Gas Chromatographic Method for Determining Methionine Hydroxy Analogue Residues in Cow Milk, Urine, and Tissues Using a Flame Photometric Detector

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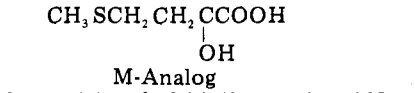
A method is described for determining residues of methionine hydroxy analogue (M-analog) in milk, urine, and tissues of cows. The procedure is based on the gas chromatographic measurement of the silyl derivative of M-analog using a sulfur-sensitive flame photometric detector. Sensitivity of the method is about 0.05 ppm based on a 50-g sample. Recoveries of M-analog added to the various substrates averaged better than 95% over the range of 0.05 to 5.0 ppm.

The calcium salt of methionine hydroxy analogue (M-analog) is the active ingredient in du Pont's Hydan Feed Supplement. A sensitive analytical method for determining residues of the M-analog in cow milk, urine, and tissues is described herein. This method is a mod-



M-Analog
[2-hydroxy-4-(methylthio)butanoic acid]

ification of a procedure for determining M-analog in rumen fluid (Alicino and Katz, 1972). It is based on the gas chromatographic measurement of the silyl derivative of M-analog using a sulfur-selective flame photometric detector. The method is sensitive to about 0.05 ppm. Quantitative recoveries are obtained on both milk and tissue samples; somewhat lower recoveries (average 83%) are obtained on urine.

This paper reports only an analytical residue method. Recent work done in our laboratories using this method plus other studies with ^{14}C-labeled methionine and M-analog are reported elsewhere (Belasco et al., 1978; Belasco, 1972).

EXPERIMENTAL SECTION

Apparatus and Reagents. The Model MT-220 Gas Chromatograph (Tracor, Inc., Austin, Tex.), equipped with a flame photometric detector with interference filter for spectral isolation of sulfur emission at 394 mμ, was used. The chromatographic column was 5% OV-7 on 80–100 mesh Supelcoport (Supelco, Inc., Bellefonte, Pa.), 6 ft glass, $^1/_4$ in. o.d., $^3/_{16}$ in. i.d.

Homogenization and extractions were conducted using a blender–centrifuge bottle and adapter base as described by Holt and Pease (1976). It is not necessary to construct this specialized equipment unless desired. Conventional blender bottles and centrifuge tubes may be used but are somewhat more time-consuming. Centrifugation was carried out with an International Size 1, Type SB Centrifuge capable of accommodating the 250-mL bottle used.

The filtering apparatus was a Coors porcelain, perforated plate Buchner funnel, size 2 with a 250-mL filter flask.

The reference standard of the calcium salt of methionine hydroxy analog was obtained from the Biochemicals Department, Agrichemicals Marketing Division, E. I. du Pont de Nemours & Co., Inc., Wilmington, Del. The solvents used were distilled-in-glass, purchased from Burdick and Jackson Laboratories, Inc., Muskegan, Mich. BSA [*N,O*-bis(trimethylsilyl)acetamide] (Supelco, Inc., Bellefone, Pa.), was the silylating reagent.

Gas Chromatographic Calibration. The chromatograph was equilibrated under the following conditions: inlet temperature, 250 °C; detector temperature, 180 °C; column temperature, 175 °C; helium carrier gas flow, 70 cm^3/min; oxygen flow, 20 cm^3/min; air flow, 40 cm^3/min; hydrogen flow, 180 cm^3/min. The chromatographic column was conditioned by maintaining the temperature at 175 °C with carrier gas flowing for at least 48 h. The flame photometric detector was operated according to instructions furnished by the manufacturer. Aliquots (1 to 5 μL) of standard calibration solutions of M-analog, prepared in ethyl acetate to contain 2, 5, 10, 15, 20, and 25 μg/mL, were injected so that the peak would not exceed full-scale deflection. The calibration solutions were prepared by extracting solutions of the calcium salt standard with ethyl acetate and subsequent derivatization with BSA as described in the method for treated samples. The retention time for the M-analog is about 3 min. A typical gas chromatographic scan of a standard solution is shown in Figure 1. A calibration curve was constructed by plotting micrograms of the M-analog injected vs. peak height. Log–log paper was used to obtain a straight line. One or more calibration solutions was chromatographed daily to ensure that the calibration curve remained accurate.

