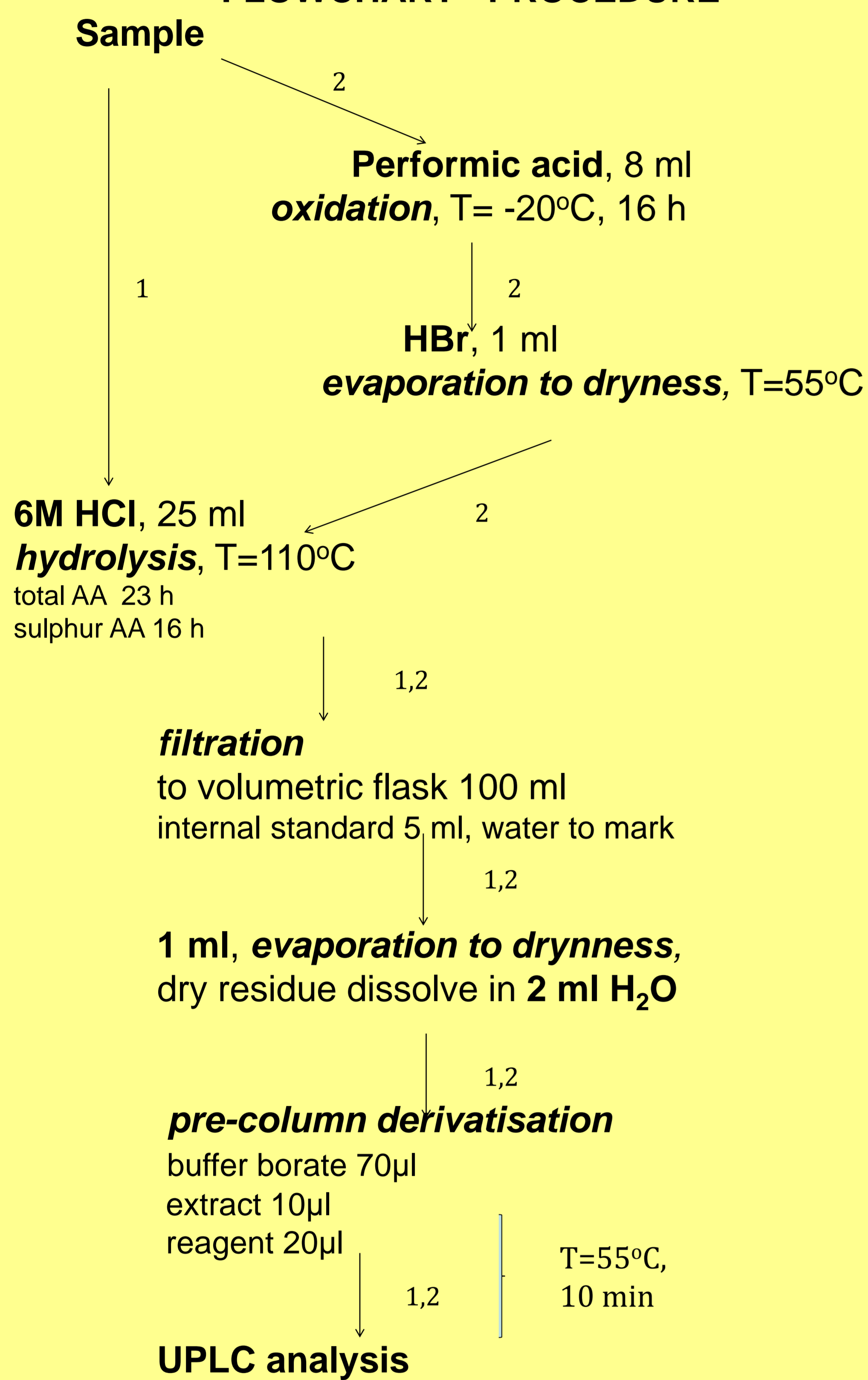




AIM OF THE PAPER

The aim of the presentation was to validate *in house* ultra-performance liquid chromatography (UPLC) method for quantifying of 17 amino acids AA (histidine, serine, arginine, glycine, aspartic acid, glutamic acid, threonine, alanine, proline, lysine, tyrosine, valine, isoleucine, leucine, phenylalanine, cystine, methionine) in feeds and to compare obtained results with performance characteristics of the official ion-exchange chromatographic method published in the regulation (EC) No 152/2009 (2)

