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PRACTITIONER'S REPORT

Method validation for determination of amino acids in feed by UPLC

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Abstract The paper presents results of validation of an analytical procedure based on ultra-performance liquid chromatography technique (UPLC) for determination of 17 amino acids in different feeds. The following performance characteristics were determined for the investigated feeds: relative standard deviations of repeatability and intermediate precisions ranged from 0.4 % to 4.6 %, and from 0.8 % to 7.9 %, respectively; recovery rates ranged from 87 % to 104 %, and limit of detection was from 0.06 g kg^{-1} (methionine) to 0.72 g kg^{-1} (glutamic acid). Two approaches were used to estimate measurement uncertainty giving values in a range of 5.1 % to 5.5 %. These performance characteristics are in agreement with the values reported in Commission Regulation (EU) No 152/2009 for the ion exchange chromatography with spectrophotometric detection.

Keywords Feed · Amino acid analysis · UPLC · Validation parameters · Uncertainty

Introduction

Proteins, the basic component of living organisms, consist of amino acids (AAs). Exogenic amino acids, namely arginine (Arg), phenylalanine (Phe), histidine (His), isoleucine (Ile), leucine (Leu), lysine (Lys), methionine (Met), threonine (Thr), tryptophan (Trp) and valine (Val), are not synthesized by the organism and must be provided in the diet in order to cover the requirement. The remaining AAs, called endogenic AAs, such as alanine (Ala), cystine (Cys), aspartic acid (Asp), glutamic acid (Glu), proline (Pro), serine (Ser), tyrosine (Tyr) and glycine (Gly), are synthesized by the organism.

Certain exogenic AAs, Lys, Met, Thr and Trp have been used in animal feeding for a long time as feed additives. Recently, more AAs have been authorized as feed additives, including Arg, Val, Leu and His. The regulations include requirements regarding the obligatory declaration of the content of Lys and Met on the label of a feed mixture as its analytical constituents; the remaining amino acids added are usually declared on the label of a feed mixture as feed additives [1].

The recipes of feed mixtures are optimized regarding the requirement for AAs, depending on the animal species, its age and productivity. Therefore, it is necessary to have a reliable and validated method for determination of AAs in feed materials and feed mixtures. The classical method of AAs analysis is based on ion exchange chromatography with spectrophotometric detection (IEC-Vis) in which AAs are separated on the chromatographic column and subjected to post-column derivation with ninhydrin [2]. IEC-Vis is recommended for the internal control and the official feed control in numerous countries [3, 4]. The method makes it possible to examine all amino acids with the exception of Trp which is determined by high-performance liquid chromatography method (HPLC) with fluorometric detection [3].

Commission Regulation (EU) No 152/2009 accepts the use of the HPLC methods beside of the IEC-Vis method for determination of AAs in feed; however, these methods

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should be validated and validation parameters should comply with those obtained by the official IEC-Vis method [3].

A number of different HPLC methods have been developed for determination of amino acids in animal feeds [5], honey [6], acacia seeds [7], pharmaceutical products [8], rumen fluid [10], food and feed [12]. Unlike the official IEC-Vis method [3, 4], these methods usually include pre-column derivatization on following acid hydrolysis with the use of *ortho*-phthaldialdehyde (OPA) [5, 8], 9-fluorenylmethyl chloroformate (FMOC) [5], phenyl isothiocyanate (PITC) [6]. According to Liu et al. [12], the use of 6 aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) as a pre-column derivatization reagent is advantageous because AQC reacts quickly, without considerable matrix interferences, and forms stable derivatives with primary and secondary AAs. Moreover, the use of ultra-performance liquid chromatography (UPLC) allows rapid (16 min) and effective separation of the investigated AAs in protein-rich food and feed [9]. In comparison, the time of the classical chromatographic analysis of AAs (total and sulphur) is about 4 h, following hydrolysis that usually takes 16 h to 23 h [3, 4].

