Interactions of Sulfanilamide and 2-Thiobarbituric Acid with Malonaldehyde: Structure of Adducts and Implications in Determination of Oxidative State of Nitrite-Cured Meats

Ronald B. Pegg,† Fereidoon Shahidi,*,† and Chet R. Jablonski‡

Departments of Biochemistry and Chemistry, Memorial University of Newfoundland, St. John's, Newfoundland, Canada A1B 3X9

Reaction of malonaldehyde (MA) with 2-thiobarbituric acid (TBA) in model systems resulted in the formation of the typical pink adduct (TMT) with absorption maxima at 278, 372, and 532 nm. Reactions of MA with sulfanilamide (SA) produced a bright yellow adduct (SMS) with absorption maxima at 256, 332, and 396 nm. Addition of TBA to the MA–SA system resulted in the disappearance of the characteristic absorption bands of SMS and the appearance of the characteristic absorption bands of TBA–MA as well as a new band at 472 nm. Similarly, addition of SA to the TBA–MA system resulted in the appearance of the 472-nm band without the loss of the previously observed maxima. Reaction of MA, TBA, and SA in model systems resulted in the formation of an adduct (SMT) with absorption maxima at 278, 372, 472, and 532 nm. The structures of these compounds, recovered as crystalline products, were elucidated using ultraviolet–visible (UV–vis), Fourier transform infrared (FTIR), nuclear magnetic resonance (NMR), and mass spectroscopic (MS) techniques. Implications of addition of SA to cured meats in the assessment of their oxidative state are discussed.

INTRODUCTION

The oxidation of unsaturated lipids of meat has been extensively studied since it is related to the deterioration of muscle foods. Although sensory analyses for acceptability of fatty foods is commonplace, objective tests for the estimation of their deterioration are highly desirable. One of the primary pathways of lipid degradation in meats is autoxidation. Autoxidation occurs by a free-radical chain reaction. The primary oxidation products, hydroperoxides, are unstable and degrade to form low molecular weight compounds such as carbonyls, alcohols, esters, and hydrocarbons which are responsible for the oxidized flavor or rancidity of food lipids (Wong, 1989).

A major carbonyl decomposition product of autoxidation is malonaldehyde (MA). Its presence and concentration in foodstuffs is commonly monitored as a marker of lipid peroxidation by the 2-thiobarbituric acid (TBA) test (Shahidi and Hong, 1991). This spectrophotometric determination, described by Tarladgis et al. (1960), involves the reaction of MA in oxidized foods with the TBA reagent to form a pink adduct with a distinctive absorption maximum at 532 nm. Various procedures have been employed for performing the TBA test; however, they generally involve heating the food product with an acid to liberate MA from its precursor(s) as well as to hasten condensation of MA with TBA. TBA reagent may be added with an acid to food directly and the mixture then heated for a sufficient period of time to obtain maximum color development. The pink pigment formed during heating may be extracted into butanol or a butanol–pyridine mixture and then quantified (Placer et al., 1966; Uchiyama and Mihara, 1978). Moreover, the TBA test may be carried out on a trichloroacetic acid extract of a foodstuff (Siu and Draper, 1978). The concentration of the pink pigment formed is then determined spectrophotometrically using a precursor of MA such as 1,1,3,3-tetramethoxypropane (TMP) or its tetraethoxy analogue (TEP) as standard. The latter procedure given by Siu and Draper (1978) often affords more realistic results than other methodologies by preventing the overestimation of TBA reactive substances (TBARS). However, the presence of colored additives such as the cooked cured-meat pigment as well as turbidity of the extracts due to soluble proteins or fat droplets may interfere with accurate determination of the colored chromogen(s) of TBARS–TBA. Conversely, a steam-distillation methodology may be used to recover MA from the acidified food product. An aliquot of the distillate is reacted with TBA reagent, and the intensity of the chromophore is again determined spectrophotometrically. Unfortunately, the distillation method generally affords higher values of TBARS due to artifact formation resulting from further breakdown of labile hydroperoxides. However, addition of antioxidants/chelators to mixtures prior to distillation has proven beneficial in some cases (Rhee, 1978; Shahidi and Hong, 1991). The pink pigment of the TBA–MA reaction was first isolated and characterized by Sinnhuber et al. (1958), who showed it to be a condensation product of one molecule of MA with two molecules of TBA. Nair and Turner (1984) elucidated the crystalline structure of the TBA–MA complex by IR, UV–vis and NMR methodologies and showed that it existed in two prominent tautomeric forms.

