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Simultaneous liquid chromatographic determination of methionine hydroxy analogue and DL-methionine in feed formulations

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ABSTRACT

A liquid chromatographic method for the simultaneous determination of methionine hydroxy analogue and DL-methionine in compounded feed samples is described. Samples are subjected to a simple extraction procedure followed by quantification on a LiChrospher reversed-phase column with ultraviolet detection at 214 nm. The reproducibility and recovery were determined for a range of typical European feed formulations. An excellent correlation was found between the data obtained by this method and by conventional methods.

INTRODUCTION

The use of synthetic amino acids as supplements in compounded feeds is essential in today's feed industry and intensive animal husbandry to formulate diets which meet the animal's nutrient requirements at the minimum cost. These additives allow the biological value of the proteins fed to be improved and the level of proteins in the feed to be reduced.

Considering the economic importance of the essential amino acid levels of feed formulations on animal performance, an accurate method for the determination of synthetic methionine, lysine and threonine sources is a key factor in feed quality control.

Synthetic methionine is added either as DL-methionine or its methionine hydroxy analogue (Alimet[a], MHA[a]). The presence of these feed additives can be determined by several methods. Methods for determining DL-methionine include high-performance ion-exchange chromatography in combination with pre- or postcolumn derivatization or an amino acid analyser[1–5]. These methods are not applicable to the

[a] Alimet and MHA are registered trade marks of Monsanto Co., St. Louis, MO, U.S.A.

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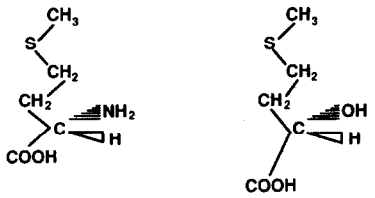

Fig. 1. Structures of DL-methionine and methionine hydroxy analogue (Alimet).

analysis of methionine hydroxy analogue because it contains an α-hydroxy instead of an α-amino group, as shown in Fig. 1.

A gas chromatographic method for the determination of the methionine hydroxy analogue was reported by Day *et al.*[6] and modified by Feit *et al.*[7]. A capillary isotachophoresis technique was utilized by Vinjamoori and Schisla[8]. The development of high-performance liquid chromatographic (HPLC) methods, allowing substantial simplification of the preparative steps, was reported by Baudichau *et al.*[a], Balschukat *et al.*[10] and Gerstl and Ranfft[11].

Because the above-mentioned feed supplements are both used to correct methionine deficiencies in animal feeds, a technique allowing for both compounds to be determined in a single analysis is much needed. The existing methods for the determination of methionine on one hand and methionine hydroxy analogue on the other are different in terms of both sample preparation and the actual determination itself. Consequently, none of them allows an accurate simultaneous determination of both compounds.

An HPLC technique has been developed for the simultaneous determination of both synthetic methionine sources. The method has been tested for its recovery and accuracy on a range of typical European feed formulation samples. The results obtained have been compared with those obtained by conventional methods.

EXPERIMENTAL

Apparatus and reagents

A Waters Assoc. HPLC system was used, consisting of two Model 510 pumps, a Model 712 WISP automatic sample injector, a Model 481 LC spectrophotometer and a workstation running Baseline 810 software. The column was a 250 × 4 mm I.D. Merck LiChrospher 100 RP-18 (5 μm) reversed-phase column.

For eluent preparation, water and methanol of HPLC grade (Baker, Deventer, The Netherlands), 85% orthophosphoric acid (analytical-reagent grade) (Merck, Darmstadt, F.R.G.) and sodium hydroxide pellets (analytical-reagent grade) (Baker) were used.

The apparatus included a centrifuge, an automatic shaker and a feed grinder to facilitate sample preparation.

Sample preparation

A sample of feed is ground to a mean particle size of 600 μm and 6.0 ± 0.1 g are accurately weighed into a vial. To this are added 30 ml of distilled water at *ca.* 90°C.

The sample is then shaken on an automatic shaking machine (Z 620 Vortex Station; Zymark, Hopkinton, MA, U.S.A.) for 10 min in order to extract both sources of methionine. After the sample has been shaken as prescribed, a minimum of 30 min are allowed for settling. The sample is then centrifuged for ca. 10 min at 2000 g (Heraeus Christ Labofuge 6L; Heraeus, Osterode, F.R.G.) to clear the solution. A portion of the supernatant is placed in an HPLC sample vial and 15 μl are injected onto the HPLC column.

