Detection and Stability of *N*-Acetylmethionine in Model Food Systems

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A procedure was developed to measure *N*-acetylmethionine (NAM) based on reaction with acylase followed by high-performance liquid chromatography. This procedure was used to evaluate the stability of NAM in solution and in model food systems. In systems undergoing Maillard browning, NAM showed only slight instability, and the recovery was much greater for NAM than it was for free methionine. In a system containing oxidized oil, NAM was less stable than methionine. The conversion of NAM sulfoxide was greater than the conversion of methionine to methionine sulfoxide.

The protein value of foods limiting in methionine can be improved through fortification with free methionine, as evidenced by increased protein efficiency ratios in rats (Jenkins et al., 1975) and increased nitrogen retention in humans (Kies et al., 1975; Zezulka and Calloway, 1976). The potential benefits of methionine fortification may not be fully realized, however, due to degradation of the added amino acid during processing or storage. Maillard browning or Strecker degradation reactions involving the amino group of methionine may lead to nutritional losses and to the formation of objectionable flavor compounds (Shemer and Perkins, 1974, 1975; Ballance, 1961). In addition, oxidation of the methionine sulfide to the sulfoxide or sulfone form may reduce the nutritional availability of methionine (Cuq et al., 1973).

So that the nutritional losses and flavor problems associated with methionine fortification can be overcome, the use of a methionine derivative, *N*-acetyl-L-methionine (NAM), has been recommended (Damico, 1975) and approved as a food additive ("Code of Federal Regulations", 1979). NAM is cleaved by a widely distributed mammalian kidney enzyme (acylase) to form methionine and acetate (Birnbaum et al., 1952; Endo, 1978). The nutritional and metabolic equivalency of L-NAM and L-methionine has been demonstrated with rats (Boggs et al., 1975; Rotruck and Boggs, 1975) and with humans (Zezulka and Calloway, 1976; Stegink et al., 1980).

The protected amino group of NAM is relatively stable to Strecker degradation (Damico, 1975) and offers higher sensory thresholds than methionine in amino acid fortified foods (Damico, 1975; Hippe and Warthesen, 1978). While the amide bond between methionine and the acetyl group appeared stable to hydrolysis at pH 7 (Damico, 1975), the stability of this bond has not been fully evaluated under other conditions that might be found in food processing. If the acetyl group on NAM is hydrolyzed, the amino group would be susceptible to reactions in the Maillard browning scheme, leading to nutritional losses and off-flavor production. Furthermore, if the sulfide group of NAM were to become oxidized to the sulfoxide or sulfone form, NAM may become less nutritionally available.

This paper will present a procedure based on high-performance liquid chromatography (HPLC) that was developed to quantify NAM and NAM oxidation products in model food systems. In addition, it was the objective of this study to evaluate the influence of pH and heat on the stability of the amide bond of NAM. The stabilities of NAM and methionine were also compared in systems undergoing Maillard browning or containing oxidized oil.

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MATERIALS AND METHODS

Reaction of NAM and Acylase. NAM solutions required reaction with acylase to form methionine and acetate. Methionine thus formed could be derivatized with dansyl chloride and detected by HPLC. Quantitative enzymatic conversion of NAM to methionine and acetate was therefore an essential part of this method. Experiments were run to determine the reaction time and enzyme concentration necessary for complete recovery of methionine from NAM. Unless stated otherwise, hog kidney acylase I (EC 3.4.1.14, Sigma Chemical Co., 1845 units/mg) was freshly prepared at a concentration of 0.362 mg/mL in 0.01 M phosphate buffer (pH 7.0). This enzyme preparation was allowed to react with an equal volume of L-NAM (Sigma Chemical Co.) solution adjusted to pH 7 at a concentration of not greater than 0.67 μmol/mL.

Preparation of Oxidized NAM. To determine if acylase was active on the oxidized derivatives of NAM, we reacted NAM with hydrogen peroxide to form the sulfoxide and sulfone derivatives using a procedure similar to that used by Lipton et al. (1977). An acidic solution of NAM was treated with hydrogen peroxide for 60 min at 50 °C, neutralized with NaOH, and reacted with catalase (Sigma Chemical Co.) to remove residual hydrogen peroxide. Control samples were carried through the procedure without the hydrogen peroxide treatment. A portion of the H_2O_2-treated NAM was treated with acylase and another portion was retained as a control. These samples were subsequently analyzed by HPLC following formation of the dansyl derivatives. A similar set of oxidized samples was prepared by using methionine as the starting material.

