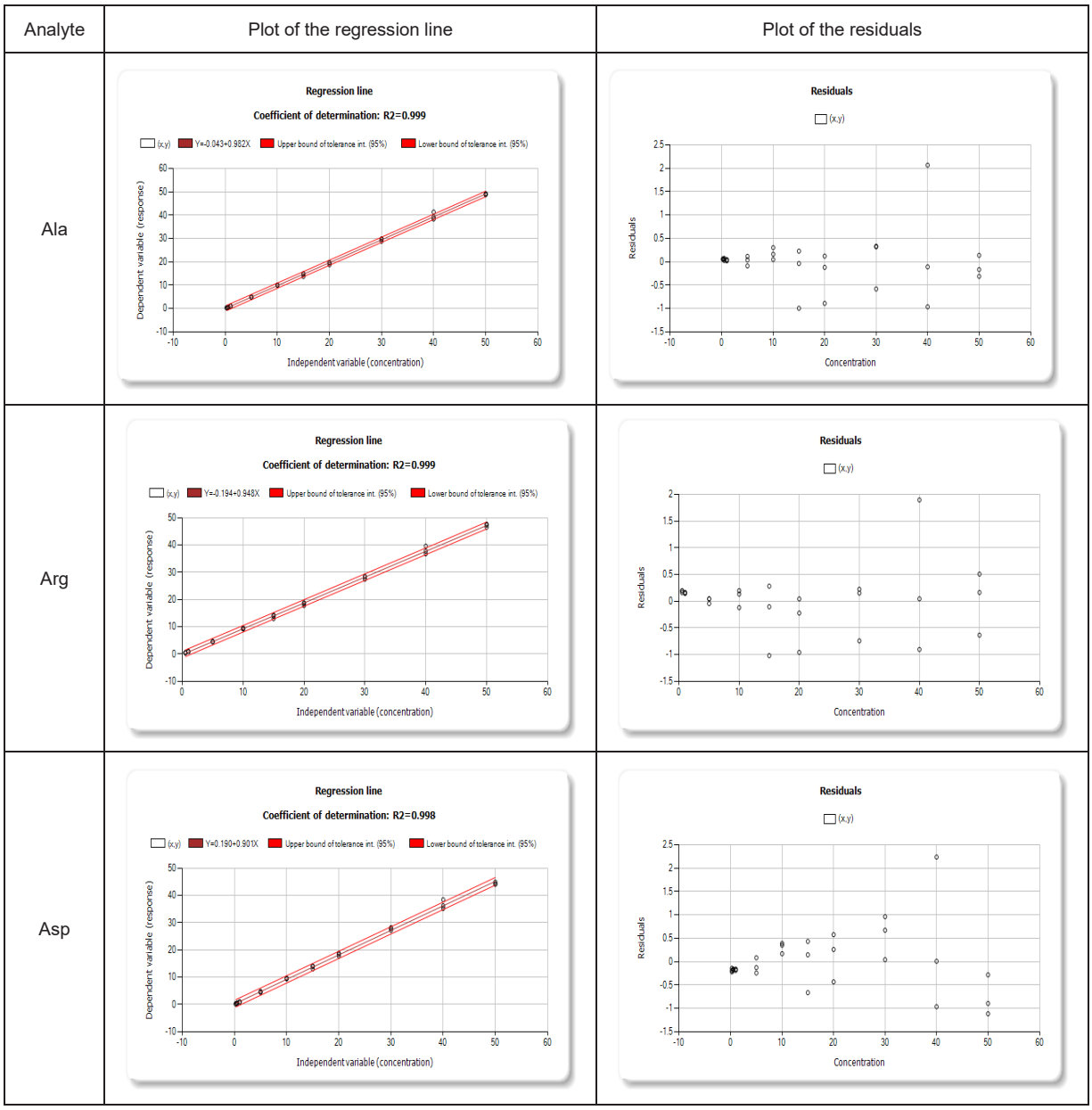


Figure 1. Linearity

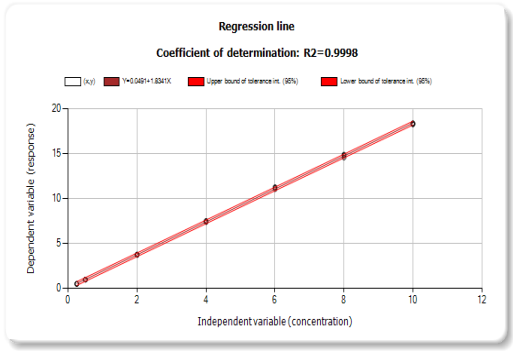
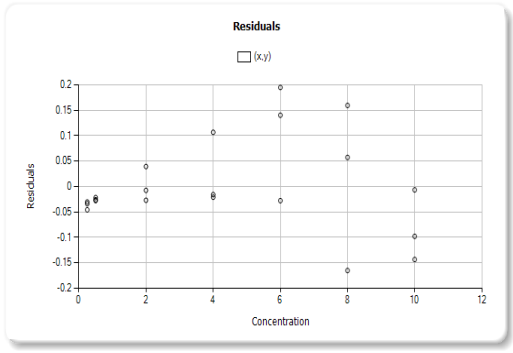
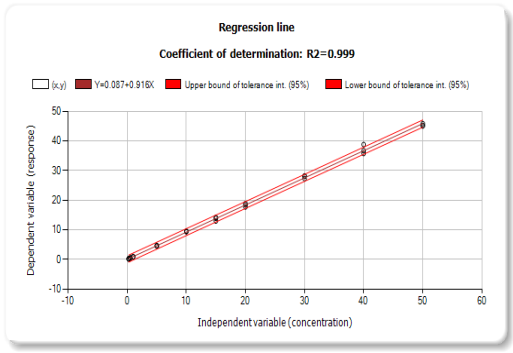
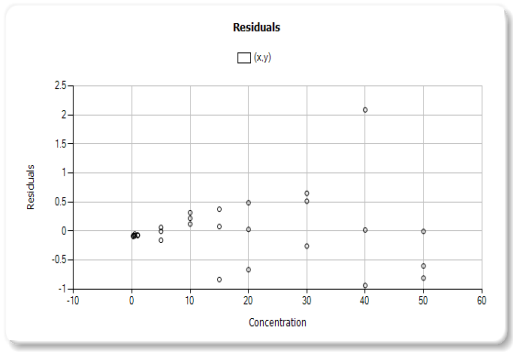
