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Convenient method of threonine, methionine and their related amino compounds by high-performance liquid chromatography and its application to rumen fluid

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Abstract

A high-performance liquid chromatographic procedure for the quantitative determination of cysteine (Cys), homocysteine (Hcys), methionine sulfoxide (MSO), methionine sulfone (MSO_2), homoserine (Hser), glycine (Gly), threonine (Thr), 2-aminobutyric acid (2AB), methionine (Met), cystathionine (Cysta) and its application to rumen fluid are described. The samples containing Thr, Met and other related amino compounds were derivatized with 9-fluorenylmethyl chloroformate. The separation of compounds was accomplished with a methanol gradient in 25 m*M* sodium citrate buffer (obtaining pH 6.40 and 3.80 by addition of 25 m*M* citric acid). All derivatized compounds were separated on a Mightysil RP-18 GP (150×4.6 mm I.D., 5 μm particle size) column. All analytes were detected at 265 nm with UV detection. The limits of detection (μ*M*) (*S*/*N* ratio, 3:1) and quantification (μ*M*) (*S*/*N* ratio, 10:1) of Cys, Hcys, MSO, MSO_2, Hser, Gly, Thr, 2AB, Met and Cysta were 0.50 and 1.68; 1.76 and 5.85; 0.85 and 2.88; 0.92 and 3.09; 1.04 and 3.52; 0.76 and 2.52; 0.65 and 2.18; 0.39 and 1.36; 0.31 and 1.03; 0.17 and 0.58, respectively. The recoveries of all compounds in rumen fluid were 97.93–102.3% in the within-day study and 94.52–98.69% on different day (6 days) studies. The average contents (μ*M*) of Cys, Gly, Thr, 2AB, Met and Cysta were 1.72, 45.6, 20.0, 4.3, 2.11 and 3.42 before morning feeding. The concentration of Thr, 2AB and Cysta in rumen fluid tended to increase with time after feeding whereas Met showed the opposite tendency.

Keywords: Derivatization, LC; Threonine; Methionine; Amino acids; 9-Fluorenylmethyl chloroformate

1. Introduction

In the rumen, the first digestive organ of ruminants, a variety of microorganisms are present which can be characterized by their high potency not only for digesting fibrous materials of the plants which are consumed by ruminants, but also synthesizing all amino acids including the essential ones required by the host animals.

Threonine (Thr) and methionine (Met) are amino acids which are essential to ruminants as well as other mammals [1,2]. In microorganisms and plants, these amino acids are synthesized by a common pathway, called the aspartate pathway in which homoserine (Hser) acts as a common precursor. It is phosphorylated to *O*-phosphohomoserine which is then converted to Thr and/or *O*-succinylated to *O*-succinylhomoserine to synthesize Met [3]. Homocysteine (Hcys), another key intermediate, is synthesized from *O*-succinylhomoserine and cysteine

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(Cys) through cystathionine (Cysta) by coupled reactions of cystathionine γ-synthase (EC 4.2.99.9) and cystathionine β-lyase (EC 4.4.1.8) in *Salmonella* [4] and *Escherichia coli* [5]. Finally, Hcys is methylated to methionine either by a vitamin B_{12}-dependent Hcys-methyltransferase enzyme (EC 2.1.1.13) [6] or by a vitamin B_{12}-independent enzyme (EC 2.1.1.14) [7]. It was also found that Thr can be converted to Glycine (Gly) and acetate by *Trypanosoma brucei* [8].

Onodera and Migita [9] reported that Met was oxidized by mixed rumen ciliate protozoa to methionine sulfoxide (MSO) and methionine sulfone (MSO_2) enzymatically and nonenzymatically. They also reported that 2-aminobutyric acid (2AB) was produced by rumen protozoa from Thr and Met via 2-oxobutyric acid. 2AB had an inhibitory effect on the growth of some strains of *Eubacterium ruminantium* and accumulated high concentrations of isoleucine in the medium [10], which suggests that this compound may have an important role in the regulation of the rumen ecosystem.