Isolation. *Milk Samples.* Fifty grams of a representative milk sample was weighed into the 250-mL blender–centrifuge bottle, and 4.2 mL of concentrated hydrochloric acid and 4 g of ammonium sulfate were added. The mixture was blended for 5 min at medium fast speed, then filtered using vacuum, on Whatman No. 42 paper (7.0 cm) and a size 2 Coors porcelain Buchner funnel, into a 250-mL filter flask. The blender bottle was rinsed with 10 mL of 1 N HCl, and this rinse was used to wash the cake remaining on the filter paper. Vacuum was continued until filtration was completed. (Filtration time usually requires 40–50 min). The filtrate was transferred to a 100-mL volumetric flask using 2× 15-mL portions of 1 N HCl as wash. The volume was adjusted with 1 N HCl and mixed well. Fifty milliliters of the acid mixture was tranferred by pipet to a 250-mL separatory funnel. Fifty milliliters of *n*-hexane was added and the separatory funnel was shaken gently for 1 min. The phases were allowed to separate. (If necessary, centrifuge to obtain a clean separation.) After discarding the hexane layer, the hexane wash was repeated using a second 50-mL portion of solvent. The hexane layer was discarded as before.

Fifty milliliters of ethyl acetate was then added to the aqueous phase and shaken for 2 min. (Emulsion may be formed; therefore, to maintain a uniformity of procedure, all extractions should be centrifuged to give a clean separation.) The ethyl acetate phase was filtered through a 1.5 in. bed of anhydrous sodium sulfate into a 250-mL

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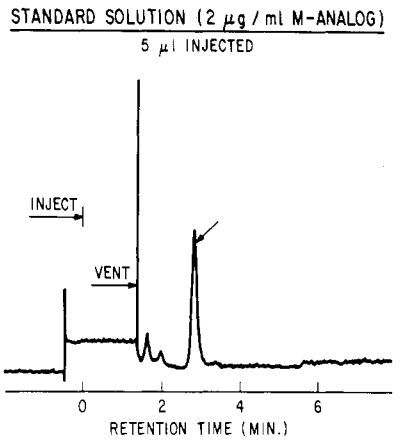

Figure 1. Standard solution of M-analog.

round-bottomed flask. This extraction was repeated two more times, each time using 50-mL portions of ethyl acetate.

The combined ethyl acetate extracts were concentrated to about 5 mL using a vacuum rotary evaporator at 60 °C and then transferred to a 10-mL graduated tube using several small quantities of ethyl acetate as rinse. The tube was placed in a 40 °C water bath and concentrated to dryness using a gentle stream of nitrogen. A pipet was used to add 0.5 mL of pyridine, rinsing down the sides of the tube during addition. To ensure complete dissolution, the mixture was mixed well. A pipet was also used to add 0.5 mL of BSA (silylating reagent). The mixture was mixed well and allowed to stand for at least 30 min before gas chromatographic analyses.

Urine Samples. Ten grams of a representative urine sample was transferred into a 250-mL separatory funnel and 2.1 mL of concentrated hydrochloric acid added. The volume was then diluted to 25 mL with distilled H_2O. Twenty-five milliliters of *n*-hexane was added and the solution gently shaken for 1 min. The phases were allowed to separate, and the hexane layer was discarded. The hexane cleanup was repeated a second time using 25 mL of additional hexane. The hexane layer was discarded as before.

The aqueous phase was next extracted with three 25-mL portions of ethyl acetate, using 2-min shaking periods for each extraction, and the ethyl acetate extracts were collected in a 150-mL beaker. The aqueous layer was discarded after the extractions and the combined ethyl acetate extracts returned to the separatory funnel. Small volumes of ethyl acetate were used as rinse.

The ethyl acetate was backwashed with 25 mL of 1 N HCl using a 2-min shake. The phases were allowed to separate, and the lower aqueous layer was discarded. The ethyl acetate layer was then transferred to a 250-mL round-bottomed flask, using small portions of ethyl acetate as wash. The solvent extract was next concentrated to approximately 25 mL using a vacuum rotary evaporator at 60 °C. The concentrate was transferred to a 250-mL separatory funnel and extracted wih two 25-mL portions of 0.1 N NaOH using 2-min shaking periods for each extraction. The aqueous layer was collected in a 100-mL beaker and the ethyl acetate discarded. [Note: Extra cleanup steps are necessary for urine analyses; they are not needed for milk analyses.]

The combined NaOH extracts were returned to the separatory funnel using small volumes of 0.1 N NaOH as wash. Fifty milliliters of *n*-hexane was added and the mixture shaken for 2 min. After the phases had separated, the hexane layer was discarded.

