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Journal of Chromatography B, 710 (1998) 17–25

JOURNAL OF
CHROMATOGRAPHY B

Quantitative determination of aromatic amino acids and related compounds in rumen fluid by high-performance liquid chromatography

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Received 16 December 1997; received in revised form 12 February 1998; accepted 13 February 1998

Abstract

A rapid method for the quantitative determination of tyrosine (Tyr), phenylalanine (Phe), *p*-hydroxybenzoic acid (HBA), *p*-hydroxyphenylacetic acid (HPA), benzoic acid (BZA), *p*-hydroxyphenylpyruvic acid (HPY), phenylacetic acid (PAA), phenyllactic acid (PLA), tryptophan (Trp), indoleacetic acid (IAA), phenylpyruvic acid (PPY), phenylpropionic acid (PPA) and cinnamic acid (CNA) in goat rumen fluid was established by high-performance liquid chromatography (HPLC). The mobile phase used for isocratic elution was 50 m*M* sodium phosphate buffer (pH 6.5)–methanol (97:3, v/v). The flow-rate was 1.0 ml/min; column temperature 40°C and compounds were monitored at 215 nm with a UV absorbance detector after injection of 10 μl of filtered rumen fluid. Analysis was completed within 40 min. The minimum detectable limits of quantification (μ*M*) of these compounds were Tyr, 2; Phe, 3; HBA, 1; HPA, 2; BZA, 2; HPY, 8; PAA, 3; PLA, 4; Trp, 2; IAA, 2; PPY, 15; PPA, 8 and CNA, 4. Detectable levels of Tyr, Phe, HPA, BZA, HPY, PAA, PLA, Trp and PPA were found in the deproteinized rumen fluid of goat fed a haycube and concentrate mixture. PAA was the predominant compound before and after feeding. The concentrations of HPA, BZA, PAA, PLA and PPA in the goat rumen fluid increased after feeding, while the concentration of Tyr decreased. Phe, HPY and Trp were minor components at all times. PPY, IAA and CNA were not detected and HBA was not completely resolved in the goat rumen fluid. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Amino acids, aromatic; Rumen fluid

1. Introduction

Phenylalanine (Phe) and tryptophan (Trp) are known as essential aromatic amino acids in mammals. Another aromatic amino acid, tyrosine (Tyr) is not essential when sufficient Phe is available [1], because the amino acid is normally produced from Phe in the liver [2].

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In ruminant animals, it has been shown that rumen bacteria could synthesize Tyr by the transamination to *p*-hydroxyphenylpyruvic acid (HPY) produced by the reductive carboxylation of *p*-hydroxyphenylacetic acid (HPA) [3]. However, it has not been clarified whether or not the rumen protozoa can produce Tyr by the same pathway as rumen bacteria or if the rumen bacteria can produce Tyr from Phe. In the study of phenylalanine (Phe) metabolism by rumen bacteria and protozoa, a relatively large

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PII: S0378-4347(98)00103-0

amount of an unknown substance was detected at the same retention time as Tyr and HPA using high-performance liquid chromatography (HPLC) [4]. However, the substance was not identified, because the peaks of Tyr and HPA were not separated [5]. Chalupa [6] and Smith [7] suggested that Tyr could be synthesized from Phe by the rumen bacterial population, but Allison [8] found no synthesis of Tyr from Phe or its immediate precursors. Thus to date, there is no direct evidence for the production of Tyr from Phe by rumen microorganisms.

Tyr has been shown to be catabolized to 3-phenylpropionic acid (PPA) and small amounts of HPA by mixed rumen microorganisms [9], but it has not been demonstrated whether or not these substances were produced by rumen bacteria or protozoa alone. To perform detailed studies on the metabolism of Tyr and its related compounds by rumen microorganisms, it was necessary to develop a simple and accurate method for the determination of the compounds related to Tyr metabolism.

Several investigators have reported HPLC methods for the determination of Tyr [10–14], Phe [10–20], phenylacetic acid (PAA) [21–25], HPA [22] and benzoic acid (BZA) [24–26] in blood plasma and urine. Recently a method was established for determination of Phe and related compounds in rumen fluid [5]. However, as far as we are aware, no methods have been reported for the simultaneous determination of *p*-hydroxybenzoic acid (HBA), HPA, HPY, Tyr, Phe and their other related compounds. In the present paper, a quantitative determination method will be described for the aromatic amino acids, including Tyr, Phe, Trp and 11 related compounds. Special attention was given to the separation of Tyr and HPA. The established method was applied to the determination of those substances in the goat rumen fluid.

