thod requires the electrode's hydrophobic gas-permeable membrane to be in contact with the extract for a longer period than if the extract were added after the standard. Nonaqueous solvents eventually wet the membrane and destroy its gas-permeable nature. We found that up to 15 determinations could be carried out without decrease in electrode performance, providing contact time of the alcohol extract with the electrode's membrane was minimal and the electrode was placed in aqueous solutions between determinations. The CV for tissue extracts containing about 6 μmol/g endogenous ammonia was 2.0% (n = 5). Tissue extracts containing low levels of ammonia were enriched by trapping the ammonia from the extract on a cationic Dowex 50W-X8 column; ammonia was washed from the column with a small volume of 1 M KCl, resulting in near-quantitative recovery.

Enzymatic Determination. Ammonia was specifically detected in tissue extracts with the enzyme glutamate dehydrogenase. Endogeneous enzymes present in the tissue apparently are not extracted and do not interfere with the assay. Methanol present in the extract is diluted with aqueous reagents and does not interfere with the enzyme assay. The CV for extracts containing 10 μmol/g or less ammonia was less than 2.5%.

Distillation. Higher values were obtained when ammonia in aged meat samples was determined by the modified Kjeldahl method (Gerhart and Quang, 1979) than by either the enzyme or ASE method (Table III) on extracts of the same meat. The higher values probably resulted from cleavage of amino groups in the protein as a result of the high temperature and alkaline pH used for the distillation. In addition, the distillate volume of the Kjeldahl reaction was found to affect the ammonia determination. For example, a 75% increase in the distillate volume resulted in a 52% increase in the ammonia value.

Apparent Total Volatile Amine Content. Subtracting the values obtained by enzymatic assay from those obtained by ASE gave values for the apparent total volatile amine content of the sample. The ratio of total volatile amine content to ammonia content for six ground beef samples varied between 5 and 12% and averaged 8.1% (Table IV). Of the food investigated, processed ham had the highest concentration of ammonia plus volatile amines and shrimp the highest ratio of volatile amines to ammonia (101.4%). The volatile amine values are approximate since the electrode response to volatile amines is not the same as its response to ammonia. For example, the electrode response determined by standard addition for several volatile amines (methyl-, ethyl-, dimethyl-, diethyl-, and butylamine) present in beef ranged between 0.3 and 3.5 times the response to ammonia. However, these same volatile amines did not interfere in the enzymatic assay of ammonia.

These results demonstrate that ammonia can easily be extracted quantitatively from ground tissue with aqueous methanol. This procedure is more rapid and less tedious than the acid precipitation procedure commonly used. Also, for samples containing low concentrations of ammonia, a cation-exchange resin can be used to concentrate ammonia in the alcohol extract, with no loss of precision in the subsequent ammonia determination. The enzyme assay is more accurate than the ASE method for determining ammonia in tissue extracts, since volatile amines do not interfere. The enzyme assay does not require high pH and elevated temperatures, which may lead to high ammonia values by the Kjeldahl method. Such high values result from cleavage of amino groups in protein in the meat samples during the Kjeldahl distillation, and the extent of cleavage varies with pH and distilate volume. The ASE responds both to ammonia and volatile amines. Since the electrode's response to volatile amines is not the same as its response to ammonia, only the apparent total volatile amine content of the extract can be obtained by comparing the enzyme and ASE methods.

ACKNOWLEDGMENT

We thank W. Fiddler and D. P. Schwartz for their helpful suggestions and J. Poccia and A. DeFelice for their technical assistance.

Registry No. Ammonia, 7664-41-7.

LITERATURE CITED

Brooks, G. M.; Ammerman, G. R. *J. Food Sci.* **1978**, *43*, 1348.
Gerhart, U.; Quang, T. Dam *Fleischwirtschaft* **1979**, *59* (7), 946.
Henry, R. J. In "Clinical Chemistry Principles and Technics"; Harper and Row: New York, 1964; p 325.
Reichelt, K. L.; Kuamme, E.; Tuett, B. *Scand. J. Clin. Lab. Invest.* **1964**, *16*, 433.
Sigma Chemical Co. Technical Bulletin 170-uv, 1980.