Table I. Recovery Study (Milk) (50-g Sample)

Residue level, ppm	μg of M-analog		
	Added	Found	% recov
0.05	2.5	3.4	136
0.05	2.5	3.5	140
0.05	2.5	3.5	140
0.10	5.0	4.7	94
0.10	5.0	4.8	96
0.10	5.0	4.0	80
0.10	5.0	7.0	140
0.10	5.0	5.9	118
0.10	5.0	3.5	70
0.10	5.0	5.0	100
0.20	10	9.2	92
0.20	10	7.0	70
0.20	10	11	110
0.20	10	8.4	84
0.30	15	19	127
0.30	15	16	107
0.30	15	13	87
0.40	20	21	105
0.40	20	17	85
0.50	25	21	84
1.0	50	45	90
2.0	100	81	81
4.0	200	160	80
		Av	101%
	μg of methionine		
5.0	250	0[a]	0
25	1250	0	0

[a] Demonstrates no interference to M-analog method from methionine.

The pH of the aqueous layer was adjusted to pH 1 by carefully adding 5 mL of concentrated hydrochloric acid. (Add more if needed to reach the desired pH.)

The pH-adjusted aqueous layer was then extracted with three 50-mL portions of ethyl acetate, using 2-min shaking periods for each extraction. The ethyl acetate was filtered through a 1.5 in. bed of anhydrous sodium sulfate into a 250-mL round-bottomed flask.

The combined ethyl acetate extracts were next concentrated to about 5 mL using a vacuum rotary evaporator at 60 °C, and the concentrated extract was quantitatively transferred to a 10-mL graduated tube using several small quantities of ethyl acetate as rinse. The tube was placed in a 40 °C water bath in a well-ventilated hood, and concentration was continued to dryness or an oily residue using a gentle nitrogen stream. A pipet was used to add 0.5 mL of pyridine, rinsing down the sides of the tube during addition. To ensure complete dissolution, the mixture was mixed well. A pipet was also used to add 0.5 mL of BSA (silylating reagent). After mixing well, the solution was allowed to stand for at least 30 min before gas chromatographic analyses.

Tissue Samples. Twenty-five grams of a representative tissue sample was weighed into the 250-mL blender–centrifuge bottle, 75 mL of 1 N HCl was added, and the mixture was blended for about 5 min. The acid mixture was then centrifuged at 1500 rpm for 10 to 15 min and the acid extract carefully decanted through cotton, collecting 50 mL in a graduated cylinder. The 50-mL volume was quantitatively transferred to a 100-mL volumetric flask and diluted to volume with 1 N HCl. The acid mixture was mixed well.

A 50-mL aliquot was transferred to a 250-mL separatory funnel, 50 mL of *n*-hexane was added, and the mixture was gently shaken for 1 min. The phases were allowed to separate (centrifuge, if necessary, to obtain a clean separation), and the hexane layer was discarded. The hexane

Table II. Recovery Study (Tissues) (25-g Sample)

	Residue level, ppm	μg of M-analog Added	Found	% recov
Liver	0.08	2.0	1.6[a]	80
	0.20	5.0	3.8[a]	76
	0.20	5.0	4.1[a]	82
	0.40	10	7.9[a]	79
	1.0	25	21[a]	84
	2.0	50	45[a]	90
			Av	82%
Kidney	0.08	2.0	2.3[b]	115
	0.08	2.0	2.1[b]	105
	0.20	5.0	5.4[b]	108
	0.40	10	7.9[b]	79
	1.0	25	21[b]	84
	2.0	50	42[b]	84
			Av	96%
Lean meat	0.08	2.0	3.0	150
	0.20	5.0	4.2	84
	0.60	15	13	87
	1.0	25	23	92
			Av	103%
Subcutaneous fat	0.08	2.0	3.1	155
	0.20	5.0	5.8	116
	0.20	5.0	5.1	102
	1.0	25	21	84
			Av	114%

[a] Corrected for average blank, 3.1 μg (0.12 ppm). [b] Corrected for average blank, 1.7 μg (0.07 ppm).