This paper presents the validation study of the analytical procedure for the determination of 17 AAs, including sulphur AAs (Met and Cys) but (excluding tryptophan) in feed, based on UPLC technique with AQC for pre-column derivatization. Three types of feeds of varied AAs content, two types of feed materials, rye and pork haemoglobin, and a feed mixture were investigated. Validation parameters of the procedure were determined, including the sample preparation step [22], as well as measurement uncertainty in accordance with the requirements of the ISO/IEC 17025:2005 standard [13]. The validation parameters obtained for the analytical procedure based on the UPLC technique are compared with the parameters of the official method recommended by the Regulation 152/2009 [3].

Materials and methods

Chemicals and reagents

The standard solution of amino acids (AAs) mixture, which contained His, Ser, Arg, Gly, Asp, Glu, Thr, Ala, Pro, Lys, Tyr, Val, Ile, Leu, Phe and Met in the same concentration of 2.5 mmol L^{-1} and Cys in the concentration of 1.25 mmol L^{-1}, DL-2-aminobutyric acid (internal standard IS), hydrobromic acid and formic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Reagents for pre-column derivatization of amino acids, borate buffer (reagent 1), AQC (reagent 2A), acetonitrile (reagent 2B) and acetonitrile of gradient purity for HPLC were purchased as the AccQ-Tag reagent kit from Waters (Milford, MA, USA). Also other reagents, concentrated AccQ-Tag Ultra Eluent A (ammonium formate 84 %, formic acid 6 %, acetonitrile 10 %, by volume), AccQ-Tag Ultra Eluent B (acetonitrile with addition 2 % of formic acid, by volume) were obtained from Waters. Deionized water (DW) was purified using a Milli-Q system (Millipore, Bedford, MA, USA).

Apparatus

Protein hydrolysis of analysed feed samples was performed in a drying oven with temperature control (Merazet, Poznan, Poland). The ACQUITY UPLC system (Waters, Milford, MA, USA) consisting of thermostat, autosampler, high-pressure binary pump and photodiode array detector PDA (an optical detector in the range ultraviolet–visible light that operates between 190 nm and 700 nm) was used for the analysis of 17 AAs. Chromatographic separation was obtained with the AccQ-Tag Ultra C-18 column (2.1 mm×100 mm; 1.7 μm).

Preparation of standard solutions

Internal standard solution of DL-2-aminobutyric acid at the concentration of 0.4 mg mL^{-1} was prepared by dilution with 0.1 mol L^{-1} hydrochloric acid. True concentration of the internal standard was calculated taking into account analytical portion and purity.

Working standard solution of AAs with internal standard was prepared by transferring 400 μL aliquot of the AAs standard solution and 250 μL aliquot of the internal standard solution into 10-mL volumetric flask and filling up with DW to the mark. The concentration of each AA in the working standard solution was 0.1 μmol mL^{-1} and for Cys was 0.05 μmol mL^{-1}. The concentration of each AA in mg mL^{-1} should be expressed to the nearest 0.00001 mg mL^{-1}. The concentration of internal standard in this solution was about 0.01 mg mL^{-1}.

Preparation of performic acid

At first, hydrogen peroxide and formic acid were placed separately into a boiling water bath for 7 min. These pre-heated constituents were then mixed (1:9, by volume) and placed in a water bath at 80 °C for 3 min to obtain performic acid. The mixture was finally cooled to ambient temperature before further use.

Analytical procedure

The flowchart of analytical procedure for neutral and acidic AAs and sulphur AAs is presented in Fig. 1. Sample