HPLC Analysis of Methionine. The formation of dansyl derivatives of methionine and methionine oxidation products was accomplished by using the method of Tufte and Warthesen (1979). Methionine solutions or NAM solutions treated with acylase were reacted with dansyl chloride and then analyzed by HPLC. Sample analysis was performed by using a Waters Associates (Milford, MA) HPLC system consisting of two Model 6000A pumps, a Model 660 solvent programmer, and a Model 440 absorbance detector with a 254-nm filter. All injections were made by using a Rheodyne injection unit equipped with a 10-μL sample loop. A Hewlett-Packard 3380A recorder-integrator was used to monitor detector output.

The mobile phase and columns used for separation of methionine derivatives varied depending on the mode of degradation under study. Where Maillard browining was the main mechanism of degradation, analysis was performed with an Ultrasil-Octyl column (Altex Scientific Co., 10 μm, 25 cm × 4.6 mm i.d.) and an isocratic mobile phase composed of 70% phosphate buffer (0.01 M, pH 7.0) and 30% acetonitrile. Dansylmethionine eluted in about 4 min

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when a flow rate of 2.0 mL/min was used.

For the analysis of samples containing the dansyl derivatives of methionine sulfoxide and methionine sulfone, separation was achieved with a Zorbax ODS column (Du Pont Instruments, 15 cm × 4.6 mm i.d.) and a solvent gradient beginning with 80:20 phosphate buffer–acetonitrile and changing to 64:36 over 5 min, curve 10. Dansylmethionine sulfoxide eluted in 2.6 min, followed by methionine sulfone at 4.0 min and dansylmethionine at 6.3 min, when a flow rate of 2.0 mL/min was used.

External standards reacted with dansyl chloride under the same conditions as the samples were used to quantify methionine, methionine sulfoxide, and methionine sulfone. For quantification of NAM, an NAM standard was prepared and reacted with acylase and then with dansyl chloride in the same manner as the samples. To quantify the oxidation products of NAM, we used methionine sulfoxide and methionine sulfone as standards. Quantification with the external standards was based on peak area. The NAM used in this study was determined to be free of methionine by reacting NAM with dansyl chloride prior to treatment with acylase. NAM treated in this manner gave no HPLC response for methionine, indicating the absence of significant amounts of free methionine.

Amide Bond Stability in Solution. The stability of the amide bond of NAM was evaluated in solution by adjusting the pH to different levels and then heating. NAM solutions (0.1 mg/mL) were prepared in 0.1 M citrate–phosphate buffers adjusted to maintain a pH of 3, 5, or 7 and in 0.1 M borate buffer to maintain a pH of 9. Duplicate samples were autoclaved at 121 °C for 15 min or were heated in a 95 °C water bath for 12 h. Heated samples were compared to control samples that received no heat treatment. Following adjustment to pH 7.0, aliquots of each sample and control were reacted with acylase. Loss of the *N*-acetyl group would lead to the formation of methionine, which could be detected by HPLC without prior reaction with acylase. A second aliquot of each sample and control was given no acylase treatment so that any methionine formed due to amide bond cleavage could be quantified.

Amino Acid Extraction. Model systems (described below) were used to study the stability of NAM and methionine to browining and oxidation. So that analysis problems caused by interference from protein and other material in the sample could be eliminated, the following procedure was used to precipitate the protein and extract the added amino acids from the model systems studied. Two grams of the model system was added to 30 mL of distilled water and mixed for 45 s in a Sorvall omnimixer. Following quantitative transfer to a 50-mL centrifuge tube, the pH was adjusted to about 4.5 by the addition of 0.05 mL of glacial acetic acid. The sample was then centrifuged at 500*g* for 10 min. The pellet was centrifuged 2 more times with 10 mL of 0.1 M, pH 4.5, acetate buffer, and the supernatants were collected in a 100-mL volumetric flask following each washing. The pH of the supernatant was adjusted to 7.0 with 5 N NaOH and brought to volume with pH 7.0, phosphate buffer. Methionine-containing samples required no further preparation prior to reaction with dansyl chloride. NAM-containing samples were given the enzyme treatment described above. The recovery of NAM or methionine through the extraction and HPLC quantitative procedure was 94 ± 2% (mean ± standard deviation).