2. Experimental

2.1. Apparatus

A liquid chromatography pump (980 PU), variable-wavelength ultraviolet detector (875 UV) and column oven (860 CO) were obtained from Japan Spectroscopic (Tokyo, Japan); the data analyzer (Chromatopac, C-R6A) and spectrophotometer (BioSpec-1600) with a thermal printer (Type II, DPU-411) were from Shimadzu (Kyoto, Japan); the autosampler (AS 8000) was obtained from Tosoh (Tokyo, Japan), and the LiChrospher 100 RP-18 column (250×4 mm I.D.) of 5 μm particle size, was from Kanto (Tokyo, Japan) under license from Merck (Darmstadt, Germany).

2.2. Chemicals

Sodium phosphate (monobasic and dibasic), sodium acetate, sulfosalicylic acid (SA), *p*-hydroxybenzoic acid (HBA), L-phenylalanine (Phe), L-tyrosine (Tyr), benzoic acid (BZA), indole-3-acetic acid (IAA) and *trans*-cinnamic acid (CNA) were purchased from Nacalai Tesque (Kyoto, Japan). L-3-Phenyllactic acid (PLA), phenylpyruvic acid (PPY) and 3-phenylpropionic acid (PPA) were from Aldrich (Milwaukee, WI, USA). *p*-Hydroxyphenylpyruvic acid (HPY), phenylacetic acid (PAA), L-tryptophan (Trp), perchloric acid (PA) and ethanol were from Wako (Osaka, Japan). *p*-Hydroxyphenylacetic acid (HPA) was from Tokyo Chemical (Tokyo, Japan) and methanol was obtained from Kanto. Ultra-pure water made with Milli-Q Labo (Nihon Millipore, Tokyo, Japan) was used to prepare the mobile phases and other solutions.

2.3. Standard solutions

Standard solutions (0.25 m*M*) of Tyr, Phe, HBA, HPA, BZA, PAA, PLA, PPA and CNA in water and of Trp, IAA, PPY and HPY in 25% ethanol were mixed with an equal volume of 5% (v/v) PA [13] and filtered through a 0.45-μm membrane filter before HPLC analysis. Ten-μl aliquots of the filtrate were directly injected into the HPLC system via the autosampler.

2.4. Chromatography

The mobile phase used for isocratic elution was prepared by mixing methanol and 50 m*M* sodium phosphate (monobasic and dibasic) buffer (pH 6.5) in a ratio of 3:97 (v/v). Before use, the mobile phase was filtered (membrane filter HV 0.45-μm, Nihon Millipore Kogyo K.K., Tokyo, Japan) and degassed

by ultrasonication. The flow-rate was 1.0 ml/min; column temperature, 40°C. Compounds were monitored at 215 nm with a UV absorbance detector using a LiChrospher 100 RP-18 (250×4 mm I.D.) column of 5 μm particle size.

2.5. Sample preparation

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 $BW^{0.75}$) and concentrate mixture (8 g DM/kg $BW^{0.75}$) provided in two equal portions at 09:00 and 17:00. The rumen contents were collected before feeding, and 1, 3, 6 and 9 h after feeding, and strained through four layers of surgical gauze. One ml of the sample was mixed with the same volume of 5% PA, kept at 4°C overnight and then centrifuged at 27 000 *g* for 20 min. The supernatant fluid was filtered (membrane filter, 0.45 μm) and a 10-μl aliquot of the filtrate was injected directly into the HPLC system.

3. Results and discussion

3.1. Choices of wavelength and mobile phase composition

The absorbance spectra of the 13 analytical compounds dissolved in solvents (water or 25% ethanol), 5% PA solution and the buffer used as mobile phase were examined with the spectrophotometer. Considering the selectivity and sensitivity a wavelength of 215 nm was chosen for the subsequent experiments.

The methanol content and pH values of the mobile phase influenced the retention time of the 13 compounds. A higher methanol percentage reduced the retention time, while a low pH increased the retention time for all compounds. Similar results were reported by Amin et al. [5] in case of methanol–50 m*M* sodium acetate buffer (pH 6.5) (8:92, v/v) for separation of Phe and related compounds of the goat rumen fluid. The methanol content and the pH of the mobile phase were varied from 0 to 10% and from 5.0 to 8.0, respectively. Optimal results were obtained with 3% methanol at pH 6.5.