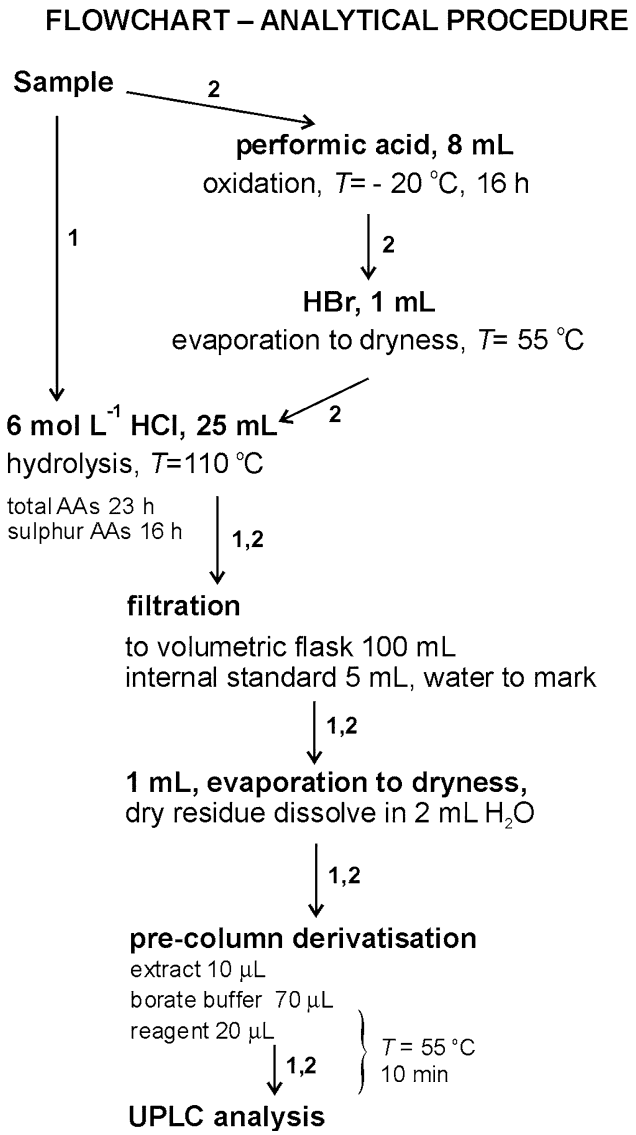

Fig. 1 Flowchart—overview of analytical procedure for neutral and acidic AAs (*1*) and sulphur AAs (*2*)

hydrolysis was based on the AOAC Official Method 994.12 [14] with some modifications (see Fig. 1, way 1). Analytical portions should contain about 35 mg of protein for all tested AAs. Hydrolysis for sulphur AAs Met and Cys was performed after sample oxidation with performic acid (see Fig. 1, way 2).

The chromatographic separation of AAs was performed on the AccQ-Tag Ultra column. Detection of AAs was carried out with the use of the optical PDA detector, with the wavelength of 260 nm. The mobile phase consisted of two eluents, A and B. The separation was carried out for 12 min in the gradient elution (see Fig. 2) with a flow rate of 0.6 mL/min. The injection

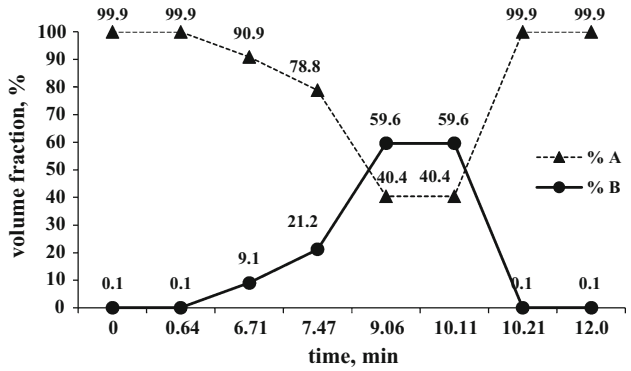

Fig. 2 Gradient eluent programme during chromatographic separation of AAs derivatized by AQC; eluent A—ammonium formate 84 %, formic acid 6 %, acetonitrile 10 %, by volume; eluent B—acetonitrile 98 %, formic acid 2 %, by volume; A, B volume fraction before mixing

volume was 1 µL, and column temperature was 55 °C (see Fig. 2).