Stability to Maillard Browning. The comparative stabilities of NAM and methionine were studied in model systems designed to favor Maillard browing reactions. The basic model formulation was 10% isolated soy protein (Promine D, Central Soya Co.), 4% D-glucose, 0.5% L-methionine or 0.64% L-NAM, and microcrystalline cellulose to make 100%. The soy protein, glucose, and microcrystalline cellulose were dry blended and then slurried with a solution containing the desired level of either NAM or methionine. The slurry was then frozen and freeze-dried. The samples were mixed in a Waring blender, placed in desiccators containing saturated calcium nitrate, and allowed to equilibrate to a water activity of 0.56 (Rockland, 1960). Five-gram portions were sealed in retortable foil pouches and heated for intervals of up to 5 h in a 95 °C water bath. Five replicates were prepared for each heating period. Control samples received no heat treatment. After removal from the bath, samples were cooled in cold water and stored at −18 °C until extracted and analyzed.

Since the pHs of the amino acid solutions used to slurry the dry blended model sytems were not equivalent (pH 3.6 in the case of NAM and 6.4 in the case of methionine), the model systems were not at the same pH. The pH of the NAM slurry was 5.7 while the pH of the methionine slurry was 6.9. To test whether this pH difference had a major influence on amino acid destruction in the dried system, we prepared a second model system. Before the system was slurried, the pH of the NAM solution was adjusted with 5 N NaOH to equal the pH of the methionine solution. Sample preparation was completed as described above. Five replicates were processed for 5 h at 95 °C, and five control replicates received no heat treatment.

For determination of the role of glucose in NAM loss, a glucose-free model system was prepared. Glucose in the basic formulation above was replaced with microcrystalline cellulose. Five replicate samples were processed at 95 °C for 5 h, and five replicate samples serving as controls received no heating.

For calculation of the amino acid stability during processing in these three model systems, the methionine or NAM following processing was computed as a percentage of the methionine or NAM present in the control (unheated) samples.

Stability to Oxidation. The comparative stability of methionine and NAM to oxidation was studied in a heated system that contained highly oxidzed corn oil. Stripped corn oil (Eastman Kodak Co.) was oxidized by bubbling oxygen through the oil for approximately 24 h. The peroxide value of the oxidized oil was 145 mequiv/kg (Association of Official Analytical Chemists, 1975). A model system consisting of 20% oxidized oil, 0.5% L-methionine or 0.64% L-NAM, 15% distilled water, and microcrystalline cellulose to 100% was prepared by thorough hand mixing. Five-gram portions were sealed in retortable foil pouches. Five replicate samples were heated for intervals up to 5 h in a 95 °C water bath. Control samples received no heat treatment. After being heated, the samples were cooled in cold water and stored at −18 °C until analyzed. Approximately 3% contaminating sulfoxide was present in the NAM and methionine used in these model systems. The sulfoxide content of the control samples was subtracted from the sulfoxide content of heated samples. The percentage of methionine or NAM that was oxidized to form the sulfoxide derivative was calculated on a mole basis.

RESULTS AND DISCUSSION

Reaction of NAM and Acylase. HPLC was chosen as the method of methionine or NAM analysis because the procedure is relatively rapid and is also capable of quantifying the oxidation products of methionine (O'Keefe and

Table I. Influence of Acylase Reaction Time on Recovery of Methionine from NAM

time[a]	% recovery	time[a]	% recovery
5 min	100	30 min	97
10 min	98	60 min	98
15 min	97	16 h	99

[a] 37 °C; pH 7; 3 mL of acylase solution (0.362 mg/mL of phosphate buffer) combined with 3 mL of NAM solution (0.128 mg/mL of water).

Warthesen, 1978; Tufte and Warthesen, 1979). The reaction of dansyl chloride with the amino group forms dansyl derivatives that are separated and detected by HPLC. NAM and related oxidized derivatives, *N*-acetylmethionine sulfoxide and *N*-acetylmethionine sulfone, do not have free amino groups and consequently do not form dansyl derivatives when reacted with dansyl chloride. An essential part of this method, therefore, is the reaction of NAM or oxidized NAM with acylase. Acylase cleaves the amide bond, which frees the amino group and allows dansyl derivative formation.