Nitrite curing inhibits meat flavor deterioration and rancidity development in cooked meats. However, residual nitrite present in cured products interferes with the TBA test (Zipser and Watts, 1962). This interference is believed to be due to the nitrosation of MA, which renders all or a portion of it unreactive in the TBA–MA test, thus resulting in underestimation of TBARS (Zipser and Watts, 1962; Shahidi et al., 1985; Kolodziejska et al., 1990). For nitrite-cured products, Zipser and Watts (1962) modified the TBA test by adding sulfanilamide (SA) prior to the distillation step to hinder nitrosation of MA. Added SA reacts with residual nitrite to yield a diazonium salt, therefore permitting MA to react quantitatively with TBA. These authors concluded that SA addition allows accurate quantification of MA in nitrite-cured meat products within

† Department of Biochemistry.
‡ Department of Chemistry.

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the limits of precision of the TBA method. However, SA itself may give rise to the formation of condensation products with reactive MA. Shahidi et al. (1991) reported that although addition of SA to meats cured with at least 100 ppm of nitrite played a positive role in evaluating the oxidative state of cured products, the measured TBA values generally suffered from errors ranging between 6 to 20%. The formation of condensation products of enamine/imine nature between MA and SA was believed to be a contributing factor. Further research has revealed that novel interactions between MA, TBA, and SA exist (Shahidi and Pegg, 1990).

In this paper, interactions between MA, TBA, and SA in model systems were investigated through spectroscopic studies to elucidate possible interactions and their implications in the TBA test when the oxidative state of cured meats is evaluated.

MATERIALS AND METHODS

Materials. Most chemicals and solvents used in this study were of ACS reagent grade. The malonaldehyde precursor, 1,1,3,3-tetramethoxypropane, 2-thiobarbituric acid, dimethyl sulfoxide (DMSO), and chemicals used in NMR experiments, namely tetramethylsilane (TMS), deuterated water (D_2O) and deuterated dimethyl sulfoxide (DMSO-d_6), were acquired from Sigma Chemical Co. (St. Louis, MO). Sulfanilamide, hydrochloric acid, sodium hydroxide, silver nitrate, and potassium bromide were purchased from Fisher Scientific Co. (Ottawa, ON).

Synthesis and Purification of TMT. The TMT adduct was synthesized in a model system of TMP and TBA in 1.45 M HCl solution as described by Sinnhuber et al. (1958) with slight modifications. TMP (0.00625 mol) and TBA (0.0125 mol) were transferred to a 500-mL boiling flask, equipped with a condenser, to which 250 mL of 1.45 M HCl solution was added. The mixture was refluxed for 90 min. After the mixture cooled to room temperature, pigment crystals were suction filtered on a fine sintered-glass funnel. Crystals were washed with 100 mL of 0.6 M HCl, briefly with hot water, subsequently with 20 mL of 95% (v/v) ethanol and 100 mL of 1:1 (v/v) ethanol and ethyl ether, and finally with 100 mL of ethyl ether. The dark purple crystals were dried on a watch glass in a vacuum oven at 60 °C for 24 h.

To purify the product, 1 g of the finely ground pigment was boiled in 200 mL of 0.6 M HCl for 40 min, cooled to 60 °C, and then suction filtered on a fine sintered-glass funnel. Crystals were washed with 100 mL of 0.6 M HCl, 25 mL of cold water, 25 mL of ethanol, and finally 100 mL of ethyl ether. The dark purple crystals were again dried on a watch glass in a vacuum oven at 60 °C for 24 h.

Synthesis and Purification of SMT. The SMT adduct was synthesized and purified in a similar manner as described for TMT except that the reaction flask contained TMP, TBA, and SA in an equimolar (0.00625 mol) ratio.

