Journal of Chromatography, 623 (1992) 63–67
Elsevier Science Publishers B V , Amsterdam

CHROM 24 412

Determination of diaminopimelic acid in biological materials using high-performance liquid chromatography

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(First received March 3rd, 1992, revised manuscript received June 2nd, 1992)

ABSTRACT

A method for the determination of diaminopimelic acid (DAPA) concentrations in feeds and rumen digesta by reversed-phase high-performance liquid chromatography using precolumn derivatization with *o*-phthaldialdehyde and fluorimetric detection was developed Samples were oxidized and hydrolysed prior to analyses by HPLC Hydrogen peroxide and formic acid were used for oxidation, hydrolyses were performed using 3 *M* hydrochloric acid under vacuum at 120°C for 17 h Oxidation allowed more space for DAPA–OPA peak elution and hydrochloric acid hydrolysis reduced sample clean-up and extended the column life Hydrolysates were diluted, adjusted to pH 7 and filtered A Beckman Model 507 autosampler with a precolumn derivatization cassette was used for the derivatization process and fluorimetric detection was used to measure the OPA derivatives Samples were prepared in order to have on-column DAPA concentrations in the range 10–100 pmol The relative recovery of the standard solutions added to the feed samples ranged from 98 4 to 102 8% The reproducibility of the method was evaluated by the analysis of eight alfalfa hay samples and eight alfalfa hay samples incubated in the rumen for 48 h and they yielded relative standard deviations of 2 04% and 2 02%, respectively

INTRODUCTION

Diaminopimelic acid (DAPA) is a component of bacterial cell walls It has been established that in bacteria the DAPA/protein ratio is relatively constant [1] Therefore, DAPA has been used as a indirect marker for the measurement of bacterial contamination in biological materials, *e g* , silages and rumen contents [2,3] The traditional method for determining DAPA in digesta or in feeds employed ion-exchange chromatography and ninhydrin [4,5] This method requires an expensive amino acid analyzer and high levels of methionine in the sample can interfere with DAPA determination Czerkawski [1] proposed a method that is less costly, but it is labour intensive and interference from proline may occur The method proposed by Webster *et al* [6] using high-performance liquid chromatography (HPLC) requires costly purification of samples and the individual sample running time is similar to that in the normal amino acid analyzer method

The objective of this study was to develop a rapid, inexpensive, sensitive and reliable HPLC method for determination of the DAPA content in biological samples with utilization of oxidation, hydrolysis, precolumn *o*-phthaldealdehyde (OPA) derivatization and fluorimetric detection

EXPERIMENTAL

Reagents and standards

HPLC-grade methanol, 2-mercaptoethanol and

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0021-9673/92/$05 00

tetrahydrofuran were purchased from Merck (Darmstadt, Germany), sodium acetate from BDH (Poole, UK) and DL-α,ε-diaminopimelic acid, OPA and ethanolamine from Sigma (St Louis, MO, USA) All other chemicals were purchased from POCH (Gliwice, Poland)

HPLC configuration

A System Gold (Beckman, San Ramon, CA) HPLC system was employed The apparatus consisted of a Model 126 gradient solvent-delivery module, a Model 507 autosampler with precolumn derivatization cassette, a Waters (Milford, MA, USA) Model 420-AC fluorescence detector (excitation filter, 338 nm interference, emission filter, 425 nm long pass), a Model 166 UV detector and a Model 406 analogue interface Analytical method development, data collection and data integration were performed by using Beckman Gold Chromatograph software on a PC-AT computer Data from the fluorescence and UV detectors were collected simultaneously The column used was a Eurospher 80 C_{18}, 5 μm (250 × 4 6 mm I D) (Knauer, Berlin, Germany) in conjunction with a Eurospher 80 C_{18} precolumn (Knauer)