The results in Table I show that acylase cleaves the *N*-acetyl group of NAM rapidly, with quantitative recovery of methionine within 5 min. If the reaction is allowed to proceed longer, such as overnight, recovery does not change. For this work, it was convenient to allow overnight (approximately 16 h) reaction of the samples with acylase. As long as excess acylase was present, conversion of NAM to methionine and acetate was quantitative.

For evaluation of the activity of acylase on the oxidized derivatives of NAM, NAM was treated with hydrogen peroxide at an acid pH to promote sulfide oxidation. Under these conditions, small amounts of NAM sulfone were formed (2%) but NAM sulfoxide was the major product. Following acylase conversion, 80% of the NAM was recovered as methionine sulfoxide or methionine sulfone. No methionine was found, indicating the absence of NAM in the oxidized samples. When methionine was subjected to the hydrogen peroxide treatment, methionine sulfoxide was the major product. No methionine remained in the samples, and 88% was recovered as either sulfoxide or sulfone. Recovery of less than 100% suggests that some of the NAM or methionine was oxidized or converted to products not detected by this analysis. Although both NAM and methionine were extensively oxidized by the hydrogen peroxide, the lower recovery of oxidized products from NAM relative to the recovery of oxidized products of methionine may mean slightly more of the NAM was oxidized to undetected compounds.

The results of this study with oxidized NAM show that acylase cleaves the *N*-acetyl group from NAM, NAM sulfoxide, and NAM sulfone to form methionine, methionine sulfoxide, and methionine sulfone, respectively. Products thus formed will react with dansyl chloride to form derivatives that can be measured by HPLC. The procedure is capable not only of NAM quantification but also of simultaneous determination of the sulfoxide and sulfone forms of NAM.

Stability of the *N*-Acetyl Group. The *N*-acetyl group on NAM offers amino group protection as long as the amide bond is stable to storage and processing conditions that may be encountered. If it is not stable, a free amino group becomes available to participate in degradative reactions, resulting in nutritional losses or off-flavor production. Therefore, the stability of the amide bond to various pH and heat treatments was studied. Since control samples that were not heated and not reacted with acylase did not contain methionine, any methionine detected in the heat-treated NAM samples would be due to the loss of the *N*-acetyl group.

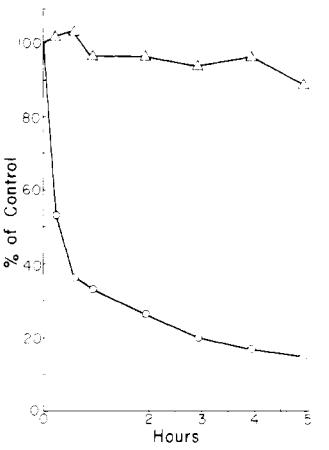

Figure 1. Recovery of NAM (Δ—Δ) and methionine (O—O) in a model system containing glucose heated at 95 °C.

The amide bond of NAM in solution was found to be relatively stable to the pH and heating conditions studied. No hydrolysis of the bond was evident at pH 5, 7, or 9 when NAM solutions were subjected to either 121 °C for 15 min or 95 °C for 12 h. In the samples adjusted to pH 3, however, the presence of methionine prior to reaction with acylase demonstrated some instability of the amide bond between the acetyl group and methionine. Autoclaving at 121 °C for 15 min at pH 3 caused the hydrolysis of 5% of the amide bonds. Sixteen percent of the NAM was hydrolyzed to methionine after 12 h at 95 °C. The results suggest that conversion of NAM to methionine is possible to a very limited extent during the heat processing of NAM-fortified foods if the pH is relatively low.

Stability of NAM and Methionine to Maillard Browning. The model systems containing protein and glucose were equilibrated to a water activity of 0.56 and heated at 95 °C to promote Maillard browning. In this model system, methionine loss was rapid. As shown in Figure 1, half of the added methionine was lost after only 20 min of heating. After the sample was heated, for 5 h, only 12 ± 0.6% of the added methionine remained. The same figure illustrates that NAM was relatively stable throughout the heating period. After 5 h at 95 °C, 82 ± 1.1% of the added NAM remained. These results indicate that NAM, with a protected amino group, offers much greater stability to Maillard browning than methionine. This agrees with the earlier work of Damico (1975) that indicated NAM to be much more resistant than methionine to Strecker degradation.

