ANALYSIS OF AMINO ACIDS IN PLANT-BASED PROTEINS AND PET FOODS – MODIFICATION OF AOAC 2018.06 TO FIT FOR NOVEL FOODS AND INGREDIENTS

Jinchuan Yang, Paul Rainville, and Stephanie Harden (contact: Jinchuan_yang@waters.com)
Waters Corporation, 34 Maple St., Milford MA 01757

INTRODUCTION

The AOAC International Official Method 2018.06 specifies an analytical procedure for the analysis of amino acids (AA) in infant formula, adult nutrition and dairy products. This procedure is based on the pre-column derivatization with 6-aminoquinoline succinic carbamate (AQC) and the reversed-phase ultra-performance liquid chromatography with UV detection.

When we applied this AOAC Method to analyze AA in pet foods, however, we found a limitation of this method in the target AA list. Specifically, hydroxyproline (HyPro), a common AA in pet foods, exhibited poor retention on the column, making it unmeasurable using the AOAC Method. HyPro is a marker for potential food adulteration using hydrolyzed animal products to artificially boost protein levels in plant-based proteins. HyPro is also a key indicator for animal-derived ingredients like gelatin and collagen, which are important for detecting the presence of these substances in halal or kosher foods. Therefore, it is necessary to develop an analytical method capable of effectively separating and detecting HyPro alongside other AA.

OBJECTIVE

The goal of this work is to modify the chromatographic conditions of AOAC Method 2018.06 to make it suitable for applications to other foods or ingredients.

EXPERIMENTAL

Samples

Pet foods include dry dog food, dry cat treat, dry cat food, wet dog food, wet cat food, and chicken feed. Plant-based protein powders include pea protein, brown rice protein, pumpkin seed protein and soy protein.

Sample preparation

The pet foods were first prepared by mixing 5.0 g of samples with water to form 80 g of mixtures (recorded mass to 0.01 g), then homogenized thoroughly using a blender. The plant protein powders were first prepared by mixing 1.0 g powder with water to form 40 g of mixtures (recorded mass to 0.01 g). Then, 800 mg of the homogenized sample-water mixtures underwent the sample preparation as described in AOAC Method 2018.06 (including an acid hydrolysis, a neutralization, and a derivatization step).

LC conditions

LC System: ACQUITY™ Premier System (BSM) with a PDA Detector

Detection: UV (260 nm)

Inlet tubing: 0.004 mm ID, 10.5 in tubing assembly (Waters p/n 430001784) between column and detector.

Software: Empower™ 3 CDS

Column: ACQUITY UPLC™ BEH™ C18 Column, 1.7 µm, 2.1 mm × 150 mm (Waters p/n 186002353). Or AccQ·Tag™ Ultra C18 Column, 1.7 µm, 2.1 mm × 150 mm (Waters, p/n 186009954).

Col. inline filter: ACQUITY Column In-Line filter 0.2 µm (Waters p/n 205000343)

Col Temp.: 50 °C

Mobile phases: A: Mixture of AccQ·Tag Ultra Eluent A Concentrate (Waters p/n 186003838) and water at 1:20 (v/v). B: AccQ·Tag Eluent B (Waters p/n 186003839)

Sample loop size: 1 µL

Injection vol.: 1 µL

Injection mode: Partial loop with needle overfill (PLNO) with 6 times overfill volume.

Weak needle wash sol.: 95:5 (v/v) Water:acetonitrile

Strong needle wash sol.: 5:95 (v/v) Water:acetonitrile

Seal-wash solvent: 50:50 (v/v) Water:acetonitrile

Flow rate: 0.4 mL/min

Gradient program:

Elution Segment	Time, min	A, %	B, %	Curve
Initial	Initial	99.9	0.1	Initial
1	5.05	99.9	0.1	6
2	9.55	92.0	8.0	7
3	20.47	77.9	22.1	5
4	21.26	39.9	60.1	6
5	21.29	8.9	91.1	6
6	22.84	8.9	91.1	6
7	26.00	99.9	0.1	6
8	32.00	99.9	0.1	6