Analytical solvents and gradient composition

A binary gradient was used Solvent A was tetrahydrofuran–methanol–buffer (5 95 900) and solvent B was methanol The buffer in solvent A was prepared from 0 1 *M* sodium acetate adjusted to pH 7 0 with phosphoric acid All components of solvent A were mixed and the pH was adjusted to 7 0 Solvents were filtered (0 45 μm) and flushed with helium prior to use The gradient composition is shown in Table I

Preparation of standard and derivatizing solutions

DL-α,ε-Diaminopimelic acid stock standard solution was prepared at a concentration of 2 63 m*M* (0 5 mg ml^{-1}) in 0 1 *M* sodium acetate adjusted to pH 7 0 and stored at 4°C The stock standard solution was further diluted with 0 1 *M* sodium acetate

Ethanolamine was dissolved in 0 1 *M* sodium acetate so that the concentration was 1 μ*M* and the pH was adjusted to 7 0 This solution was used for final sample dilution

The derivatizing solution was prepared by dissolving 50 mg of OPA crystals in 1 25 ml of methanol To this solution 11 2 ml of 0 4 *M* borate buffer (pH 9 5) and 50 μl of 2-mercaptoethanol were added The contents were mixed, transferred into a dark flask and flushed with nitrogen

TABLE I

GRADIENT COMPOSITION

Time (min)	Flow-rate (ml min^{-1})	Composition (%)		Gradient duration (min)[a]
		A	B	
0 00	1 00	70	30	
1 00	1 00	54	46	16 00
17 00	1 00	0	100	5 00
24 00	1 00	70	30	1 00
28 00[b]	1 00	70	30	

[a] Isocratic pumping if cell is empty
[b] Sample injection

Sample preparation

Feeds and rumen incubated feeds [7] were dried and finely ground prior to analysis Samples corresponding to 2 mg of nitrogen were weighed into 50-ml round-bottom flasks and oxidized with 2 ml of oxidizing reagent (0 2 ml of 30% hydrogen peroxide + 1 8 ml of 98% formic acid) The oxidizing reagent was allowed to stand at 50°C for 3 min before being added to the sample Oxidation proceeded at 50°C for 15 min and was terminated by adding 0 3 ml of 40% bromic acid before the oxidizing mixture was evaporated on a Buchi rotary evaporator The oxidized samples were hydrolyzed in the same flasks with 5 ml of 3 *M* hydrochloric for 17 h at 120°C Prior to hydrolysis the flasks were jointed with a glass adapter flow control (Kontes, Vineland, NJ, USA) and air was evacuated from the flasks with a vacuum pump Following hydrolysis, the vacuum was released from the flasks Hydrolysates were transferred into 50-ml beakers and adjusted to pH 7 with 10 *M* NaOH and made up to 50 ml The hydrolysates were stored at −27°C until analysis A 1-mol volume of the hydrolysate was mixed with 4 ml of 0 1 *M* sodium acetate, adjusted to pH 7 0 and filtered though a 0 22-μm filter into the autosampler vial

Sample derivatization

Derivatization of DAPA and other amino acids was performed with the Beckman Model 507 auto-

sampler using a precolumn derivatization cassette A 50-μ volume of sample from the vial was mixed with 50 μl of derivatizing solution and after reaction for 2 min it was injected into the analytical column

RESULTS AND DISCUSSION

The use of hydrochloric acid for sample hydrolysis followed by pH adjustment resulted in a much longer column life in comparison with the same procedure using methanesulphonic acid Comparison of these two hydrolyzing agents showed that column life was approximately tripled when using the former procedure

In regular amino acid analysis, tyrosine is eluted prior to DAPA and methionine, consequently, methionine often overlaps with DAPA This is particularly true with non-oxidized samples where the DAPA peaks are eluted very close to methionine and hence are difficult to integrate In order to allow more elution space for the DAPA peaks and because the DAPA–OPA derivative peaks were very small, oxidation of the samples was performed This caused the tyrosine peak to disappear and methionine to be converted into methionine sulphone, which is eluted much earlier This allowed for better integration of the DAPA peaks in comparison with non-oxidized samples It was found that oxidation did not change the DAPA content in oxidized samples, the mean recovery was 103 6 ± 2 8% in comparison with non-oxidized samples In summary, the oxidation of the samples overcame the problems discussed above and also allowed the analysis time to be decreased to less than 30 min (Table I)