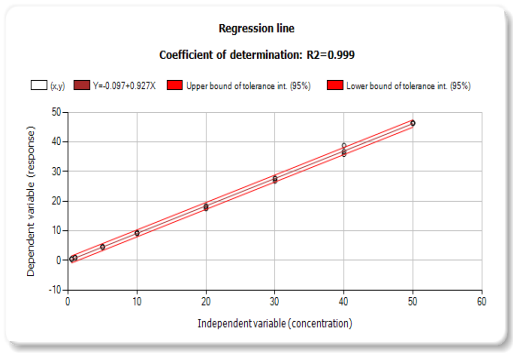
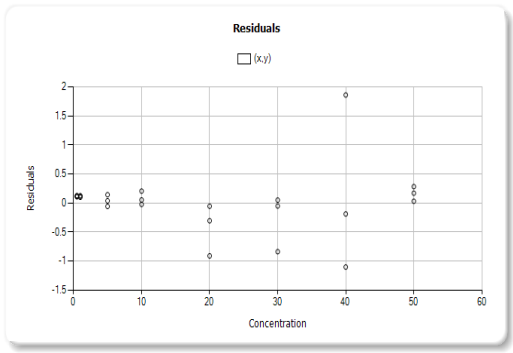
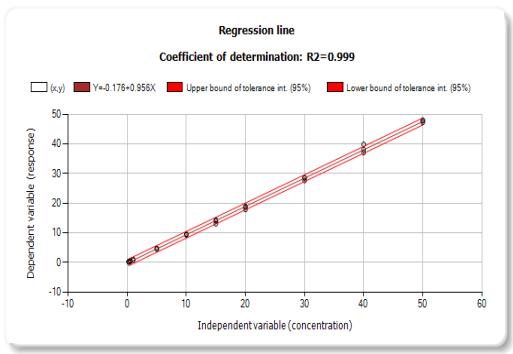
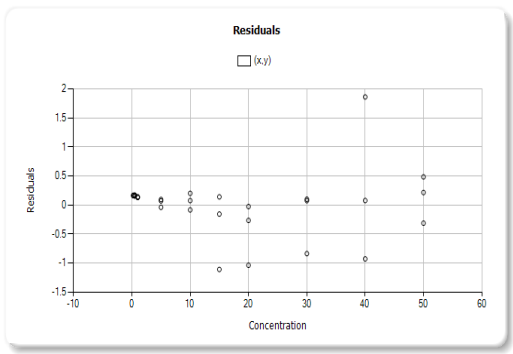
Cys2		
Glu		
Gly		
His		