In this regard, however, it has not yet been examined if Hser and Hcys can be used as substrates for producing Thr and Met by rumen microorganisms. In order to clarify these subjects in more detail, at first, a determination method for Thr, Met and their related amino compounds such as Cys, Hcys, MSO, MSO_2, Hser, Gly, 2AB and Cysta, was necessary.

The use of high-performance liquid chromatography (HPLC) for the quantitative determination of amino acids in biological samples has received wide popularity within the last two decades due to its speed of analysis and good detection limits in comparison with classical ion-exchange chromatography. Pre-colunm methods using HPLC are based on the derivatization of amino acids with reagents that show strong UV absorbance or fluorescence, such as OPA (*o*-phthaldialdehyde) [11–15], phenylisothiocyanate [15], dansylchloride [15], and FMOC-Cl (9-fluorenylmethyl chloroformate) [15–21]. Of these derivatizing agents, recently, FMOC-Cl has been widely used for the analysis of amino acids, because it can react rapidly with both primary and secondary amino groups at room temperature and form stable derivatives and it is relatively less sensitive to matrix interference than the other agents [18–20].

Many investigators have reported HPLC methods for the determination of Thr [11,15,17–20], Met [11,15,17–20], Cys [11,13], Hcys [11,13], MSO [12], Hser [11], Gly [11,15,17–20], 2AB [11,15], and Cysta [14]. However, none of these methods encompass all the compounds mentioned above. In this paper, we described a sensitive and selective HPLC method for the simultaneous determination of the 10 compounds of interest by using FMOC-Cl in rumen fluid to conduct further investigation related to the metabolism of Thr and Met.

2. Experimental

2.1. Apparatus

A liquid chromatography pump (PU-980), variable-wavelength ultraviolet detector (UV-970) and a three-line degasser (DG-980-50) were from JASCO (Tokyo, Japan); a data analyzer (Chromatopac, C-R6A) was from Shimadzu (Kyoto, Japan). A column oven was from Hitachi (Tokyo, Japan). The Mightysil RP-18 GP column (150×4.6 mm I.D., 5 μm particle size) and guard column (5.0×4.6 mm, 5 μm particle size) were obtained from Kanto (Tokyo, Japan). A horizontal type shaking machine (JK MS1 Minishaker IKA, Wilmington, NC, USA) and 1.5-ml Eppendorf tubes (Reaktionsgefäβe 3810, Hamburg, Germany) were used for derivatization.

2.2. Reagents and chemicals

Methanol (LC-grade) and dehydrated acetonitrile were obtained from Kanto. Trisodium citrate dihydrate, citric acid monohydrate, 5-sulfosalicylic acid dihydrate (SSA), potassium hydroxide, tetrahydrofuran (THF), ethylenediaminetetraacetic acid tripotassium salt (EDTA-3K), boric acid, metaphosphoric acid, perchloric acid, L-cysteine hydrochloride, L-threonine, DL-homocysteine, L-(+)-cystathionine, L-2-aminobutyric acid and FMOC-Cl were obtained from Nacalai Tesque (Kyoto, Japan). Iodoacetic acid was from TCI (Tokyo, Japan). L-Homoserine and L-methionine were obtained from Wako (Osaka, Japan), L-methionine sulfoxide and L-methionine sulfone were purchased from Sigma (St. Louis, MO, USA). All other amino acids, e.g.,

Asp, Glu, Asn, Gln, Ser, Cit, Arg, His, Pro, Ala, Val, Trp, Tyr, Phe, Ileu, Leu, Orn and Lys were also purchased from Nacalai Tesque, Wako, Sigma and Aldrich (Milwaukee, WI, USA). Ultrapure water made with Milli-Q Labo (Nihon Millipore, Tokyo, Japan) was used to prepare the mobile phases and other solutions.

2.3. Preparation of borate buffer, derivatization reagent, and standard solutions

The borate buffer solution was prepared by dissolving 0.8 *M* of boric acid, pH 9.90, adjusted with 5.0 *M* potassium hydroxide solution. Iodoacetic acid was mixed with this borate buffer at 0.75 g/100 ml (w/v) and kept at 4°C.

9-Fluorenylmethyl chloroformate was dissolved as 10 m*M* in dehydrated acetonitrile and kept in a freezer (−20°C); suitable for 5 days.

