Microbial Conversion of Methionine to Methionine Hydroxy Analogue and Its Natural Occurrence in Various Foods and Feed Products

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Analysis of food and feed products that include a fermentative process step, such as various cultured milk products, bread, sauerkraut, beer, distillers' mixed grains, and corn silage, revealed the presence of methionine hydroxy analogue (M-analog) as a naturally occurring ingredient at concentrations ranging up to 60 ppm. The microbial conversion of ^{14}C-labeled methionine to M-analog was further evaluated using strains of *Saccharomyces cerevisiae*, *Lactobacillus lactis*, *Lactobacillus bulgaricus*, *Streptococcus lactis*, and *Bacillus subtilis* as inocula in milk. All were capable of this conversion, with *S. cerevisiae* being the most efficient.

The use of the calcium salt of the hydroxy analogue of methionine [M-analog; 2-hydroxy-4-(methylthio)butanoic acid, Ca salt] as a nutrient supplement in commercial broiler feeds is a well-established practice. In recent years, there has been considerable interest in improving the protein nutritional response of ruminant animals to maximize meat, milk, and wool production. One of the promising routes for improving this response has been M-analog supplementation of ruminant rations. Earlier data from this laboratory (Belasco, 1972) indicate the greater resistance of M-analog, compared to methionine, to rumen microbial degradation, possibly explaining the reported nutritional efficacy of M-analog supplemented ruminant feeds in improving milk and milk fat production (Chandler et al., 1976; Griel et al., 1968; Polan et al., 1970) and in improving the growth of lambs and heifers (Burroughs and Trenkle, 1969a,b).

Recently, a sensitive residue method for determining M-analog in milk and meat has been developed (Pease et al., 1978). In the course of this development, M-analog was detected in control milk samples, which had inadvertently spoiled for lack of refrigeration.

A review of the literature revealed that M-analog arises from the action of certain common bacteria and fungi on methionine. Akobe (1936) reported that *Oidium lactis* and *Bacillus subtilis* are capable of converting methionine to M-analog. Similarly, Ballio et al. (1961) found that a strain of *Penicillium chrysogenum*, when grown on a medium containing ^{35}S-labeled methionine, gave rise to M-analog. Maw and Coyne (1966) reported that methionine is converted to M-analog by the action of *Saccharomyces cerevisiae* in a glucose medium where methionine was the source of sulfur. In 1965, Galsworthy and Metzenburg observed the presence of M-analog in a nutrient medium in which a mutant of *Neurospora crassa* was grown.

Additional studies were undertaken to further evaluate the pervasiveness of the microbial conversion of methionine to M-analog and to analyze certain milk products, foods, and feed ingredients, suspected of containing detectable, naturally occurring amounts of M-analog. This investigation of [^{14}C]methionine or its incorporation into cellular protein.

EXPERIMENTAL METHODS

L-[1-^{14}C]Methionine was obtained from Amersham/Searle Corp., Arlington Heights, Ill., having a radiochemical purity of 98% and a sp act. of 60 mCi/mmol.

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Microorganisms. *Bacillus subtilis* (6051), *Saccharomyces cerevisiae* (9763), *Lactobacillus lactis* (12315), and *Lactobacillus bulgaricus* (11842) were obtained from the American Type Culture Collection, Rockville, Md.

Culture Media. Stock cultures of the lactobacilli were carried in Bacto-Micro Assay Culture Agar and the inocula were prepared in Bacto-Micro Inoculum Broth. The *B. subtilis* culture was maintained on Bacto-Nutrient Agar, and the inocula were prepared in Bacto-Nutrient Broth. *S. cerevisiae* was maintained in Bacto-Czapek Solution Agar, supplemented with a vitamin mixture. Bacto-Czapek-Dox Broth, supplemented with vitamins, or the nutrient medium described by Maw (1963) was used for the preparation of inocula. In tests to evaluate methionine conversion, the sodium sulfate in the medium (10 mg of sulfate sulfur/L) was replaced with an equivalent amount of methionine. In those tests, where the conversion of methionine to M-analog in milk was evaluated, sterile skim milk was used as medium for inoculum preparation.