Chromatographic conditions

The separation is carried out using a linear gradient programme with the following eluents: solvent A, 0.03 *M* H_3PO_4, adjusted to pH 3 with 0.1 *M* NaOH; solvent B, 0.03 *M* H_3PO_4–methanol (9:1), adjusted to pH 3 with 0.1 *M* NaOH. The programme is as follows: 0–8 min, 100% A; 8–9 min, linear change to 100% B; 9–44 min, 100% B; 44–45 min, linear change to 100% A; 45–60 min, 100% A.

The flow-rate used is 0.6 ml/min, detection wavelength 214 nm and detector attenuation 0.2 a.u.f.s.

Calibration and calculation

Standard solutions containing both methionine sources are prepared by weighing *ca.* 20.0, 40.0 and 60.0 mg of DL-methionine and Alimet (accurate to 0.1 mg) into a 100-ml volumetric flask, diluting with distilled water to the mark and agitating. The shelf-life of the standards is 1 month.

A 15-μl volume of each standard solution is injected onto the HPLC column and peak heights and areas are calculated. Two calibration graphs can be generated by plotting either peak-area counts or peak height against the amount of DL-methionine or the amount of Alimet injected. The standards need to be injected just before each series of feed samples to be analysed. At the end of a series of analysis the standards are reinjected and if the results obtained differ more than 5% from the initial values the whole analysis is rejected.

Good linear relationships exist for peak-area counts *versus* the amount of DL-methionine at levels of 0.01–0.40% (w/w) ($r = 0.9967$) and for Alimet at levels of 0.05–0.40% (w/w) ($r = 0.99952$). These ranges cover the normal ranges of concentrations applied in supplemented feeds.

RESULTS AND DISCUSSION

The modification of the preparative steps and the actual HPLC analysis in comparison with conventional methods is obvious.

The conventional extraction procedure shows marked differences for both sources: Alimet undergoes an aqueous extraction, and the extraction of DL-methionine from feeds is done with hydrochloric acid. Experiments have shown the latter reagent to be unsuitable for Alimet extraction.

Kabwe wa Mupenda[12] demonstrated that DL-methionine extraction recoveries obtained with hydrochloric acid and with distilled water at ambient temperature are not statistically different. Further, the solubility of DL-methionine in aqueous solutions increases 5-fold (176.0 *vs.* 33.8 g/l) when the water temperature is increased from 25 to 100°C[13,14]. For these reasons the extraction of both sources was conducted with distilled water at *ca.* 90°C.