Figure 1. ***(continued)***

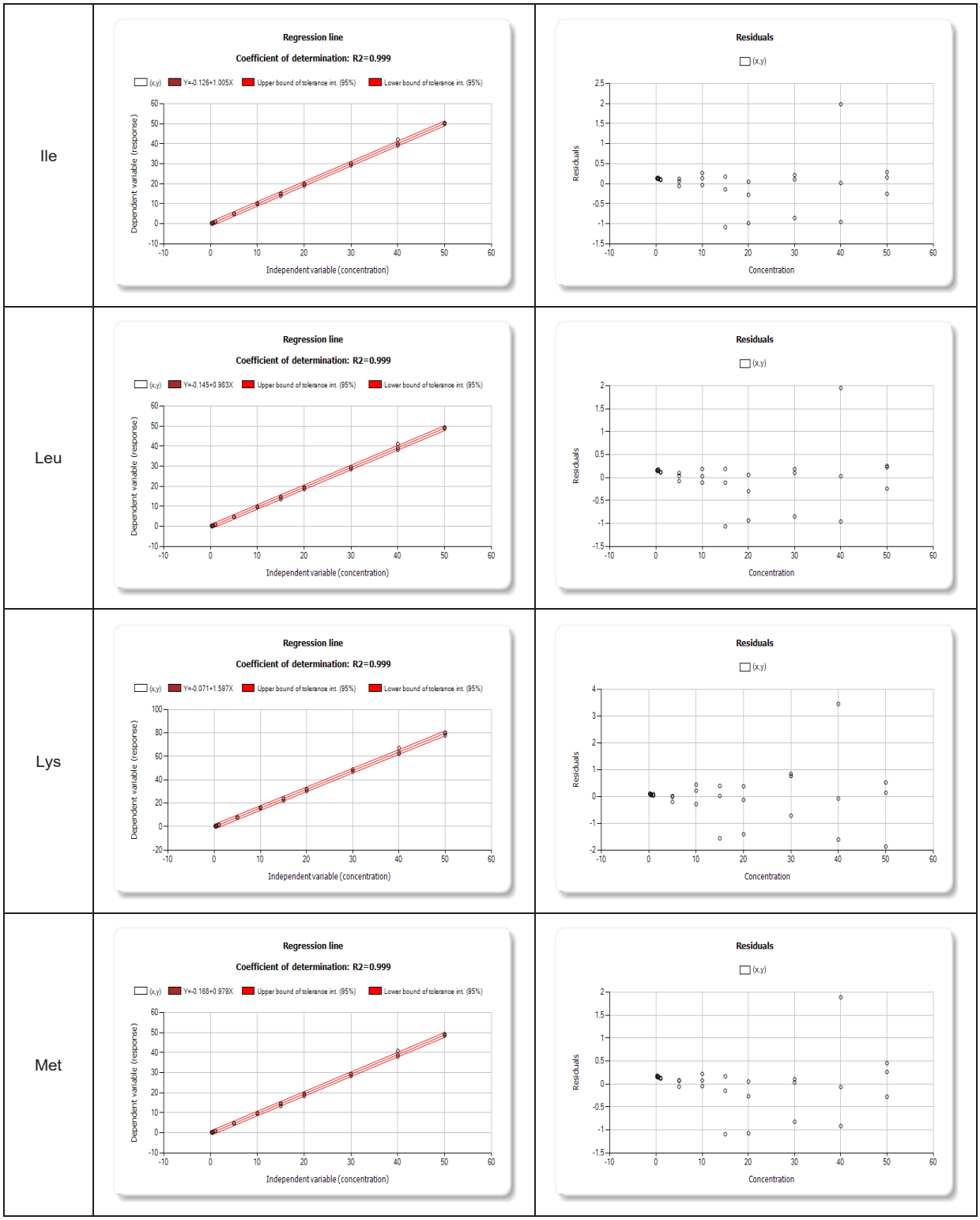


Figure 1. ***(continued)***

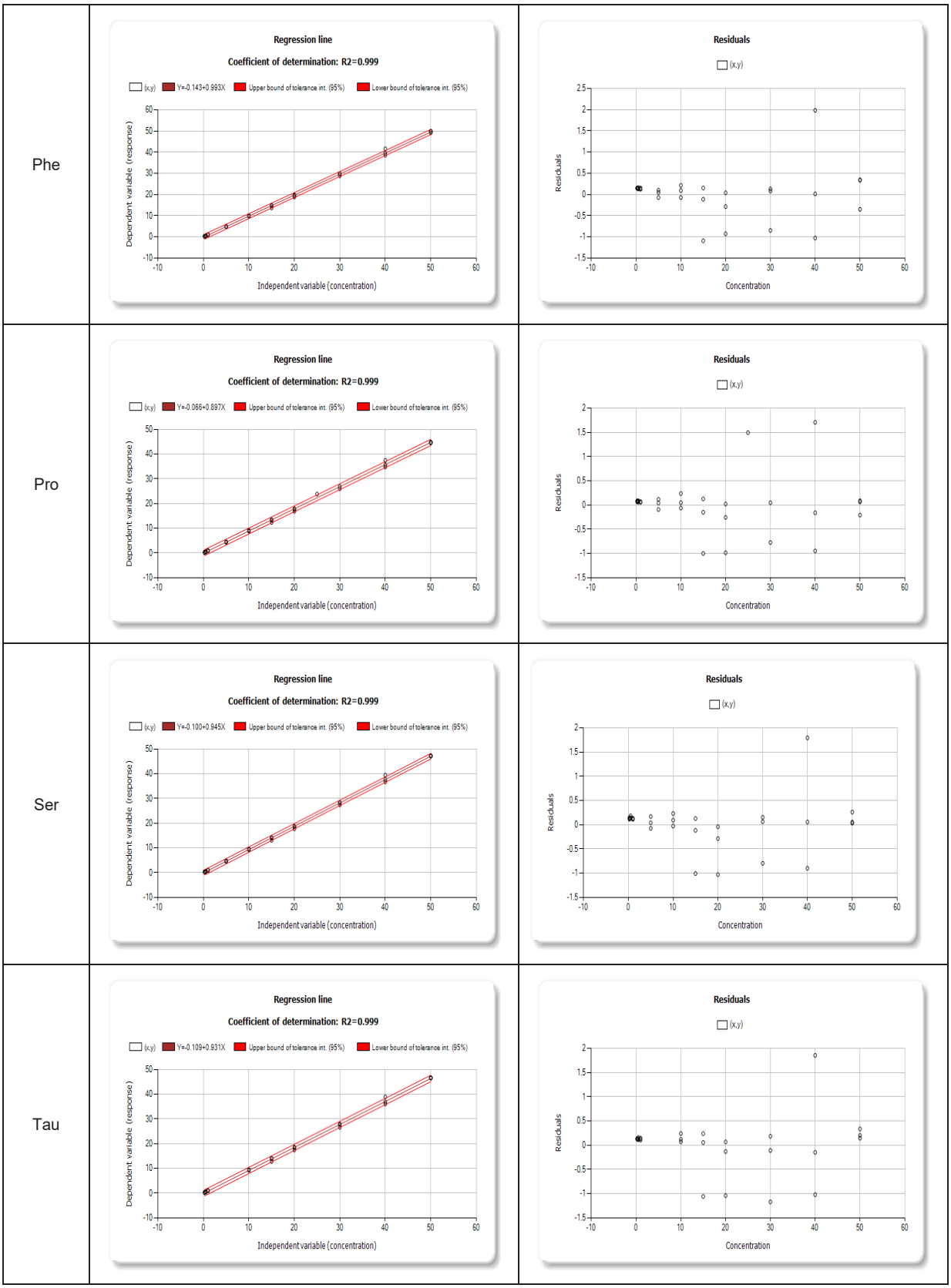


Figure 1. ***(continued)***

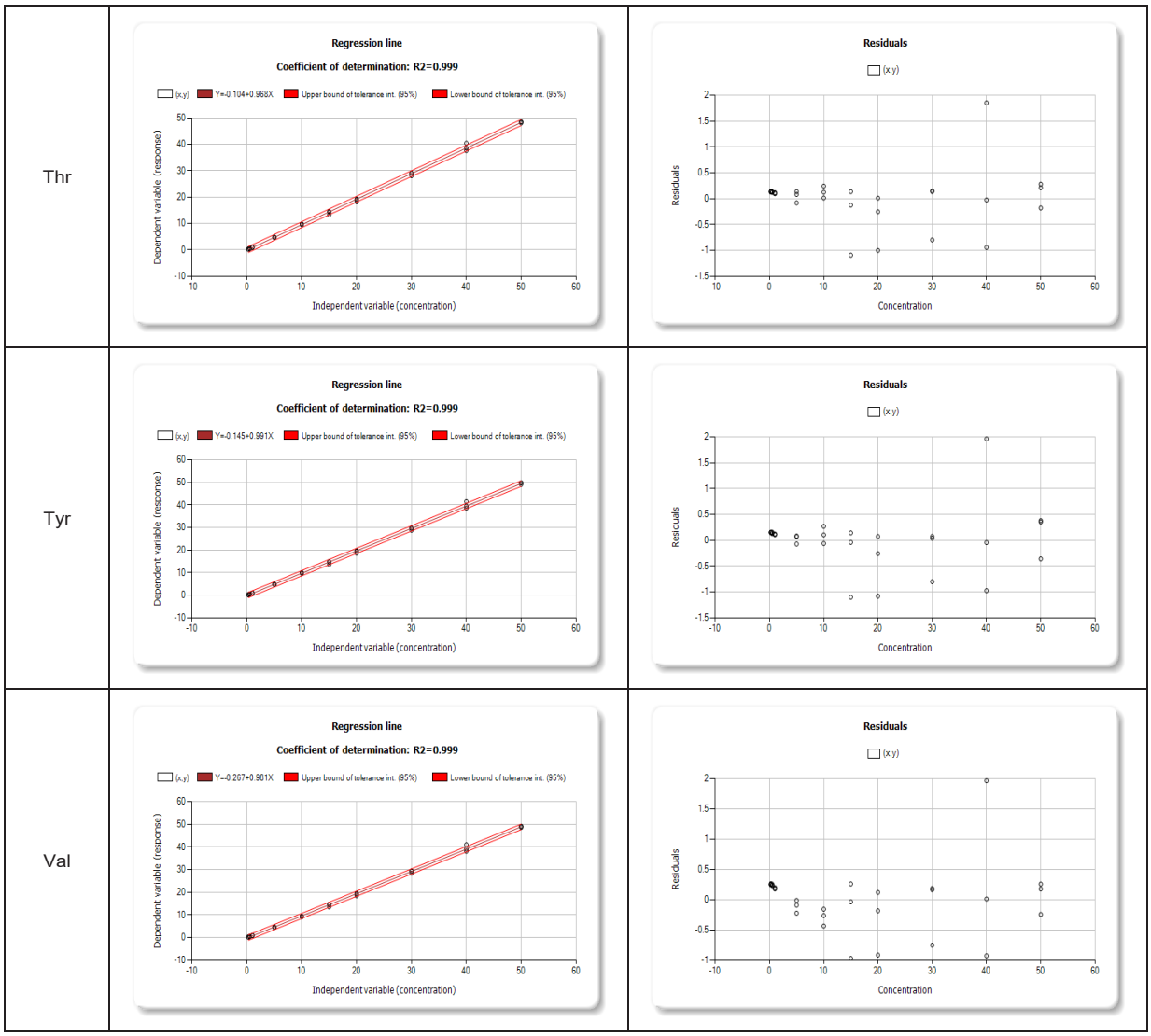


Figure 1. ***(continued)***

mg/100 g reconstituted final product for each amino acid (*see* Table 5). Comparing those values with the results obtained with the SPIFAN kit (Table 4 indicated that 2% of the measured values were above the calibration range. Of note, these were all in adult nutritional RTF products. No value was below the calibration range.

Experimental Plan and Sample List

Samples were analysed in duplicate on each of six days by three different analysts on two different ACQUITY UPLC™ systems. Recoveries were calculated based on single spike analyses in each matrix. For NIST SRM 1849A, recoveries were also calculated based on the reference values from the certificate of analysis.

Note: Spike standard solutions can be added at the hydrolysis step in place of required water volume.

Products were numbered following the list detailed in the SPIFAN II SLV kit (*see* Table 6**).**

Results

Data were obtained without nitrogen sparging. No significant difference was observed upon sparging (*see* Appendix J), but this step was added to the method to minimize unwanted oxidation. Average concentrations expressed in mg/100 g reconstituted product are detailed in Table 4 for each analyte/matrix pair. Values are based on robust statistics. Appendix B contains the same values converted to mg/100 g product. Values are identical between Appendix A and Appendix B for ready-to-feed (RTF) products (3, 4, 5, 17, 18, 19).