Sample Preparation. Aseptic procedures were used for withdrawal of samples from the incubating mixtures for thin-layer or gas–liquid chromatographic analysis. Samples were transferred to microcentrifuge tubes (1.5-mL capacity) and centrifuged in a Brinkmann centrifuge, Model 3200, for 5 min to remove the bacteria or yeast. In those instances where skim milk was the test medium, it was also necessary to coagulate the major fraction of the milk protein by treatment with 1 N HCl in the amount of 0.1 mL/mL of milk prior to centrifugation. After centrifugation and withdrawal of supernatant, the solids were washed with 0.1 N HCl and the tube and its contents were recentrifuged. The wash supernatant was then combined with the original supernatant.

Thin-Layer Chromatographic Procedure. The supernatant sample was reduced in volume under nitrogen to approximately 0.5 mL and a 50-μL aliquot was applied to a cellulose TLC plate (Brinkmann, Celplate 22, 100 μm thickness) as a streak. The plate was spotted with standard solutions of L-methionine and the calcium salt of M-analog and developed in a mixture of 1-butanol–acetic acid–water (12:3:5, v/v/v) for a distance of 15 cm. The position of the methionine and M-analog standards was ascertained by spraying a 1:1 aqueous mixture of 0.066 M KI and 0.0033 M H_2PtCl_6 (Winegard et al., 1948). Methionine and M-analog had R_f values of 0.45 and 0.87, respectively. The plate was scanned on a Varian Aerograph/Berthold Model 6000-2 Automatic/Integrating TLC Radioscanner and then subjected to autoradiography to locate the bands of residual ^{14}C-labeled methionine and the ^{14}C-labeled M-analog resulting from the microbial conversion. These areas of radioactivity were then re-

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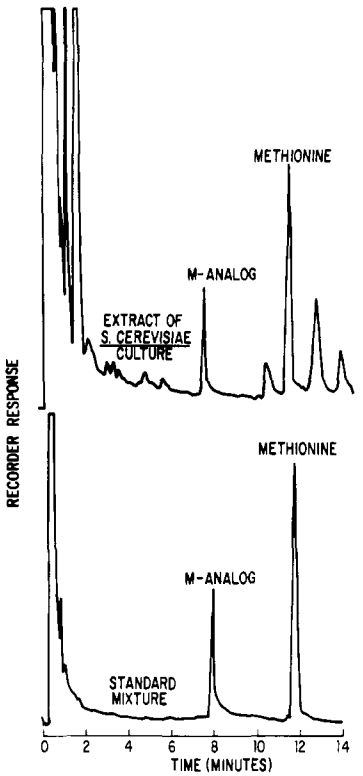
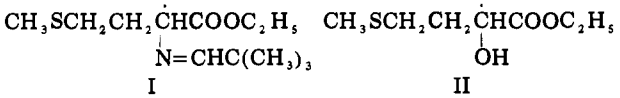


Figure 1. Gas chromatograms of derivatized methionine and M-analog from a methionine supplemented culture medium inoculated with *Saccharomyces cerevisiae*, compared with that of the standard.

moved from the plate and suspended in a mixture of 3.5 mL of water and 11.5 mL of Aquasol (New England Nuclear) and radioassayed in a liquid scintillation counter (Nuclear Chicago Isocap, Model 300).

Gas–Liquid Chromatographic Procedure. A sample (3 to 5 mL) free of bacteria and milk protein was introduced into a 50-mL Erlenmeyer flask equipped with 19/38 ground glass joint and taken to dryness on a rotary evaporator under vacuum at 80–85 °C. The free ^{14}C-labeled methionine and M-analog were then derivatized by the procedure described by Zscheile et al. (1972) to yield the neopentylidene ethyl ester of methionine (I) and the ethyl ester of M-analog (II). The samples were then



chromatographed using a Model 810 F&M gas chromatograph equipped with a flame ionization detector. A 9:1 splitter channeled the major fraction of the column effluent into vials containing scintillation solution, thus permitting simultaneous radioassay of the various volatile fractions of the injected sample as indicated by the response on the recorder. A 6-ft glass column (7 mm o.d., 4 mm i.d.), packed with 3% OV-17 on 80–100 mesh Chromosorb W (HP), was used for the GLC separation. The detector and injection port temperatures were 250 °C. The initial column temperature was 100 °C which was programmed at a rate of 6 °C/min to a final temperature of 220 °C. The helium flow rate was 60 mL/min. The respective retention times of M-analog and methionine derivatives under these conditions were 7.8 and 11.8 min (Figure 1).

