Carbonyl–Amine Reaction Products as a Possible Source of Nitrosatable Nitrogen

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The possibility of forming *N*-nitrosamines by reaction of sodium nitrite with compounds produced from aliphatic aldehydes and primary amines was investigated. Sodium nitrite was combined with an aldehyde–amine mixture, with imines formed by aldehyde–amine condensation, and with a model dry food system designed to promote nonenzymatic browning reactions between aldehydes and amino groups. Reaction products were tested for apparent nitrosamine content with Griess reagent, and, whenever possible, were characterized by boiling point, infrared spectrophotometry (ir), and gas chromatography–mass spectrometry (GC–MS). *N*-Nitrosamine formation was not demonstrated in aqueous systems containing sodium nitrite and carbonyl–amine reaction products. Extracts of meat samples to which an arbitrary excess of sodium nitrite (2.76%) had been added were analyzed with GC–MS, but the presence of nitrosamines has not yet been confirmed.

Several *N*-nitrosamines have been shown to be carcinogenic for a wide range of animal species (Magee, 1971; Magee and Barnes, 1967). *N*-Nitrosamines may be formed readily by reaction of sodium nitrite with secondary or tertiary amines under mild conditions of pH and temperature (Hatt, 1943). Consequently, the use of sodium nitrite as a food additive has been questioned (Lijinsky and Epstein, 1970), and a number of food products, particularly cured meats and smoked fish, have been examined for nitrosamine content. The nitrosamine concentrations found have seldom been greater than 100 μg/kg and usually less than 10 μg/kg (Crosby et al., 1972; Fiddler et al., 1972; Telling et al., 1971). However, even low concentrations of *N*-nitrosamines are undesirable in view of their potential as carcinogens for humans.

N-Nitrosamine formation requires (1) a nitrosating agent, such as nitrous acid from sodium nitrite, (2) a source of nitrosatable nitrogen, and (3) proper reaction conditions. The purpose of this investigation was to examine carbonyl–amine reaction products as one possible source of nitrosatable nitrogen. Imine formation from carbonyl–amine condensations has been reviewed by Hodge (1953) and demonstrated in model dry food systems by Dugan and Rao (1971). Suspected nitrosamines were reported by Devik (1967) in products of the Maillard reaction involving glucose and amino acids. These were subsequently shown by Kadar and Devik (1970) to be pyrazines which gave interfering reactions in the test methods used. No nitrite was present in these systems to provide a basis for nitrosamine formation. The objectives of this study were to determine (1) if imines would react directly with sodium nitrite to form *N*-nitrosamines; (2) if *N*-nitrosamines would form from a reaction between nitrite and an intermediate in the synthesis of imines; and (3) if nitrosamines could be formed in a model dry food system designed to enhance nonenzymatic browning in the presence of aldehyde and/or sodium nitrite.

EXPERIMENTAL PROCEDURES

Safety Precautions. *N*-Nitroso compounds are both toxic and carcinogenic; therefore, rubber gloves were always used when working with them, and all experiments were conducted under an exhaust hood.

***N*-Nitrosamine Standards.** *N*-Nitrosamine standards not purchased from commercial sources were prepared from secondary amines by a method similar to that described by Hatt (1943). A secondary amine–hydrochloric acid solution was heated at 70–75° and maintained at pH 6.0–6.5 during dropwise addition of a 1.4 molar excess of an aqueous solution of sodium nitrite. Stirring was continued for 2 hr after addition was complete; then the mixture was fractionally distilled in vacuo. Distillation fractions which gave positive Griess and ninhydrin tests were additionally analyzed using methods which were more selective for nitrosamine content. The identities of nitrosamine standards obtained from these distillation fractions were confirmed by comparison of boiling points and infrared and mass spectra with data reported by Pensabene et al. (1972), Saxby (1972), and Williams et al. (1964).

Griess and Ninhydrin Reagents. Griess reagent was made by mixing solutions of sulfanilic acid (1% in 30% acetic acid) and α-naphthylamine hydrochloride (0.1% in 30% acetic acid) 1:1 just before using. A 0.3% ninhydrin solution was prepared using ethanol containing 2% pyridine.

Infrared Spectrophotometry (ir). A Beckman IR-12 double beam infrared spectrophotometer was used to record the spectra of neat samples (thin films on NaCl cells) with air as a reference.