The cells are formatted according to the SMPR concentration ranges as follows: 0.5–5.0 (bold, underlined), 5.0–150 (normal), and 150–2500 (italic, underlined) mg/100 g reconstituted

Table 4. Results for SPIFAN 2 sample kit[a]

	SPIFAN II SLV kit product																		
Analyte	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
His	41.2	45.3	126.5	*195.8*	27.0	34.6	22.2	31.9	35.5	27.2	44.9	37.9	44.2	28.0	25.6	33.6	28.0	129.0	*189.0*
Tau[b]	NA	NA	NA	NA	NA	**4.1**[c]	**4.7**[c]	5.2	**3.5**	**4.7**	NA	8.1	**4.0**	**3.8**	**4.7**	6.4	**4.7**	17.5	20.1
Ser	98.2	62.3	*307.8*	*448.8*	82.3	78.4	71.6	73.3	87.5	65.9	71.0	88.9	57.9	66.8	66.3	75.2	79.3	*312.0*	*461.8*
Arg	63.6	105.4	*709.3*	*244.0*	35.8	43.3	29.8	94.4	46.3	35.2	87.2	59.3	103.8	36.8	34.1	100.7	36.0	*707.5*	*237.0*
Gly	36.3	47.3	106.0	141.8	28.0	27.0	24.7	56.5	29.9	26.6	*153.3*	35.4	48.8	25.2	22.5	58.3	28.0	106.3	141.0
Asp	*158.1*	*198.1*	*503.0*	*581.3*	*155.8*	126.2	*159.4*	*173.4*	147.9	121.4	143.5	147.1	*195.9*	127.3	113.9	*173.5*	145.8	*491.8*	*583.3*
Glu	*363.5*	*244.0*	*1237.8*	*1789.0*	*302.5*	*305.4*	*241.5*	*279.1*	*334.3*	*209.8*	*243.4*	*334.9*	*239.9*	*248.5*	*244.4*	*281.4*	*287.8*	*1205.8*	*1785.5*
Thr	79.6	79.7	*252.5*	*326.5*	87.0	70.3	93.1	52.8	79.1	61.1	69.5	75.4	78.9	65.7	66.3	54.0	85.8	*252.8*	*328.5*
Ala	64.3	64.6	*218.8*	*240.8*	65.3	52.2	69.5	61.9	61.3	40.6	89.5	60.4	62.5	53.1	49.7	61.5	62.3	*214.8*	*239.5*
Pro	146.4	61.4	*519.0*	*840.5*	116.3	129.8	77.6	70.4	134.7	79.0	129.2	136.2	60.8	93.1	97.0	72.1	113.5	*510.8*	*835.8*
Lys	143.4	119.8	*491.0*	*594.8*	121.0	120.8	131.6	89.3	141.4	100.4	98.0	132.0	111.6	112.6	100.2	87.5	115.5	*484.8*	*597.8*
Tyr	75.6	93.2	*267.5*	*398.0*	55.8	64.3	37.1	49.7	68.7	57.3	69.1	72.1	95.6	52.7	49.9	52.4	56.3	*271.3*	*403.0*
xCys	17.7	22.4	59.3	26.9	23.3	14.6	33.5	16.4	20.4	24.9	16.8	17.6	23.9	23.5	18.4	16.1	22.8	56.5	25.3
Met	38.1	41.3	138.8	*196.3*	31.5	51.0	25.6	34.1	36.6	22.3	37.3	36.0	43.0	29.6	28.6	41.4	32.0	141.5	*199.5*
Val	96.9	141.4	*326.0*	*489.4*	81.8	90.8	69.0	56.5	88.9	64.9	98.4	90.7	142.5	74.1	68.8	62.3	88.3	*324.0*	*477.8*
Ile	84.6	122.8	*274.3*	*380.9*	76.8	79.7	74.4	56.0	78.5	59.2	86.8	80.5	124.6	68.8	63.4	62.6	84.8	*270.0*	*372.3*
Leu	*164.6*	*203.3*	*584.3*	*724.8*	*150.3*	139.9	138.3	109.0	*157.5*	111.6	140.6	*155.5*	*200.7*	133.3	120.6	111.3	148.8	*574.5*	*723.0*
Phe	79.2	103.1	*258.8*	*397.3*	59.3	65.5	42.0	69.0	68.9	49.4	78.0	75.5	104.9	54.7	51.1	74.1	58.8	*260.5*	*388.3*

[a] Average values of the SPIFAN II SLV kit products in mg/100 g reconstituted product.

[b] NA = samples did not have detectable levels of taurine as they were not fortified

Note: 0.5–5.0 (bold, underlined), 5.0–150 (normal), and 150–2500 (italic, underlined) mg/100 g reconstituted product.

Table 5. Converted pmol/µL (concentration of the derivatized sample) to mg/100 g reconstituted product

	MW	0.25 pmol/µL	50 pmol/µL
Ala	89.10	2.0	405.0
Arg	174.20	4.0	791.8
Asp	133.11	3.0	605.0
XCys	240.30	5.5	218.5
Glu	147.13	3.3	668.8
Gly	75.07	1.7	341.2
His	155.16	3.5	705.3
Ile	131.18	3.0	596.3
Leu	131.18	3.0	596.3
Lys	146.19	3.3	664.5
Met	149.21	3.4	678.2
Phe	165.19	3.8	750.9
Pro	115.13	2.6	523.3
Ser	105.09	2.4	477.7
Tau	125.15	2.8	568.9
Thr	119.12	2.7	541.5
Tyr	181.19	4.1	823.6
Val	117.15	2.7	532.5
		mg/100 g rec.pr.	

Table 6. SPIFAN II SLV kit

No.	Description
	Placebo products
1	Child Formula Powder
2	Infant Elemental Powder
3	Adult Nutritional RTF, High Protein
4	Adult Nutritional RTF, High Fat
5	Infant Formula RTF, Milk Based
	Fortified products
6	SRM 1849a
7	Infant Formula Powder Partially Hydrolyzed Milk Based
8	Infant Formula Powder Partially Hydrolyzed Soy Based
9	Toddler Formula Powder Milk-Based
10	Infant Formula Powder Milk-Based
11	Adult Nutritional Powder Low Fat
12	Child Formula Powder
13	Infant Elemental Powder
14	Infant Formula Powder FOS/GOS Based
15	Infant Formula Powder Milk Based
16	Infant Formula Powder Soy Based
17	Infant Formula RTF Milk Based
18	Adult Nutritional RTF High Protein
19	Adult Nutritional RTF High Fat

product). Six samples did not have detectable levels of taurine as they were not fortified and are listed as NA.