Analysis of Cultured Milk Products. The extraction and analysis of cultured milk products (Table I) were based on the procedure in which M-analog was derivatized with BSA [*N,O*-bis(trimethylsilyl)acetamide] to form the corresponding trimethylsilyl derivative described by Pease et al. (1978).

Table I. Commercial Cultured Milk Products

Purchase location	M-analog content, ppm: Cottage cheese	Sour cream	Buttermilk
Atlanta, Ga.	0.13	0.66	3.1
Houston, Tex.	<0.05	1.6	1.7
Menlo Park, Calif.	<0.05	0.32	1.4
Hopkins, Minn.	<0.05	0.69	3.5
Wilmington, Del.	0.80	1.4	2.7

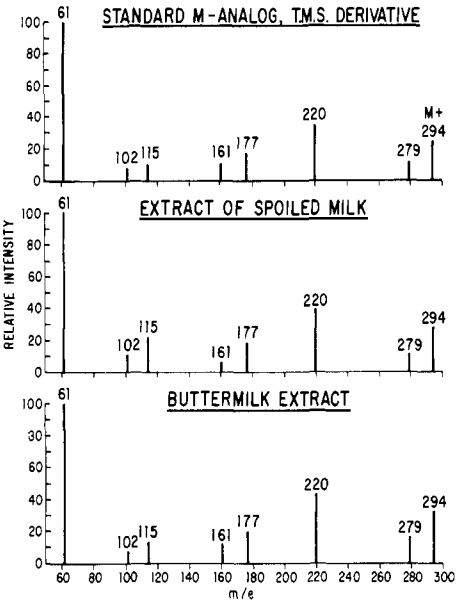


Figure 2. Selected ion mass spectra of Me_3Si derivatives of M-analog in extracts of spoiled milk and buttermilk.

Gas Chromatographic–Mass Spectrometer Analysis. Confirmation of the identity of M-analog in cultured milk products, as the silyl derivative, and in bread, corn silage, distillers' mixed grains, sauerkraut, beer, and in the various culture media as the ethyl ester was accomplished by GC–MS using a du Pont Mass Spectrometer (Model 21-492) coupled to a Perkin-Elmer Gas Chromatograph (Model 990).

RESULTS AND DISCUSSION

Samples of commercial cultured milk products (cottage cheese, sour cream, and buttermilk) from five locations were analyzed for M-analog, after finding the presence of M-analog in milk samples which had inadvertently spoiled for lack of refrigeration. The results, shown in Table I, clearly demonstrate that M-analog is a naturally occuring component in these milk products. Of the three products investigated, cottage cheese contained the lowest concentration of M-analog (<0.05 to 0.80 ppm), with sour cream next (0.32 to 1.6 ppm), while buttermilk contained the highest concentration (1.4 to 3.5 ppm). Confirmation of the identity of the trimethylsilyl derivative of M-analog found in buttermilk and spoiled milk was accomplished by GC–MS (Figure 2). The bar graph plots of eight of the major ions in the M-analog spectra, which were free from interference from other components in the extracts, conclusively show that the samples contained M-analog. Furthermore, a specific ion GC–MS analysis of spoiled milk and buttermilk extracts using the *m/e* 294 molecular ion showed a large peak at the correct retention time for the derivative. No peak at this retention time was evident with extracts of control fresh milk.

Table II. Conversion of Methionine to M-Analog by *Saccharomyces cerevisiae*

Sampling interval	% of total ^{14}C activity (TLC)		% of total ^{14}C activity in GLC column effluent	
	Methionine	M-analog	Methionine	M-analog
At start-up	90.2	0.3	92.0	0.1
1 h	81.1	7.6	83.3	4.6
4 h	42.9	44.9	57.2	19.0
7 h	12.8	73.0	16.7	67.1
24 h	2.2	84.6	2.6	81.3

Table III. Conversion of Methionine to M-Analog by *Bacillus subtilis*

Sampling interval	% of total ^{14}C activity in GLC column effluent as	
	Methionine	M-analog
At start-up	88.7	0.2
1 h	89.0	0.3
4 h	86.5	1.3
7 h	85.8	2.8
24 h	81.9	6.0
48 h	83.2	5.0

Table IV. Conversion of Methionine to M-Analog in Milk

Inoculum	% of total ^{14}C activity in GLC column effluent as	
	Methionine	M-analog
Control[a]	86.6	<0.1
Bacillus subtilis	70.9	4.5
Lactobacillus lactis	52.7	11.9
Saccharomyces cerevisiae	32.7	32.0
Pasteurized skim milk	52.3	15.7

[a] Uninoculated sterilized skim milk.