Gas Chromatography–Mass Spectrometry (GC–MS). Mass spectra were obtained using a combined GC–mass spectrometer LKB 9000 equipped with a glass column (6 ft × ⅛ in.) of 3% OV-210, with ionizing electron energies of 22.5 or 70 eV, the flash heater set 20° above the GC column temperature, the molecular separator at 230°, and the ion source at 290°. The spectra were recorded as bar graphs by means of an on-line data acquisition and processing program (Sweeley et al., 1970).

Synthesis of Imines. Imines were prepared from primary amines using a modification of the procedure by Campbell et al. (1944). Aqueous solutions of methyl- or ethylamine hydrochloride were chilled to 0° and mixed with an equimolar KOH solution, followed by the dropwise addition of an equimolar amount of hexanal or butanal. The reaction products were fractionally distilled at atmospheric pressure and then characterized by ir and MS.

Attempted *N*-Nitrosamine Synthesis Using Imines and Sodium Nitrite. An aqueous sodium nitrite solution was maintained at pH 6.0–6.5 with 6 *N* HCl during the dropwise addition of imine. Stirring was continued for 1 hr after the imine had been added. The aqueous layer was then removed, washed three times with 50-ml aliquots of ether, and combined with the organic layer for fractional distillation in vacuo. The distillation fractions were further characterized by ir spectra and by the Griess test. Based on these results, selected fractions were analyzed by GC–MS.

Attempted *N*-Nitrosamine Synthesis Using Aldehyde, Primary Amine, and Sodium Nitrite. An aqueous primary amine solution was chilled to 0° and adjusted to pH 3.0 with 6 *N* HCl. A four-fold molar excess of sodium nitrite was added, followed immediately by the dropwise

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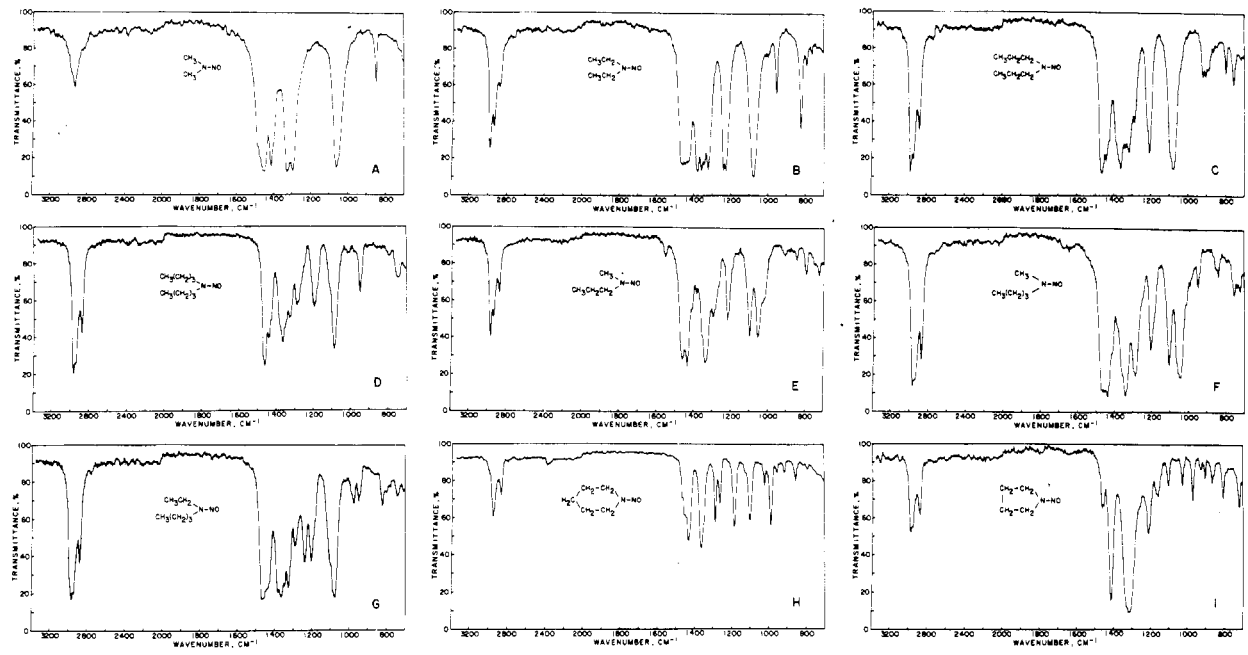

Figure 1. Infrared spectra of *N*-nitrosodimethylamine (A), -diethylamine (B), -dipropylamine (C), -dibutylamine (D), -methylpropylamine (E), -methylbutylamine (F), -ethylbutylamine (G), -piperidine (H), and -pyrrolidine (I).