Repeatability

Repeatability results (based on robust statistics) are detailed in Table 7 for each analyte/matrix pair. Values above SMPR values are highlighted in grey.

From Table 7, 99.7% of the measured values are within the requirements.

Intermediate Reproducibility

Intermediate reproducibility results (based on robust statistics) are detailed in Table 8 for each analyte/matrix pair. Values above SMPR values are highlighted in grey.

From Table 8, 79.8% of the values are within the requirements. Valine and Isoleucine show high CV_{iR}. This is most likely because those amino acids are released slower than the others upon hydrolysis. Prolonging the hydrolysis step would however, negatively affect other amino acids such as serine, tyrosine, threonine, cysteine/cystine and methionine.

Recovery

Spike recovery results (based on robust statistics) are detailed in Table 9 for each analyte/matrix pair. Values above SMPR values are highlighted in grey.

From Table 9, 92.1% of the values are within the requirements.

The last column is the average recovery value for each amino acid across all the SLV kit. Those values are between 93% and 107%.

Finally, recoveries for Product 6 (NIST 1849A) were compared with the reference values from the certificate of analysis (CoA). In addition, recoveries were compared to SRM 1869 (*See* Table 10; CoA in the drafting phase).

Note: Only seven laboratories were part of the data set for SRM 1849a, where 14 labs participated in SRM 1869. The high value for threonine observed with SRM 1849a was not seen with SRM 1869. This could be attributed to the methods used to evaluate 1849a However these details are not available from NIST.

Chromatograms

Example chromatograms for selected concentrations of the calibration curve and SPIFAN II SLV kit products are presented in Appendix I.

Answers to the ERP Comments (March 2017)

The ERP made several comments and suggestions regarding this method during the mid-year AOAC meeting in March 2017. Answers to those questions are provided in this document and also detailed in Appendix J.

Per ERP request, the working group (WG) on amino acids reconvened to review the SMPR and submitted a recommendation to the stakeholder panel for modifications. The stakeholder panel discussed this during the mid-year meeting in 2018 and requested the SMPR ranges to be reviewed once again. The WG held three meetings over May-July. A draft SMPR is scheduled to be reviewed by the stakeholder panel during the 2018 annual meeting. The long discussions on acceptance levels is a testimony to the complexity of each amino acid. The data presented herein correspond to the updated SMPR approved by the stakeholder panel during the 2018 annual meeting. The final

Table 7. Repeatability[a]

Analyte	SPIFAN II SLV kit product																		
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
	%																		
His	0.7	0.4	1.2	3.7	1.9	0.5	0.8	0.9	2.0	1.1	1.4	1.2	3.0	1.2	2.0	1.4	0.0	1.6	0.8
Tau[b]	NA	NA	NA	NA	NA	1.2	1.4	1.8	2.3	1.3	NA	1.0	1.9	1.7	2.8	1.8	1.6	2.2	2.5
Ser	0.7	0.6	0.2	0.7	0.6	1.0	1.0	0.7	1.0	0.8	0.4	0.8	2.9	1.9	2.0	0.5	0.7	1.5	1.5
Arg	0.5	0.4	0.8	1.9	1.5	1.3	1.4	1.1	1.5	1.3	1.9	1.6	1.7	1.7	1.2	1.3	1.5	1.6	2.0
Gly	0.6	1.1	1.0	1.7	1.9	0.4	1.2	1.2	1.4	0.4	1.0	1.0	1.2	0.7	0.5	0.5	0.0	1.0	1.5
Asp	0.9	1.5	1.4	2.3	0.7	0.5	0.4	0.7	0.8	1.2	0.9	0.4	1.7	0.6	1.8	0.5	0.7	0.6	1.1
Glu	0.6	0.6	0.6	2.0	1.0	0.5	0.3	0.8	0.6	0.7	0.5	0.6	1.4	0.4	0.5	0.7	0.5	0.6	1.6
Thr	0.3	0.6	0.4	0.6	0.6	0.7	0.8	0.7	0.7	1.1	0.4	0.5	1.6	1.2	1.3	0.4	0.6	1.0	1.3
Ala	0.5	0.9	1.2	1.7	0.8	0.7	0.3	0.8	0.7	0.7	0.4	0.5	1.6	0.4	0.5	0.3	0.8	0.7	1.3
Pro	0.4	0.3	0.5	1.4	0.9	0.8	0.5	1.3	0.6	0.7	0.8	0.4	1.2	0.6	0.7	0.4	0.0	0.8	1.0
Lys	1.0	0.6	0.5	3.0	0.9	1.0	0.7	1.4	1.2	0.5	1.0	1.3	1.9	0.9	0.9	0.7	0.9	0.9	1.5
Tyr	0.5	1.6	1.6	1.8	0.9	0.8	0.6	1.5	1.5	1.7	1.3	0.6	2.4	0.3	1.0	1.3	1.9	1.2	1.0
xCys	1.0	0.5	0.9	3.1	0.0	1.6	1.2	1.4	1.1	2.1	1.7	1.3	3.9	0.7	0.6	2.9	0.0	0.0	2.1
Met	0.3	0.7	0.8	1.6	1.7	1.5	1.4	1.0	1.1	1.0	1.1	1.3	0.7	0.8	0.6	1.0	0.0	1.5	2.1
Val	2.0	0.3	1.1	1.5	2.6	0.8	1.2	1.3	2.0	1.7	0.7	0.8	0.9	1.8	1.6	3.4	1.8	1.9	1.1
Ile	1.7	0.3	1.3	1.9	3.4	0.9	0.7	1.1	2.2	2.1	0.7	0.8	1.2	2.2	2.3	3.2	2.5	2.3	1.7
Leu	0.5	0.3	0.5	1.4	1.0	0.9	0.5	0.7	1.2	0.5	0.5	0.5	1.0	0.6	0.7	0.9	0.4	0.8	1.0
Phe	0.7	0.7	1.2	2.0	1.8	0.6	1.1	1.0	1.9	1.1	0.8	1.1	1.2	0.5	1.1	0.7	1.8	0.8	0.9

[a] Repeatability data, values above performance requirements are in grey.