Received for review July 26, 1982. Revised manuscript received January 28, 1983. Accepted March 21, 1983. Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

Analysis of the Free Acid of Methionine Hydroxy Analogue in Supplemented Feeds

Richard J. Day,* Diane E. Borowy, and Robert M. Schisla

A method is described for determining methionine hydroxy analogue free acid (HMB) in supplemented poultry feeds. The procedure is based on extraction of analogue from the feed, followed by gas chromatographic measurement of the silyl derivative of the HMB using a capillary column/FID system. Recoveries of HMB over the supplementation range of 0.04–0.4% by weight are 93–95%, with coefficients of variation of 4.2 ± 0.6% at 95% confidence. The method is equally applicable to the calcium salt of HMB.

The supplementation of poultry feeds with methionine-active substances has been practiced in the industry for over 25 years. Prior to 1979, bulk supplementation with solid products DL-methionine and the calcium salt of methionine hydroxy analogue [2-hydroxy-4-(methylthio)-butanoic acid calcium salt] [marketed as MHA (trademark of Monsanto Co.) and Hydan (trademark of Du Pont) feed supplements] was used exclusively. In 1979, a liquid form,

Nutrition Chemicals Division, Monsanto Company, St. Louis, Missouri 63167.

0021-8561/83/1431-0889$01.50/0

methionine hydroxy analogue free acid (HMB), was introduced by Monsanto Co. as an 88% aqueous solution under the trade name Alimet feed supplement. A similar product was sold by Du Pont under the trade name Hydan-L, but this product is no longer supplied by Du Pont. Methods for analyzing DL-methionine and assaying feeds and premixes for methionine content are well-known. They include iodometric titration for analysis of DL-methionine and assay of premixes (Fahnenstich et al., 1974) and ion-exchange chromatography for assaying premixes and complete feeds for methionine (Beck et al., 1978). These methods are not applicable to the analysis of HMB or its calcium salt. The literature methods for the calcium salt of HMB include the classical bromide/bromate titration and the McCarthy/Sullivan test, neither of which can be used on feeds (McCarthy and Sullivan, 1941). These methods are not precise in that other sulfur-containing compounds are measured as well. Gas chromatographic procedures have also been reported (Alcino and Katz, 1972; Belasco et al., 1978; Pease et al., 1978) in other applications but are not directly applicable to feed analysis.

The growth of the broiler industry and increased use of methionine hydroxy analogue, especially the free acid, provided impetus for the development of an assay method for analogue in feeds comparable in precision and accuracy to the methods for DL-methionine.

EXPERIMENTAL SECTION

Apparatus and Reagents. Both the Model 3700 (Varian, Palo Alto, CA) and Model 5880A (Hewlett-Packard, Palo Alto, CA) gas chromatographs, equipped with flame ionization detector and interfaced with a computer data system, were used in this work; other comparable instruments would work equally well. The column recommended is a 25 m × 0.3 mm i.d. fused silica capillary column coated with SE-54 (trademark of General Electric Co.) silicone gum and siloxane deactivated. The silylating reagents used were BSA [*N,O*-bis(trimethylsilyl)acetamide], HMDS (hexamethyldisilazane), and TMCS (trimethylchlorosilane). The reference standard bis(silyl) derivative of 2-hydroxy-4-(methylthio)butanoic acid was synthesized in our laboratory, purified by fractional distillation (bp 65 °C/0.05 mmHg), and verified for purity by GC analysis. It must be stored in a freezer under N_2 to ensure long-term stability. Other chemicals used are standard laboratory reagents used without further purification. The equipment used in the extraction step is standard laboratory apparatus. The Pierce Reacti-Therm stirring/heating module (Pierce Chemical Co., Rockford, IL) and the Vortex-Genie (trademark of Scientific Industries, Inc.) proved to be especially useful in the preparation of the silyl derivatives. The feeds are ground to about 60 mesh in a Moulenex coffee and spice grinder, although any comparable grinder should work equally well.