Synthesis and Purification of SMS. The SMS adduct was synthesized in a similar manner as described for TMT except that SA (0.0125 mol) was used in place of TBA. Furthermore, 0.03 M HCl was used as solvent instead of 1.45 M HCl solution for synthesis and purification of SMS, as it afforded higher yields.

Spectroscopic Analyses. *UV–Vis Spectroscopy.* Ultraviolet–visible (UV–vis) absorption characteristics of the adducts were monitored using a Hewlett-Packard 8452A photodiode array spectrophotometer. Solutions of TMT and SMT were made by transferring 10–30 mg of dried crystals to a 1-L volumetric flask, dissolving them in 10 mL of DMSO, and then filling the flask to mark with 0.1 M HCl. Solutions of SMS were made in a similar fashion without using DMSO. Aliquots of the stock solution were transferred to a 100-mL volumetric flask and the flask filled to mark with 0.1 M HCl (DMSO) solvent. UV–vis absorption characteristics of purified pigments were compared to those of adducts prepared from model systems, as described below.

A stock solution of TMP in 0.1 M HCl was used at a concentration of 10^{-3} M; 10^{-2} M solutions of TBA and SA were prepared in 0.1 M HCl, these concentrations being 10 times that of the MA precursor solution. One-milliliter aliquots of the MA solution were transferred to borosilicate glass tubes. Aliquots of the TBA and SA solution at volumes of 0, 0.5, 1, and 2 mL were added individually and in combinations to MA samples. Enough 0.1 M HCl was added to each tube such that a total volume of 10.0 mL was attained. Systems were capped, heated in a boiling water bath for 30 min, and then cooled to room temperature. A 1-mL aliquot from each system was transferred to a clean tube containing 9.0 mL of fresh solvent. Tubes were vortexed, and absorption spectra of their contents were recorded.

In another set of experiments, 1-mL aliquots of the MA stock solution were transferred to borosilicate glass tubes. Aliquots of the SA solution at volumes of 0, 0.5, 1, and 2 mL were added to the tubes as well as the 0.1 M HCl solvent. Systems were capped and heated in a boiling water bath for 30 min. Aliquots of the TBA solution at volumes of 0, 0.5, 1, or 2 mL were added to the hot samples which were then reheated for 30 min in the water bath. The final volume of the systems during the second heating was 10.0 mL. After cooling to room temperature, 1 mL from each sample was transferred to a clean tube containing 9.0 mL of fresh solvent. Tubes were vortexed, and absorption spectra of their contents were recorded. The experiment was repeated, but, this time, TBA was heated with MA and then SA was added after the initial heating.

IR Spectroscopy. Infrared (IR) spectral data of each adduct in a KBr disk were obtained using a Mattson Polaris Fourier transform IR spectrophotometer.

NMR Spectroscopy. Nuclear magnetic resonance (NMR) spectra were obtained at 300 MHz with a General Electric GN-300 spectrometer. ^1H and $^{13}C\{^1H\}$ NMR data were collected at room temperature in DMSO-d_6 or in DMSO-d_6/D_2O mixtures. Chemical shifts are reported relative to tetramethylsilane (TMS) internal standard. In addition, attached proton tests (APT) and two-dimensional heteronuclear correlation (HETCOR) NMR experiments were performed to further elucidate the chemical structure of the adducts.

MS Spectrometry. All mass spectra were measured using an electron ionization (EI) mode at 70 eV with a 7070 HS Micromass double-focusing mass spectrometer.