The standard solutions were prepared by dissolving known amounts of Cys, Hcys, MSO, MSO_2, Hser, Gly, Thr, 2AB, Met and Cysta, in water and mixing with an equal volume of 4.0% (w/v) SSA solution containing 10 m*M* EDTA-3K to obtain 0 to 500 μ*M* concentration. The solutions obtained were filtered through a 0.45-μm membrane filter and kept at −20°C when not used.

2.4. Collection and preparation of biological samples

For the analysis of rumen fluid three fistulated goats (Japanese native breed, 35±5 kg) were fed on a daily ration consisting of lucerne cubes (23 g DM/kg $BW^{0.75}$) (DM, dry matter; $BW^{0.75,}$ metabolic body weight) and concentrate mixture (8 g DM/kg $BW^{0.75}$) provided in two equal portions given at 09:00 h and 17:00 h. The rumen contents were collected before morning feeding and 2, 4 and 6 h after feeding, strained through four layers of surgical gauze. One ml of the sample was placed in an Eppendorf tube and mixed with 1 ml of 4% (w/v) SSA containing 10 m*M* EDTA-3K, kept in a refrigerator (4°C) overnight and centrifuged at 27 000 *g* for 35 min. The supernatant fluid was then filtered through a membrane filter (0.45 μm) and kept at −20°C until HPLC analysis.

2.5. Determination of analytical recovery

Samples of SSA extracts of rumen fluid were spiked with mixed standard compounds to final concentrations of 0, 62.5, 125, 250 and 500 μ*M*, respectively [21,22]. To calculate the recovery, the concentration of endogenous compound of rumen fluid, prior to spiking, was measured once and compared with equivalent standards.

2.6. Derivatization procedure

We followed our previous derivatization process [16] with slight modification. Standard solutions, rumen fluid, or mixture of standard and rumen fluid (100 μl each) were mixed with 50 μl potassium borate buffer (0.8 *M*, pH 9.90) containing iodoacetic acid (0.75 g/100 ml buffer) in an Eppendorf tube and the solution was vortex-mixed for 1 min. A 100-μl aliquot of 10 m*M* FMOC-Cl in dehydrated acetonitrile was added and the solution was immediately agitated for 1 min on a shaking machine (1800 rpm). Then, 0.5 ml of *n*-heptane was added and vortex-mixed for 30 s (1800 rpm) to remove excess reagent. The extraction was repeated twice and the heptane phases (upper portion) were discarded. The sample was ready for injection into the HPLC system.

2.7. Chromatography

Derivatized compounds were separated using a ternary gradient system. Solvent A was 25 m*M* sodium citrate, adjusted to pH 6.40 with 25 m*M* citric acid. Solvent B was 25 m*M* sodium citrate (adjusted to pH 3.80 with 25 m*M* citric acid) containing THF (1 ml/100 ml buffer). Solvent C was methanol (90% in water). Buffer was filtered through a 0.45-μm membrane filter (Nihon Millipore Kogyo K.K., Tokyo, Japan) before use. For elution, the gradient was used as given in Table 1. The flow-rate was maintained at 1.4 ml/min throughout the analysis. The column temperature was controlled at 38.5°C and peak response of compounds was monitored at 265 nm by UV detection. A 10-μl portion of FMOC-Cl derivatives of samples was injected for analysis.

Table 1
Gradient profile

Time (min)	Solvent A (%)	Solvent B (%)	Solvent C (%)
0	53	1	46
4	53	1	46
5	50	1	49
13.5	50	1	49
14.5	0	70	30
18.5	0	70	30
21	0	57	43
36	0	57	43
61	0	31	69
75	0	10	90

3. Results and discussion

3.1. Derivatization conditions

Derivatization of amino acids with FMOC-Cl occurs at room temperature under basic conditions in an aqueous solution [18,19]. The FMOC-Cl also reacts with water to yield FMOC-alcohol as a hydrolysis product which shows strong UV absorbance [16], which was detected in the chromatograms (Figs. 1, 2 and 3). Within 1 min, the derivatization of all amino acids was completed [17]. Then, excessive FMOC-Cl had to be removed by heptane extraction [16] in order to minimize the formation of the hydrolysis (FMOC-alcohol) product.