The effect of phosphate [19] and acetate [5,27] buffers were also compared. It was found that similar retention times were obtained with both buffers. However the former seemed better than the latter with respect to good separation and peak-height. In addition, 5% PA and 4% SA solution were compared as a deproteinizing agents. There was considerable overlap of the SA peak with HBA, and slight overlap with Tyr, whereas the PA peak did not coincide with the peaks of any of the 13 analytes. Finally, when methanol–phosphate buffer (3:97, v/v) at pH 6.5 was used, all compounds tested were properly separated within 40 min (Fig. 1). The first peak with retention time (t_R): 2.3 min was that of the PA used for the deproteinization of the sample. The retention times (t_R) for the 13 analytes are provided in Table 1 and the chromatogram is shown in Fig. 1.

3.2. Calibration curve and minimum detectable amounts

A linear relationship was observed between the peak-heights and sample concentrations of 15.6, 31.3, 62.5, 125.0, 250.0, 500.0 and 1000.0 μ*M* standard solutions of each of the 13 analytes. Thus the concentration of the individual compounds could be readily calculated from their respective peak-height. The correlation coefficients (*r*) for peak-height and concentration of all analytes were highly significant ($P<0.001$) (Table 1). The equation of the linear regression curve for each analyte is presented in Table 1. The minimum detectable limits of quantification (μ*M*) of 13 analytes for this method were examined. The values are provided in Table 1.

3.3. Peak identification

In order to identify the compounds in each peak, variations in retention time with changes in pH and the percentage of methanol in the mobile phase were monitored. Changes in the retention times of peaks in the rumen fluid correlated well with changes in the retention time of the standard compounds over a pH range from 6.3 to 6.7 and methanol concentrations from 0 to 5%. For all compounds the maximum difference in retention time was less than 4 s.

The peak of HBA was closely associated with an unknown compound in the rumen fluid. Three compounds that can be resolved by our method (PPY,