Result calculation

The identification of AAs was performed by the comparison of the retention time of the AA peaks in the sample extract with peaks in the standard solution. The content of the amino acid in the examined sample was calculated using the internal standard and the Empower software (Waters, Milford, MA, USA). The amino acid mass fraction (w), the response factor (R) and the calibration ratio (r_{st}) were calculated as follows:

$$w = \frac{R \cdot D}{r_{st} \cdot m} \quad (1)$$

where D is the dilution factor ($D = V \cdot f$, where V is the volume of sample hydrolysate in mL; f is the coefficient of dilution, which is the reciprocal of a part of hydrolysate (in mL) taken to analysis) and m is the mass of the analytical portion, in grams.

$$R = \frac{P_{AAs}}{P_{ISs}} \cdot c_{ISs} \quad (2)$$

where P_{AAs} is a peak area of analysed AA in a sample; P_{ISs} is a peak area of IS in a sample; and c_{ISs} is a concentration of IS in a sample, in mg mL^{-1}.

$$r_{st} = \frac{P_{AAst}}{P_{ISst}} \cdot \frac{c_{ISst}}{c_{AAst}} \quad (3)$$

where P_{AAst} is a peak area of AA in the standard solution; P_{ISst} is a peak area of IS in the standard solution; c_{ISst} is a concentration of IS in the standard solution, in mg mL^{-1}; and c_{AAst} is a concentration of AA in the standard solution, in mg mL^{-1}.

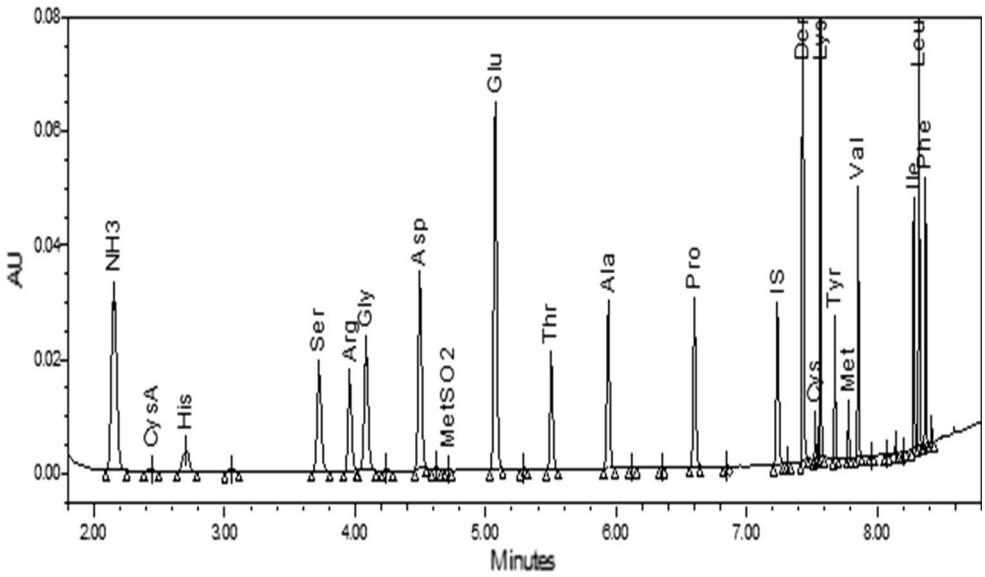

Fig. 3 Chromatogram of neutral and acidic AAs extract from feed mixture

Sulphur AA contents, Met and Cys, were calculated in the same way as neutral and acidic AAs. Therefore, regarding the sulphur AAs the calibration ratio r_{st} was corrected by dividing it, in the case of Met, by the ratio of the methionine sulphone molar mass to the Met molar mass (r_{st}/1.21) and, in the case of Cys, by the ratio of the cysteic acid molar mass to the cysteine molar mass (r_{st}/0.7).