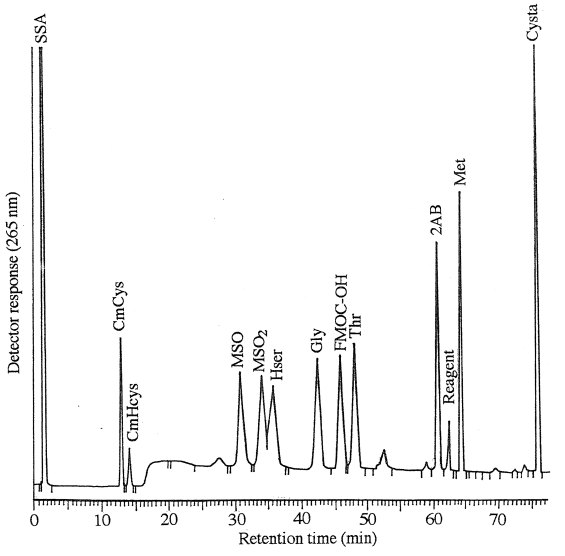

Fig. 1. Chromatogram of a standard mixture of Cys, Hcys, MSO, MSO_2, Hser, Gly, Thr, 2AB, Met (125 μ*M*); Cysta (62.5 μ*M*) by HPLC. Cys as CmCys (*S*-carboxymethyl cysteine); Hcys as CmHcys (*S*-carboxymethyl homocysteine).

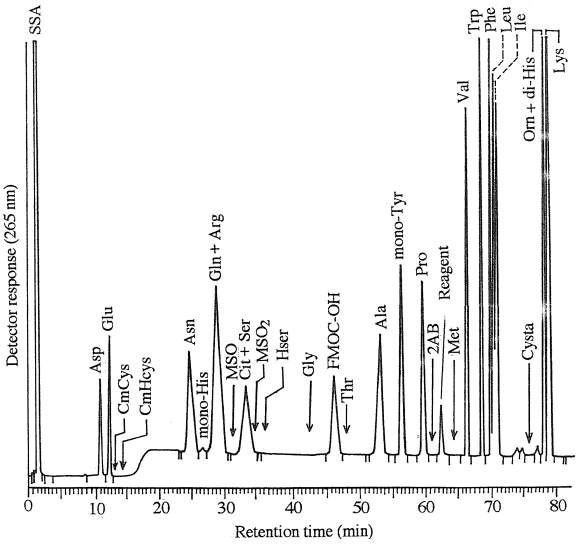

Fig. 2. Chromatogram of a standard mixture of amino acids (125 μ*M*) (Cit=62.5 μ*M*, Ser=62.5 μ*M*) without 10 analytes by HPLC. Arrows indicate the elution positions of CmCys, CmHcys, MSO, MSO_2, Hser, Gly, Thr, 2AB, Met and Cysta, respectively.

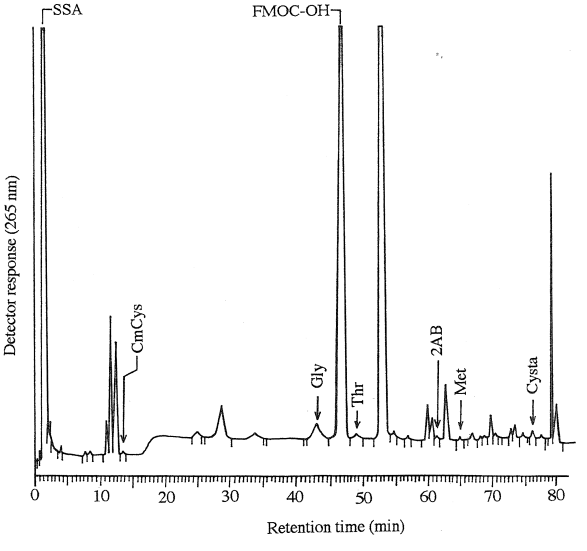

Fig. 3. Chromatogram of deproteinized goat rumen fluid (before morning feeding) by HPLC.

