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Preparation and Purification of Malonaldehyde Sodium Salt

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Malonaldehyde is not commercially available and is generally prepared when required from 1,1,3,3-tetramethoxypropane (TMP). Although sodium malonaldehyde (NaMA) is potentially a stable product that could be preprepared to meet research needs, present procedures either are tedious or have been shown to be inadequate in terms of purity. NaMA was prepared by adding 1 N HCl to TMP, stored at 4 °C for 24 h, and neutralized to pH 8.0 and the moisture removed under vacuum at <40 °C. The dry end products were selectively solubilized in anhydrous alcohol and filtered twice through neutral charcoal to remove any color compounds, the ethanol was removed under vacuum, and the residue was lyophilized. Analysis of the lyophilisate by HPLC produced a single peak, shown to be free of methanol and higher polymeric forms by infrared analysis, and its purity was determined to be >98% by NMR. The procedure developed is an improvement over previous methods, has an overall recovery of about 60%, and provides a product of sufficient purity for mutagenicity testing and as a standard for the thiobarbituric acid test (TBA) commonly used for assessing the autoxidation of fats and oils.

Malonaldehyde has come under increasing scrutiny by the health profession as a potential mutagenic agent (Basu and Marnett, 1983) that has ramifications in relation to the ingestion of oxidized lipids. The effects of malonaldehyde are difficult to study because it cannot be obtained commercially, is generally unstable in solution, and has to be prepared as required, limiting its ready availability as a standard material for mutagenicity studies or more general work such as standardizing the thiobarbituric acid test commonly used to evaluate the degree of autoxidation of fats and oils.

Malonaldehyde was first described by Claisen (1903) as a three-carbon dialdehyde resulting from the acid hydrolysis of β-ethoxyacrolein diethyl acetal. In 1941, Hüttel developed a simple procedure for producing malonaldehyde but found it to be hygroscopic, generally unstable, but could be stabilized in the form its sodium salt. The subsequent recognition that malonaldehyde was a

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primary oxidation product of polyunsaturated fatty acids (Dahle et al., 1962) and its color reaction with 2-thiobarbituric acid (TBA) have led to its extensive use as an indicator of oxidative rancidity in foods and biological systems (Tarladgis et al., 1960; Nair and Turner, 1984). Calibration of the TBA test is done indirectly through the hydrolysis of 1,1,3,3-tetramethoxypropane (TMP), assuming complete conversion, rather than with malonaldehyde directly since it is not available commercially (Tarladgis et al., 1960). The specificity of the TBA reaction, its use, and the chemical properties of malonaldehyde have been reviewed extensively by Melton (1983) and Kosugi and Kikugawa (1985). Malonaldehyde has been characterized by infrared spectroscopy (Kwon and Van der Veen, 1968; Buttkus, 1975), nuclear magnetic resonance (George and Mansell, 1968; Nair et al., 1981), and X-ray analysis (Brown et al., 1979). In addition, malonaldehyde has been isolated by various chromatographic methods such as thin-layer chromatography (Gutteridge, 1975), GLC (Gutteridge, 1975; Marnett and Tuttle, 1980), gel filtration (Kwon, 1966; Marnett and Tuttle, 1980), and HPLC (Bird et al., 1983; Csallany et al., 1984; Esterbauer and Slater, 1981; Esterbauer et al., 1984; Kakuda et al., 1981; Wade et al., 1984, 1985). Aqueous solutions of malonaldehyde at neutral pH show a maximum absorption of 267 nm with presence in four possible four forms, based on the progressive release of hydrogen ions as the pH is raised from 3.0 to 7.0 (Kwon and Watts, 1963; Saunders and May, 1963). In general, the most common aqueous form of malonaldehyde is its enol form, which has $pK_a = 4.65$ at 23 °C.