Webster *et al* [6] proposed the use of a UV detector for the determination of DAPA To determine DAPA–OPA derivatives using a UV detector, higher sample concentrations are required This results in the loading of highly concentrated samples on to the column, which decreases its performance and lifetime To avoid these problems, a special clean-up procedure is required, which increases the cost of analysis In addition, high sample concentrations resulted in more time being required to elute the sample from the column and thus increased the analysis time Even though the concentration of other amino acids was too high for their accurate determination with a fluorescence detector, it was still impossible to determine DAPA using the UV detector as was proposed by Webster *et al* [6] (Fig 1) The sensitivity of the fluorescence detector is approximately eight times higher than that of the UV detector set at 340 nm

Samples were prepared such that the on-column amino acid concentration was about ten times higher than that for the regular hydrolysate used for amino acid analyses [8] and so that the DAPA concentration was in the 10–100-pmol range In this range the response of the fluorescence detector was

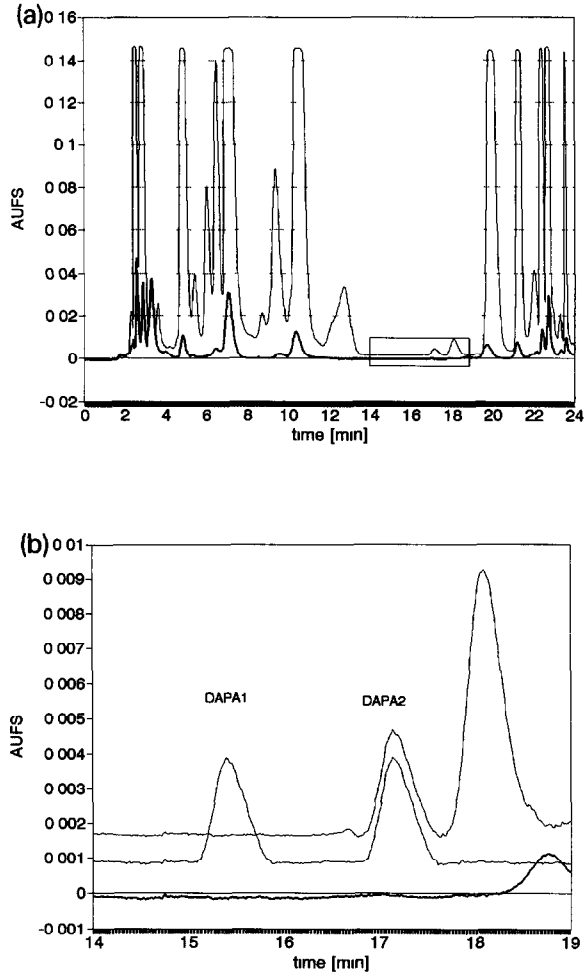

Fig 1 Chromatogram of grass hay for DAPA determination showing data from two detectors (a) Upper line, - fluorescence detection, lower line, UV detection (340 nm) (b) Expanded area from chromatogram (a) with added chromatogram of DAPA standard (20 pmol) with fluorescence detection (centre line)

linear with respect to the DAPA–OPA concentration

As reported by Webster *et al* [6], the DAPA standard was eluted in two peaks having very similar areas (Figs 1 and 2) Zanol and Gastaldo [9], using a reversed-phase column and 1-fluoro-2,4-dinitrophenylalaninamide, reported that the same standard yielded three peaks They identified them as D,L-, D,D- and L,L-DAPA In this paper the peaks will be referred as DAPA1 and DAPA2 as there are no isolated stereoisomers available commercially Both DAPA peaks were assumed to have equal fluorescence detector responses so the amount of each stereoisomer present in the standard were determined from its contribution to the peak area The form DAPA2 occurred in forages such as grass hay or alfalfa hay, which according to Webster *et al* [6] represented the D,L-stereoisomer However, in grass silage and corn silage both peaks occurred For the rumen incubated feeds, DAPA1 appeared on the chromatogram (Fig 2) and the concentration of both DAPA peaks was increased with increasing time of incubation Our observations show that bacteria colonizing the feed particles contain all DAPA isomers which is in agreement with the work of Zanol and Gastaldo [9]