The sulfhydryl group (–SH) of amino acids such as Cys and Hcys also reacts with FMOC-Cl, and the derivatives showed strong UV absorbance unlike OPA derivatives [23]. However, these compounds appeared in the chromatograms as very late eluting peaks and were difficult to separate from the peaks of Orn and Lys (figure not shown). It was therefore necessary to alter the retention of Cys and Hcys on the HPLC column. It has been reported [23–25] that by using some chemicals (e.g., iodoacetate, 4-vinylpyridine, iodoacetamide, etc.), it would be possible to alter retention of Cys and to increase the fluorescence intensity of the product by oxidizing –SH groups following OPA derivatization. Among them, for oxidizing the –SH group of Cys and Hcys, we used iodoacetate which led to the *S*-carboxymethyl derivatives of Cys and Hcys. Iodoacetate was advantageous for a rapid reaction at ambient temperature and compatible with the FMOC-Cl reaction and showed good UV absorbance. As shown in Fig. 1, Cys and Hcys appeared as the first and second eluting peaks, respectively.

It has been reported that basic borate buffer was used to maintain the basic condition during the derivatization process, but it was also necessary to neutralize acidic samples with a suitable alkali prior to the commencement of the derivatization process [26,27]. In the current method, 0.8 *M* potassium borate buffer (pH 9.90) was used during derivatization process which changed the acidic pH of rumen samples (because of the use of SSA) directly to the basic pH. Therefore, no prior pH adjustment of acidic rumen samples by another alkali was needed before the derivatization process. We also used borate buffer of different pH values but this resulted in worse yields during derivatization.

3.2. Use of deproteinizing agent

Biological samples may be injected directly into the column [28], but it caused a rapid increase in back-pressure and a deterioration of column performance due to presence of protein in the sample [29]. It was therefore necessary to deproteinize the biological samples prior to HPLC analysis. We used 3.75% (w/v) metaphosphoric acid, 4% (v/v) perchloric acid [30] and 4% (w/v) sulfosalicylic acid (w/v) for deproteinization of rumen fluid. Peak heights of the standard compounds were decreased and reproducibility was not good in case of the first two deproteinizing agents (data not shown). Sulfosalicylic acid (4%, w/v) showed an excellent efficacy. In other words, by using sulfosalicylic acid, neither the subsequent derivatization nor UV detection were unfavorably influenced.

3.3. Use of chelating agent

It is generally prudent to analyze biological samples as soon as possible after preparation. At first, it was difficult to prevent the oxidation of Cys and Hcys (in 2% SSA). By using EDTA-3K in SSA solution, the oxidation of sulfhydryl groups of some Cys and Hcys to cystine and homocystine during the sample preparation was effectively prevented. Solutions of standard Cys and Hcys in SSA–EDTA were stable at 4°C for at least 8 days without significant losses. When SSA–EDTA solution was prepared some precipitation usually occurred after leaving overnight. Therefore, it was advisable that SSA–EDTA solution should be prepared at least 1 day prior to use and kept in a refrigerator at 4°C. DeMaster and Redfern [31] used PCA (perchloric acid)–EDTA solution for this purpose, and they did not mention this type of phenomenon we observed. Another type of chelating agent, DTPA (diethylenetriaminepentaacetic acid), had been described as a more effective one than EDTA to prevent oxidation of thiols, e.g., Cys, Hcys, etc. [32]., but in our case, DTPA decreased the peak heights of Cys and Hcys and was less suitable than EDTA (data not shown).

3.4. Stability of the FMOC derivatives

To test the decomposition of FMOC derivatives over time, standard mixtures were derivatized and injected immediately and at 1.5, 2, 3, 4, 5, 6, 8, 10, 14, 16, 18 and 20 h after storing at room temperature. No degradation of the derivatives was observed up to 20 h with the exception of Hcys, Met and Cysta. In case of Hcys, if storage time was greater than 4 h, it led to the slow appearance of an unknown peak on the Hcys peak, while Met and Cysta showed a gradually degradation (i.e., after 4 h). No attempt was made to increase the stability (after 4 h) in this case. Following derivatization,

Malmer and Schroeder [19] also reported that samples could be stored at room temperature for at least 24 h before HPLC analysis without loss of response.

