An HPLC Method for the Determination of 2-Hydroxy-4-(methylthio)butanoic Acid (HMB) in Supplemented Animal Feeds

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A method using high-pressure liquid chromatography is described for the determination of 2-hydroxy-4-(methylthio)butanoic acid (HMB), a biological equivalent for methionine, in supplemented animal feeds. The method consists of extraction from the feed, basic hydrolysis to characterize all active material as monomer, adjustment of the hydrolysate pH, and analysis by reversed-phase chromatography on an amide column using an ultraviolet detector at 210 nm. A 30-min sample preparation time results in rapid sample turn around. Analyses of typical diets supplemented at levels from 0.05 to 0.40% HMB by weight have recoveries averaging 97% with an average coefficient of variation of 3.7%. Excellent correlation exists between data obtained by this method and that obtained by gas chromatographic analyses. The method is also applicable to the calcium salt of HMB and is comparable in accuracy to the analytical procedure for DL-methionine.

Methionine deficiencies in animal feeds are corrected by nutritionists by adding one of two synthetic methionine sources, DL-methionine or its hydroxy analogue, 2-hydroxy-4-(methylthio)butanoic acid (HMB). As the first limiting amino acid in poultry diets, supplemental methionine is important in maximizing protein utilization, promoting broiler growth and egg production, and improving feed conversion efficiency. The methionine source, HMB, is available as a dry calcium salt (DL-HMB-Ca) and as an 88% aqueous solution of the methionine hydroxy analogue free acid (DL-HMB-FA). The use of this methionine source in poultry rations has increased markedly as a result of the growing poultry industry and the conversion of many users from dry to liquid application.

Accurate and timely analytical methods are essential for verifying supplemental levels present in feed for economic purposes and for good quality control. Either HMB or DL-methionine can be assayed for activity by bromide/bromate titration or the McCarthy/Sullivan test (McCarthy and Sullivan, 1941). These assay methods, however, were developed for the concentrated products and lack the sensitivity needed to measure low concentrations present in supplemented feeds. The lower levels of supplemental DL-methionine in feeds can be measured by acid extraction with HPLC ion-exchange chromatography in combination with pre- or postcolumn derivatization or an amino acid analyzer (Beck et al., 1978), based on the molecule's α-amino group. These techniques are not applicable to HMB because it contains an α-hydroxy in place of the α-amino group.

A gas chromatographic method for determining HMB in supplemented feeds has been reported by Day et al. (1983) and modified by Feit et al. (1984). In this method, HMB is silated to form its bis(silyl) derivative, a volatile compound that is readily analyzed by gas chromatography. The preparative steps up to and including derivatization are lengthy and take approximately 2 days to complete. A second method for determining HMB on feeds utilizes capillary isotachophoresis (Vinjamoori and Schisla, 1984, 1986). This more rapid technique selectively measures HMB ions by their mobility in an applied electric field.

The development of a high-pressure liquid chromatographic method provides an analytical alternative that yields accurate analyses in a time-efficient manner. The derivatization step necessary for GC is eliminated since HPLC is well suited to the analysis of nonvolatile compounds. This additional analytical technique gives laboratories a greater choice of methods depending upon instrumentation available.

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EXPERIMENTAL SECTION

Apparatus and Reagents. A Spectra Physics Model SP8100 liquid chromatograph equipped with an autosampler, a Model SP4200 computing integrator, and a Kratos Spectraflow Model 757 UV detector were used in this work. Other comparable instruments would be applicable. Analysis is by reversed-phase chromatography on a 250 × 4.6 mm (i.d.) amide column of 5-μm particle size (IBM Amino-RP, Phenomenex IB-Sil 5 NH2, Alltech Amino-RSIL, or equivalent). Elution is isocratic with the mobile phase consisting of 23% acetonitrile and 77% 0.01 M aqueous phosphoric acid adjusted to pH 3.2 with ammonium hydroxide and preserved with 25 mg/L sodium azide to prevent microbial growth. All reagents, including deionized water, are HPLC grade. Reagent-grade ammonium hydroxide and potassium hydroxide may be used. Assorted laboratory equipment, including a centrifuge, an automatic shaker, a feed grinder, and a mixer, facilitate sample preparation. Disposable filter units of 0.2-μm nylon 66 (Rainin Instruments) work well for filtering samples prior to HPLC injection.