Extraction Step. Into a 250-mL narrow-neck flat-bottom flask is weighed accurately (±0.1 mg) 5 g of feed, previously ground to about 60 mesh. To this is added 50 mL of 60% v/v aqueous tetrahydrofuran (THF) and 1.5 g of 50% w/w potassium hydroxide. A stirring bar is added, a water-cooled condenser attached, and the mixture stirred on a hot plate under moderate reflux for 3 h. It is recommended that the flask be wrapped with insulation during the extraction. After the mixture is cooled, the slurry is filtered with suction through Whatman No. 1 filter paper, prewetted with 60% THF and coated with a $^1/_4$-in. cake of Hyflo Supercel (trademark of Johns-Manville Corp.) filter aid, on a small Büchner funnel. The residual reactor contents are rinsed onto the filter with three small portions of 60% THF, and the filter cake is washed with three small portions of 60% THF, breaking up the cake and recompacting during the washing procedure. The filtrate is transferred quantitatively to a tared (±0.1 mg) 100-mL round-bottom flask and allowed to stand overnight. The filtrate is then evaporated to dryness on a rotary evaporator. With the flask out of the water bath, vacuum is applied slowly until full aspirator vacuum is reached and then held for 30 min. The flask is totally immersed in the water bath at 25 °C and gradually heated to 40 °C until the film bubbles (**caution**: watch for bumping when the extract begins to film). Then the bath temperature is increased to 70 °C and held for a minimum of 1 h. The flask containing the extract is then placed in a vacuum desiccator containing Drierite (trademark of Drierite Co.) for 48 h to ensure complete drying. The extract must be dry and brittle when removed. The flask is reweighed (±0.1 mg) and the extract weight calculated. With a metal spatula, the hygroscopic extract is scraped from the walls of the flask, transferred to a 4-dram vial, and crushed to uniform particle size with a glass rod, and the vial is capped tightly.

Gas Chromatographic Analysis. *Operating Conditions.* Injection port, 250 °C; detector, 300 °C; helium carrier gas at 60 psig; column flow rate, 2 mL/min.; capillary makeup flow, 28 mL/min; split ratio, 200/1; temperature program, 125–275 °C with an 8-min hold at initial temperature, followed by a program rate of 30 °C/min with a 20-min hold at the upper limit.

Calibration. A 1.0-μL sample of bis(silyl) analogue standard is injected and analyzed for percent purity by area counts. This is repeated at least twice and the mean value used in the area correction factor (ACF) calculation. A series of calibration mixtures of the analogue standard and *n*-tetradecane internal standard in BSA are prepared, encompassing the expected analogue concentration range in the extract. At least five GC analyses are carried out at each concentration and the ACF is calculated. A calibration curve of ACF vs. analogue concentration is then plotted. In our hands, the ACF was constant over the entire concentration range studied. The calculations are as shown.

$$\text{weight of HMB} = (\text{weight of silyl deriv.})(\%\ \text{purity})(150.2)/[(100)(294.5)] \quad (1)$$

$$\text{ACF of HMB} = (\text{area of internal std})(\text{weight of HMB})/[(\text{weight of internal std})(\text{area of HMB})] \quad (2)$$