3.5. Chromatographic separation

A separation of the standard compounds (Cys, Hcys, MSO, MSO_2, Hser, Gly, Thr, 2AB, Met and Cysta) is shown in Fig. 1. Fig. 2 shows a chromatogram of a mixture of standard amino acids (Asp, Glu, Asn, Gln, Arg, Ser, Cit, Ala, Val, Pro, Trp, Phe, Leu, Ile, Tyr, His, Orn and Lys) with the exception of the standard compounds of interest. Comparison of Figs. 1 and 2 showed that none of the mixture of the amino acids interfered with the analysis of the compounds of interest. During the development of the present method, different types of buffer in different pH, ratio of mobile phase composition and several gradient profiles had been tested. At first, it was difficult to separate MSO from Ser and Cit and 2AB from Pro. Inclusion of small percentage of THF in solvent B solved these problems efficiently. At the initial stage of chromatography, higher pH 6.40 (solvent A) was important to increase the separation mode of standard compounds from the standard amino acids mixture but after 14.5 min a comparatively lower pH 3.80 (solvent C) was employed to ensure the separation of Hser, Thr and 2AB from other compounds. Finally, the present gradient profile (Table 1) was selected as the most suitable on the basis of resolution of 10 analytes. These analytes were separated from the potentially interfering amino acids and appeared within 76 min in the chromatogram (Figs. 1 and 2). After elution of Cysta, the percentage of methanol (solvent C) was rapidly and automatically increased (i.e., from 76 min, solvent C – 100%) to elute other amino acids. 85–90 min after injection, the mobile phase composition was restored to the initial value. The analytical column should be equilibrated for at least 25 min before injection of the next sample. Fig. 3 shows a typical chromatogram from a rumen fluid. In the rumen sample, the peaks with the same retention times of the authentic Cys, Gly, Thr, 2AB, Met and Cysta were observed. Peak identity was confirmed by addition of pure Cys, Gly, Thr, 2AB, Met and Cysta to the rumen fluid before chromatography. During the course of the present investigation, we discovered that other amino acids such as Asp, Glu, Asn, Pro, Trp and Phe also might be separated by the present method.

3.6. Precision, accuracy, linearity and sensitivity

The precision of the method was determined for both within-day and day-to-day variations and is expressed as the relative standard deviation (RSD). As shown in Table 2, at different concentrations of the standard samples added to the rumen fluid, the RSDs of all compounds varied between 0.83 and 3.79% in the within-day study, but from 1.17 to 5.83% on different day (6 days) studies.

The accuracy of the method was assessed by determining analytical recovery. The recoveries of all the compounds in rumen fluid were 97.93–102.3% in the within-day study and 94.52–98.69% on different day (6 days) studies (Table 2).

Linearity of the analytes was determined by the analysis of standard solution containing 0.0, 1.95, 3.90, 7.81, 15.62, 31.25, 62.5, 125.0, 250.0 and 500.0 μM of the different standard substances. A linear relationship was observed between the peak heights and sample concentrations. Thus, the concentration of the individual compounds could be readily calculated from their respective peak height by using the equation of linear regression curve presented in Table 3.

The correlation coefficients (r) of the calibration graphs for all the derivatives were highly significant ($P<0.001$) as shown in Table 3.

The values of the limit of detection (LOD) at a signal-to-noise (S/N) ratio of 3:1 and limit of quantification (LOQ) (S/N ratio, 10:1) of the compounds are provided in Table 3.