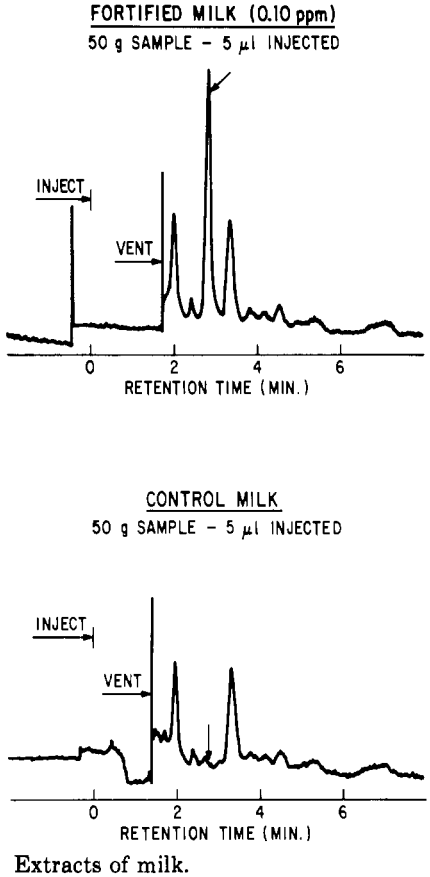

Figure 2. Extracts of milk.

wash was repeated one more time using 50 mL of solvent. The hexane layer was discarded as before.

The aqueous phase was next extracted with three 50-mL portions of ethyl acetate using 2-min shaking periods for each extraction. (Centrifuge, if necessary, to obtain a clean separation.) The ethyl acetate extracts were decanted through a 1.5 in. bed of anhydrous sodium sulfate into a 250-mL round-bottomed flask.

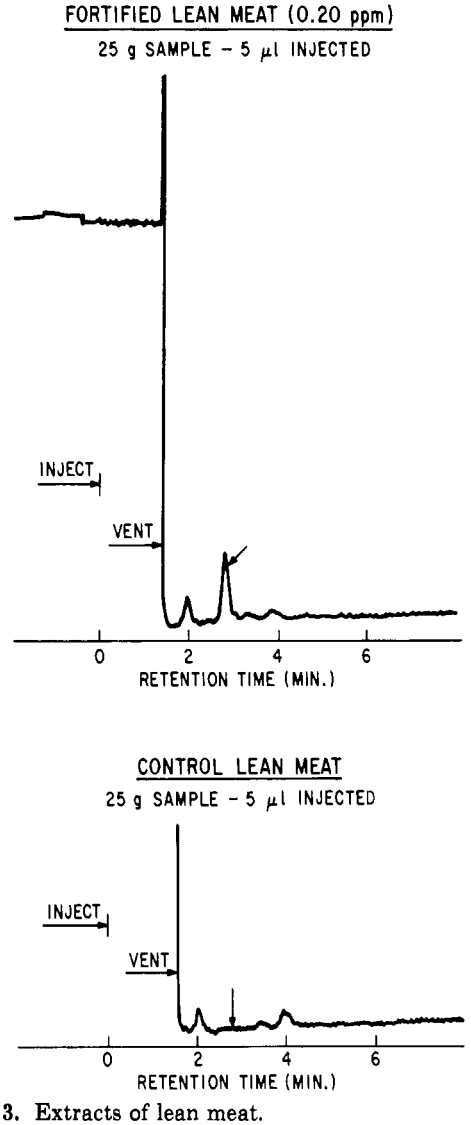

Figure 3. Extracts of lean meat.

The combined ethyl acetate extracts were concentrated to about 5 mL using a vacuum rotary evaporator at 60 °C and the concentrated extract transferred to a 10-mL graduated tube using several small quantities of ethyl acetate as rinse. The tube was placed in a 40 °C water bath and concentration was continued to dryness using a gentle stream of nitrogen. A pipet was used to add 0.5 mL of pyridine, rinsing down the sides of the tube during addition. To ensure complete dissolution, the mixture was mixed well. A pipet was also used to add 0.5 mL of BSA (silylating reagent). The mixture was mixed well and allowed to stand for at least 30 min before gas chromatographic analyses.

Gas Chromatographic Analyses. The instrument was equilibrated and aliquots (1 to 5 μL) of the sample extracts were chromatographed as described under the Gas Chromatographic Calibration section. The peak height of the M-analog was measured and the micrograms of this material in the aliquot determined by using the calibration curve previously prepared. The amount of M-analog in parts per million was calculated, correcting the micrograms found in the aliquot for both aliquot and recovery factors.