FLOWCHART - PROCEDURE



1 – Procedure for total amino acids
2 – Procedure for sulphur amino acids

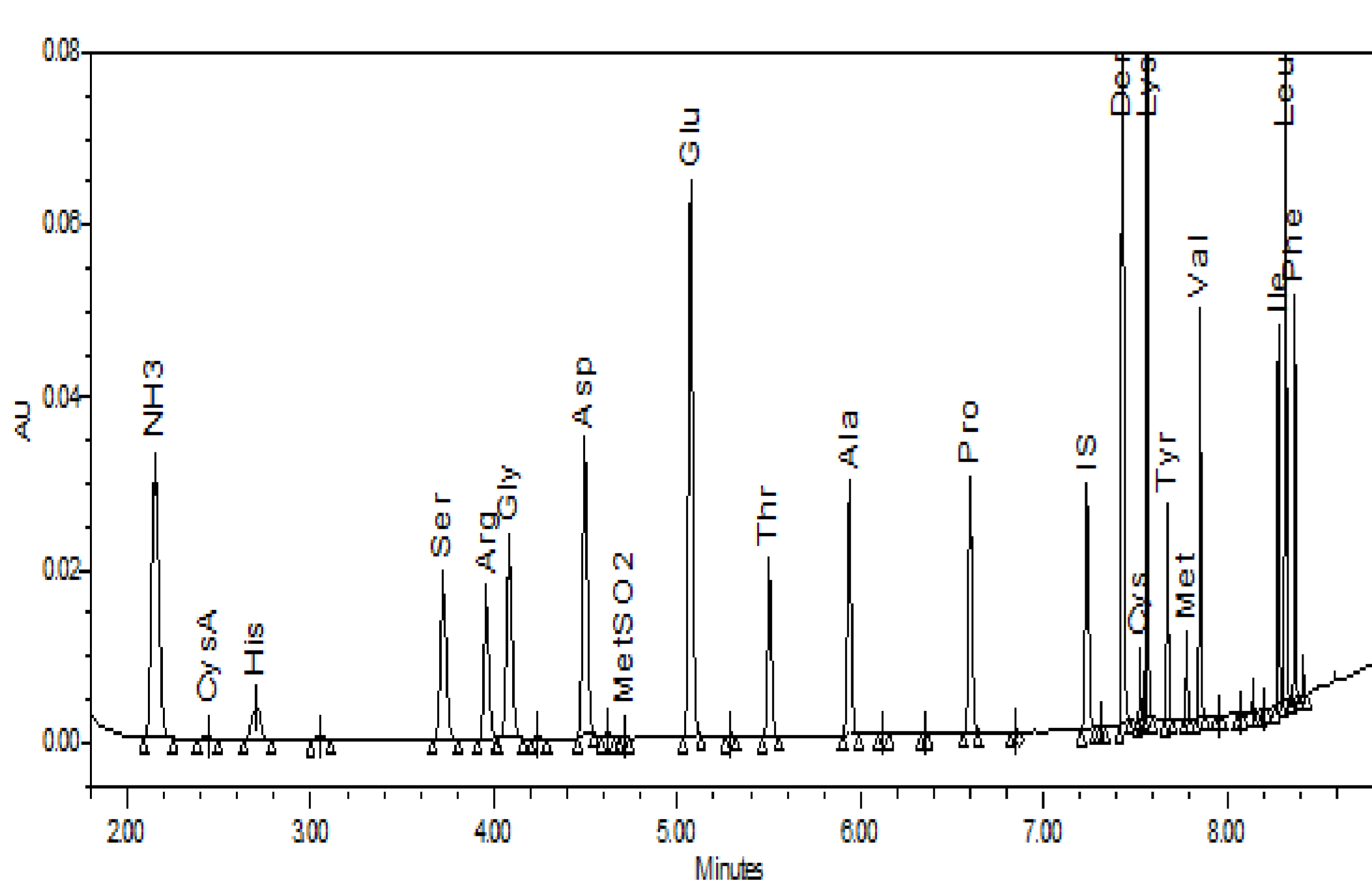


Fig. 1. Chromatogram of the extract from a feed mixture

REFERENCES

1. B. Magnusson, U. Örnemark (eds.) Eurachem Guide: Fitness for Purpose of Analytical Methods – A Laboratory Guide to Method Validation and Related Topics, 2nd ed., 2014
2. Commission Regulation (EC) No 152/2009 – OJ L54/1, 26.2.2009
3. Analytical procedure PB 59 KLP of the National Laboratory for Feedingstuffs - Determination of amino acids in feeds by UPLC – based on the Waters AccQ·Tag. Chemistry Package. Instruction Manual.

PRINCIPLE

Feed samples were hydrolyzed with hydrochloric acid, filtrated, evaporated and dissolved in water with addition of 2-aminobutyric acid as the internal standard. The 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) was used as pre-column derivatizing reagent. Cystine and methionine were oxidized to derivatives – cysteic acid and methioninesulfone, respectively. Products of pre-column derivatisation were analyzed chromatografically with using Waters ACQUITY UPLC system with reversed phase separation occurred on the AccQ·Tag Ultra column and UV detection at 260 nm.

MATERIALS AND METHOD - PERFORMANCE CHARACTERISTICS

During validation following feeds with different content of amino acids were tested: rye grains, feed mixture and porcine hemoglobin. Waters ACQUITY UPLC system with UV detector was used. Separation parameters were generated on AccQ·Tag Ultra C-18 (2,1 x 100 mm; 1,7µm) column with temperature controlled