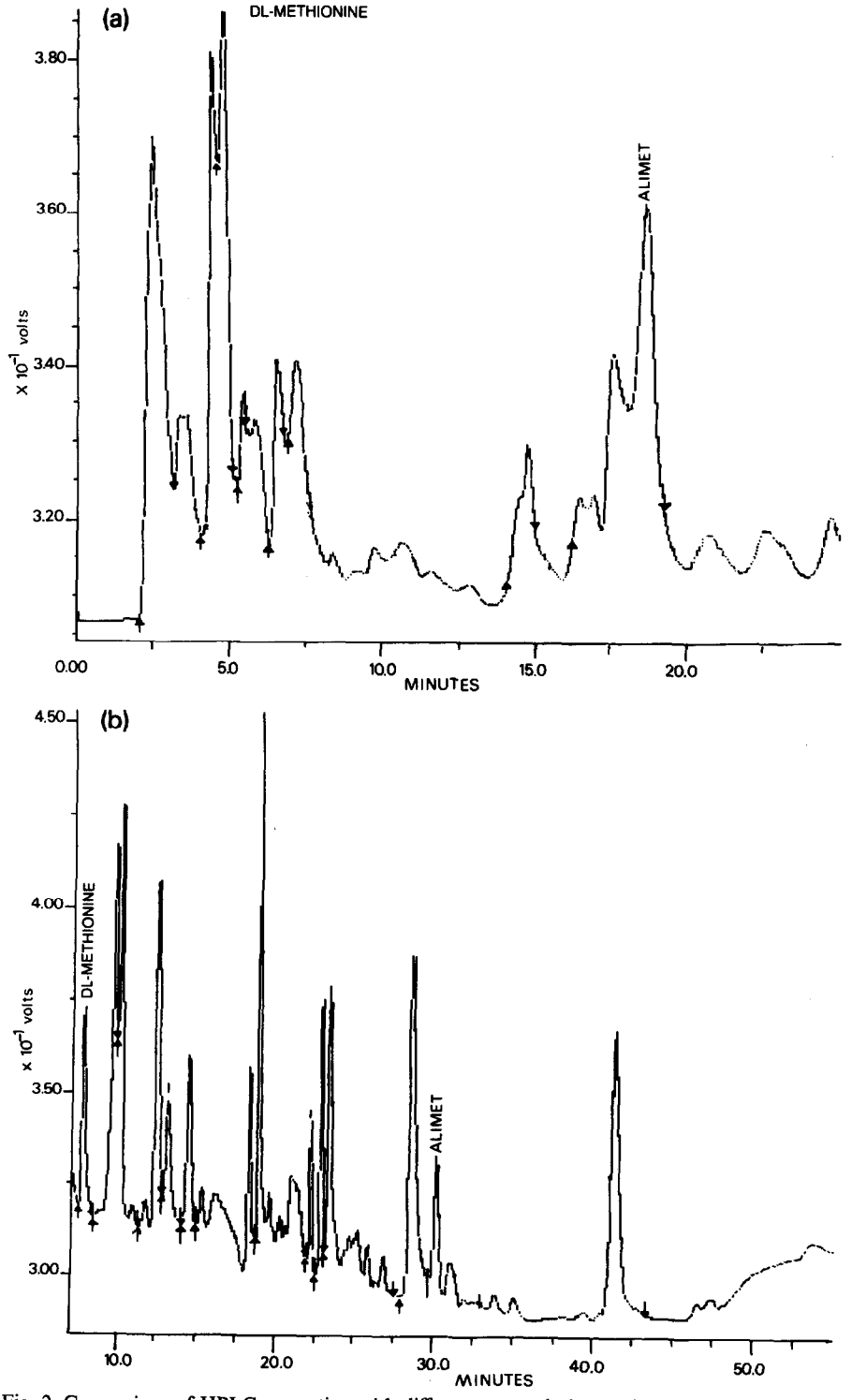

Fig. 2. Comparison of HPLC separation with different reversed-phase columns: (a) LiChrosorb; (b) LiChrospher. Both chromatograms were recorded on the same feed sample extract at 0.068% Alimet and 0.060% DL-methionine supplementation levels.

Ultraviolet scans of both compounds showed high UV absorbance at wavelengths between 200 and 220 nm. The detection wavelength was chosen as 214 nm, which yields good sensitivity and interference-free Alimet and DL-methionine detection.

The HPLC separation was carried out on a LiChrospher reversed-phase column. This type of column was selected because of its large number of theoretical plates and its specific characteristics towards polar compounds, *i.e.*, more retentive than other reversed-phase columns and avoiding poor peak shapes of these compounds[15,16]. Fig. 2 clearly demonstrates the improvement obtained by using a LiChrospher *versus* a LiChrosorb reversed-phase column.

For the eluents it must be stressed that careful control of the pH is required to ensure a good separation of the different compounds showing UV absorbance at 214 nm. Fig. 3 shows some typical chromatograms of standard solutions and feed samples separated on the LiChrospher reversed-phase column. DL-Methionine and Alimet are clearly separated, allowing their detection and determination in compounded feeds in the presence of other constituents.

Method validation

The percentage recovery and reproducibility of the method were determined by analysing spiked feeds at several supplementation levels. In addition, a wide variety of compounded feed samples representing formules commonly used in European mar-