Although malonaldehyde is not commonly available, it can be produced by acid hydrolysis of either TMP or TEP (1,1,3,3-tetraethoxypropane) as described by Protopopova and Skoldinov (1956) and Saslaw and Waravdeekar (1957), respectively. A reasonably stable malonaldehyde solution was produced by Kwon and Watts (1963) by acid hydrolysis of TEP while Brooks and Klamerth (1968) used an excess of Dowex 50 to effect hydrolysis. George and Mansell (1968) prepared malonaldehyde by hydrolysis and produced its sodium salt in solution by neutralization. Purification of the sodium salt by crystallization from ethanol/ether has been reported by Nair et al. (1981). Sodium malonaldehyde (NaMA) produced with use of Dowex 50 has been further purified by Sephadex LH-20 chromatography (Marnett and Tuttle, 1980) and has also been obtained from β-(*p*-nitrophenoxy)acrolein by Basu and Marnett (1983). Summerfield and Tappel (1978) developed a novel enzymatic method using 1,3-propanediol as a substrate for alcohol dehydrogenase to produce malonaldehyde.

Although numerous investigators have devised a variety of means to produce either malonaldehyde or sodium malonaldehyde, most forms are in solution and have limitations in terms of stability, purity, and/or complex preparation/isolation procedures. There is a need for a simple method for producing crystalline sodium malonaldehyde that is more stable, can be stored, and can be available for research purposes. Our inability to obtain good-quality crystalline malonaldehyde based on procedures in the literature led to the development of our own procedure for producing crystalline sodium malonaldehyde and is the subject of this paper.

MATERIALS AND METHODS

Preparation of Sodium Malonaldehyde. Most of the common methods used to effect hydrolysis of TMP or TEP are based on the use of HCl, although they all differ somewhat in the conditions used, i.e., temperature, time, and concentration. The procedures advocated by Kurechi et al. (1980) and Nair et al. (1981) were chosen as a starting point from which to produce malonaldehyde in solution. The hydrolysates produced by these methods were yellow, were subsequently neutralized to pH 7.0 with 5 N NaOH to generate the sodium malonaldehyde salt in solution, were lyophilized, and were subsequently analyzed.

A procedure was developed to reduce the yellow color associated with the hydrolysates obtained by Kurechi and Nair procedures and the subsequent crystallization problems we encounterted with the end products. A gentle hydrolysis of 2 g of TMP (Eastman Kodak Co., Rochester, NY) was initiated by adding 32 mL of 0.1 N HCl and the solution stirred and left to stand at 4 °C for 24 h. The hydrolysate was neutralized using 5 N NaOH to pH 8.0 and evaporated to dryness under vacuum below 40 °C, followed by the addition of 70 mL of anhydrous ethanol (J. T. Baker Chemical Co., Phillisburg, NJ) to selectively dissolve the organic material. The alcoholic solution was filtered twice through a 3-cm layer of neutral charcoal (Fisher Scientific, Fair Lawn, NJ) in a Buchner funnel supported by No. 41 Whatman filter, the ethanol removed under vacuum below 40 °C, and the residue lyophilized to remove traces of residual moisture.

An attempt was made to purify the neutralized hydrolysates produced by the Kurechi and Nair methods by ion-exchange chromatography using a 1.6 × 30 cm column packed with Sephadex DEAE-A25 (Pharmacia, Uppsala). A 1-mL sample (1 mg/mL) was applied and the elution performed in two stages, the first using 158 mL of phosphate buffer (0.01 M) at pH 7.4 and the second the same buffer containing 0.5 M NaCl. Both stages were run at a flow rate of 50 mL/h with the absorbance of 4-mL fractions monitored at 267 nm.

Assessment of Sodium Malonaldehyde Purity. (*i*) *HPLC.* The lyophilized hydrolysates prepared by the procedures developed by the Keruchi and Nair and by us were reconstituted (0.15 mg/mL) in phosphate buffer (0.02 M, pH 7.4) and subjected to HPLC analysis. HPLC gel exclusion chromatography was performed on a Waters (Model 680) chromatograph equipped with a 0.75 × 30 cm Protein-Pack C-60 column. A 0.78 × 30 cm TSK G-1000 PW column (Waters Sci. Ltd.) was also used in accordance with the method advocated by Csallany et al. (1984). For both systems, the eluant was phosphate buffer (0.1 M, pH 7.4) run at a flow rate of 1.0 mL/min and the absorbance was monitored at 267 nm on a Waters Model 490 UV/visible detector set at 1 AUFS.

(*ii*) *Aqueous Infrared Spectrometry.* Lyophilized samples were reconstituted in water (1–5% w/v) and scanned by a double-beam infrared spectrophotometer equipped with 40-μm barium fluoride cells, custom-designed for the specific purpose of obtaining quantitative infrared spectra from aqueous solutions (Mills et al., 1986). The spectra were recorded and the absorption bands analyzed to determine the functional groups present.