3.7. Application of the method

In order to test the applicability of the method to rumen fluid, samples were analyzed. As shown in Fig. 3, deproteinized rumen fluid of goat contained detectable amounts of Cys, Gly, Thr, 2AB, Met and Cysta. The average contents (μM) of Cys, Gly, Thr, 2AB, Met and Cysta were 1.72, 45.6, 20.0, 4.3, 2.11 and 3.42 before morning feeding; 0.0, 29.2, 14.6, 5.6, 9.6 and 0.78 at 2 h after feeding; 0.0, 30.7, 16.1, 7.3, 8.6 and 1.11 at 4 h after feeding; 5.04, 35.5,

Table 2
Precision and accuracy of Thr, Met and related other amino compounds in rumen fluid[a]

Compound	Concentration added (μM)	Concentration found (μM) (mean±SD)		RSD (%)		Accuracy (%)	
		WV	DV	WV	DV	WV	DV
Cys	500	504.23±5.30	493.47±5.83	1.05	1.18	100.0	98.69
	250	246.76±3.92	245.62±4.27	1.58	1.73	98.70	98.24
	125	126.4±2.76	119.2±3.76	2.18	3.15	101.1	95.36
Hcys	500	497.7±6.23	491.17±8.16	1.25	1.66	99.54	98.23
	250	249.35±2.28	243.18±4.68	0.91	1.92	99.74	97.27
	125	123.34±2.92	120.18±3.85	2.36	3.20	98.67	96.14
MSO	500	504.86±7.67	491.30±6.78	1.51	1.38	100.9	98.26
	250	250.64±3.48	239.2±2.80	1.38	1.17	100.25	95.68
	125	122.72±3.74	120.18±4.78	3.04	3.97	98.17	96.14
MSO_2	500	498.36±5.82	492.82±8.92	1.16	1.80	99.67	98.56
	250	249.42±6.32	243.3±5.92	2.53	2.43	99.76	96.92
	125	126.42±4.23	121.17±5.72	3.34	4.72	101.13	96.93
Hser	500	501.36±6.22	491.38±6.92	1.24	1.40	100.27	98.2
	250	249.72±3.82	243.3±7.32	1.52	3.00	99.88	97.32
	125	124.16±2.18	121.3±3.32	1.75	2.73	99.32	97.04
Gly	500	511.64±9.62	493.32±7.82	1.88	1.58	102.3	98.66
	250	252.16±4.72	241.23±5.62	1.87	1.32	100.8	96.49
	125	124.62±4.43	120.38±6.32	3.55	5.25	99.69	96.30
Thr	500	498.11±4.78	489.18±6.78	0.95	1.38	99.62	97.83
	250	248.32±3.67	242.4±4.83	1.47	1.99	99.32	96.96
	125	124.8± 4.73	119.16±5.62	3.79	4.71	99.84	95.32
2AB	500	499.23±4.16	492.16±9.23	0.83	1.87	99.84	98.43
	250	249.42±3.96	243.13±4.92	1.58	2.02	99.76	97.25
	125	124.16±2.16	121.32±4.67	1.73	3.84	99.32	97.05
Met	500	502.16±6.42	480.62±8.18	1.27	1.70	100.4	96.12
	250	249.16±7.48	240.60±5.66	3.00	2.35	99.66	96.24
	125	123.18±2.72	118.31±6.36	2.20	5.37	98.54	94.64
Cysta	250	248.36±4.12	242.35±4.62	1.65	1.90	99.34	96.94
	125	122.42±3.52	118.16±5.95	2.87	5.03	97.93	94.52
	62.5	61.32±1.13	59.33±3.46	1.84	5.83	98.11	94.92

[a] RSD=Relative standard deviation, WV=within-day variation (n=5), DV=day-to-day variation (n=6).

21.4, 9.6, 6.6 and 3.77 at 6 h after feeding, respectively (Fig. 4).

Among these compounds, Gly was the major component in the rumen fluid both before feeding and after feeding and the concentration at a maximum before feeding.

Cys was only present in the sample collected before feeding and 6 h after feeding. The concentrations of Thr, 2AB and Cysta in rumen fluid tended to increase with the time after feeding, whereas Met showed the opposite tendency. This is the first report that Cys, Gly, Thr, 2AB, Met and Cysta were quantitatively determined in the rumen fluid as far as we are aware.