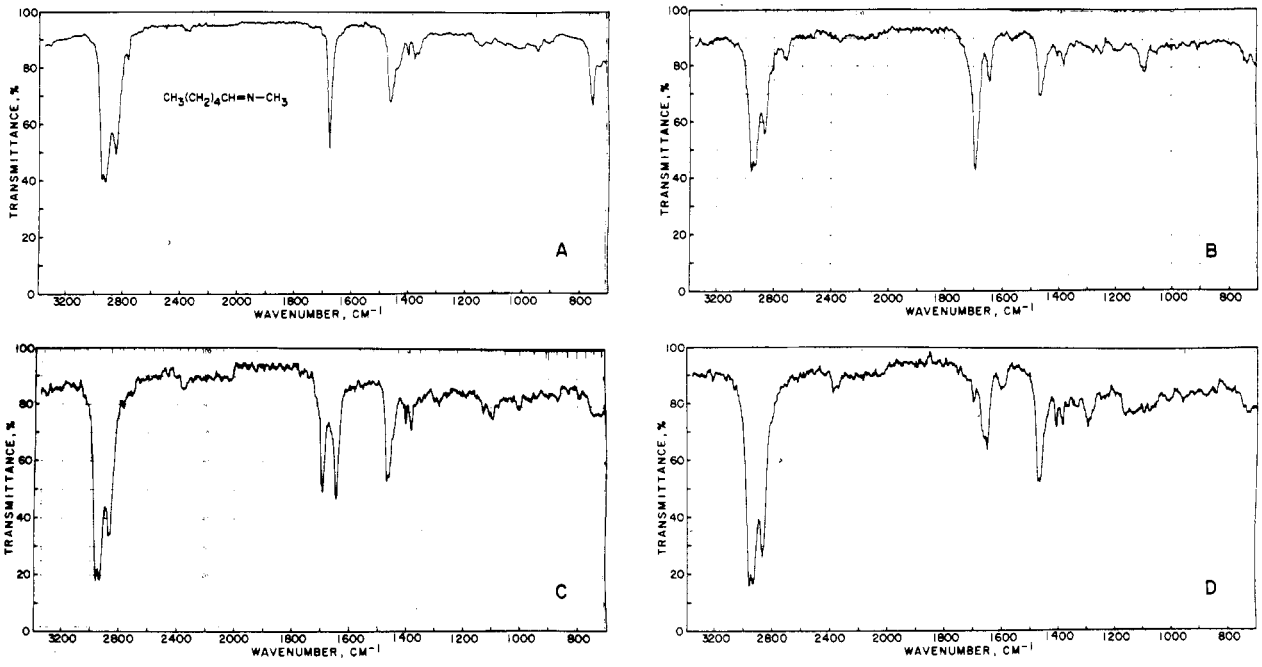

Figure 2. Infrared spectra of hexylidenemethylamine prior to addition of sodium nitrite (A) and of three distillation fractions obtained from the imine–sodium nitrite mixture, (B, C, and D, respectively) 30, 113, and 125° (17 mm).

addition of an equimolar amount of butanal. Stirring was continued for 1 hr after all the aldehyde had been added. The mixture was extracted with three 50-ml aliquots of dichloromethane, and the extract concentrated using a Kuderna-Danish evaporative concentrator. The concentrated extract was tested with Griess reagent and the ir spectrum recorded.

Ground Ham Model System. Fresh (uncured) ham muscles, trimmed free of visible fat prior to grinding, were divided into 100-g portions, and various additives were incorporated into the ham by a 60-sec blending in a 250-ml stainless steel Waring Blendor jar. Each emulsion was prepared by blending the ham with 20 ml of water and the following additives: (1) none (control); (2) 2.76 g of $NaNO_2$; (3) 2.76 g of $NaNO_2$ and 2.0 g of NaCl; (4) 2.76 g of $NaNO_2$ and 2.0 g of hexanal; (5) 2.0 g of hexanal; (6) 2.0 g of hexanal plus 2.76 g of $NaNO_2$ added just prior to extraction; and (7) 2 ml of ethanol containing 1 mg each of dimethylnitrosamine and dibutylnitrosamine. After blending, all samples were immediately frozen and then stored at −26° for 24 hr.