Sample Preparation. A feed sample is ground to a mean particle size of 600 μm through a grinding screen with 1.0-mm pores and mixed well to assure representative subsamples. An accurately weighed 2-g sample of the ground feed is shaken with 20 mL of an extraction solution consisting of 10% acetonitrile and 90% deionized water. The sample is shaken on an automatic shaker for 5 min or by hand for 80 shakes minimum. Feed samples supplemented with the dry DL-HMB-Ca require more rigorous extraction conditions than for those supplemented with the liquid DL-HMB-FA due to the time required for the dry powder to go into solution. Samples supplemented with DL-HMB-Ca should be shaken for 5 min on an automatic shaker while immersed in a 60–70 °C water bath.

Following shaking (extraction), the sample is allowed to settle. A 5-mL aliquot of the supernatant is placed in a 15-mL disposable centrifuge tube with 100 μL of 50% potassium hydroxide and shaken for 10 s. Concentrated phosphoric acid (100 μL) is added and the tube shaken for an additional 10 s and then centrifuged at a relative centrifugal force (RCF) of 1650g for 5 min. A portion of the centrifuged supernatant is used to presaturate a 0.2-mm nylon 66 filter unit, and the remainder is filtered through

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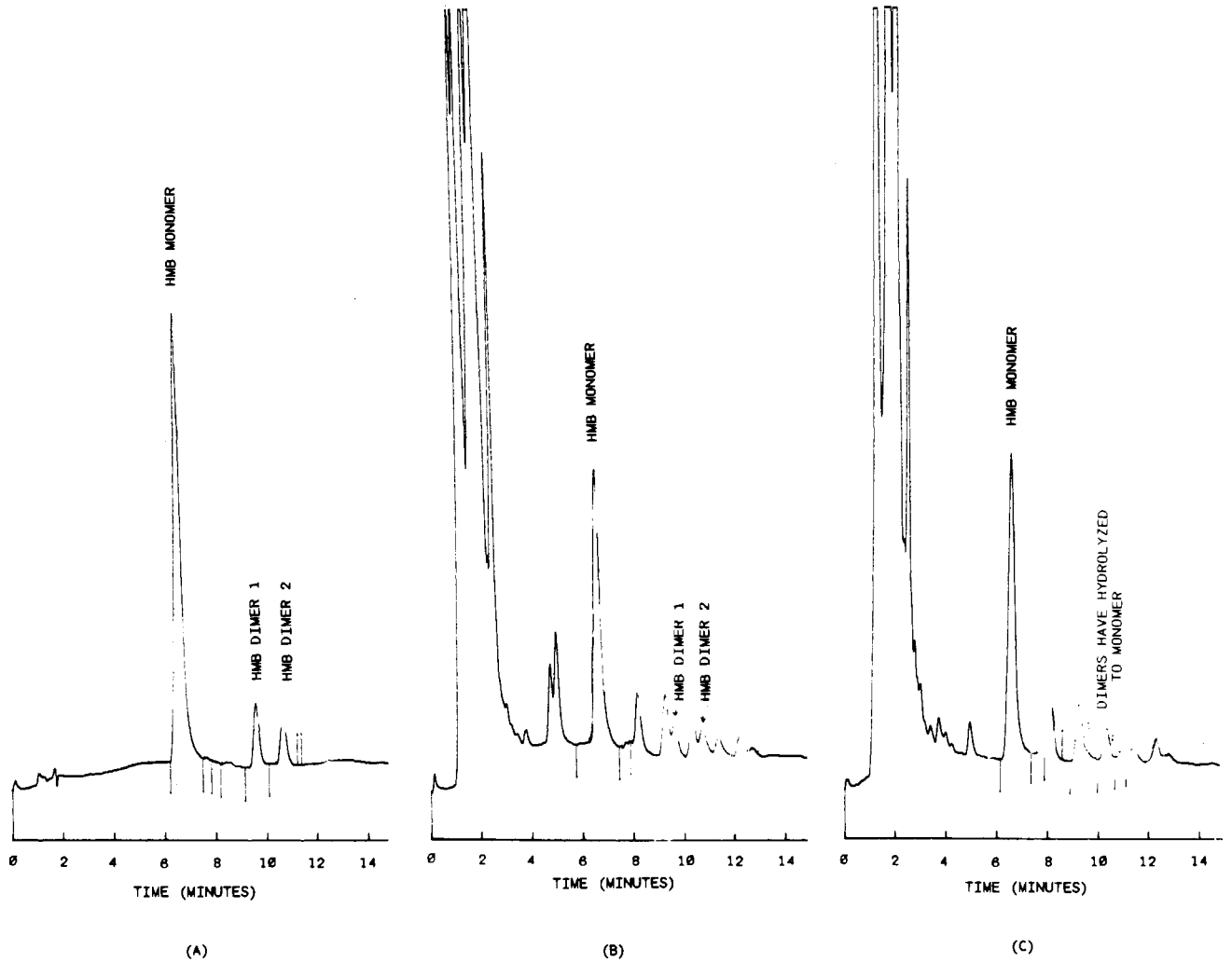