RESULTS AND DISCUSSION

Characterization of the TMT Adduct. The TMT pigment isolated from the TBA–MA model system yielded purple needlelike crystals with no definite melting point up to 350 °C, similar to that reported by Sinnhuber et al. (1958). The UV–vis spectrum of TMT dissolved in the 0.1 M HCl/DMSO solvent exhibited a pink chromogen with an absorption maximum at λ 532 nm (ϵ = 125 000). This π–π^* transition is diagnostic of a highly conjugated system. However, the absorption spectrum of the TBA–MA model system in 0.1 M HCl revealed three absorption bands at 278, 372, and 532 nm. The UV absorption spectrum of the TBA parent molecule revealed an absorbance maximum at 278 nm. Thus, the 278-nm band in the spectrum of the TBA–MA mixture, which contained an excess of TBA, is characteristic of unreacted TBA. A broad band between 370 and 380 nm with a maximum at 372 nm in the TBA–MA model system is most likely due to an intermediate in the TBA–MA reaction such as a 1:1 adduct of TBA and MA as opposed to the typical 2:1 complex. The 1:1 adduct is formed as an intermediate reaction product in the proposed mechanism of TMT formation as described by Nair and Turner (1984) and as illustrated by Pegg and Shahidi (1991). Absence of the 372-nm signal in the UV–vis spectrum of purified TMT suggests that the adduct is quite stable in the acid medium. Furthermore, when a 0.1 M $AgNO_3$ solution was added to dissolved TMT in deionized H_2O, no precipitate was formed, thus suggesting that the adduct did not exist as a hydrochloride salt. The proposed chemical structure of the TMT adduct is presented in Figure 1.

The FTIR spectrum of TMT exhibited bands characteristic of the group frequencies associated with the

