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The Advanced Analytical Method Through the Quantitative Comparative Study of Taurine in Feed Using LC-MS/MS

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Abstract : Taurine is a type of sulfur-containing amino acid having a sulfate functional group, that is biosynthesized from cysteine. It is mainly distributed in high concentrations in animal tissues and is known to have various effects such as osmotic pressure control, calcium control, anti-inflammatory, antioxidant, and hepatocellular protection. Also, taurine deficiency causes a variety of symptoms, including visual impairment. In particular, in the case of cats, taurine is not biosynthesized and must be supplied through food, so it is classified as an essential amino acid. In this study, an analysis method using mass spectrometry was developed instead of the commonly used derivatization method to quickly, environmentally, and precisely analyze taurine in various animal feeds. The developed analytical method showed good linearity ($R^2 > 0.99$), accuracy (81.97-105.78%), and precision (0.07-12.37%). In addition, the developed method was further verified through quantitative comparison with the derivatization method. This developed method was used in the determination of taurine in 20 animal feed samples obtained from South Korea. The levels of taurine found ranged from 81.53 to 6,743.53 mg/kg. The developed analysis method will be used for the detection and quantification of taurine in domestic feed.

Keywords : taurine, feed, LC-MS/MS, amino acid analyzer, comparative study

Introduction

Amino acids are components of proteins and are essential for animal growth and maintenance of physiological functions. There are about 20 kinds of amino acids that make up proteins, and they are classified into essential amino acids and non-essential amino acids. Essential amino acids are amino acids that must be supplied from the outside to sustain animal life and include lysine, leucine, methionine, phenylalanine, taurine, threonine, tryptophan, and valine.[1-3] Among them, taurine (β-amino ethane sulfonic acid) is a type of sulfur-containing amino acid having a structure in which an amino group is bonded to β-carbon and a sulfate group is bonded to α-carbon.[4,5] Taurine is biosynthesized from cysteine and mainly distributed in high concentrations in animal tissues (muscle, heart, brain, retina).[2,6] The physiological action of taurine is known to have various effects such as cell proliferation, osmotic pressure regulation, calcium regulation, glucose metabolism promotion, nerve excitability regulation, anti-inflammatory, antioxidant, and hepatocellular protection.[7-12]

Amino acids in the feed are used as supplements added to feed to increase their utility. The addition of taurine to high-fat diets can improve serum total cholesterol and triglyceride levels without affecting the productivity of laying hens,[13] and in the case of finishing pigs has shown that it increased growth and decreased serum and liver total cholesterol levels.[14] In particular, in the case of cats, taurine cannot be biosynthesized and must be supplied through food, so it is classified as an essential amino acid. The minimum taurine content required for adult cats in pet food is set at 25 mg/100 kcal for dry food and 50 mg/100 kcal for wet food.[15] Currently, taurine is classified as an supplementary feed in the domestic feed management law, and it is mainly used by adding it to the feeds of animals that need taurine, such as cats.[16] Although there is no registration standard for taurine, an analysis method that can accurately quantify trace amounts of taurine contained in the feed ingredient and compound feed is needed to prevent taurine deficiency and excess.

A representative analysis method for analyzing taurine in feed is the Association of Official Analytical Chemists

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(AOAC) official method, which is an internationally recognized analysis method. In this analysis method, taurine is quantified in feed using LC-FLD through fluorescence derivatization after hydrolysis with hydrochloric acid and reaction with dansyl chloride.[17] Several studies have been conducted to quantify taurine in sports drinks and dairy products based on AOAC internationally recognized analytical methods.[18-21] Amino acids including taurine have an amino group ($-NH_2$) and a carboxyl group (-COOH) structure, and since absorption does not occur in the ultraviolet and visible light regions, the fluorescence derivatization process is absolutely necessary.[22-24]