Two treatments were used in this model study: (1) frozen ham samples were transferred directly into a forced air oven and heated at 70° for 15 hr; (2) ham samples were freeze-dried and then heated as in (1).

After heating, the ham muscles were extracted by blending for 5 min in 200 ml of dichloromethane. The extract was filtered through Whatman Sharkskin filter paper into

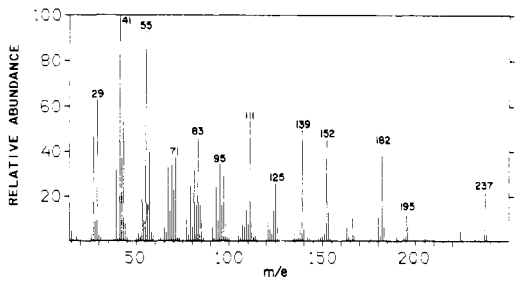

Figure 3. Mass spectrum of the 113° (20 mm) fraction distilled from a mixture of HMI and sodium nitrite.

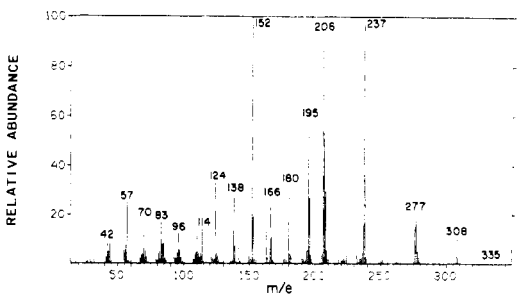

Figure 4. Mass spectrum of the 125° (20 mm) fraction distilled from a mixture of HMI and sodium nitrite.

a 500-ml distillation flask, boiling chips and several KOH pellets were added, and the flask was arranged for distillation at atmospheric pressure. Distillation was terminated when the flask appeared dry and liquid no longer distilled. The distillate was first concentrated to about 4 ml using a Kuderna-Danish evaporative concentrator, and then concentrated to 0.5 ml under a slow stream of nitrogen.

The concentrated ham extracts were initially analyzed for compounds which would give a nitroso-type colorimetric reaction with Griess and ninhydrin reagents. This initial sample screening was done by spotting 40-μl aliquots on thin-layer plates (precoated with 0.25-mm silica gel G), developing the plates with hexane–diethyl ether–dichloromethane (4:3:2), and visualizing the sample with either Griess or ninhydrin reagents. Sample extracts which tested positive were additionally analyzed by GC–MS.

RESULTS AND DISCUSSION

Attempted *N*-Nitrosamine Synthesis Using Imines and Sodium Nitrite. The ir spectra of aliphatic *N*-nitrosamines are distinguished by absorption bands at 1425–1460 cm^{-1} for N=O and at 1030–1150 cm^{-1} for N-N stretching (Williams et al., 1964). These absorption bands are present in the ir spectra of nine standard nitrosamines shown in Figure 1. These spectra were recorded from thin films of neat samples on NaCl cells; therefore, the large solvent peaks present in spectra previously reported by Pensabene et al. (1972) do not appear.

Figure 2 contains the ir spectrum of *N*-hexylidenemethylamine (HMI) prior to addition of sodium nitrite and the spectra of three distillation fractions obtained from the imine–sodium nitrite reaction mixture. All of these spectra show absorption between 1450 and 1470 cm^{-1}. However, since the imine, spectrum A, also absorbs in this region, these large peaks were not considered to be evidence of nitrosamine formation. Spectrum B does contain a small absorption band at 1095 cm^{-1} which could be due to N–N stretching. However, the distillation fractions and the distillation residue all were negative for nitrosamine content when tested with Griess reagent. The shift in the large imine-absorption band at 1675 cm^{-1}, spectrum A, to 1695 cm^{-1}, spectrum B, may be due to a change of the C=N– group to a charged species, $C{=}N{=}^{+}$, as a result of attack on the nitrogen by either H^+ or ^+NO (Smith, 1965). Similar ir spectra were obtained from a reaction mixture containing sodium nitrite and *N*-butylidene–ethylamine.