Feed samples were divided and ground according to EN ISO 6498 [21]. Validation of the multianalyte procedure included evaluation of the performance parameters such as limit of detection (*LOD*), limit of quantification (*LOQ*), precision (repeatability and within-laboratory reproducibility), recovery and uncertainty [15–18]. *LOD* was calculated as three times of standard deviation ($LOD = 3s$) of relatively low concentration of AAs determined in rye sample and *LOQ* as six times of the standard deviation ($LOQ = 6s$, $n = 8$). Results of uncertainty calculated according to GUM [16] were compared to

Table 1 Performance characteristics of AAs determination by UPLC method

Amino acid	*LOD* (g kg^{-1})	RSD_r (%)	RSD_{Rw} (%)	Recovery (%)	Reg. 152/2009 [3]	
					RSD_r (%)	RSD_R (%)
His	0.18	1.3–3.1	3.5–5.1	94.2–98.9		
Ser	0.30	0.8–2.4	1.1–2.6	95.7–104.4		
Arg	0.57	0.4–4.6	2.8–5.2	98.6–101.9		
Gly	0.42	0.8–3.5	1.6–4.0	92.9–99.7		
Asp	0.33	1.1–1.8	1.6–2.8	96.6–100.2		
Glu	0.72	0.8–1.3	1.3–1.7	93.5–100.4		
Thr	0.18	0.6–1.9	1.1–2.3	98.2–103.6	1.9–2.1	4.1–5.2
Ala	0.12	0.7–1.2	1.2–1.9	99.2–101.2		
Pro	0.54	1.2–2.1	1.3–2.3	100.6–103.0		
Lys	0.21	1.5–2.2	2.6–3.2	100.2–102.1	2.1–2.8	3.2–5.4
Tyr	0.24	3.0–4.4	3.2–7.9	95.1–102.3		
Val	0.21	1.6–2.1	1.8–2.5	97.3–100.9		
Ile	0.15	1.6–3.5	2.2–5.2	93.1–101.2		
Leu	0.27	0.5–1.7	0.8–2.6	101.2–103.6		
Phe	0.51	1.4–4.3	2.9–5.1	96.6–98.9		
Cys	0.15	1.5–3.7	4.0–5.7	93.0–97.1	2.8–3.3	8.8–9.9
Met	0.06	0.8–1.9	2.2–6.8	87.1–99.3	3.1–3.4	7.0–10.9
Total range		0.4–4.6	0.8–7.9	87.1–104.4		

RSD_r relative standard deviation of precision, RSD_{Rw} relative standard deviation of within-laboratory reproducibility, RSD_R relative standard deviation of reproducibility

Table 2 Comparison of uncertainty results for 17 AAs in feed mixtures calculated according to three different approaches

Amino acid	w (g kg^{-1})	GUM			Eurolab TR [19, 20]			Horwitz [23]
		$u(RSD_{Rw})$ [%]	u(rec) [%]	$u(w)$ [%]	RSD_{Rw} [%]	b [%]	$u(w)$ [%]	$u(w)$ [%]
His	6.71	2.5	4.7	5.3	3.6	7.5	8.3	4.2
Ser	13.5	1.4	5.9	6.1	2.0	4.3	4.7	3.8
Arg	17.2	2.3	5.1	5.6	3.3	3.1	4.5	3.7
Gly	11.2	1.8	1.8	2.5	2.6	2.4	3.6	3.9
Asp	27.3	1.6	6.3	6.5	2.3	4.2	4.7	3.4
Glu	50.0	1.2	6.2	6.3	1.7	5.8	6.0	3.1
Thr	10.7	1.3	6.4	6.5	1.8	3.4	3.8	4.0
Ala	12.1	1.1	7.0	7.1	1.5	4.2	4.5	3.9
Pro	15.1	1.2	3.6	3.8	1.7	3.1	3.5	3.8
Lys	14.5	1.9	2.0	2.8	2.7	3.3	4.2	3.8
Tyr	8.90	3.4	4.7	5.8	4.8	11.4	12.4	4.1
Val	11.8	1.5	3.6	3.9	2.1	5.2	5.6	3.9
Ile	11.0	1.6	3.5	3.8	2.2	6.1	6.5	3.9
Leu	20.8	1.1	5.0	5.1	1.5	2.3	2.8	3.6
Phe	13.3	2.0	4.7	5.1	2.9	4.2	5.1	3.8
Cys	3.40	2.7	3.9	4.7	4.0	5.5	6.7	4.7
Met	5.43	2.0	5.6	5.9	2.8	6.6	7.2	4.4
Total range		1.1-3.4	1.8-7.0	2.5-7.1	1.5-4.8	2.3-11.4	2.8-12.4	3.1-4.7
Average				5.1			5.5	3.9