It is apparent, therefore, that those microorganisms which survive pasteurization, as well as those used in the manufacture of cultured milk products, are capable of this biochemical formation of M-analog from any free methionine in milk or that freed by microbial action. For further proof, a laboratory investigation was undertaken to evaluate the biochemical conversion of ^{14}C-labeled methionine to the correspondingly labeled M-analog.

Initially, a synthetic nutrient medium (Maw, 1963), where the sulfate was replaced with radiolabeled methionine, was inoculated with *Saccharomyces cerevisiae* (ATCC 9763) and incubated over a 24-h period at 30 °C. Samples for analysis were withdrawn after 1, 4, 7, and 24 h. Radioscans of thin-layer chromatograms on cellulose, followed by radioassay of plate scrapings, revealed rapid conversion of methionine to M-analog (Table II). After 24 h, 85% of the total radioactivity on the TLC plate was M-analog while only 2% was methionine. Radio-gas chromatographic analysis on duplicate samples, which had been derivatized, gave values which were in good agreement with those obtained by TLC. The identity of the derivatized methionine and M-analog was confirmed by GC–MS. The derivatized extract showed a peak for the ethyl ester of M-analog at the correct retention time on a 3-ft 10% OV-17 column, with the mass spectrum confirming its identity. There was excellent agreement between the mass spectra obtained with the ester of standard M-analog and the derivatized extract (Figure 3). A prominent molecular ion was obtained at m/e 178. The base peak at m/e 61 in the spectrum is due to the characteristic fragment $CH_3SCH_2^+$. The prominent fragments at m/e 104 and 76 are likely due to $CH_3SCH_2CH{=}O$ and $CH_3SCH_2CH_3$, respectively, both resulting from hydrogen rearrangements.

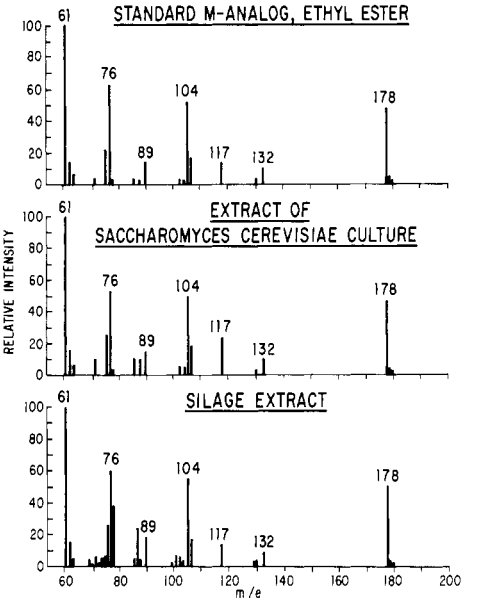


Figure 3. Mass spectra of ethyl ester derivatives of M-analog in a *Saccharomyces cerevisiae* culture and in corn silage.

A synthetic nutrient medium, consisting of 10 g of glucose, 1.5 g of K_2HPO_4 and 1 g of NH_4Cl per liter and supplemented with ^{14}C-labeled methionine (1.32 mM), was inoculated with *B. subtilis* (ATCC 6051) culture. Samples were removed at time intervals up to 48 h for GLC analysis. The results (Table III) confirmed the findings of Akobe (1936), showing that *B. subtilis* is capable of producing M-analog from methionine. The M-analog accounted for 6 and 5% of the total radioactivity after 24 and 48 h incubation, respectively, which is considerably less than that observed with *S. cerevisiae*.