METHOD DEVELOPMENT

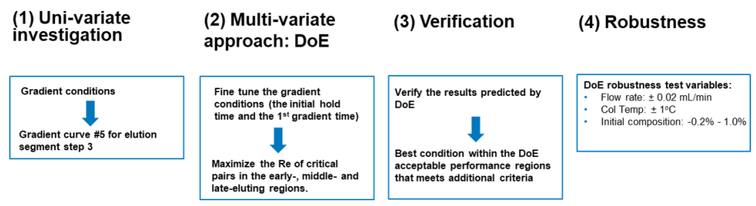

Figure 1. *Schematic of method development strategy.*

Table 1. *Experiment Design and USP resolution response results*

Run No.	Initial Hold Time (min)	1st Gradient Time (min)	Rs (Asp/MetSO2)	Rs (Xcys/Met)	Rs (Ser/Arg)
1	3.5	4	2.053	1.984	5.814
2	5.5	4	1.969	2.198	3.053
3	3.5	6	2.084	1.784	2.26
4	5.5	6	1.952	2.03	4.51
5	3.5	5	2.124	1.897	1.464
6	5.5	5	1.995	2.102	3.928
7	4.5	4	2.045	2.071	1.734
8	4.5	6	2.047	1.893	3.489
9	4.5	5	2.038	1.999	2.719
10	4.5	5	2.047	1.987	2.736
11	4.5	5	2.04	1.995	2.714

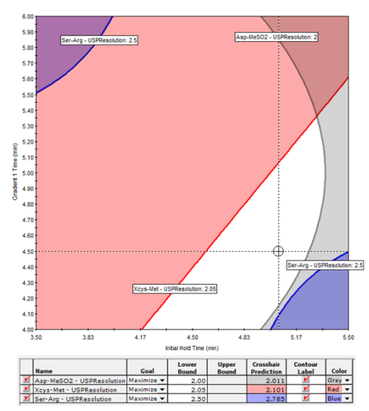

Figure 2. *Response graph showing the acceptable performance region with Lower Bounds at 2.05, 2.00, and 2.50 for pairs CysX/Met, Asp/MetSO2, and Ser/Arg, respectively.*

Table 2. *Robustness evaluation parameters and the corresponding instrument specs.*

Variables	Units	Target	Range	Safe operating space[a]	Instrument specs or volume tolerance
Column Temp.	°C	50	49.0 - 51.0	49.3 - 51.0	Temp. error < 0.5
Flow rate	ml/min	0.40	0.39 - 0.41	0.39 - 0.41	Flow rate error < 0.004
Starting A composition	%	99.9	99.7 - 100	99.7 - 100	Volumetric flask tolerancer[b] < 0.10

[a]: The operating region where Rs of at least 2.0 can be obtained. [b]: The volume tolerance (%) for class A volumetric flasks (50 mL) used in preparation of mobile phases.

RESULTS

1) Chromatography optimization

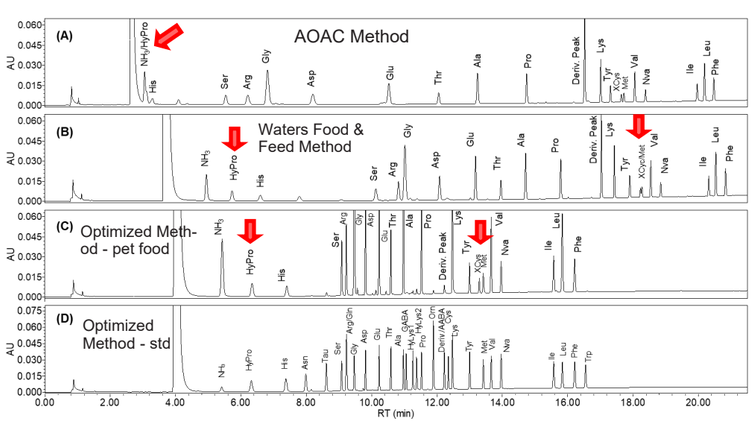

Figure 3. *Chromatograms of AA in a pet food or in a standard solution under various gradient conditions: (A) AA in a pet food under the chromatographic conditions specified in the AOAC Method 2018.06; (B) AA in a pet food under the conditions in the Waters Food and Feed Solution; (C) AA in a pet food under the optimized gradient elution condition developed in this study; (D) AA standards under the optimized gradient elution conditions in this study.*