$u(m_s)/m_s = 0.035$ %; $u(V_s) = 0.04$ %; b—bias

uncertainty calculated on the basis of the practical approach presented in the Eurolab Technical Report No 1/2007 [19] and results obtained in proficiency testings (PTs), organized by Austrian Agency for Health and Food Safety (AGES) [20]. Due to the lack of feed CRM, the accuracy of the developed procedure was checked on the basis of recovery and analyses of feed reference materials obtained from AGES [20].

Results and discussion

Elution profile of 17 AAs derivatized with AQC and separated on the AccQ-Tag Ultra C-18 column is presented in Fig. 3. Good chromatographic separation was obtained for all AAs, including sulphur AAs, which is an advantage of proposed procedure, as not all sulphur AAs and secondary AAs were determined with the use of pre-column derivatization [5, 11]. The validation of analytical procedure for determination of AAs in rye, pork haemoglobin and feed mixture samples converted determination of all parameters required in the feed control area. *LOD* values ranged from 0.06 g kg^{-1} to 0.72 g kg^{-1} (Table 1). Similar *LOD* values were obtained by Jajić et al. [5] during an analysis of 14 AAs in feeds with HPLC method (0.02 g kg^{-1} to 1.30 g kg^{-1}) and by Shim et al. [11] (0.07 g kg^{-1} to 0.59 g kg^{-1}) analysing 16 AAs in infant formula by UPLC method using AQC for pre-column derivatization.

The precision of AAs measurement (repeatability and within-laboratory reproducibility) in investigated rye, pork haemoglobin and feed mixture samples was evaluated using relative standard deviation of replicated measurements ($n = 10$). Repeatability for all AAs in all samples ranged from 0.4 % to 4.6 %. Within-laboratory reproducibility values were higher and ranged from 0.8 % to 7.9 % for the same samples. These results comply with the criteria set for the official control method [3], according to which repeatability for Lys, Thr, Met and Cys in feed mixtures, commonly used as feed additives, should be less than 4 % and reproducibility less than 11 % (Table 1).

As no commercial CRM was available, spiked samples were analysed to evaluate bias. Satisfactory recovery rates were obtained for the various matrices investigated, ranging from 87 % to 104 % (Table 1). These values confirm the bias values observed in the AGES IAG-Feedingstuffs [20] proficiency tests (ranging from 2.3 % to 11 %), (Table 2).

Two approaches were used for the assessment of uncertainty measurements (Table 2). The "bottom-up approach" according to GUM was applied to all the feeds

investigated [16, 17]. The second one, based on interlaboratory data (within-laboratory reproducibility calculated during validation study) and interlaboratory data (bias calculated from PT results), was applied only to feed mixture.

Many sources contribute to the combined standard uncertainty $u(w)$ of a given AA. However, only sources providing the largest contribution such as within-laboratory reproducibility $u(RSD_{Rw})$ and recovery $u(rec)$ were taken into consideration. The contributions of the standard uncertainty for the sample mass $u(m_s)$ and the standard uncertainty for the sample preparation $u(V_s)$ were smaller (Table 2). Obtained standard uncertainties ($k = 1$) of AAs measurement calculated according to this approach ranged for feed mixture from 2.5 % (Gly) to 7.1 % (Ala), 5.1 % on average.

Uncertainties of measurement of AAs in feed mixture according to GUM were compared with the uncertainties calculated on the basis of the Eurolab Technical Report No 1/2007 [19]. Standard uncertainties of AAs measurement in feed mixtures, calculated on the Eurolab approach, ranged from 2.8 % (Leu) to 12.4 % (Tyr), 5.5 % on average. Obtained values were in agreement with uncertainties calculated on the basis of "bottom-up approach" for the feed mixture sample (Table 2, 5.1 % on average), indicating that both approaches gave similar results. Calculation of uncertainty from the Horwitz equation [23, 24] by doubling target standard deviation gave comparable uncertainty results to the GUM approach for rye sample (low levels of AAs); however, in the case of feed mixture (higher levels of AAs) uncertainties calculated from Horwitz equation were lower or equal to those calculated on the basis of the GUM approach.