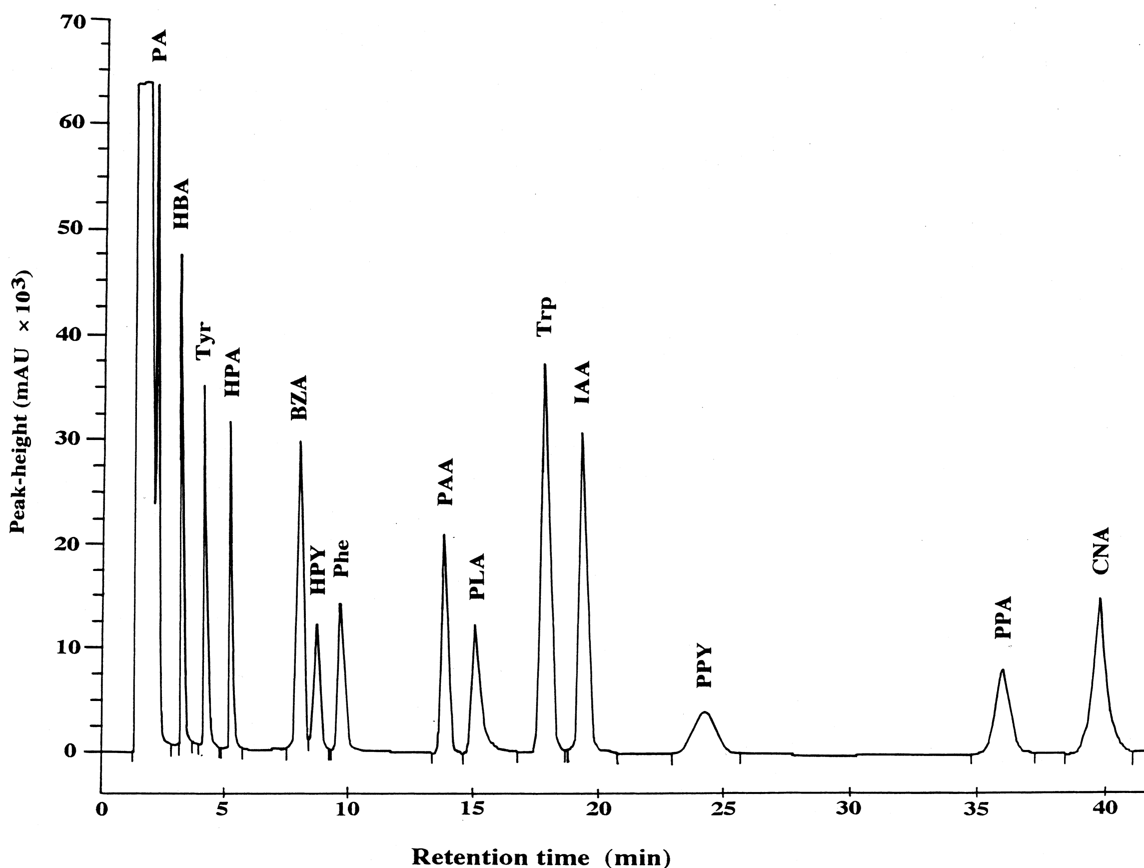

Fig. 1. Chromatogram of standard tyrosine (Tyr), phenylalanine (Phe), *p*-hydroxybenzoic acid (HBA), *p*-hydroxyphenylacetic acid (HPA), benzoic acid (BZA), *p*-hydroxyphenylpyruvic acid (HPY), phenylacetic acid (PAA), phenyllactic acid (PLA), tryptophan (Trp), indolacetic acid (IAA), phenylpyruvic acid (PPY), phenylpropionic acid (PPA) and cinnamic acid (CNA) by HPLC. Column: 250×4 mm I.D.; flow-rate: 1.0 ml/min; mobile phase: methanol–50 m*M* sodium phosphate buffer (pH 6.5) (3:97, v/v).

IAA and CNA) were not detected in the rumen fluid. When these three compounds were added to the rumen fluid in control experiments, peaks appeared with retention times as expected for each compound at corresponding pH values (6.3 to 6.7) and methanol percentages (0 to 5%). Thus, with the exception of only one peak (HBA), all of the compounds of the rumen fluid assayed by this method were identified.

The purity of the compound in each peak was confirmed by comparing the peak-height ratios of the authentic compounds and of the identified peaks at different wavelengths (215, 220 nm), according to Lefeng et al. [15] and Amin et al. [5]. The ratios of absorbance at the two wavelengths for the rumen samples were in good agreement with the ratios found for the respective pure compounds (Table 2). This means that at the selected wavelength there were no significant contributions to the peak-heights from compounds in the rumen fluid other than selected analytes.

3.4. Analytical recovery, precision and accuracy

In the direct injection analyses, standard solutions of different concentrations, rumen fluid and mixtures of the two were used to determine the analytical recovery of all compounds. Standard compounds of different concentrations were added to the rumen fluid and then the concentrations were measured. Recovery was calculated by subtracting the con-

Table 1
Retention time (t_R), limit of quantification (LOQ) and calibration curve of aromatic amino acids and related compounds

Compound (n=7)	t_R (min)	LOQ (μM)	Linear regression line (equation)	r
p-Hydroxybenzoic acid (HBA)	3.3	1	$y=28.388x+0.0479$	0.99998***
Tyrosine (Tyr)	4.2	2	$y=17.488x+0.0397$	0.99996***
p-Hydroxyphenylacetic acid (HPA)	5.3	2	$y=15.895x-0.0038$	0.99997***
Benzoic acid (BZA)	8.1	2	$y=14.661x+0.0304$	0.99987***
p-Hydroxyphenylpyruvic acid (HPY)	8.7	8	$y=7.659x+0.0309$	0.99971***
Phenylalanine (Phe)	9.7	3	$y=9.191x+0.0023$	0.99995***
Phenylacetic acid (PAA)	13.6	3	$y=11.745x-0.1127$	0.99976***
Phenyllactic acid (PLA)	14.8	4	$y=8.332x-0.0252$	0.99975***
Tryptophan (Trp)	17.3	2	$y=24.485x+0.0008$	0.99992***
Indolacetic acid (IAA)	18.6	2	$y=19.588x+0.3011$	0.99968***
Phenylpyruvic acid (PPY)	24.6	15	$y=2.969x+0.0103$	0.99993***
Phenylpropionic acid (PPA)	35.8	8	$y=5.198x-0.0312$	0.99993***
Cinnamic acid (CNA)	39.8	4	$y=8.498x+0.0109$	0.99994***

n=Number of concentrations (15.6~1000.0 μM), r=correlation coefficient for peak-height and concentration, y=peak height (cm), x=concentration (mM). Column: 250×4 mm I.D.; flow-rate: 1.0 ml/min; mobile phase: methanol–50 mM sodium phosphate buffer (pH 6.5) (3:97, v/v).
*** Significant, $P<0.001$.

centration of rumen fluid from the mixture of standard solution and rumen fluid and comparing with the concentration of the standard compound. The recoveries are shown in Table 3.