2) Analytical performance

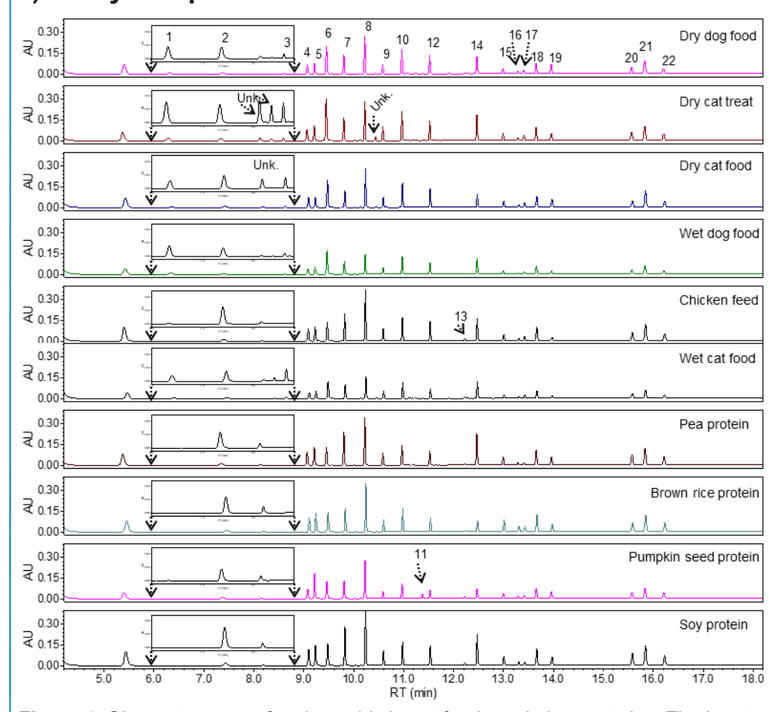

Figure 4. *Chromatograms of amino acids in pet foods and plant proteins. The inserts are the enlarged portion of the chromatogram from 6.0 to 8.8 min. The peaks are 1. Hyp; 2. His; 3. Tau; 4. Ser; 5. Arg; 6. Gly; 7. Asp; 8. Glu; 9. Thr; 10. Ala; 11. HyLys. 12. Pro; 13. Derivatized peak; 14. Lys; 15. Tyr; 16. XCys; 17. Met; 18. Val; 19. Nva; 20. Ile; 21. Leu; 22. Phe.*

Table 3. *Repeatability, intermediate reproducibility for RT and resolution, sensitivity, and linearity.*

Compound	Repeatability, n=10				Reproducibility (intermediate), n=4				Sensitivity	Calibration
	RT		Re*		RT		Re*		LOQ	R^2
	Avg. (min)	RSD (%)	Avg.	RSD (%)	Avg. (min)	RSD (%)	Avg.	RSD (%)	(µM)	Avg. (n=3)
HyPro	6.33	0.78	-	-	6.01	6.2	-	-	0.04	0.9999
His	7.40	0.73	8.29	1.5	6.93	7.7	7.03	20.3	0.06	0.9999
Asn	8.00	0.49	5.72	1.2	7.71	4.4	6.27	9.1	-	0.9996
Tau	8.62	0.30	8.21	0.7	8.48	2.0	8.36	5.0	0.02	0.9999
Ser	9.09	0.22	8.26	0.6	8.97	1.5	7.55	10.9	0.08	0.9998
Arg	9.23	0.16	2.81	5.2	9.14	1.2	2.73	8.2	0.02	0.9992
Gly	9.47	0.17	4.66	1.4	9.38	1.1	4.49	5.2	0.03	0.9998
Asp	9.82	0.13	7.81	0.6	9.76	0.8	7.67	3.3	0.05	0.9992
Glu	10.23	0.10	10.80	1.0	10.19	0.6	10.38	4.6	0.04	0.9999
Thr	10.59	0.07	9.69	0.5	10.56	0.6	9.52	2.9	0.06	0.9999
Ala	10.97	0.07	10.19	0.2	10.94	0.6	9.89	3.5	0.05	0.9997
GABA	11.06	0.07	2.24	0.7	11.03	0.6	2.19	2.9	-	0.9988
HyLys1	11.26	0.04	5.37	2.1	11.23	0.6	5.36	2.2	0.16	0.9999
HyLys2	11.38	0.05	2.97	3.2	11.34	0.6	3.07	2.1	0.16	0.9999
Pro	11.53	0.06	3.78	2.2	11.49	0.6	3.90	7.5	0.02	0.9999
Orn	11.89	0.05	8.83	0.8	11.85	0.6	8.70	2.5	0.07	0.9997
AMQ (Deriv.)	12.23	0.06	7.87	2.1	12.19	0.6	8.17	4.8	-	-
Cys	12.35	0.05	2.82	1.6	12.31	0.5	2.69	12.6	0.07	0.9999
Lys	12.47	0.04	2.99	0.7	12.43	0.6	2.81	11.8	0.10	0.9992
Tyr	13.00	0.07	11.85	1.6	12.96	0.6	12.06	1.9	0.06	0.9998
Xcys	13.30	0.06	6.47	0.2	13.25	0.7	6.25	4.0	-	0.9979
Met	13.42	0.06	2.43	1.1	13.38	0.6	2.29	16.2	0.04	0.9975
Val	13.66	0.06	4.78	0.7	13.62	0.7	4.63	3.7	0.02	0.9999
Nva	13.97	0.06	5.84	0.2	13.93	0.7	5.72	2.9	-	-
Ile	15.57	0.05	28.13	0.3	15.53	0.7	27.46	2.7	0.06	0.9998
Leu	15.84	0.06	4.22	0.2	15.79	0.8	4.14	2.6	0.03	0.9999
Phe	16.22	0.07	5.82	0.6	16.16	0.8	5.60	4.7	0.03	0.9998
Trp	16.55	0.08	5.00	0.7	16.49	0.7	4.76	6.5	-	0.9999