Conclusion

The performance characteristics of the investigated UPLC method are similar to those of the official IEC-VIS method [3]. UPLC is therefore a suitable method for the determination of 17 AAs in low- and high-protein feed materials, feed mixtures and complementary feed. However, Commission Regulation 152/2009 [3] prescribes for official control the use of ion exchange chromatography after post-column derivatization; hence, the UPLC method presented is an alternative and efficient method.

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References

1. Regulation (EC) No 767/2009 of 13 July 2009 on the placing on the market and use of feed, O.J. L 229, 01.09.2009
2. Moore S, Spackman DH, Stein WH (1958) Anal Chem 30:1185–1190
3. Commission Regulation (EC) No 152/2009 of 27 January 2009 laying down the methods of sampling and analysis for the official control of feed, O.J. L 54, 26.02.2009
4. AOAC Official Method 994.12, Amino Acids in Feeds. Official Methods of Analysis of AOAC Int. 18th Ed, 2005, Rev. 4. 2011, Chapter 4, pp 9–19
5. Jajić I, Kristović S, Glamočić D, Jakšić S, Abramović B (2013) J Serb Chem Soc 78(6):839–850
6. Spano N, Piras I, Ciulu M, Floris I, Panzanelli A, Pilo MI, Piu PC, Sanna G (2009) J AOAC Int 92:1145–1152
7. Falade OS, Adewusi RA (2013) J AOAC Int 96:102–109
8. Bartolomeo MP, Maisano F (2006) J Biomol Tech 17:131–137
9. Meussen BJ, van Zeeland ANT, Bruins ME, Sanders JPM (2014) Food Anal Methods 7:1047–1055
10. Wadud S, Onodera R, Or-Rashid MM, Amin MR (2000) J AOAC Int 83:8–15
11. Shim YS, Yoon WJ, Ha J, Seo D, Lee KW, Lee WY, Kwon KI, Kang TS, Lee JH, Kim HJ, Kwak HJ, Lee SP, Kim SJ, Yun WK, Lee J, Hwang JB (2013) Food Sci Biotechnol 22(6):1567–1571
12. Liu HJ, Chang BJ, Yan HW, Yu FH, Liu XX (1995) J AOAC Int 78:736–744
13. EN ISO/IEC 17025:2005 General requirements for the competence of testing and calibration laboratories
14. AOAC. Official Method of Analysis of AOAC Int. 18th Ed. Method 994.12. Association of Official Analytical Communities, Arlington, VA, USA (2005)
15. Konieczka P, Namieśnik J (2009) Quality assurance and quality control in the analytical chemical laboratory: a practical approach. CRC Press, Boca Raton
16. Guide to the expression of uncertainty in measurement (GUM), ISO Geneva; also JCMG 100, 2008. http://www.bipm.org/en/publications/guides/gum
17. Ellison SLR, Williams A (eds) (2012) Eurachem/CITAC guide: Quantifying uncertainty in analytical measurements. www.eurachem.org
18. Chudzińska M, Dębska A, Barałkiewicz D (2012) Accred Qual Assur 17:65–73
19. EUROLAB Technical Report 1/2007, Measurement uncertainty revisited: Alternative approaches to uncertainty evaluation. http://www.eurolab.org/
20. AGES IAG-Feedingstuffs. http://www.ages.at/en/service/servicies-feed
21. EN ISO 6498:2012 Animal feeding stuffs—Guidelines for sample preparation
22. Korol W, Bielecka G, Rubaj J, Walczyński S (2015) Accred Qual Assur 20:61–66
23. Horwitz W (2003) J AOAC Int 86:109–111
24. Horwitz W, Albert R (2006) J AOAC Int 89:1095–1109