The amounts of DAPA found in feed samples incubated in the rumen are shown in Table II The concentration of DAPA increased despite the digestive processes which occurred during rumen incubation During rumen fermentation ingested feedstuffs are digested and converted into microbial biomass Adherence of these microorganisms to the feed particles causes an increase in DAPA concentration It was found that the relationship between DAPA-N (μg g^{-1} DM) and time of incubation in the rumen of cows was best described by the exponential regression $y = 6\,81 + 10\,42\,[1 - \exp(-0\,05t)]$, where t (h) is the time of incubation in the rumen According to this equation, the onset of bacterial contamination of grass hay occurred largely within the first 12 h of rumen incubation It is interesting that the same type of regression was used to describe the rate of disappearance of feedstuffs in the rumen [10]

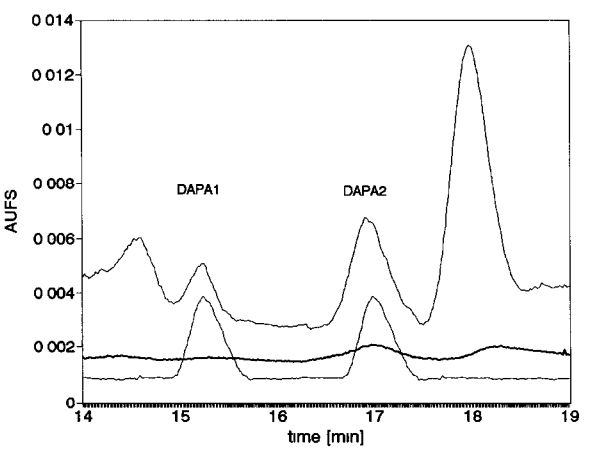

Fig 2 Part of chromatogram with DAPA peaks of grass hay incubated in the rumen for 24 h Upper line, fluorescence detection, centre line UV detection (340 nm), lower line, fluorescence detection for DAPA standard

TABLE II

CONTENT OF DIAMINOPIMELIC ACID IN GRASS HAY INCUBATED IN THE RUMEN OF COWS

Rumen incubation (h)	DAPA (nmol g^{-1} DM)[a]	DAPA-N (μg g^{-1} DM)[a]
0	243 2 ± 23 5	6 81 ± 0 65
2	274 2 ± 7 3	7 68 ± 0 20
4	299 2 ± 8 1	8 38 ± 0 23
8	395 2 ± 12 3	11 06 ± 0 35
12	408 5 ± 18 6	11 43 ± 0 52
24	474 1 ± 1 4	13 27 ± 0 04
48	615 4 ± 11 4	17 23 ± 0 32

[a] Mean ± standard error from five different grass hays used

Recovery of DAPA was performed by adding the DAPA standard to the feed samples and rumen incubated feed samples prior to oxidation and hydrolysis The mean recovery of the standard solution added to the feed samples was 99 8 ± 1 4% and ranged from 98 4 to 102 8% for seven feeds To check the accuracy of the autosampler, ethanolamine (internal standard) was added to the samples at the time of injection It must be noted that this causes an increase in analysis time The reproducibility of the method was evaluated by analyzing eight alfalfa hay samples (227 9 ± 4 67 nmol g^{-1} DM) and eight samples of alfalfa hay that had been incubated for 48 h in the rumen (940 14 ± 19 02 nmol g^{-1} DM), which yielded relative standard deviations of 2 04% and 2 02%, respectively

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