at 55°C and operated with a flow rate of 0,6 ml/min. The gradient elution system was used. Mobile phase was AccQ·Tag Ultra Eluent A and AccQ·Tag Ultra Eluent B (Waters). Detection was performed at 260 nm (3). Following performance characteristics were evaluated during validation study: limit of detection (LOD = 3 x SD); limit of quantification (LOQ = 6 x SD); working range; trueness (bias, recovery); precision (repeatability, intermediate precision); measurement uncertainty (from Horwitz eq. and according to GUM); robustness (1).

RESULTS

The validation parameters for some amino acids (commonly used as feed additives) are summarized in the Table 1. Obtained performance characteristics were satisfactory and generally fit for purpose. Robustness was checked in the scope of derivatisation parameters: time (10-15 min), temperature 55°C±1.0°C and pH 8.8 ±0.5. All investigated parameters were robust in the tested ranges. LOQ for analyzed amino acids were from 0.2 g/kg (for methionine) to 2.2 g/kg (for glutamic acid). The precision parameters were acceptable and generally lower than 5%, like in the case of the official method published in the Regulation (EC) No 152/2009 (2). Recoveries ranged from 87.1% to 102%; 95,3% on average. Uncertainties (expanded, k=2) calculated on the basis of GUM approach were generally lower than calculated from Horwitz eq. (tab. 1). Obtained performance characteristics of the UPLC method were agree with the official method parameters (2), (Table 2)

Table 1. An example of performance characteristics some aminoacids tested by the UPLC method