Disadvantages of such an analysis method are that it takes a lot of time for sample preparation such as hydrolysis and derivatization, and LC-FLD analysis through derivatization is not suitable for accurate quantitative analysis because of its relatively low analytical sensitivity compared to LC-MS/MS. In addition, since the concentration of taurine is relatively low compared to other amino acids, the above pretreatment method to liberate all amino acids may be affected by other amino acids when taurine is separated from the column. Therefore, in this study, an analysis method using ultrasonic extraction and mass spectrometry that can analyze taurine without such a derivatization process was developed. In order to confirm the extraction efficiency of the developed method and the accuracy and precision of the instrumental analysis, a comparative experiment with the derivatization method based on the AOAC method was performed. Comparative experiments were performed using certified reference materials (CRM). It was confirmed that the analytical method developed through the comparative experiment between the analytical methods had no significant difference from the existing analytical method in quantifying taurine, and then the validity was confirmed within and between laboratories. Taurine analysis was performed on 20 feed samples using the finally developed analysis method.

Experimental

Chemicals and reagents

Taurine (99%) used as a standard material was a high-purity reagent from Sigma-Aldrich (St. Louis, MO, USA). Distilled water was prepared using a Milli-Q Direct 8 model manufactured by Merck Millipore (MA, USA). As for methanol, Merck (Darmstadt, Germany) product was used as HPLC grade, and formic acid (98%) was used by Thermo Fisher Scientific (Waltham, USA). Ammonium acetate ($NH_4CH_3CO_2$) used for solvent and extraction was manufactured by Thermo Fisher Scientific (Waltham, USA), and a syringe filter (Whatman, Maidstone, UK) for filtering the sample was 13 mm made of PTFE (polytetrafluoroethylene), 0.2 μm size was used. Hydrochloric acid was EP-S grade, manufactured by Chemitop (Korea), and Ethanol, manufactured by Merck (Darmstadt, Germany) was used as HPLC grade. The mobile phase solvent is buffer aqueous solution No. 05112 of KANTO Chemical (Tokyo, Japan). Buffer for protein hydrolysate PH-1 (Sodium citrate dihydrate 0.62%, Sodium chloride 0.56%, Citric acid monohydrate 1.97%, Ethanol 10.20%, β-Thiodiglycol 0.55%, 25% Brij-35 0.40%, n-Octanoic acid 0.01%, pH 3.3), ninhydrin reagent Reagent (R) 1 (Propylene glycol monomethyl ether 979 mL, Ninhydrin 39 g, Sodium borohydride 81 mg), Reagent (R) 2 (Distilled water 336 mL, Lithium acetate dihydrate) 204 g, Glacial acetic acid 123 mL, Propylene glycol monomethyl ether 401 mL) was purchased using a Ninhydrin Coloring Solution Kit from FUJIFILM Wako Pure Chemical (Osaka, Japan).

Linearity and calibration curve

The standard preparation for LC-MS/MS analysis is as follows. Taurine was dissolved in 10 mM ammonium acetate to prepare a standard stock solution at a concentration of 1,000 mg/L. To prepare a calibration solution for quantitative analysis, a standard solution was mixed with an untreated sample extract in a ratio of 9:1 to prepare a concentration of 20, 50, 100, 200, 500, 1,000, and 2,000 μg/L. The preparation of standard materials for the amino acid analyzer is as follows. Taurine was dissolved in 10 mM ammonium acetate to prepare a standard stock solution at a concentration of 1,250 mg/L. For quantitative analysis, standard solutions were prepared with 10 mM ammonium acetate at concentrations of 1,250, 2,500, 6,250, and 12,500 μg/L.

Sample preparation

For the untreated samples to verify the validity of the taurine analysis conditions, a compound feed for growing pigs and pets and a soybean ingredient feed were selected. In addition, experiments were conducted using SRM (NIST 3290) and pet dog food as samples to investigate the quantitative ability of the taurine analytical method.

Ultrasonic extraction method for LC-MS/MS

0.5 g of the homogenized sample was precisely weighed and placed in a 50 mL centrifuge tube, 50 mL of a 10 mM aqueous ammonium acetate was added, followed by ultrasonic extraction for 30 min, followed by centrifugation at 4°C, 4,000 *g* for 10 min. The extracted sample solution was filtered with a 0.2 μm (PTFE, Whatman Inc., Maidstone, UK) syringe filter, and then used as the sample solution.