Figure 1. Typical chromatograms: (A) DL-HMB-FA in water; (B) unhydrolyzed feed extract; (C) hydrolyzed feed extract.

the unit into an HPLC vial, ready for injection.

HPLC Analysis. Chromatographic conditions: isocratic mode; flow rate, 2 mL/min; wavelength, 210 nm; AUFS, 0.05; run time, 20–30 min (varies with each individual column); injection volume, 10 μL; peak width, 8; peak threshold, 40; chart speed, 0.5 cm/min; attenuation, 8 mV FS; HMB retention time, 4.3 min on IBM and Phenomenex columns, 6.6 min on Alltech column. (The retention time is dependent not only on the brand of column used but also on the exact pH of the eluent; i.e., an increase in pH of 0.1 unit may increase retention time by 0.2 min.)

Calibration. Standards are prepared by supplementing a typical commercial starter diet of corn, soybean meal, vitamin–mineral premix, and fat with varying levels of HMB. A calibration curve is generated by plotting peak area counts against the amount of HMB present in these standards, which are extracted and treated according to the sample preparation procedure each day a set of samples is to be analyzed. An excellent linear response is obtained for these feeds supplemented at levels from 0.05 to 1.00% by weight (the range of concentrations typically seen in supplemented feeds). A typical regression equation for the calibration curve when using a Phenomenex column is $y = 26.2x - 217$, $R = 0.999$.

When DL-HMB-FA is added to feed to make standards, the mixtures should sit for about 3 days before being used to generate a standard curve. The slope and y intercept of the curves generated each day these feed mixtures are analyzed should remain essentially constant after 3 days of "equilibration time". As a matter of practice, new feed standard mixtures are prepared approximately every 2 months as most feed samples requiring analysis are less than 2 months old.

Column Care. New columns are equilibrated with miscible HPLC-grade solvents in a stepwise fashion to displace any nonpolar solvents with the polar mobile phase. A guard column helps maximize column life by trapping components in the sample or mobile phase that might normally collect on the head of the analytical column. Amide columns tend to be very sensitive to eluent composition changes and may be longer lived if major composition changes are avoided.

RESULTS AND DISCUSSION

An ultraviolet scan of methionine hydroxy analogue free acid (DL-HMB-FA) shows maximum UV absorbance occurs between 200 and 215 nm. Within this region, and under the specified column conditions, HMB elution occurs free of interferences due to any other extracted feed components that absorb UV at this end of the spectrum.