Amino acid content	Validation parameter	Material			Amino acid content	Validation parameter	Material		
		Rye grains (1)	Feed mixture (2)	Porcine hemoglobin (3)			Rye grains (1)	Feed mixture (2)	Porcine hemoglobin (3)
Lys 1 – 3.09 g/kg 2 – 14.52 g/kg 3 – 71.36 g/kg	Range	0.6 – 77.0 g/kg			Cys 1 – 1.79 g/kg 2 – 3.40 g/kg 3 – 5.69 g/kg	Range	0.5 – 7.0 g/kg		
	LOD	0.3 g/kg				LOD	0.2 g/kg		
	LOQ	0.6 g/kg				LOQ	0.4 g/kg		
	Repeatability	2.2 %	1.5 %	1.7 %		Repeatability	2.6 %	3.7 %	1.5 %
	Inter. precision	3.2 %	2.6 %	2.6 %		Inter. precision	4.2 %	4.0 %	5.7 %
	Recovery	92.8 %	93.2 %	95.4 %		Recovery	95.3 %	96.6 %	93.0 %
Thr 1 – 3.06 g/kg 2 – 10.69 g/kg 3 – 29.67 g/kg	Uncertainty 1	9.6 %	7.6 %	6.0 %	Met 1 – 1.67 g/kg 2 – 5.43 g/kg 3 – 7.31 g/kg	Uncertainty 1	10.4 %	9.4 %	8.6 %
	Uncertainty 2	10.0 %	8.0 %	8.0 %		Uncertainty 2	6.0 %	4.0 %	4.0 %
	Range	0.4 – 31.0 g/kg				Range	0.2 – 9.0 g/kg		
	LOD	0.2 g/kg				LOD	0.1 g/kg		
	LOQ	0.4 g/kg				LOQ	0.2 g/kg		
	Repeatability	1.9 %	1.7 %	0.6 %		Repeatability	1.0 %	0.8 %	1.9 %
Cys 1 – 1.79 g/kg 2 – 3.40 g/kg 3 – 5.69 g/kg	Inter. precision	2.3 %	1.8 %	1.1 %	Met 1 – 1.67 g/kg 2 – 5.43 g/kg 3 – 7.31 g/kg	Inter. precision	2.2 %	2.8 %	6.8 %
	Recovery	98.2 %	99.8 %	102.0 %		Recovery	93.5 %	97.0 %	87.1 %
	Uncertainty 1	9.6 %	7.8 %	6.8 %		Uncertainty 1	10.4 %	8.8 %	8.4 %
	Uncertainty 2	4.0 %	6.0 %	3.5 %		Uncertainty 2	3.0 %	6.0 %	5.0 %

Uncertainty 1 – calculated from Horwitz Eq. ; Uncertainty 2 – GUM approach

Table 2. Comparison performance characteristics of the UPLC method with the characteristics of the official method (2) for some amino acids

AA	Range, g·kg ⁻¹	UPLC, repeat.%	UPLC, int.prec.%	UPLC, Recovery,%	U (k=2) %	Repeat. 152/09,%	Reprod. 152/09,%
Thr	0.4-31.0	0.6-1.9	1.1-2.3	98.2-102.0	6.8-9.6	1.9-2.7	3.8-5.2
Lys	0.6-77.0	1.5-2.2	2.6-3.2	92.8-95.4	6.0-9.6	2.1-2.8	3.0-5.4
Cys	0.4-7.0	1.5-3.7	4.0-5.7	93.0-96.6	8.6-10.4	2.6-3.3	8.8-12.3
Met	0.2-9.0	0.8-1.9	2.2-6.8	87.1-97.0	8.4-10.4	2.2-3.4	7.0-13.0

CONCLUSIONS

The UPLC method for determination of amino acids in feeds is fit for purpose and can be used in feed monitoring during internal feed producer control. Accuracy of the UPLC method was confirmed in the proficiency testing organized by AGES, Austria: IAG - Feedingstuffs 2014-2015 and satisfying results were obtained (mean z-score equal to 0.37; from -1.1 to 0.7). The UPLC method is characterized by validation parameters similar to the official method and can be used for the official control purpose.