Table 3
Retention time (t_R), limit of detection (LOD), limit of quantification (LOQ) and linear regression equation of Thr, Met and their related amino compounds[a]

Compound	t_R (min)	LOD (μM)	LOQ (μM)	Linear regression equation	r
Cysteine (Cys)	13.1	0.50	1.68	$y=357.5+81\,650x$	0.99986***
Homocysteine (Hcys)	14.4	1.76	5.85	$y=168.73+19\,757x$	0.99918***
Methionine sulfoxide (MSO)	31.1	0.85	2.88	$y=411.4+51\,411x$	0.99951***
Methionine sulfone (MSO_2)	34.4	0.92	3.09	$y=480.67+46\,969x$	0.9992***
Homoserine (Hser)	35.9	1.04	3.52	$y=281.07+43\,046x$	0.99969***
Glycine (Gly)	42.8	0.76	2.52	$y=308.4+62\,368x$	0.99964***
Threonine (Thr)	48.3	0.65	2.18	$y=463.4+67\,334x$	0.99949***
2-Aminobutyric acid (2AB)	61.1	0.39	1.36	$y=526.42+1.2182\cdot10^5x$	0.99976***
Methionine (Met)	64.7	0.31	1.03	$y=446.22+1.5529\cdot10^5x$	0. 99993***
Cystathionine (Cysta)	76.1	0.17	0.58	$y=688+4.9166\cdot10^5x$	0.99993***

[a] y=Peak height (μV), x=concentration (mM), r=correlation coefficient for peak height and concentration.
*** Significant, $P<0.001$.

4. Conclusions

A selective, sensitive and reproducible HPLC method based on pre-column derivatization using FMOC-Cl has been developed and evaluated for the simultaneous analysis of Cys, Hcys, MSO, MSO_2, Hser, Gly, Thr, 2AB, Met and Cysta in rumen fluid with UV detection. In this method, basic condition for derivatization was maintained by using 0.8 M potassium borate buffer, pH 9.90, therefore, no prior pH adjustment of acidic rumen samples by other alkali was needed before derivatization process. The

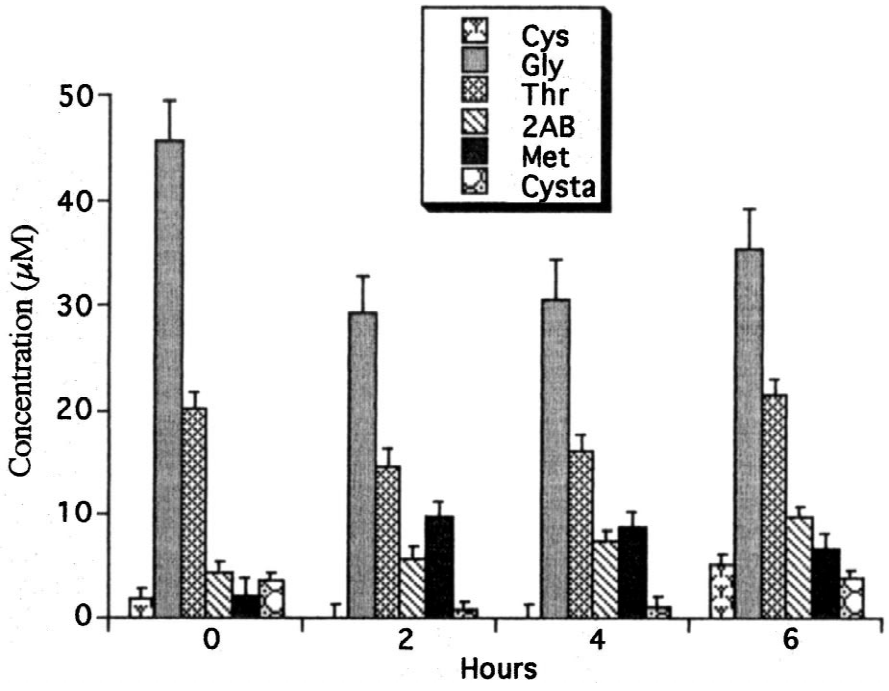

Fig. 4. Average concentration (μM) of Cys, Gly, Thr, 2AB, Met and Cysta in the goat rumen fluid before morning feeding and 2, 4 and 6 h after feeding.

ability of this assay to measure 10 analytes simultaneously, may be applicable to further studies on the metabolism of Thr, Met and their related amino compounds (mentioned earlier) by rumen microorganisms.

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