The recovery of BZA and HPY in rumen fluid was satisfactory at the lower concentrations (<0.5 mM), but not at higher concentrations (>0.5 mM) due to close retention time of these compounds. HBA could not be recovered well due to its association with the peak of an unknown compound of rumen fluid. The recoveries (%) of all other compounds in the rumen fluid were good (ranging from 97.49±4.27 to 100.58±2.70 in a single day and from 97.09±4.36 to 101.16±2.72 on different days).

Table 2
Peak-height ratio of the compounds at 215 and 220 nm

Compound	Peak-height ratio (215/220)	
	Standard sample	Rumen sample
Tyr	1.015	1.019
HPA	1.236	1.211
BZA	1.439	1.446
HPY	1.330	1.328
Phe	1.702	1.697
PAA	1.552	1.547
PLA	1.471	1.473
Trp	0.967	0.961
PPA	1.628	1.670

Abbreviations and HPLC conditions are given in Table 1.

The precision, accuracy and reproducibility of the analytical procedure were determined for both within-day and day-to-day variations (Table 3). Precision is expressed as the coefficient of variation (C.V.) and accuracy as the relative error (R.E.). At different concentrations of the standard samples the C.V. of all compounds varied between 1.28 and 5.09% on the same day, but from 2.26 to 5.44% on different-day studies (Table 3).

The accuracy was assessed by analyzing known amounts of analytes. The observed concentrations were in good agreement with the actual concentrations. The R.E. ranged from 0.14 to 3.57% on the same day and from 0.05 to 4.02% between days (Table 3). These data demonstrated that the method established for the measurement of the compounds in the rumen fluid was accurate.

3.5. Applications

The established method has been applied to the analysis of 45 rumen samples from three goats at different times after feeding. A typical chromatogram of deproteinized rumen fluid is shown in Fig. 2. As stated previously, detectable amounts of Tyr,

Table 3
Precision, accuracy and recovery of aromatic amino acids and their related compounds in rumen fluid

Compound n=6	Concentration added (nM)	C.V. (%).		R.E. (%)		Mean recovery[a] (%)	
		S.V.	D.V.	S.V.	D.V.	S.V.	D.V.
Tyr	0.125	1.28	5.44	1.68	0.15	98.69	100.79
	0.250	1.64	2.58	0.81	0.79	±1.58	±3.51
	0.500	2.02	3.10	1.20	1.33		
HPA	0.125	2.13	3.78	0.64	3.44	98.79	97.09
	0.250	2.82	5.41	2.18	1.48	±3.44	±4.36
	0.500	5.09	4.15	0.78	3.80		
BZA	0.125	2.97	2.88	0.19	0.05	98.94	100.53
	0.250	2.41	3.12	0.16	0.16	±2.63	±2.72
HPY	0.125	3.01	5.09	0.76	1.67	100.58	97.15
	0.250	2.70	4.06	0.39	4.02	±2.70	±4.56
Phe	0.125	3.16	4.28	0.78	0.17	99.32	98.80
	0.250	3.15	3.80	0.14	2.37	±3.11	±4.25
	0.500	3.41	5.02	1.39	1.03		
PAA	0.125	3.36	3.77	2.47	1.60	98.12	99.45
	0.250	2.79	4.55	1.88	1.49	±2.82	±4.00
	0.500	2.68	3.51	1.29	1.43		
PLA	0.125	2.98	2.60	0.99	0.09	98.87	101.16
	0.250	3.24	3.32	2.28	2.01	±3.19	±2.72
	0.500	2.69	2.26	2.09	1.39		
Trp	0.125	3.59	2.28	0.25	0.48	100.12	100.18
	0.250	3.88	3.37	0.18	0.54	±3.12	±2.75
	0.500	2.19	2.86	0.44	0.60		
PPA	0.125	4.10	3.77	2.55	0.51	97.49	99.40
	0.250	4.34	3.65	3.57	0.11	±4.27	±3.55
	0.500	4.31	3.84	0.97	1.23		

C.V.=Coefficient of variation, R.E.=relative error, S.V.=same day variation, D.V.=day-to-day variation. Abbreviations and HPLC conditions are given in Table 1.
[a] Mean of the recoveries of all concentrations.