Derivatization and Analysis of Extract. A total of 0.5 g of extract and 0.010 g of *n*-tetradecane (±0.1 mg) is weighed accurately (±0.1 mg) into a 4-dram vial fitted with a polyethylene-lined cap, and 4 drops (0.30–0.33 g) of 50% KOH is added with a medicine dropper. A $^5/_8$ in. × $^5/_{16}$ in. stirring bar is added and the mixture stirred for 1 min at room temperature. A total of 2.5 mL of pyridine is added and the mixture is stirred for 10 min. A total of 1.5 mL of TMCS is added, mixing well and breaking up any remaining solid lumps with a melting point tube (**caution**: it is essential that all solid lumps be broken up and dispersed before proceeding further). An additional 0.5 mL of TMCS is added, then 2.5 mL of HMDS is added cautiously with stirring, and finally another 0.5 mL of TMCS is added. The vial is capped and placed in the stirring/heating module at 65–70 °C for 30 min or until the sample is completely dissolved. The vial is removed every 10 min and mixed on the Vortex-Genie to aid the derivatization. The vial is removed and allowed to stand for solids to settle. A total of 1.5 μL of the supernatent liquid of the

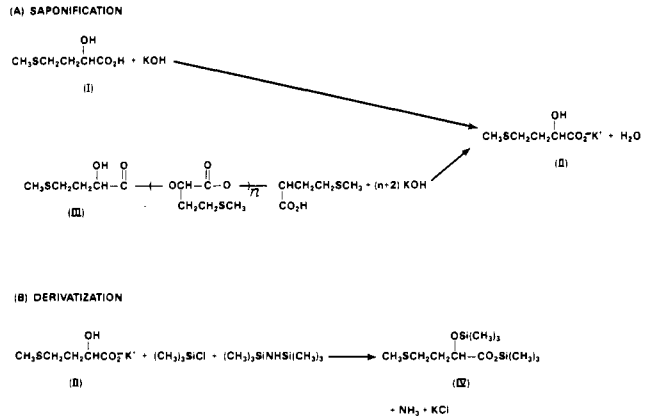

Figure 1. Equations for saponification and derivatization of analogue monomer and oligomers. The value of n is 0 for dimer, 1 for trimer, 2 for tetramer, etc.

derivatized extract is injected into the chromatograph. The percent analogue in the extract and in the starting feed are calculated according to the equations

$$\% \text{ HMB in extract} = (\text{ACF})(\text{area of HMB})(\text{weight of internal std}) \times (100)/[(\text{weight of sample})(\text{area of internal std})] \quad (3)$$

$$\% \text{ HMB in feed} = (\% \text{ HMB in extract}) \times (\text{weight of extract})/(\text{weight of feed sample}) \quad (4)$$

The same derivatization and chromatographic procedure are used to analyze for the calcium salt in a feed sample. The calculations for Ca-HMB are as follows:

$$\% \text{ Ca salt in extract} = (\text{ACF})(\text{area of HMB}) \times (\text{weight of internal std})(100)(169.2)/[(\text{weight of sample})(\text{area of internal std})(150.2)] \quad (5)$$

$$\% \text{ Ca salt in feed} = (\% \text{ Ca salt in extract}) \times (\text{weight of extract})/[(\text{weight of feed sample})] \quad (6)$$

RESULTS AND DISCUSSION

The challenges presented in developing the method were 3-fold: (1) release of the adsorbed or bonded analogue from the feed; (2) quantitative recovery of the analogue; (3) specific analysis of the analogue by standard high-recision techniques. The key factors which answered these challenges were (1) the use of 60% aqueous THF as the extraction solvent, (2) addition of 50% KOH to the solvent to release the analogue from the feed, (3) rigorous drying of the extract, (4) silylation of the extract, and (5) gas chromatographic analysis of the silyl derivative.

The derivatization procedure first converts the analogue (free acid or calcium salt) (I) to the potassium salt (II) and saponifies polymeric esters (III) present in the extract. The salt is then converted to the bis(silyl) derivative (IV) by using a mixture of hexamethyldisilazane (HMDS) and trimethylchlorosilane (TMCS) (Figure 1). The silyl derivative is quantitatively determined by GC analysis against an internal standard. Figure 2 shows a typical chromatogram.