[b] NA = samples did not have detectable levels of taurine as they were not fortified

Table 8. Intermediate reproducibility[a]

Analyte	SPIFAN II SLV kit product																		
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
	%																		
His	4.9	7.8	4.6	11.0	6.8	4.7	7.5	7.6	9.2	5.6	7.6	6.4	3.7	6.3	12.0	7.5	4.3	7.5	9.3
Tau[b]	NA	NA	NA	NA	NA	2.3	4.2	4.2	10.2	2.2	NA	6.3	7.1	3.7	7.7	6.6	6.2	3.9	4.4
Ser	7.4	10.6	6.9	5.7	5.8	9.0	4.2	5.3	2.3	2.4	3.5	7.4	6.3	3.0	5.0	7.9	5.3	8.8	9.7
Arg	3.7	2.1	2.0	7.9	7.6	2.7	6.4	3.0	8.1	6.4	5.1	8.2	4.3	6.4	8.0	8.1	2.0	6.6	7.2
Gly	3.0	3.9	2.6	5.5	2.5	1.0	1.9	3.1	4.3	5.0	5.0	7.2	6.4	3.7	4.1	9.4	0.0	6.2	6.0
Asp	2.0	3.4	1.7	2.1	4.2	3.3	4.2	3.2	4.3	3.3	1.1	4.3	2.3	1.2	6.8	5.2	3.9	1.8	3.8
Glu	1.4	3.7	1.7	2.0	3.2	1.2	3.8	2.3	3.0	2.9	2.2	4.4	3.5	3.2	5.6	5.1	3.1	1.5	4.5
Thr	3.1	2.1	2.6	3.9	3.1	2.7	1.3	1.6	3.6	1.9	0.8	4.8	2.8	0.9	2.6	5.4	2.5	4.9	5.1
Ala	2.0	1.3	3.5	3.4	3.7	1.3	4.3	3.4	3.5	2.7	1.9	5.3	4.0	2.3	4.6	4.5	2.5	4.2	5.6
Pro	0.6	2.8	1.8	2.4	1.9	1.1	1.4	2.1	4.2	3.2	3.0	4.0	2.3	2.7	3.8	5.5	2.1	4.6	5.3
Lys	1.4	3.2	1.6	2.8	3.5	3.0	7.4	7.6	2.3	3.9	3.9	3.4	3.9	4.9	11.3	8.9	6.2	5.1	3.1
Tyr	4.3	5.5	2.5	6.2	3.8	2.9	5.2	5.6	5.8	2.5	6.1	4.3	3.7	1.8	7.1	9.2	4.4	4.9	7.4
xCys	4.2	10.6	5.1	3.8	5.1	3.4	3.9	5.2	6.5	2.4	3.4	3.5	10.5	3.7	8.1	8.1	5.2	2.6	6.1
Met	5.7	3.4	1.4	9.1	7.7	2.3	2.1	4.1	4.1	1.9	1.8	1.4	3.3	2.5	4.0	6.3	1.9	6.8	7.2
Val	10.0	0.8	9.4	5.5	13.3	6.7	9.4	13.8	13.9	7.4	4.3	11.9	2.5	6.1	12.8	15.0	6.5	11.4	12.2
Ile	10.2	0.5	9.6	8.3	15.7	8.6	10.8	12.6	14.5	8.8	7.9	13.7	3.5	8.0	13.7	14.1	6.2	11.8	12.1
Leu	1.8	3.7	2.4	2.3	1.8	1.3	2.7	2.6	5.2	4.4	3.4	0.9	3.1	2.3	6.2	7.9	1.8	4.3	7.5
Phe	5.5	5.2	4.0	5.2	3.3	2.5	8.3	6.4	5.5	6.1	5.2	5.5	5.8	4.4	9.4	8.5	3.3	3.7	5.8

[a] Intermediate reproducibility data, values above performance requirements are in grey. These are components with historically known hydrolysis concerns.

[b] NA = samples did not have detectable levels of taurine as they were not fortified.

Table 9. Recovery (spikes)