HPA, BZA, HPY, Phe, PAA, PLA, Trp and PPA were found in the deproteinized rumen fluid. As shown in Fig. 3, the concentration of Tyr did not change significantly (54–71 μM) and was only slightly higher before feeding. Amin et al. [5] did not find Tyr in the goat rumen fluid, because their method did not separate Tyr from HPA [5]. Phe, HPY and Trp were minor components of the rumen fluid at all times tested (7–40, 13–27 and 3–10 μM, respectively). Similar results were reported previously for Phe [5]. This is the first report on the concentration of Tyr, HPA, HPY and Trp in the rumen fluid.

Large differences in the concentrations of HPA (0–60 μM), BZA (79–127 μM), PAA (310–551 μM), PLA (0–72 μM) and PPA (21–236 μM) were found before and after feeding. Most of the aromatic acids were present at their highest concentrations 1 h after feeding, except PPA, which reached a maximum 3 h after feeding. Similar values of BZA and higher values of PAA, PLA and PPA were found in

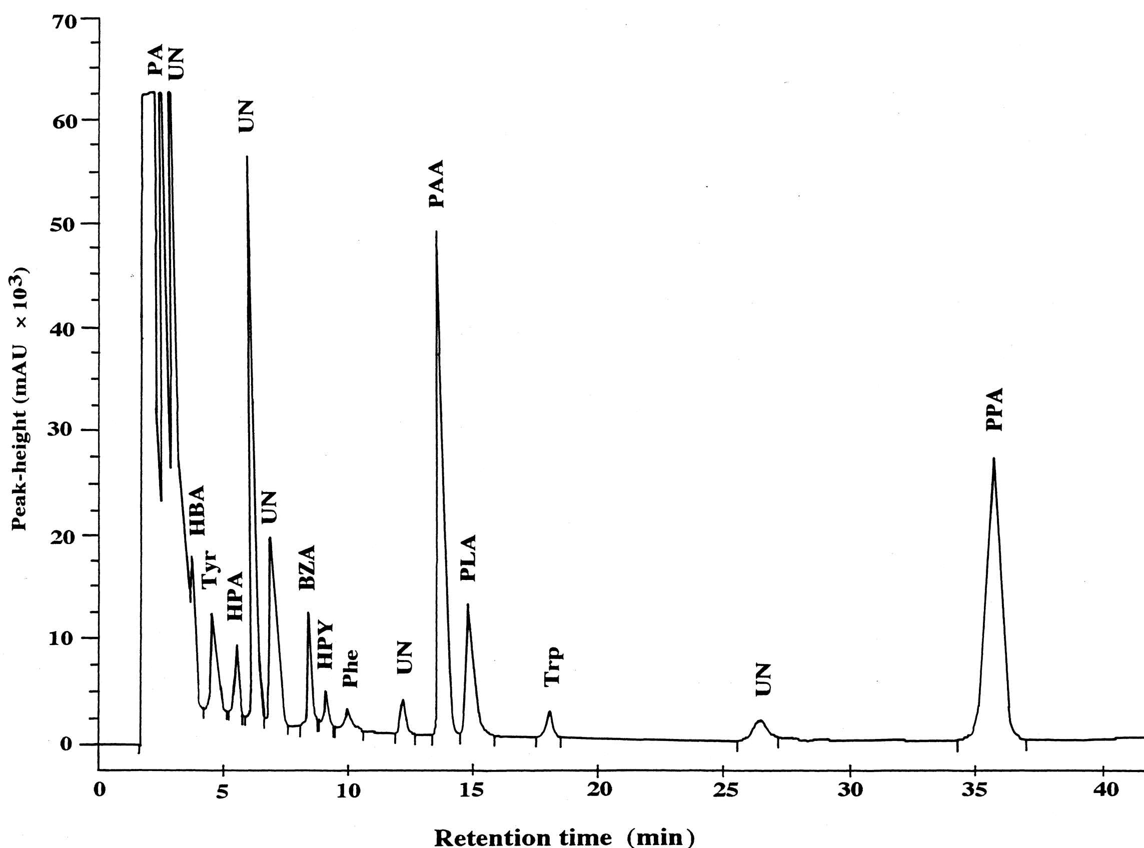

Fig. 2. Typical chromatogram of deproteinized goat rumen fluid by HPLC. Abbreviations of the compounds and all conditions of HPLC as Fig. 1. UN=unknown peak.