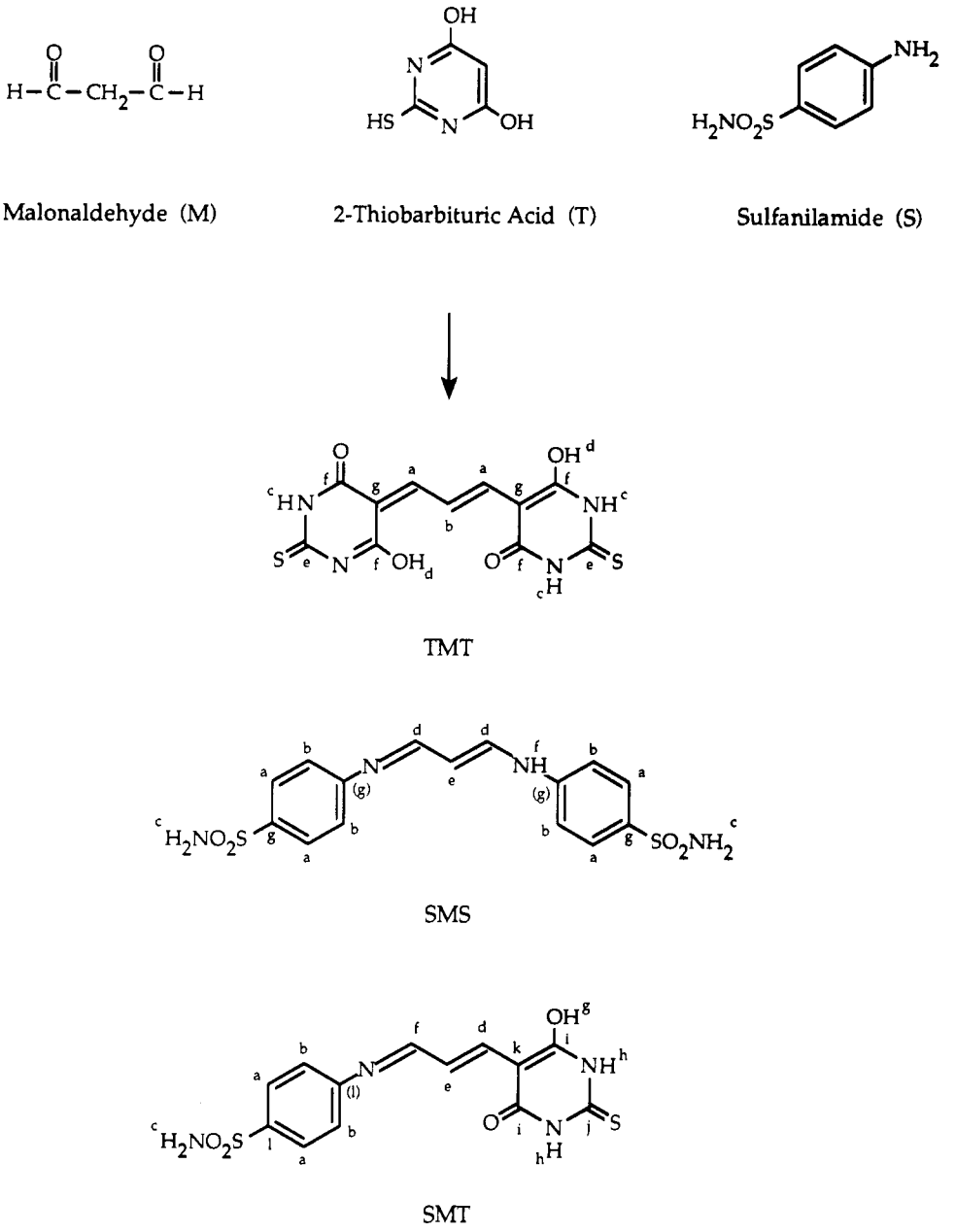

Figure 1. Formation of TMT, SMS, and SMT from malonaldehyde (M), 2-thiobarbituric acid (T), and sulfanilamide (S).

proposed molecule (Table I). The three vibrational bands diagnostic of secondary amides were present (i.e., C=O stretch, 1630 cm^{-1}; N—H bend, 1496 cm^{-1}; and C—N stretch, 1299 cm^{-1}) as well as the C=S stretch (1127 cm^{-1}) of the thioamide moiety. A strong absorption at 1163 cm^{-1} in the TBA parent molecule was interpreted as the C=S stretch of the many resonance contributors (Goel et al., 1985). In addition, a weak vibrational band at 2550 cm^{-1} in TBA, characteristic of S–H stretching, was not detected in the FTIR spectrum of the crystalline TMT adduct. Although S–H groups were not detected in the solid state, they may exist as resonance contributors of the molecule in solution. Raman spectroscopy may offer a means of clarifying the nature of this functional group as it can show a strong response for vibrational bands of sulfhydryl groups.

The 300-MHz ^1H NMR spectrum of the TMT adduct in DMSO-d_6 revealed four types of resonances. A doublet and a triplet at δ 7.71 and 8.55, with a relative integration equivalent to two and one proton(s), respectively, and a coupling constant of 13.8 Hz, were diagnostic of the *trans*-vinyl protons in TMT. A broad temperature- and concentration-dependent resonance at δ 5.1 which rapidly exchanged with D_2O can be assigned to the amide NH protons of the substituted pyrimidine moieties. A sharp peak at δ 11.5 with a relative integration equivalent to four protons was assigned to OH groups of the TMT crystalline adduct; however, integration of only two protons was expected. Again, addition of D_2O resulted in the disappearance of the signal, indicating that these protons were exchangeable.

The $^{13}C\{^1H\}$ NMR spectrum revealed five resonances for the proposed 11-carbon adduct. The APT spectrum showed that three of the five nonequivalent carbons in the molecule were quaternary. Carbon resonances at δ 158.0 and 118.1 correlated with vinyl ^1H resonances at δ 7.71 and 8.55, respectively. The broad, but weak, signal at δ 162.5 was assigned to the thioamide group as reported by Nair and Turner (1984). Signals of the quaternary carbons at δ 175.8 and 101.5 were assigned as the amide carbons and the remaining two equivalent ring carbons, respectively. ^{13}C NMR signals of substituted pyrimidine groups in TMT are supported by ^{13}C NMR data obtained for the parent TBA molecule. TBA exhibited five signals

Table I. FTIR (in KBr) Data for Adducts of 2-Thiobarbituric Acid (T) and Sulfanilamide (S) with Malonaldehyde (M)[a]