For comparison purposes, sterile skim milk, fortified with ^{14}C-labeled methionine (7.0 mM) was inoculated with *B. subtilis*, *L. lactis*, *S. cerevisiae*, and with pasteurized, but unsterilized skim milk and incubated for 72 h. The samples were then analyzed and radioassayed by GLC. The data in Table IV show the conversion of methionine to M-analog with each of the microbial inocula. Again *S. cerevisiae* appeared the most efficient, with 32% of the radioactivity in the column effluent characterized as

Table V. Microbial Conversion of Methionine to M-Analog in Milk

Interval, h	Distribution (%) of total radioactivity when incubated with					
	S. cerevisiae		*B. subtilis*		*L. bulgaricus*	
	Methionine	M-analog	Methionine	M-analog	Methionine	M-analog
0			90.1	<0.1		
7	77.2	7.4	85.2	2.4	78.7	2.6
24	59.4	19.3	84.0	4.6	75.4	5.0
48	57.7	23.5	75.5	8.1	70.2	13.6
72	41.3	19.6	74.3	8.3	46.1	18.6

Table VI. M-Analog Content in Selected Feed and Food Products

Item analyzed	M-analog content (ppm) as analyzed by GC-S detector	GC-MS[a]
Bread	<0.1	0.08
Beer	0.2	0.2
Corn silage	10	18
Distillers' mixed grains (acid hydrolyzed)	88	60
Sauerkraut	1.3	1.0

[a] Specific ion monitoring used (m/e 178).

M-analog. Probably the discrepancy in the extent of M-analog formation in the synthetic nutrient medium vs. the skim milk medium can be attributed to the fact that the former contained no sulfur other than that contributed by the ^{14}C-labeled methionine. In the skim milk medium, the milk protein probably supplied a large fraction of the bacterial sulfur–amino acid requirements and therefore the conversion of the supplemental, radiolabeled methionine was less efficient. The use of pasteurized skim milk as the inoculum resulted in M-analog formation, accounting for 15.7% of the radioactivity in the column effluent. These data substantiate the finding of M-analog in unrefrigerated, spoiled milk and indicate that microbial contaminants of milk, surviving the pasteurization step, are capable of this biochemical conversion. *L. lactis* also demonstrated this capability. Its use as the inoculum resulted in M-analog, making up 11.9% of the ^{14}C activity in the column effluent. *B. subtilis* was equally as effective in the skim milk medium as in the synthetic nutrient medium in the formation of M-analog, accounting for 4.5% of the total ^{14}C activity.

The formation of M-analog by *S. cerevisiae*, *B. subtilis*, and *L. bulgaricus* was also monitored at time intervals over a 72-h period (Table V). The extent of M-analog formation with *S. cerevisiae*, approximating 20% of the total effluent ^{14}C activity, was somewhat less than that found in the previous tests, but the difference was not considered significant. The results obtained with *B. subtilis* are in good agreement with the earlier data (Tables III and IV). This test demonstrated that *Lactobacillus bulgaricus* (ATCC 11842) also possessed the capability of this biochemical conversion, with 18.6% of the ^{14}C activity of the GC column effluent accounted for as M-analog after 72 h of incubation.

These findings pointed to the possibility that certain beverages, foods, and feed ingredients subjected to microbial action in their manufacture might also contain M-analog in detectable amounts. Accordingly, a sample of bread, beer, sauerkraut, distillers' mixed grains, and corn silage were analyzed for M-analog. The mass spectrum of M-analog in the corn silage extracts as its ethyl ester showed the same characteristic pattern as found with synthetic M-analog and with that found in a yeast fermentation (Figure 3), as discussed earlier. The level of M-analog in the beer and bread extracts was too low relative to the other components to obtain definitive mass spectral scans. Quantitative data on the M-analog content of these selected samples of beer, bread, sauerkraut, distillers' mixed grains, and corn silage were obtained by using a GLC instrument with a sulfur detector (Pease et al. 1978) and by specific ion GC–MS analysis, employing the m/e 178 molecular ion. The data in Table VI demonstrate that M-analog is a naturally occurring component in all the above items, which go into human and animal consumption. Bread, beer, sauerkraut, corn silage, and acid-hydrolyzed distillers' mixed grain had M-analog concentrations of 0.08, 0.2, 1.0, 18, and 60 ppm, respectively, based on GC–MS analysis. Results obtained by GLC using a sulfur-selective detector were in agreement.

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