goats by Amin et al. [5]. The concentration of BZA found in this study was much higher than the values (4 and 10 μ*M*) found in sheep [9,28]. According to Martin [28], the concentration of PAA in sheep rumen varied from 0 to 257 μ*M*. The concentration of PPA found in this study was close to the concentration of PPA in sheep (313 μ*M*) [28], but lower than that found in cow (660 μ*M*) [29]. In the present study, PAA was the major aromatic acid before and after feeding which is similar to the results of Amin et al. [5] found in goats, but different from steers [30], cows [31] and sheep [28,32] where the major aromatic acid was PPA. HPA and PLA were only found after feeding.

In summary, we described here a highly sensitive, reproducible and reliable method for the simultaneous separation of Tyr, Phe, HPA, BZA, HPY, PAA, PLA, Trp, IAA, PPY, PPA and CNA in rumen fluid. Only basic HPLC equipment with isocratic elution is needed and no specialized sample preparation or detection system is required for the analysis of these aromatic amino acids and their related compounds.

Acknowledgements

The authors are extremely grateful to Professor H. Ogawa, the University of Tokyo, for inserting permanent rumen fistula on goats. The present study was financially supported by research grants from Kaken Pharmaceutical Co. Ltd. and Daiichi Seiyaku Co., Japan. We would like to thank the Ministry of Education, Science, Sports and Culture of Japan (Monbusho) for the award of a research scholarship

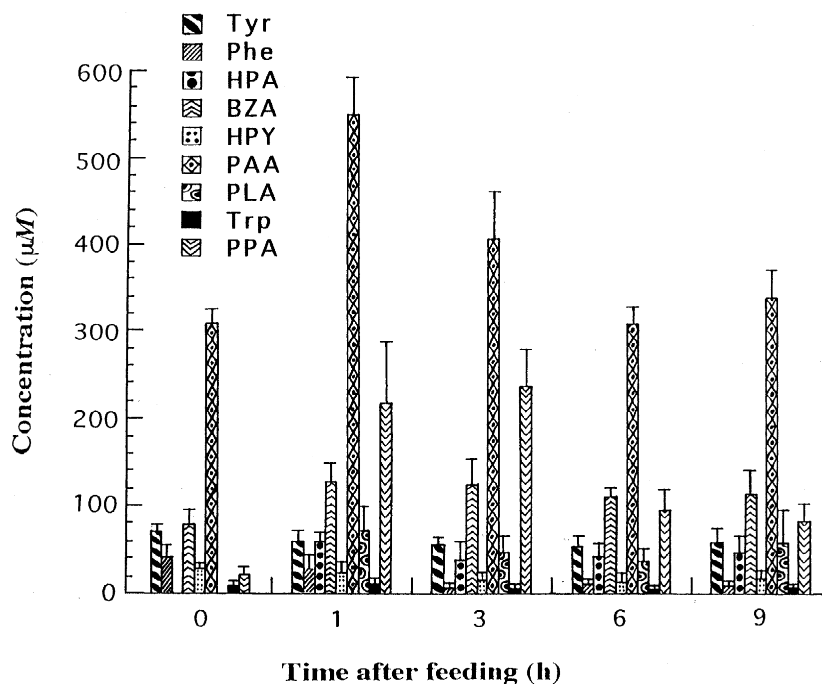

Fig. 3. Concentration (μM) of tyrosine (Tyr), phenylalanine (Phe), *p*-hydroxyphenylacetic acid (HPA), benzoic acid (BZA), *p*-hydroxyphenylpyruvic acid (HPY), phenylacetic acid (PAA), phenyllactic acid (PLA), tryptophan (Trp), and phenylpropionic acid (PPA) in goat rumen fluid before feeding and 1, 3, 6 and 9 h after feeding.

to R.I. Khan and N. Mohammed since 1996 and to M.R. Amin since 1992. The authors are indebted to Professor K.M. Karrer, Marquette University, USA, for her thorough editing of this manuscript and many other valuable suggestions to improve its quality.

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