	SPIFAN II SLV kit product																			
Analyte	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	Avg.
	%																			
His	98	100	99	90	100	93	100	109	95	103	95	102	96	103	98	97	98	99	97	98
Tau	100	101	99	94	96	97	97	98	96	100	96	110	100	99	98	98	99	100	99	99
Ser	109	103	97	91	80	104	93	95	98	95	93	102	93	94	97	96	92	96	99	96
Arg	97	98	101	91	92	95	92	93	93	92	94	104	90	93	94	97	96	100	96	95
Gly	100	99	100	94	98	98	98	97	97	104	98	107	106	102	97	98	101	99	99	100
Asp	113	110	103	101	108	105	105	108	114	107	104	112	109	109	107	104	105	102	106	107
Glu	107	104	106	94	104	103	99	102	110	104	100	110	103	104	101	100	101	99	98	103
Thr	100	99	99	92	90	100	95	96	97	97	95	105	97	97	97	97	96	97	98	97
Ala	102	102	102	96	100	100	98	101	104	102	98	108	102	102	100	99	100	100	99	101
Pro	101	100	104	94	97	100	96	99	101	99	94	106	99	99	98	97	99	99	97	99
Lys	108	106	107	99	100	101	97	106	113	101	97	104	102	102	103	102	104	104	100	103
Tyr	100	100	98	94	93	96	93	94	96	98	94	102	96	97	97	97	97	99	97	97
xCys	102	103	103	94	96	99	97	100	100	101	102	101	99	100	101	100	102	96	97	100
Met	89	90	95	90	90	95	90	88	90	96	90	102	94	95	93	94	93	94	91	93
Val	94	101	100	85	102	92	94	98	100	98	95	108	101	99	98	97	100	103	93	98
Ile	94	101	100	87	101	90	94	98	99	101	96	108	100	100	98	98	100	102	95	98
Leu	100	101	102	91	99	97	96	99	102	100	96	108	102	100	99	98	100	102	96	99
Phe	100	101	100	93	98	97	97	96	96	100	96	108	102	100	99	98	99	101	97	99

Table 10. Recovery

	NIST SRM 1849a		NIST SRM 1869
		Recovery vs. SRM COA	
Analyte	Spike recovery,	Recovery vs. CoA,	Recovery vs. CoA,
His	93	100	102
Tau	97	100	97
Ser	104	97	103
Arg	95	97	95
Gly	98	101	101
Asp	105	106	105
Glu	103	106	101
Thr	100	124	99
Ala	100	103	102
Pro	100	98	99
Lys	101	108	109
Tyr	96	113	110
xCys	99	102	99
Met	95	NA	94
Val	92	108	100
Ile	90	109	100
Leu	97	100	100
Phe	97	102	101

stakeholder approval was at the AOAC March 2019 mid-year meeting.

SLV Author Conclusion

Method performance versus requirements (SMPR AOAC 2014.013):

Analytical range.—Criteria = 0.5–2500 mg/100g in ready-to-feed form. This method has a range of 2.5–2500+ (with dilution). No samples were found to have amounts below 18 mg/100 g (3 mg/100 g Taurine). The method meets the needs of the infant formula/adult nutrition matrices.

Limit of quantitation (LOQ).—Criteria = ≤0.5 mg/100 g in ready-to-feed form. This method has an LOQ of 2.5. However, no samples (even unfortified) were found to have amino acid levels below 18 mg/100 g (3 mg/100 g Taurine). The method meets the needs of the infant formula/adult nutrition matrices.

Repeatability (RSD_r).—Criteria = 0.5–5.0 mg/100g ≤7%; 5.0–150 ≤5%; 150–2500 ≤3% in ready-to-feed form. This method met the requirements in 99.7% of the cases. The only exceeding value was an RSD_r of 3.7%.

Reproducibility (RSD_R).—Criteria = 0.5–5.0 mg/100 g ≤11%; 5.0–150 ≤8%; 150–2500 ≤5% in ready-to-feed form. The data presented in this report is as (RSD_{iR}) intermediate reproducibility. The actual method reproducibility is to be determined during an MLT study.

This method met the requirements in 79.8% of the cases. Of note, over 4000 data points are included in this calculation. Additionally, the reactive impacts of hydrolysis to any and all of the amino acids. Of greater concern are serine and threonine and of course total loss of tryptophan under acidic conditions. Additionally, valine and isoleucine are found to have better hydrolysis at >24 hr and >110°C. The method presented here represents a compromise for the best simultaneous hydrolysis of all amino acids (except tryptophan).

System suitability.—Response for mid-point calibration passes <2% RSD for all analytes. Additionally, an ERP requested example is provided in Appendix covering resolution of methionine.

Reference material accuracy.—Method accuracy was proven by analysis of both Reference Material (SRM 1849a and 1869) as well as by recovery rates within 90–110 %.

The results prove that the method is a good candidate for further validation by Multi Laboratory Testing in order to grant Final Action status. A multilaboratory Testing will take place. with Greg Jaudzems as Study Director.

Appendices

Appendix A.—Average values of the SPIFAN II SLV kit products in mg/100 g reconstituted product.

Appendix B.—Average values of the SPIFAN II SLV kit products in mg/100 g product.

Appendix C.—Repeatability performance requirements for each analyte/sample pair according to the concentration of the analyte in the reconstituted product.

Appendix D.—Repeatability data obtained with the SPIFAN II SLV kit products, color-coded with respect to performance requirement values.

Appendix E.—Reproducibility performance requirements for each analyte/sample pair according to the concentration of the analyte in the reconstituted product.

Appendix F.—Intermediate reproducibility data obtained with the SPIFAN II SLV kit products, color-coded with respect to reproducibility requirement values.

Appendix G.—Recovery performance requirements for each analyte/sample pair according to the concentration of the analyte in the reconstituted product.

Appendix H.—Recovery data obtained with the SPIFAN II SLV kit products, color-coded with respect to performance requirement values.

Appendix I.—Example chromatograms for selected concentrations of the calibration curve and SPIFAN II SLV kit products.

Appendix J.—Additional information requested by AOAC SPIFAN ERP during mid-year AOAC meeting (March 2017).

References

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(2) Waters UPLC Amino Acid Analysis Solution System Guide, 71500129702 - Revision B Waters Corporation 2007. https://www.waters.com/webassets/cms/support/docs/71500129702rb.pdf (accessed on June 2019)

(3) Jacobs, W., & Feng, P., et al. (2015) *J. AOAC Int.* **98**, 1073–1076