(*iii*) *Determination of NaCl.* NaMA was assessed for residual salt by the AOAC silver nitrate method (AOAC, 1984).

(*iv*) *Nuclear Magnetic Resonance.* ^1H NMR analysis of NaMA was performed on a Bruker Model WH-400 NMR spectrometer coupled to an Aspect 2000 computer. Fifty-milligram samples of the lyophilisate were solubilized either in D_2O or in dimethyl sulfoxide (DMSO-d_6) and scanned.

RESULTS AND DISCUSSION

Both the Keruchi and Nair procedures resulted in yellow to orange solutions, which upon lyophilization produced a dark orange powder. The development of these colors is generally considered to be an indication of polymerization (Kwon and Watts, 1964; Buttkus, 1975). Subsequent Protein Pack HPLC analysis of the Kurechi hydrolysate or lyophilisate showed the presence of four peaks, and a typical chromatogram is presented Figure 1. The infrared scan of the Kurechi lyophilisate reconstituted in distilled water shown in Figure 2A was compared to the scans obtained by Bacon et al. (1965) and Buttkus (1975). The infrared scans indicated the presence of both polymers of malonaldehyde and an appreciable quantity of methanol in both the Kurechi and Nair

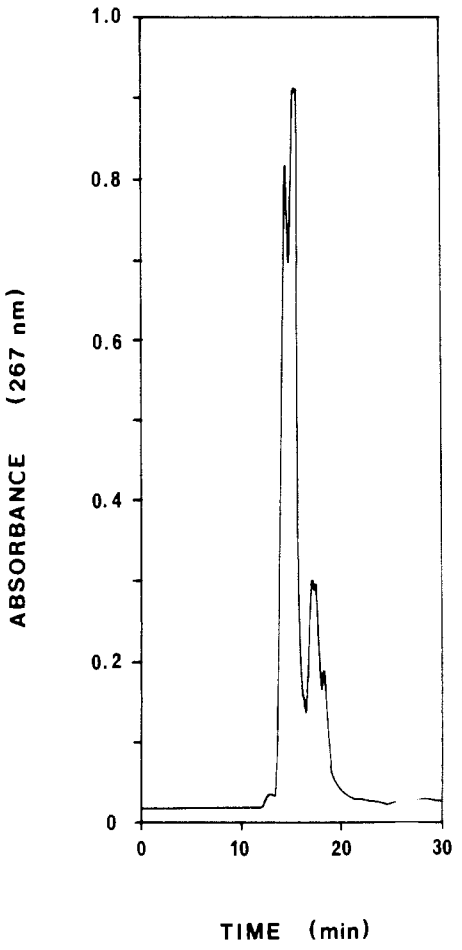

Figure 1. Typical HPLC Protein Pack C-60 chromatogram of NaMA hydrolysate prepared by the procedure of Kurechi et al. (1980).