The method has been validated on laboratory-prepared corn–soy mixtures supplemented with HMB at levels of 0.04, 0.10, 0.20, and 0.40% by weight. However, levels as low as 0.01% have been determined in feeds and as high as 5% in premixes. It is emphasized that basal (nonsupplemented) feeds must be analyzed as controls or blanks, a standard analytical procedure. The experimental feeds were prepared by mixing 88% aqueous HMB (Alimet liquid feed supplement) in the proper proportions with a 50/50 mixture of cornmeal and soybean meal.

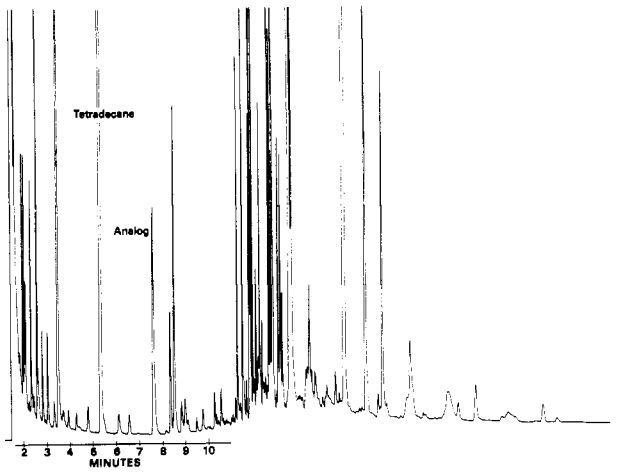

Figure 2. Typical gas chromatogram of derivatized extract at 0.15% analogue supplementation level. The tetradecane standard at 5.3 min and analogue at 7.6 min are identified.

Table I. Recovery, Standard Deviation, and Coefficient of Variation Results on Supplemented Test Feeds

theory applied, %	mean recovered,[a] %	% recovered	SD	C_v, %
0.040	0.037	92.5	0.00158	4.3
0.101	0.095	94.1	0.00455	4.8
0.201	0.190	94.5	0.00715	3.8
0.406	0.384	94.6	0.01390	3.6

[a] Based on five replicate total analyses at each supplemental level. The mean recovered values are corrected for a blank value of 0.011 in the unsupplemented feed.

The analytical data from the experimental feeds were statistically evaluated to give mean recovery of the applied analogue free acid, standard deviations (SD), and coefficients of variation (C_v). The data in Table I show an average recovery of about 93–95% of theory, with coefficients of variation of 4.2 ± 0.6%. This is comparable to data reported for analysis of DL-methionine in feeds. In addition, over 200 samples of commercial broiler and turkey feeds, including practical starter, grower, and finisher diets, as well as premixes have been analyzed, supporting the utility of the method for determining HMB in supplemented feeds.

ACKNOWLEDGMENT

Thanks are extended to the Applied Sciences Department of Monsanto Industrial Chemicals, especially to G. M. Gasser and T. W. Hunter, for assistance and helpful suggestions.

Registry No. I, 583-91-5; I·0.5Ca, 4857-44-7.

LITERATURE CITED

Alcino, N. J.; Katz, S. E. *J. Dairy Sci.* **1972**, *55* (2), 272.
Beck, A.; Schmidtburn, H.; Spendler, M.; Tanner, J. "Literature Digest for the Feedstuffs Industry"; Degussa AG: Federal Republic of Germany, 1978.
Belasco, I. J.; Pease, H. L.; Reiser, R. W. *J. Agric. Food Chem.* **1978**, *26* (2), 327.
Fahnenstich, R.; Mose, W.; Tanner, H. *Landwirtsch. Forsch.* **1974**, *27*, 95.
McCarthy, T. E.; Sullivan, M. X. *J. Biol. Chem.* **1941**, *141*, 871.
Pease, H. L.; Prince, J. L.; Johnson, E. F. *J. Agric. Food Chem.* **1978**, *26* (2), 331.

Received for review June 28, 1982. Accepted March 30, 1983. The material included in this paper was presented in part at the Poultry Science Association Meeting, Aug 1981, Vancouver, B. C., Canada.