Derivatization analysis method for amino acid analyzer

0.2 g of the homogenized sample into a 50 mL centrifuge tube, add 20 mL of 6 M HCl, and then hydrolyze at 110°C for 20 hours. After cooling the sample solution to room temperature, put it in a 100 mL volumetric flask and add water to adjust the total volume to 100 mL. Take 1 mL of supernatant, concentrate, and then re-dissolve in 10 mM aqueous ammonium acetate.[17] The extracted sample solution was filtered with a 0.2 μm (PTFE, Whatman Inc., Maidstone, UK) syringe filter, and then used as the sample solution.

LC-MS/MS analysis

LC-MS/MS 8060 manufactured by Shimadzu (Tokyo, Japan) was used, and Hypersil Gold C18 (5 μm, 4.6 × 150 mm) was chosen as the analytical column. The flow rate was 0.5 mL/min. The injection volume was 5 μL and the

Table 1. Multiple reaction monitoring (MRM) conditions for taurine.

Compound	Precursor ion		Product ion					
			Quantitative ion			Qualitative ion		
	m/z	Q1 pre bias (V)[a]	m/z	CE (V)	Q3 pre bias (V)[b]	m/z	CE (V)[c]	Q3 pre bias (V)
Taurine	124.2	-13	80.1	22	11	124.2	2	12

[a] Voltage promotes the ionization of the precursor ion.
[b] Voltage promotes the ionization of the product ion.
[c] Collision energy.

Table 2. Amino acid automatic analyzer L-8900 gradient conditions for taurine.

Time (min)	%B1[a]	Flow (mL/min)	%R1[b]	%R2[c]	%R3[d]	Flow (mL/min)
0.0	100	0.400	55	45	0	0.350
3.2	100	0.400	55	45	0	0.350
3.3	100	0.400	0	0	100	0.350
6.2	100	0.400	0	0	100	0.350
6.3	100	0.400	55	45	0	0.350
23.2	100	0.400	55	45	0	0.350

[a] Water solution contains following substances; Sodium citrate dihydrate 0.62%, sodium chloride 0.56%, citric acid monohydrate 1.97%, ethanol 10.20%, β-thiodiglycol 0.55%, Brij-35 (dissolve 25 g into 100 mL of distilled water.) 0.40%, and n-octanoic acid 0.01%, pH 3.3.
[b] Propylene glycol monomethyl ether 979 mL, ninhydrin 39 g, and sodium borohydride 81 mg.
[c] Distilled water 336 mL, lithium acetate dihydrate 204 g, glacial acetic acid 123 mL, and propylene glycol monomethyl ether 401 mL.
[d] Water solution contains 50 mL ethanol.

temperature was maintained at 40°C. The mobile phase is water containing 50 mM ammonium acetate as mobile phase A, and methanol containing 0.1% formic acid as mobile phase B. The gradient elution method was optimized. 0 min 97%(A) 3%(B), 5 min 97%(A) 3%(B), 6 min 3%(A) 97%(B), 7.5 min 3%(A) 97%(B), 8.5 min 97%(A) 3%(B), 12 min 97%(A) 3% (B). For detailed conditions of the mass spectrometer, the negative ion mode of the electrospray ionization (ESI) method was used, and the interface temperature 300°C, DL temperature 250°C, nebulizing gas 3 L/min, heating gas 10 L /min, drying gas 10 L/min. Multiple monitoring mode (MRM) conditions were established as shown in Table 1.

Amino acid analyzer analysis

Amino acid analyzer AAA L-8900 from Hitachi (Tokyo, Japan) was used, and the analysis column was chosen Hitachi ion exchange resin 855-4506 (Na type, 4.6 × 60 mm). The reaction column was Hitachi reaction column 852-3540 (4.6 × 60 mm). The flow rates of 0.4 mL/min (pump 1), 0.35 mL/min (pump 2), and injection volume were 20 μL. The analysis column temperature was 57°C, and the reactor temperature was maintained at 135°C. The gradient conditions of the mobile phase are shown in Table 2.