Selected distillation fractions were analyzed by GC–MS. Figures 3 and 4 are the mass spectra of the 113° (20 mm) and 125° (20 mm) distillation fractions, respectively, from a mixture of HMI and sodium nitrite. These and other spectra obtained from various distillation fractions were compared with the spectra of 25 standard nitrosamines (Pensabene et al., 1972), but were not identified with any of these.

Attempted *N*-Nitrosamine Synthesis Using Aldehyde, Primary Amine, and Sodium Nitrite. An aqueous solution of sodium nitrite was mixed with primary amines and aliphatic aldehydes to determine whether nitrite would react with some intermediate, formed during the carbonyl–amine reactions, to produce nitrosamines. The results obtained by adding nitrite to imine precursors were the same as those obtained by adding nitrite to the preformed imine, i.e., negative Griess tests and a shift in the large ir band at 1675 to 1695 cm^{-1}. It was concluded that imines and imine precursors would not combine with sodium nitrite to form nitrosamines in these aqueous systems.

Table I. R_f Values of Apparent Nitrosamines Found in the Extract from "Wet" and "Dry" Model Food Systems as Indicated by Griess and Ninhydrin Reactions on Silica Gel G TLC Plates Developed with Hexane–Diethyl Ether–Dichloromethane (4:3:2)

	Treatments	
Additives	Freeze-dried	Non-freeze-dried
Control (water)		
Nitrite		0.30
Nitrite + NaCl		0.30
Nitrite + aldehyde		0.30, 0.73
Aldehyde		
Aldehyde + nitrite added just before extraction		
Dimethyl- and dibutylnitrosamine	0.30, 0.60	0.30, 0.60

Ground Ham Model Systems. When aqueous mixtures of sodium nitrite and carbonyl–amine reaction products could not be shown to form nitrosamines, an attempt was made to learn if nitrosamines would be formed by combining these reactants in a model dry food system. A nonaqueous system would be less likely to cause hydrolytic cleavage of the imines prior to their being nitrosated. Ham muscles were chosen as the matrix upon which aldehyde and sodium nitrite were to be distributed, because it is a food normally consumed after being cured with sodium nitrite, and because ham muscles contain proteins which would supply free amino groups for reaction with aldehyde. Aldehydes, from the oxidative decomposition of fatty acids, have been shown to react with the amino group of phospholipids to form imines (Dugan and Rao, 1971).

Various combinations of sodium nitrite, sodium chloride, and hexanal were included in both the "wet" (non-freeze-dried) and "dry" (freeze-dried) systems. The "dry" meat emulsions were freeze-dried in order to reduce the possibility of decomposition of nitrosamines or nitrosamine intermediates as a result of hydrolysis during the prolonged heat treatment. The amounts of aldehyde and nitrite added to the model system were based on an estimate of

the millimoles of free amino groups in 100 g of ham muscle. Enough hexanal for a 1:1 stoichiometric reaction with the amino groups, a twofold excess of sodium nitrite, and 2% sodium chloride were added to the appropriate model system samples. To ensure that nitrosamines were not forming during the extraction procedure, sodium nitrite was added to the baked emulsion of some of the samples at the beginning of the extraction procedure. The negative result obtained from this variable (see Table I) indicates that nitrosamine formation did not occur during the extraction procedure. The minimum level of nitrosamine standards detectable on the thin-layer chromatography (TLC) plates with the Griess and ninhydrin reagents was 1 μg; therefore, a negative result in Table I means that less than 1 μg of apparent nitrosamine was present in the 40 μl of concentrated extract used for each TLC determination. Thus, for these model systems, the minimum detectable nitrosamine concentration was 125 ppb. Dimethyl- and dibutylnitrosamine standards incorporated into control samples at a concentration of 10 ppm (1 mg of nitrosamine/100 g of muscle) could be recovered consistently.