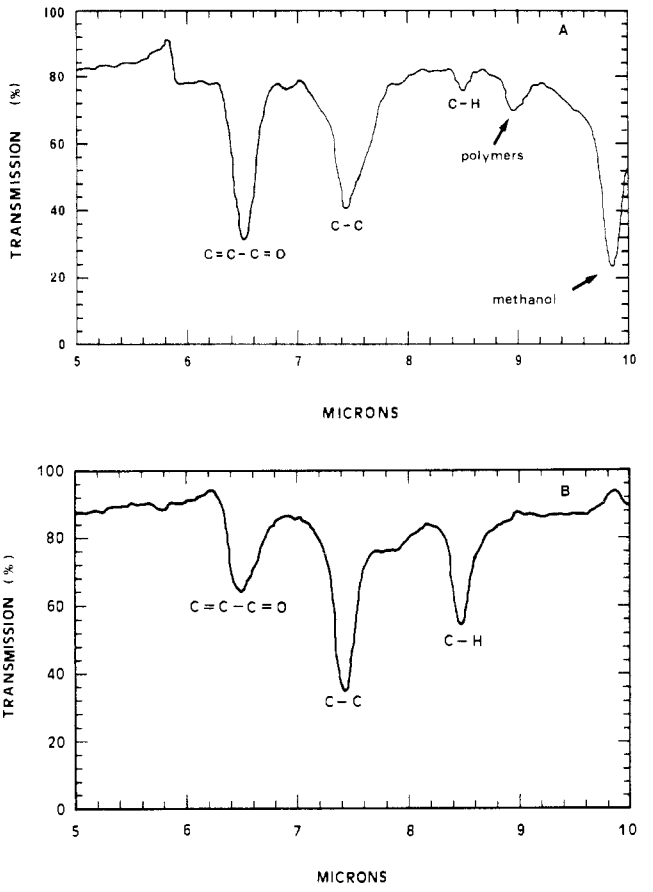

Figure 2. Infrared spectra of (A) aqueous hydrolysate of TMP obtained by the method of Kurechi et al. (1980) and (B) aqueous hydrolysate of NaMA prepared by our procedure.

hydrolysates. The presence of a methanol band at 9.8 μm reflects the hydrolysis mechanism for TMP, which produces four molecules of methanol for each molecule of malonaldehyde (Apaja, 1980). Protein Pack HPLC analysis of the Nair hydrolysate also showed one major peak and four minor ones (Figure 3A); however, the components are present in a slightly different proportion than that of the Kurechi hydrolysate. Overall, the presence of color, the polymer bands in the infrared spectra, and the multiple HPLC peaks confirm Marnett and Tuttle's (1980) inference that products other than NaMA are present in the hydrolysates of tetraalkoxypropanes.

Nair et al. (1981) described the further purification of sodium malonaldehyde by crystallization, using ethanol/ether, presumed to be in a 1:1 proportion, as no specific solvent ratio was presented in their paper. Considerable effort was expended in trying to obtain a crystalline material by this method, including varying the solvent ratio and crystallization conditions; however, no suitable crystals could be obtained. Crystalline NaMA was obtained with use of anhydrous ethanol and acetone as proposed by Protopopova and Skolinov (1956); however, the yield was too low for practical purposes. The utilization of diethyl ether or acetone alone resulted in the unselective precipitation of constituents from TMP hydrolysates while classic crystallization techniques based on hot organic solvents (Pavia et al., 1982) were also ineffective.

An alternate hydrolysate purification procedure was attempted with gradient ion-exchange column chromatography (Sephadex DEAE-A25), which produced the elution chromatogram presented in Figure 4, showing a major peak and a large shoulder. Fractions collected from the main peak (Figure 4) and resubjected to Protein Pack HPLC produced a single clean peak (Figure 3B) with an absorption maximum of 267 nm.

Glycine, which is homologous in both molecular configuration and molecular weight to malonaldehyde, was also chromatographed on the Protein Pack column and gave a similar retention time (Figure 5). This indirect indication that purified NaMA could be obtained was encouraging; however, the procedure is only of academic interest, as the process is tedious, the concentrations are low, and the material is still in solution, rather than being crystalline.

On the basis of impurities in the hydrolysates indicated by HPLC and infrared analysis, plus the failure to reproduce Nair's crystallization process, and the problems associated with the other crystallization procedures, we were led to devise our own procedure by trial and error. Efforts were directed toward minimizing polymerization by reducing the initial concentration of the starting material and changing the hydrolysis temperature. Using 2 g of TMP, half of that used by Nair, and a hydrolysis temperature of 4 °C reduced color formation during the reaction period. Upon completion of the reaction, adjustment of the pH to 8.0 retarded polymerization during the evaporation step. After the moisture was removed under vacuum, the residue was selectively resolubilized in anhydrous ethanol as opposed to methanol and neutral activated charcoal used to remove any traces of color formed during the process. The end product was then lyophilized, and product recovery was ~60% based on the weight of the starting material.