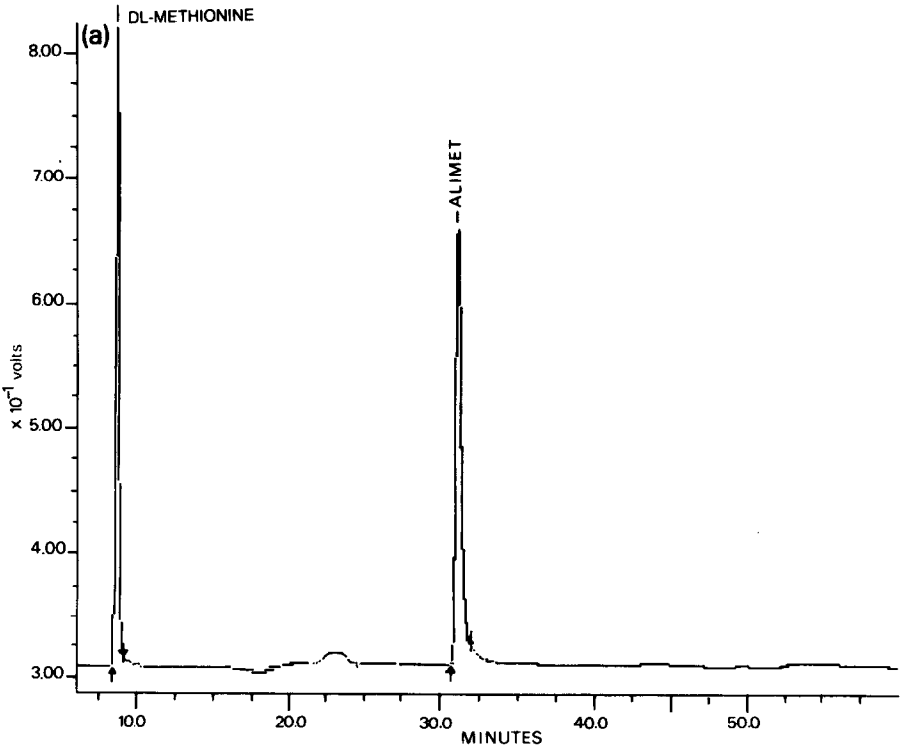

Fig. 3.

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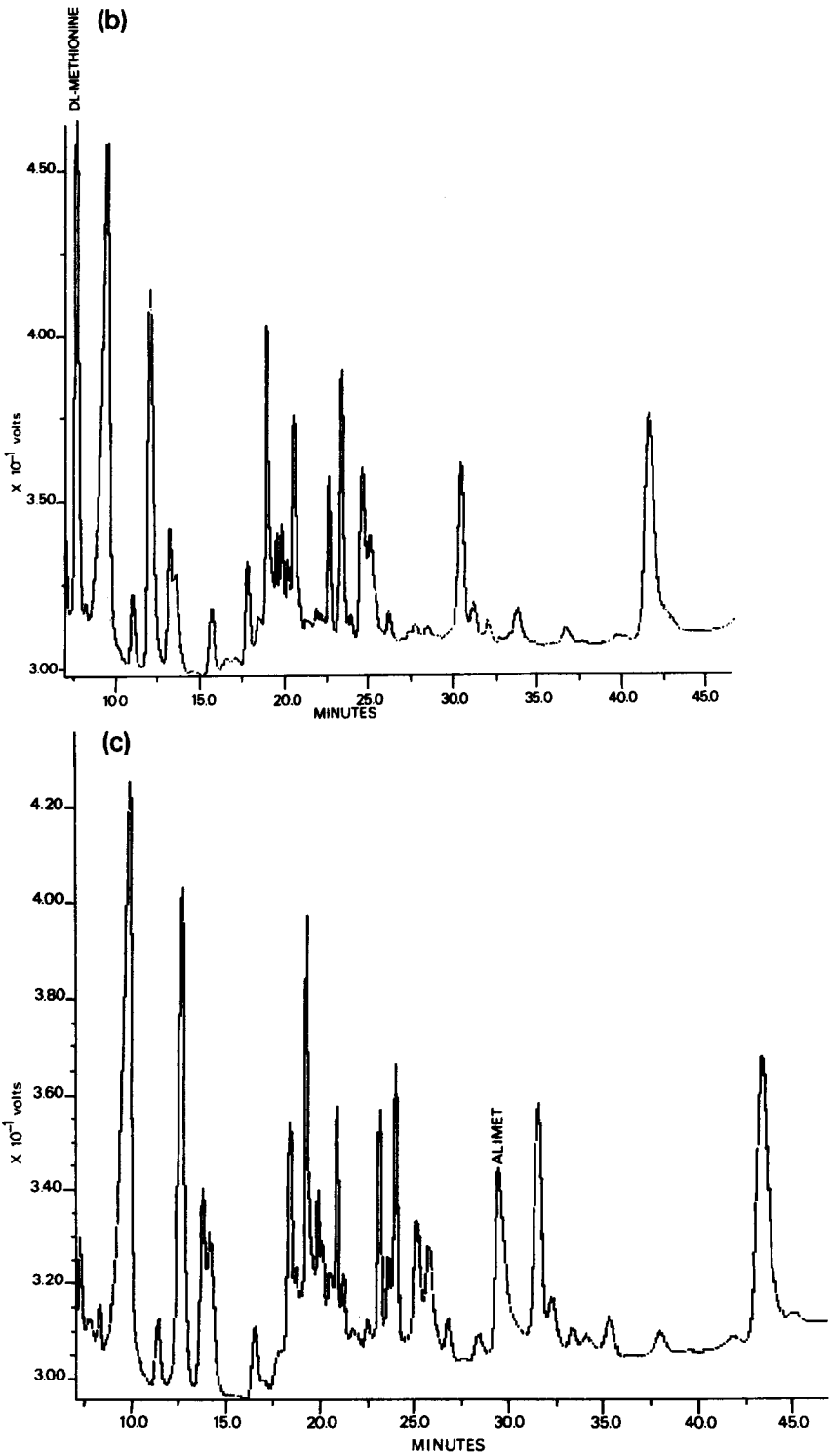