A positive Griess and/or ninhydrin test was not considered as conclusive evidence for the presence of nitrosamines, since these colorimetric reagents are not selective for nitrosamines. Accordingly, the compounds responsible for the development of colored spots (see Table I) are referred to as "apparent" nitrosamines. All of the "wet" treatment samples which contained nitrite, alone or in combination with other variables, gave positive Griess and ninhydrin reactions. Some of the nitrite-containing dry-system samples also gave positive Griess *or* ninhydrin reactions, but R_f values were not recorded in Table I unless both tests were positive. The R_f values of the apparent nitrosamines were the same for both wet and dry systems, 0.30 and 0.73. Based on the identical R_f values, it seems probable that the same colorimetric-positive compounds were formed in both the wet and dry systems, but were present in larger quantities in or were more easily extracted from the wet (non-freeze-dried) system. The lower incidence of positive colorimetric tests for the dry ham emulsion extracts could represent either compounds which formed prior to freeze-drying, i.e., while the system still contained moisture, or residual levels resulting from losses of volatile compounds during the freeze-drying and/or heating steps. The surface of the wet samples developed extreme case-hardening during the 15-hr heating in circulating dry air, and this may have helped retain the colorimetric-positive compounds during the heat treatment.

Samples which contained apparent nitrosamines, based on positive Griess and ninhydrin reactions, were further analyzed by GC–MS. The spectra obtained were compared with the spectra of 25 standard nitrosamines (Pensabene et al., 1972), with 101 pyrazines (Maga and Sizer, 1973), and with imines known to result from carbonyl–amine reactions (Marshall, 1974), but could not be identified with any of these.

Formation of nitrosamines in the non-freeze-dried ham muscles would seem reasonable in view of the excessive nitrite level used, which was 138 times the legal maximum. Fiddler et al. (1972) have shown that nitrosamine formation will occur in meat emulsions, provided an excessive nitrite concentration is present. However, none of the apparent nitrosamines found in the model food system extracts were confirmed to be nitrosamines upon examination of their mass spectra. Furthermore, there was no evidence that the addition of components to meat which would enhance the probability of nonenzymatic browning in the system contributed to the presence of nitroso-positive reactants in the extracts.

LITERATURE CITED

Campbell, K. N., Sommers, A. H., Campbell, B. K., *J. Am. Chem. Soc.* **66,** 82 (1944).

Crosby, N. T., Foreman, J. K., Palframan, J. F., Sawyer, R., *Nature (London)* **238,** 342 (1972).

Devik, O. G., *Acta Chem. Scand.* **21,** 2302 (1967).

Dugan, L. R., Jr., Rao, G. V., U.S. Army Natick Laboratories, Natick, Mass., Technical Report 72-27-FL, 1971.

Fiddler, W., Piotrowski, E. G., Pensabene, J. W., Wasserman, A. E., 18th Meeting, Meat Research Workers, Guelph, Ontario, 1972.

Hatt, H. H., in "Organic Syntheses", Blatt, A. H., Ed., Collect. Vol. II, Wiley, New York, N.Y., 1943, pp 211–213.

Hodge, J. E., *J. Agric. Food Chem.* **1,** 928 (1953).

Kadar, R., Devik, O. G., *Acta Chem. Scand.* **24,** 2943 (1970).

Lijinsky, W., Epstein, S. S., *Nature (London)* **225,** 21 (1970).

Maga, J. A., Sizer, C. E., *Crit. Rev. Food Technol.,* 39–115 (1973).

Magee, P. N., *Food Cosmet. Toxicol.* **9,** 207 (1971).

Magee, P. N., Barnes, J. M., *Adv. Cancer Res.* **10,** 163 (1967).

Marshall, J. T., Jr., Ph.D. Dissertation, Michigan State University, East Lansing, Mich., 1974.

Pensabene, J. W., Fiddler, W., Dooley, C. J., Doerr, R. C., Wasserman, A. E., *J. Agric. Food Chem.* **20,** 274 (1972).

Saxby, M. J., *J. Assoc. Off. Anal. Chem.* **55,** 9 (1972).

Smith, P. A. S., "The Chemistry of Open-Chain Organic Nitrogen Compounds", Vol. I, W. A. Benjamin, New York, N.Y., 1965, pp 297–300.

Sweeley, C. C., Ray, B. D., Wood, W. I., Holland, J. F., Kritchevksy, M., *Anal. Chem.* **42,** 1505 (1970).

Telling, G. M., Bryce, T. A., Althorpe, J., *J. Agric. Food Chem.* **19,** 937 (1971).

Williams, R. L., Pace, R. J., Jeacocke, G. J., *Spectrochim. Acta* **20,** 225 (1964).

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