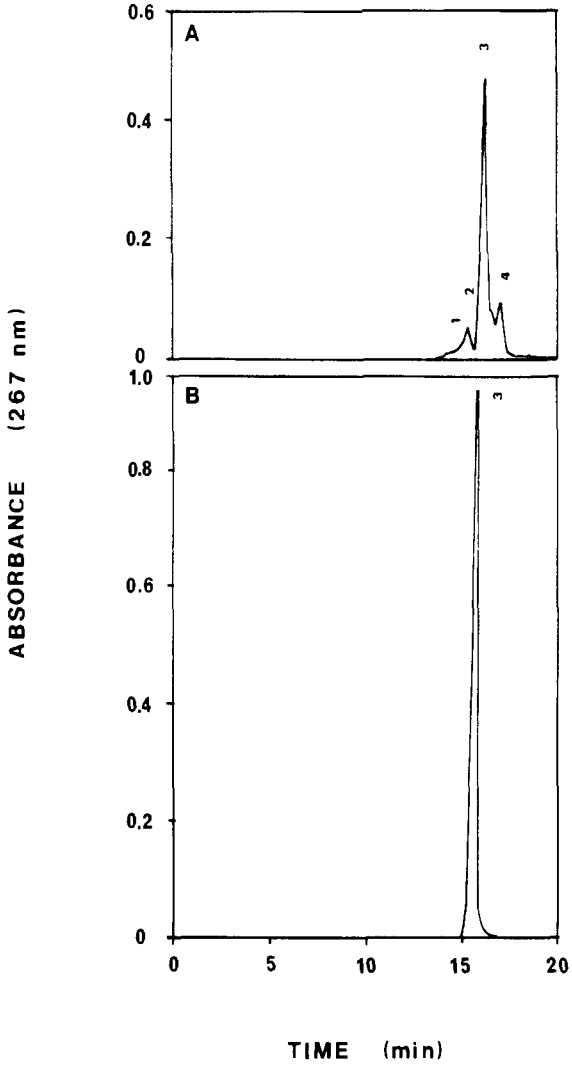

Figure 3. Protein Pack C-60 HPLC chromatograms of NaMA: (A) end product obtained by the method of Nair et al. (1981); (B) the same product obtained from the main fraction obtained after purification using Sephadex DEAE-A25.

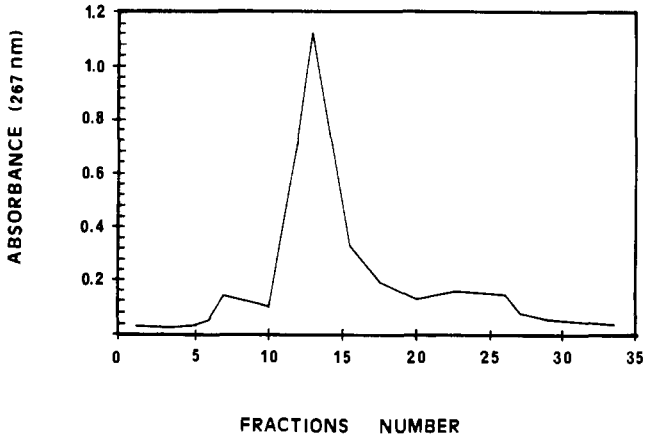

Figure 4. Sephadex DEAE-A25 chromatogram of TMP hydrolysates obtained by the procedure of Nair et al. (1981).

HPLC gel exclusion analysis of the lyophilized material obtained by our procedure, using both the Protein Pack C-60 and TSK G-1000 PW columns, produced a single peak, the latter chromatogram being presented in Figure 6. The Protein Pack chromatogram matched the HPLC chromatogram obtained for hydrolysates prepurified by Sephadex DEAE-A25 chromatography (Figure 3B). Infrared analysis of our end product showed that