*: Resolution between the previous and the current peaks.

3) Sample analysis results

Table 4. *Amino acid profiles of pet foods and plant-based proteins analyzed in this study.*

Sample \ Conc. (g/100g)[a]	HyPro	His	Tau	Ser	Arg	Gly	Asp	Glu	Thr	Ala	HyLys	Pro	Orn	Lys	Tyr	Cys[b]	Met	Val	ILe	Leu	Phe
Dry dog food	2.25	2.25	0.30	3.98	6.74	7.46	7.62	16.12	3.44	6.88	0.19	7.13	0.09	5.51	3.41	1.79	2.70	4.90	4.08	8.60	4.56
Dry cat treat	3.09	2.90	1.13	3.87	7.45	8.89	7.16	12.84	3.87	6.47	0.18	5.91	0.18	6.37	3.30	1.42	2.80	4.79	4.11	7.16	4.26
Dry cat food	1.46	2.38	0.75	4.28	6.22	7.04	6.72	16.07	3.45	6.69	0.14	7.21	0.08	4.08	4.02	2.03	3.02	4.78	4.06	10.22	5.30
Wet dog food	3.16	2.52	0.30	3.74	6.90	9.46	7.83	12.51	3.53	7.52	0.26	6.66	0.15	7.38	3.02	1.68	2.25	5.38	3.55	7.88	4.29
Chicken feed	0.17	2.82	0	4.46	7.28	4.31	9.30	18.49	3.64	5.59	0	6.40	0	6.02	3.56	1.82	2.55	5.25	4.45	8.83	5.06
Wet cat food	1.70	2.65	1.23	3.85	7.01	6.69	8.32	13.47	3.89	6.88	0.16	5.15	0	8.13	3.41	1.65	3.55	5.39	4.51	7.99	4.39
Pea protein	0	2.53	0	4.82	9.02	3.92	10.85	16.39	3.50	4.56	0	4.36	0	8.02	4.13	1.56	1.24	5.52	5.08	8.72	5.77
Brown rice Protein	0	2.51	0	4.80	9.44	4.26	7.72	16.71	3.32	5.56	0	4.54	0	3.01	6.19	3.33	3.00	6.38	4.45	8.68	6.04
Pumpkin seed protein	0	2.39	0	4.68	17.01	4.92	7.83	17.16	2.75	4.44	1.63	3.60	0	3.50	3.72	2.15	2.58	5.14	3.90	7.29	5.31
Soy protein	0	2.65	0	4.85	8.27	3.93	10.62	18.65	3.53	4.49	0	5.18	0	6.62	3.99	1.89	1.48	5.15	4.99	8.22	5.48

[a]: Expressed as gram of AA in 100 grams of total AA; [b]: Cystine is converted from Xcys concentration.

CONCLUSION

- **Excellent separation resolution for all amino acids has been achieved with excellent repeatability and intermediate reproducibility in RT.**
- **Amino acids in six pet foods and four common plant-based proteins have been successfully analyzed.**
- **This modified AOAC Method offers a reliable and practical solution for the analysis of amino acids in pet foods and plant-based proteins.**

Scan the code to download literature.

ACQUITY, UPLC, BEH, Empower, AccQ.Tag are trademarks of Waters Technologies Corporation.

TO DOWNLOAD A COPY OF THIS POSTER, VISIT WWW.WATERS.COM/POSTERS

720009022EN
©2025 Waters Corporation