Fig. 3. Typical chromatograms of (a) DL-methionine-Alimet standard solution; (b) feed sample extract at 0.120% DL-methionine supplementation level; (c) feed sample extract at 0.092% Alimet supplementation level.

TABLE I

RECOVERY AND PRECISION OF ANALYSES ON SUPPLEMENTED TEST FEED SAMPLES BASED ON FIVE INDEPENDENT ANALYSES AT EACH SUPPLEMENTATION LEVEL

Alimet added (%)	*DL-Methionine added (%)*	*Recovery (%)*	*R.S.D. (%)*
0.07	–	96.7	2.8
0.35	–	98.6	4.2
–	0.06	95.0	3.0
–	0.40	97.4	1.6

TABLE II

RECOVERIES FOR COMMERCIAL FEED SAMPLES

Origin	*Theoretical Alimet applied (%, w/w)*	*Theoretical DL-methionine applied (%, w/w)*	*Recovery (%)*	*Recovery conv.[a] (%)*
F.R.G.	–	0.010	90.0	
	–	0.040	86.3	
	–	0.060	98.3	
	–	0.080	101.3	
	–	0.100	89.0	
Spain	0.125	–	90.4	97.6
	0.193	–	99.0	114.5
	–	0.170	101.7	94.1
	–	0.230	115.3	108.6
France	0.068	–	94.1	101.4
	0.068	–	100.0	101.4
	0.140	–	110.0	100.0
	–	0.060	95.0	110.0
	–	0.140	110.7	
Portugal	0.0092	–	90.2	85.9
	–	0.129	100.0	
	–	0.177	94.9	
Netherlands	0.120	–	106.6	100.8
	0.129	–	103.8	100.0
	0.129	–	94.5	107.7
	–	0.125	105.8	
	–	0.312	111.2	

[a] Recovery obtained by conventional methods, *i.e.*, HPLC determination for Alimet and DL-methionine separately[1,5,9,11].

keting areas were examined for potential interferences and recoveries.

Recovery tests. Recovery tests were performed by adding known amounts of Alimet and DL-methionine to a compounded feed. A between-day reproducibility of the assay was obtained by executing five repeated determinations on feed sample extracts. The precision was determined by calculating the relative standard deviations (R.S.D.) for each set of five analyses (Table I). The R.S.D.s obtained are comparable to those reported for the determination of both substances by conventional methods[9,17,18].

Validation on commercial feed samples. In order to evaluate the effect of a typical sample matrix in terms of potential interferences, numerous feed samples

originating from Belgium, The Netherlands, Spain, Italy, France, F.R.G., U.K., Portugal, Poland and Israel were qualitatively examined. None of these samples exhibited a peak eluting at or near the retention time of either DL-methionine or its hydroxy analogue.

A quantitative evaluation of commercial poultry and pig feeds ranging in supplementation levels from 0.010 to 0.312% (w/w) and originating from different countries was made using the proposed method. The results in Table II demonstrate that in each instance the amount of Alimet or DL-methionine is within a range of 85–115% of the theoretically expected levels. Moreover, excellent agreement between the results obtained by the proposed simultaneous analysis method and the conventional methods[1,5,9,11] was recorded.

CONCLUSION

A reliable method for the simultaneous determination of Alimet and DL-methionine in compounded feed samples has been developed. It involves a simple extraction procedure followed by a gradient HPLC analysis on a LiChrospher reversed-phase column. The separation is excellent for a wide variety of samples, and the procedure yields accurate results and shows excellent correlation with conventional methods.

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