RESULTS AND DISCUSSION

This gas chromatographic method is sensitive to about

Table III. Recovery Study (Urine) (10-g Sample)

Residue level, ppm	μg of M-analog Added	Found[a]	% recov
0.2	2.0	1.4	70
0.2	2.0	1.2	60
0.5	5.0	2.4	48
0.5	5.0	4.6	92
0.5	5.0	4.0	80
0.5	5.0	5.7	114
1.0	10	6.8	68
1.0	10	7.9	79
1.0	10	11	110
2.0	20	20	100
2.0	20	11	55
5.0	50	59	118
		Av	83%

[a] Corrected for the appropriate blank of untreated control; blank varied from 0.6 to 3.4 μg (0.06 to 0.34 ppm).

0.05 ppm in milk based on a 50-g sample. Because of interferences encountered on untreated urine and tissue samples, smaller sample sizes were analyzed and sensitivities of 0.2 and 0.08 ppm, respectively, were obtained. The procedure is a modification of the M-analog in rumen fluid method of Alicino and Katz (1972); additional clean-up steps were included in our isolation steps to further purify the extract before derivatization. BSA [*N*,*O*-bis(trimethylsilyl)acetamide] was used as the silylation reagent instead of the reagent (Tri-Sil) used by Alicino and Katz. In addition, the gas chromatographic determinations were made using a 6-ft glass column packed with 5% OV-7 on 80/100 mesh Supelcoport. To obtain a highly selective measurement of the desired compound, a sulfur-sensitive flame photometric detector was used. The previous workers used a different GC column (1.5% OV-210 on Gas-Chrom Q) and a flame ionization detector. It was necessary to make these modifications in the published method in order to apply it to this work.

Recoveries of M-analog added to untreated control samples are summarized in Tables I, II, and III. As shown in Table I, methionine, if present in the sample, would not interfere with the analyses. Blanks (positive readings) were encountered on untreated liver and kidney samples. The recovery factors from these tissues have been corrected for the respective blanks. Lower and more erratic recoveries were obtained from 10-g urine samples. These data have also been corrected for blanks (positive readings) obtained on untreated control urine.

Gas chromatographic scans representing control milk, milk fortified at 0.1 ppm, control lean meat, and lean meat fortified at 0.2 ppm are shown in Figures 2 and 3.

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Occurrence of Methylguanidine and Agmatine, Nitrosatable Guanidino Compounds, in Foods

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Among naturally occurring guanidines, nitrosated methylguanidine (MG) has been reported to be strongly mutagenic and carcinogenic and nitrosated agmatine (AG) to be moderately mutagenic. A survey has been conducted on the MG and AG contents of various fresh and processed foods. No appreciable amount or trace amounts of MG could be detected in fresh beef, pork, chicken, and various fish and shellfish; in addition, almost the same low levels of MG were detected in various processed foods, except for the cases of smoked-dried fish products called "Katsuo-bushi" and "Kezuri-bushi" in Japanese, and the MG values for these products ranged from 18 to 178 mg/kg. Comparatively high concentrations of AG were detected in fresh abalone and top-shell muscles ranging from 40 to 200 mg/kg among the fresh foods tested, while fairly high concentrations of AG could be detected in some processed foods in which dried squid was found to contain as high as 650 mg/kg of AG.

Endo et al. (1974a) examined the mutagenicity of various nitrosated guanidine derivatives which are structurally similar to *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), a well-known mutagen and a potent gastric carcinogen. Among naturally occurring guanidines, nitrosated methylguanidine (MG) has been reported to be the most mutagenic and nitrosated agmatine (AG) to be moderately mutagenic. Mirvish (1971) and Endo and Takahashi (1972) demonstrated that MG was nitrosated under acidic conditions to give methylnitrosocyanamide and methylnitrosourea. The oral and intragastric administrations of these *N*-nitroso compounds have been confirmed to induce gastric and esophagal cancers in experimental animals (Druckrey, 1972; Endo et al., 1974b). MG has long been considered to occur widely in nature, especially in fresh beef and various fish. Concerning the occurrence of MG in foodstuffs, however, most of the studies were conducted in the 1930's and seemingly many problems exist as to the methods of isolation and identification of MG in the test materials. AG, a decarboxylated product of arginine, has been isolated from a few invertebrate sources, notably from the sponge, *Geodia gigas*, from several cephalopods (Baldwin, 1963), and from putrefied material (Hayashi, 1955). No data on the occurrence of AG in fresh foods and various processed foods so far have been available.

Recently we have developed a method for the determination and identification of some basic guanidino compounds, i.e., MG, AG, and guanidine in foods, and a

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