Validation

The validity of the established analytical method was verified according to the guidelines of the Ministry of Food and Drug Safety (MFDS): selectivity, linearity, accuracy, and precision.[25] To check the linearity, a matrix matched calibration standard solution was prepared so as to be 20-2,000 μg/L, and the coefficient of correlation (R^2) of the calibration curve was obtained. In order to confirm the accuracy and precision of the analysis method, a recovery rate experiment was performed by adding a standard solution to the untreated sample. The recovery rate was repeated three times at LOQ, 2LOQ, and 5LOQ concentrations, respectively, to calculate the accuracy and relative standard deviation (%RSD, relative standard deviation). The LOQ of the analytical method was selected as the lowest concentration that satisfies the recovery criteria according to the validation guidelines of SANTE/12682/2019.[26] For more precise validation, cross-validation between laboratories was performed. The matrix effects were calculated by compairing the slope of the calibration curve of the taurine standard prepared by dissolving in a pure solvent and the slope of the calibration curve of the matrix-matched standard, respectively, using the following formula.

$$\text{Matrix effect}(\%) = \left(\frac{\text{Slope of calibration curve in matrix}}{\text{Slope of calibration curve in solvent}} - 1\right) \times 100$$

Results and discussion

LC-MS/MS condition

The optimal multiple reaction monitoring (MRM) was established in the negative ion mode of electrospray ionization (ESI). Precursor ions were identified in full scan mode, and product ions were selected in consideration of the ratio of each ion. Among them, m/z 80.5, the product ion with the best sensitivity, was selected as the quantitation ion, and the ion showing the next highest sensitivity, m/z 124.2, was selected as the confirmation ion (Table 1).

Selectivity and linearity

For linearity evaluation, seven standard solution concentrations ranging from 20 to 2,000 μg/L in matrix extracts were analyzed to determine the linearity. In general, the coefficient of determination (R^2) was higher than 0.99 in all matrices, indicating suitable linearity. And as a result of analyzing the samples without treatment of the single feed and the compound feed by establishing the MRM conditions, high selectivity and resolution were confirmed (Figure 1).

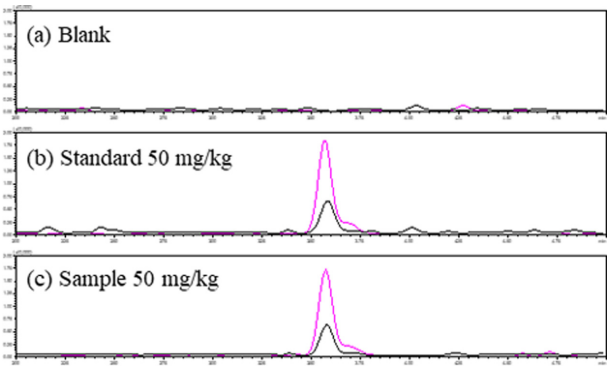

Figure 1. Representative chromatogram of (a) blank sample, (b) standard 50 mg/kg, and (c) sample 50 mg/kg.

Matrix effect

As a result of confirming the matrix effect on the pig, dog, and soybean feed used for validation, pig feed (-35.61%), dog feed (-44.31%), and soybean feed (-43.98%) were shown. In all samples, the signal of the analyte showed a tendency to decrease (suppression), and the matrix effect was within -50%. Therefore, it was confirmed that the application of matrix matched calibration is necessary for accurate quantification.

LOQ, precision, and accuracy

The limit of quantitation of the analytical method was selected as 10 mg/kg, which is the lowest stock concentration at which the recovery result satisfies the criteria for validation guidelines of SANTE/12682/2019.[26] In order to evaluate the accuracy and precision of the developed analysis method, breeding pigs, pet dog compound feed, and soybean ingredient feed were used. The recovery rate experiment was repeated three times with LOQ, 2LOQ, and 5LOQ concentrations, respectively. As a result, the accuracy and precision were 81.97-105.78 % and 0.07-12.37 %, confirming that the recommended standard was satisfied (Table 3).

Method comparison

The established analytical method is a simplified method

Table 3. LOQ, accuracy, and precision of taurine in feed ingredient (soybean) and compound feeds (pig and dog).