A direct HPLC injection of DL-HMB-FA diluted 0.025 g/100 mL in water results in a chromatogram as shown in Figure 1A. As is typical with liquid α-hydroxy acids, monomer and dimer are both present (Holten, 1971). The monomer of HMB elutes at 6.6 min, with two dimer peaks eluting at 9.6 and 10.7 min. Chromatogram 1B is typical of the unhydrolyzed extract of a feed sample supplemented with 0.1% HMB. The monomer of HMB again elutes at 6.6 min. Several peaks that vary with feed type elute in

Table I. Accuracy (% Recovery) and Precision of HMB Analyses of Supplemented Test Feeds Based on 15 Independent Analyses at Each Supplemental Level

	HMB, %			
N	theory	found ± 1 SD	recovered, %	CV, %
15	0.05	0.048 ± 0.002	96	4.2
15	0.10	0.096 ± 0.004	96	4.2
15	0.20	0.195 ± 0.006	98	3.1
15	0.40	0.397 ± 0.013	99	3.3
			97[a]	3.7[a]

[a] Average value.

the region of 9–11 min. These peaks make it difficult to quantify any dimer that might be present. In this case, the dimer peaks are seen as shoulders on the interfering peaks. Chromatogram 1C is characteristic of a feed sample, supplemented with 0.1% HMB, taken through the entire extraction and hydrolysis procedure. The potassium hydroxide added to the feed extract rapidly hydrolyzes any oligomers present to monomer. Total quantitation is achieved with all of HMB characterized as monomer in a region of the chromatogram free from interferences. Addition of phosphoric acid lowers the pH of the hydrolyzed sample to pH 4–5, a range allowing for good column separation.

Accuracy (percent recovery) and precision of the method were determined by analyzing laboratory-supplemented feeds for DL-HMB-FA at four concentration levels ranging from 0.05 to 0.40%. By the method of calibration described, HMB recoveries of 15 independent analyses at each supplemental level averaged 97% of theory (Table I). Precision was determined by calculating the coefficient of variation for each set of 15 analyses. This CV averaged 3.7%. These statistics are comparable to the GC and ITP methods for determining HMB in supplemented feeds and also to that reported for analysis of DL-methionine in feeds. Analyses of feed samples supplemented with radiolabeled DL-HMB-FA confirm an extraction efficiency of 98–100%.

In addition to these analyses, 54 commercial broiler, layer, and turkey feeds ranging in concentration from 0 to 0.6% DL-HMB-FA were analyzed by HPLC and also by the GC procedure (Day et al., 1983). The excellent agreement between the two methods is illustrated by a linear regression performed on the pairs of data ($R = 0.98$). Samples analyzed for DL-HMB-Ca correlate equally well. Under the conditions described in this paper, HMB levels as low as 0.008% can be determined (signal/noise 2).

In summary, the HPLC method for the analysis of DL-HMB-Ca and DL-HMB-FA has proven to be rapid and reliable, providing analyses in a time-efficient manner. The procedure yields accurate and precise results and exhibits excellent correlation with the GC procedure. Since sample preparation is simple and the HPLC instrumentation is accessable in many laboratories, the method could be an effective quality control technique for many feed operations worldwide.

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Protein Value of Dry Bean Cultivars: Factors Interfering with Biological Utilization

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The protein content and protein nutritive value of cooked dry beans from 12 cultivars were studied. Methionine and cysteine were consistently the most limiting amino acids. Best values for methionine (0.96–1.99 g/100 g of crude protein) were obtained by BrCN reaction with bean flour followed by GLC of methyl thiocyanate formed and for cysteine (0.5–2.2 g/100 g of crude protein) by GLC of the amino acid derivatives. Methionine determined by BrCN/GLC correlated well ($r = 0.820^{**}$, $p = 0.01$) with bioavailable methionine (0.23–0.77 g/100 g of crude protein). PER (0.79–1.16) showed linear positive correlation ($r = 0.718^{**}$, $p = 0.01$) with total sulfur (120–270 mg/100 g of whole bean) and a weak negative correlation ($r = -0.511$, $p = 0.10$) with total tannin (470–570 mg/100 g of whole bean).

Dry beans (*Phaseolus vulgaris*, L.) have been used in human diets mainly as a source of protein and carbohydrate. They furnish about 28% of the protein and 12% of the calories in the Brazilian diet (Sgarbieri and Garruti, 1986).

The nutritive properties of the dry bean protein have been studied by a large number of investigators (Jaffé, 1950a; Bressani et al., 1961; Evans and Bandemer, 1967; Sgarbieri et al., 1982), among others. It has been shown that limiting sulfur amino acids and the presence of protein

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