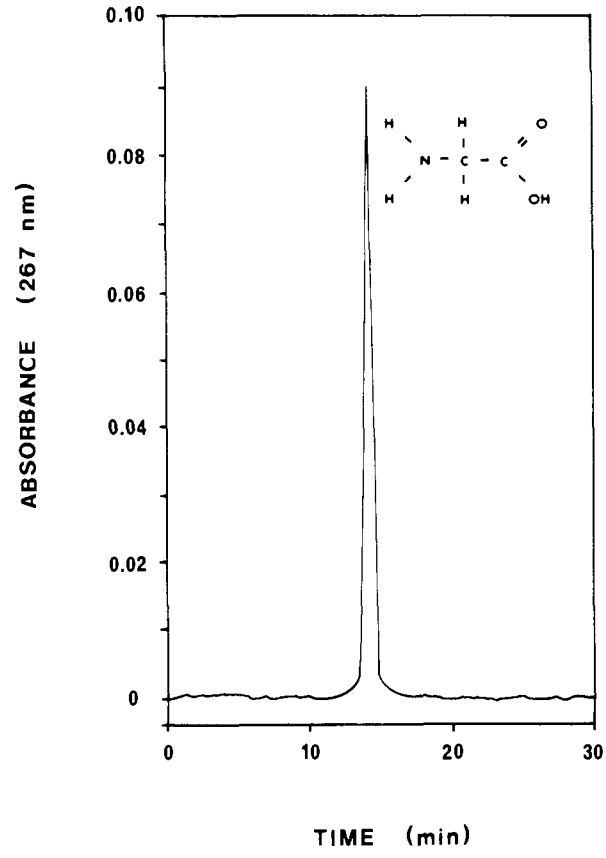

Figure 5. Molecular structure and HPLC chromatogram of glycine elution on Protein-Pack C-60 column.

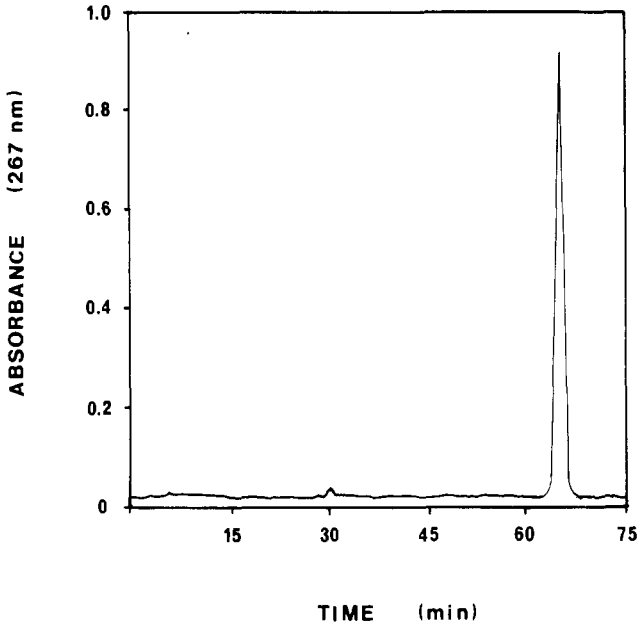

Figure 6. Gel exclusion HPLC chromatogram of NaMA prepared by our procedure and analyzed on a TSK G-1000 PW column.

the methanol and polymer bands previously present were now absent (Figure 2, A vs B), and a salt analysis indicated that only a minor residue of NaCl (1–2%) was associated with the end product.

Subsequent ^1H NMR spectroscopy of the lyophilized NaMA produced using our protocol and dissolved in D_2O (Figure 7) produced a triplet at 5.32 ppm representing a vinyl proton as well as a doublet at 8.65 ppm representing a formyl proton. In DMSO-d_6 additional minor