Analyte	LOQ (mg/kg)	Recovery Conc.[a] (mg/kg)	Accuracy (Precision, %)					
			Intra-Lab (n = 3)			Inter-Lab (n = 3)		
			FI[b] (soybean)	CF[c] (pig)	CF (dog)	FI (soybean)	CF (pig)	CF (dog)
Taurine	10	10	83.39 (12.37)	95.98 (6.80)	105.78 (1.47)	98.15 (0.07)	94.30 (0.45)	87.90 (8.37)
		20	88.87 (8.26)	91.20 (7.54)	102.41 (6.82)	95.70 (1.63)	95.85 (1.70)	91.85 (2.08)
		50	81.97 (5.77)	88.92 (2.16)	102.20 (1.45)	92.95 (3.88)	91.75 (1.62)	94.15 (0.23)

[a] In the case of dog compound feed, taurine was detected in all blank samples, and the recovery concentration was adjusted to 20, 50, and 100 mg/kg.
[b] Feed ingredient.
[c] Compound feed.

Table 4. Comparison of quantitative values (%) and % RSD of taurine in CRM and real samples according to extraction method and analysis instrument.

Extraction method / Instrument	CRM[a]		Real sample	
	Hydrolysis Extraction	Ultrasonic Extraction	Hydrolysis Extraction	Ultrasonic Extraction
Amino acid analyzer	0.19 (0.78)	0.19 (19.57)	0.19 (0.88)	0.21 (18.02)
LC-MS/MS	0.20 (9.94)	0.23(10.27)	0.21 (4.80)	0.23 (8.34)

[a] The taurine content of the certified standard material was 0.24%.

Table 5. Content levels of taurine in 20 feed samples in South Korea.

Sample	No. of samples	Minimun (mg/kg)	Median (mg/kg)	Maximum (mg/kg)	Recommend level[a)] (mg/kg)
Cat feed	10	1,059.11	1,625.58	6,743.53	1,000
Dog feed	10	81.53	349.43	2,008.69	-

[a)] Official Publication for taurine levels in dog and cat feed according to Association of American Feed Control Officials (AAFCO).

of the commonly used taurine pretreatment method, and since LC-MS/MS, which is different analytical equipment from the existing analytical method, was used, an experiment was performed to compare it with the existing amino acid analyzer. Therefore, we compared the results using LC-MS/MS and an automatic amino acid analyzer (Hitachi L-8900) for the sample solutions extracted with taurine by the conventional extraction method through HCl hydrolysis and the established extraction method. As a result, when ultrasonic extraction and LC-MS/MS were used, the results were closest to the quantitative value of CRM (Table 4).

Real sample analysis

The established method was applied in the determination of taurine in 20 pet feed samples obtained in various regions of South Korea. The presence of a positive sample was confirmed by comparing the retention time and product ion ratio obtained with the calibration standards. Taurine was detected at levels from 81.53 to 6,743.53 mg/kg in 20 feed samples (Table 5). According to the Association of American Feed Control Officials (AAFCO) official publication, recommend level of taurine in cat feed is defined as 1,000 mg/kg[15]. In this study, all cat feeds were found to be present in taurine at levels between 1,059.11 and 6,743.53 mg/kg and observed above the AAFCO recommended level of 1,000 mg/kg. It was confirmed that the developed method is capable of quantification and qualification of the taurine in animal feeds by ultrasonic extraction without the derivatization process, which is a traditional extraction method.

Conclusions

As a result of comparing the new analysis method for taurine analysis in feed with the existing general analysis method, it was confirmed that extraction was possible only with simple ultrasonic extraction without the existing derivatization process. Accordingly, it is expected that the time and cost of taurine analysis in feed can be greatly reduced. In addition, it is expected that a trace amount of taurine in the compound feed can be confirmed more accurately and precisely by checking the sensitivity and resolution more precisely than the existing equipment through the advancement of the analysis equipment. As can be seen from the monitoring results for real samples, it was confirmed that a small amount of taurine was detected. In conclusion, through this study, the analysis method related to the analysis of taurine in feed has been further advanced, and it is expected that more precise and accurate analysis will be possible through this study.

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