NAM is more acidic than methionine, and one reason for the higher resistance of NAM to Maillard browning could be the lower pH of the NAM model system. Because the more alkaline pH of the methionine system would favor Maillard browning (Reynolds, 1963), an experiment was conducted in which the NAM solution was adjusted to the same pH as the methionine prior to incorporation into the respective model systems. The stability of NAM in this model system was equal to the stability in the unadjusted model system (82% recovery), indicating that the lower pH of the NAM solution relative to that of methionine was not a factor in the increased stability of NAM.

The 18% loss of NAM after 5 h of heating was further investigated to determine if this decrease was due to loss of the *N*-acetyl group and subsequent reaction of the free amino group with glucose or some other mechanism. No methionine was present in the heated samples prior to treatment with acylase, suggesting that if methionine were

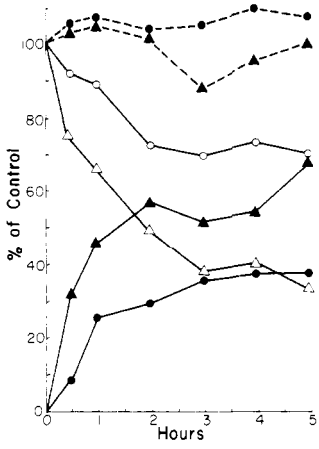

Figure 2. NAM, methionine, sulfoxides, and total recovery in a model system containing oxidized oil heated at 95 °C. Methionine sulfoxide(●—●); NAM sulfoxide (▲—▲); methionine (○—○); NAM (△—△); total methionine and methionine sulfoxide (●---●); total NAM and NAM sulfoxide (▲---▲).

formed from NAM during heating, it must have been subsequently degraded in the Maillard reaction. In a NAM model system prepared without glucose, no methionine was found after heating 5 h, and recovery of NAM was 100%. This indicates that the heating alone did not cause the loss of the *N*-acetyl group and that glucose plays a role in the 18% loss of NAM. This loss mechanism apparently does not involve heat-induced conversion of NAM to methionine followed by condensation with glucose in the Maillard reaction. While NAM appears to be nonreactive in the Maillard reaction, another mechanism such as reaction of carbohydrate with the sulfide as proposed by Lipton and Bodwell (1976) may be responsible for some of the NAM loss in the model system containing glucose.

Comparative Stability of NAM and Methionine to Oxidation. Model systems with oxidized corn oil were used to determine the comparative stabilities of NAM and methionine under oxidizing conditions. In model systems prepared with oxidized oil and then heated, the oxidation of both methionine and NAM was evident. The sulfoxide derivative of both amino acids was formed and increased in concentration with increases in heating time. As shown in Figure 2, the increase in the sulfoxide forms was accompanied by decreases in the levels of methionine or NAM. Conditions were apparently severe enough to cause sulfoxide formation but not formation of the more highly oxidized sulfone.

NAM appears to be more susceptible to oxidation than methionine under the conditions used in this experiment. Within the first 2 h, the amount of NAM oxidized was approximately 2 times more than the amount of methionine oxidized. After 5 h at 95 °C, 63.5 ± 5.1% of the NAM was oxidized to NAM sulfoxide while 39.7 ± 1.2% of the methionine was oxidized to methionine sulfoxide. One reason for the higher rate of oxidation of NAM could be that the acetyl group increases the electrophilicity of the amino acid, and the overall effect of this could be a more reactive sulfide. Another explanation for the higher rate of NAM oxidation could be based on the difference in polarity between methionine and NAM. In the model system, the amino group of methionine would exist in the ionic form and would be more polar than the *N*-acetyl group on NAM. Methionine therefore would tend to associate with water or polar components of the system rather than with the nonpolar lipid components. NAM, being less polar, is more likely to be associated with the nonpolar lipid material that is probably the site of oxidation in this system.

The unoxidized amino acid and the sulfoxide derivative levels for each amino acid were combined to determine if total recovery of both amino acids was attained. These data, presented in Figure 2, show that recovery was near 100% for all heating times. The high recovery suggests that sulfoxide is the only major product forming from the amino acids and also confirms that the acylase is effective in quantitatively converting NAM sulfoxide to methionine sulfoxide.

While the use of NAM as a replacement for methionine would mean a more stable amino acid during Maillard browning, this would not be the case during oxidative losses of these amino acids. NAM appears to be more sensitive to sulfoxide formation than methionine. The nutritional significance of sulfoxide formation in NAM is difficult to determine since there is not agreement on the nutritional value of methionine sulfoxide for humans and apparently no data are available on the nutritional value of NAM sulfoxide.

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