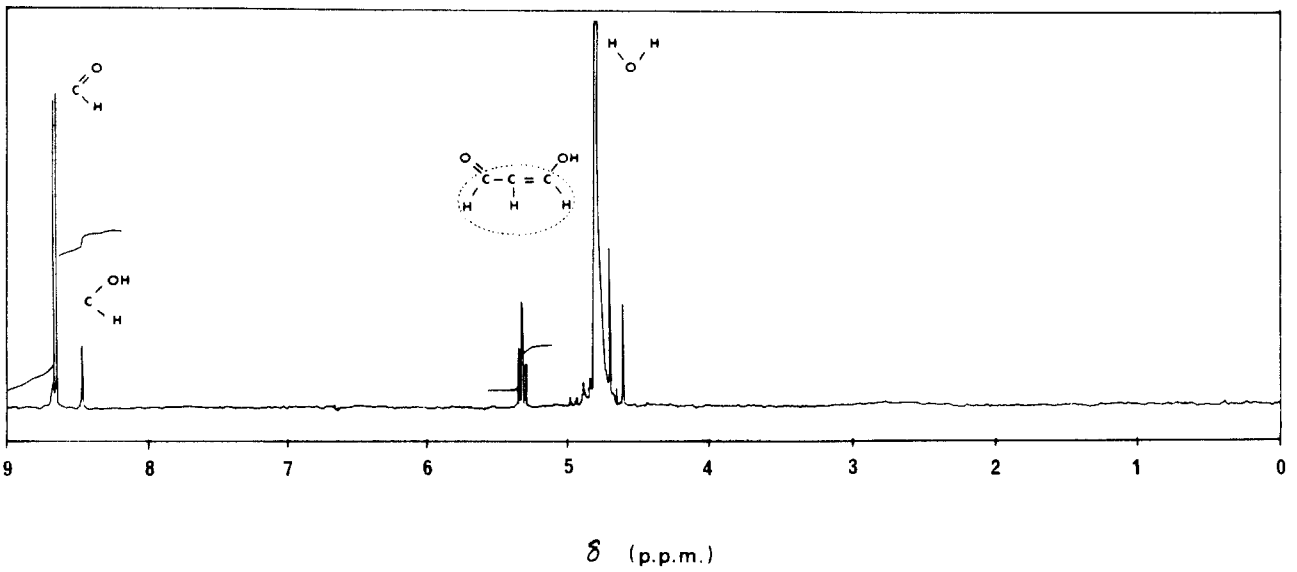

Figure 7. ^1H NMR spectrum of NaMA in D_2O.

signals at 8.46 and 4.8 ppm appeared, indicating that a small amount of the enolic anion was also present, along with some residual bound water. The spectra generally agree with those previously reported by George and Mansell (1968), Marnett and Tuttle (1980), and Kikugawa and Ido (1984). On the basis of NMR data, the purity of NaMA was estimated to be ~99%.

CONCLUSION

Generally it has been accepted that malonaldehyde must be prepared from TMP when required. Our investigation of the procedures for producing NaMA indicated that the methods developed to date lack simplicity and efficacy and had purity problems, or some combination of these limitations. A simple procedure was devised to produce crystalline sodium malonaldehyde in reasonable quantities (~60% recovery) in purities better than 98%. This procedure should be useful for the production of sodium malonaldehyde for mutagenicity studies or as a reference material for the calibration of the TBA test.

Registry No. NaMA, 24382-04-5; malonaldehyde, 542-78-9.

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Multiresidue Method for the Determination of Sulfonamides in Pork Tissue

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A multiresidue technique for the isolation and liquid chromatographic determination of eight sulfonamides from pork muscle tissue is described. Sulfanilamide, sulfathiazole, sulfadiazine, sulfamerazine, sulfamethazine, sulfamethoxazole, sulfisoxazole, and sulfadimethoxine standards were fortified into pork tissue (0.5 g) and blended with C_{18} (octadecylsilyl-derivatized silica) packing material (2 g). The blended C_{18}/muscle tissue matrix was used to prepare a column that was washed with hexane (8 mL). Sulfonamides were eluted with methylene chloride (8 mL). Sample extracts contained sulfonamide analytes (62.5–2000 ng/g) that were free from interfering compounds when examined by HPLC utilizing photodiode array detection at 270 nm. Correlation coefficients of standard curves of sulfonamides isolated from fortified pork tissue ranged from 0.994 (±0.006) to 0.999 (±0.001). Percentage recoveries (70.4–95.8%) and intra- (3.46–6.17%) and interassay variabilities (4.04–14.05%) for the eight sulfonamides were indicative of a suitable quantitative method for the analysis of these compounds in a muscle tissue matrix.

The use of antibiotics as chemotherapeutic agents in animal production has increased in the last decade. Antibiotics such as sulfonamides have become an integral part of the livestock production industry and function to prevent disease and/or increase feed efficiency. However, residues of these drugs in foods derived from treated animals could pose a health threat to consumers, and the constant exposure of some microorganisms to these drugs may manifest itself in the development of drug-resistant strains. Recent evidence has implicated sulfamethazine as a possible carcinogen (Littlefield, 1988), which has magnified risk assessment concerns. These concerns have prompted the U.S. Department of Agriculture/Food Safety Inspection Service to include sulfamethazine, sulfathiazole, and five other sulfonamides in the Compound Evaluation and Analytical Capability National Residue Program Plan (USDA, 1988). Regulatory agencies have established withdrawal periods for such drugs for animals treated prior to slaughter, as well as maximal residue levels allowable in foods (USDA, 1988), to minimize their impact.

The widespread use of sulfonamides in animal production necessitates the development of multiresidue techniques by which residue levels can be monitored. Techniques used for sulfonamide determinations include, but are not limited to, microbiological, high-performance liquid chromatographic, gas chromatographic, and/or mass

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