compd	wavenumber, cm^{-1}	assignment
TMT	1630, 1671 (sh) (vs)	ν C=O (amide I)
		ν C=C
	1496 (vs)	δ N—H (amide II)
	1360 (vs)	δ_{ip} O—H
	1299 (s)	ν C—N (amide III)
	1214 (s)	ν C—O
	1176 (m)	ν C—O
	1127 (s)	ν C=S
	1002 (m)	δ_{op} =C—H
SMS	3168 (s)	ν =C—H
	3060 (s)	ν =C—H
	1637 (vs)	δ NH_2
	1579, 1600 (sh) (vs)	ν C=C of aromatic ring
	1491 (m)	ν C=C of aromatic ring
	1336 (vs)	ν_{as} SO_2
	1197 (m)	ν C=C of aromatic ring
	1152 (vs)	ν_s SO_2
	909 (m)	ν S—N
	835 (m)	ν_{op} =C—H
	612 (s)	δ SO_2
SMT	3369 (m)	ν_{as} NH_2
	3209 (s)	ν =C—H
	3087 (s)	ν =C—H
	1638 (s)	ν C=O (amide I)
		ν C=C
	1489, 1510 (sh) (vs)	δ N—H (amide II)
	1385, 1412 (sh) (vs)	ν C—O
	1337 (s)	ν_{as} SO_2
	1302 (s)	ν C—N (amide III)
	1196, 1185 (sh) (s)	ν C=C of aromatic ring
		ν C—O
	1153 (vs)	ν_s SO_2
	1130 (vs)	ν C=S

[a] vs, very strong; s, strong; m, medium; sh, shoulder, ν, stretching; δ, bending; ν_{as}, asymmetric stretching; ν_s, symmetric stretching; δ_{ip}, in plane bending; δ_{op}, out of plane bending.

for the three nonequivalent carbon atoms at δ 82.0, (162.2, 166.0), and (175.1, 180.8). Signals at δ 162.2 and 166.0 are most probably based on tautomeric forms of the thioamide group, while signals at δ 175.1 and 180.8 are due to tautomeric forms of the amide functional group. In contrast to TBA, five NMR signals were displayed for the five nonequivalent carbons in TMT, thus conceivably denoting the existence of a limited number of tautomers. A summary of the ^1H and ^{13}C NMR assignments for TMT is presented in Table II.

The normalized EI mass spectrum of TMT ($C_{11}H_8N_4O_4S_2$) revealed that the base peak had a mass to charge ratio (i.e., m/z) of 144, thus signifying that the major fragment ion was the TBA parent molecule. The molecular ion was detected at m/z 324 with a modest intensity of 1.5%. Furthermore, another fragment ion was detected at m/z 338 during some scans with an intensity of 0.3% but could not be assigned. Major fragment ions included m/z 180 (61%), 170 (11%), 156 (10%), 137 (9%), 122 (13%), 116 (47%), 109 (17%), 93 (31%), 86 (12%), 69 (49%), 65 (15%), 64 (22%), 59 (68%), 43 (81%), and 42 (95%).

Characterization of the SMS Adduct. Addition of SA to the MA precursor yielded a bright yellow compound conceivably due to the formation of condensation product(s) of an enamine/imine or N,N′-disubstituted 1-amino-3-iminopropene structure. Shahidi et al. (1991) reported that the adduct from the MA–SA system exhibited fluorescent activity due to the presence of chromophoric –N=C—C=C—N– system containing 6π electrons whose conjugation may be extended by delocalization of electrons

Table II. 300-MHz ^1H and 75-MHz ^{13}C NMR (in DMSO-d_6) Data for Adducts of 2-Thiobarbituric Acid (T) and Sulfanilamide (S) with Malonaldehyde (M)[a]

^1H data			^{13}C data	
δ, TMS		assignment	δ, TMS	assignment
	TMT			
5.1 (s)	br, ex, var	c	101.5	g
7.71 (d, 2 H)	J = 13.8 Hz	a	118.1	b
8.55 (t, 1 H)	J = 13.8 Hz	b	158.0	a
11.5 (s, 4 H)	ex	d	162.5	e
			175.8	f
	SMS			
3.43 (s)	br, ex, var	f	100.2	e
6.56 (t, 1 H)	J = 11.3 Hz	e	117.7	b
7.44 (s, 4 H)	ex	c	127.7	a
7.57 (d, 4 H)		b	141.1	g
7.92 (d, 4 H)		a	159.6	d
8.92 (d, 2 H)		d		
	SMT			
3.44 (s)	br, ex, var	h	103.5	k
7.34 (s, 2 H)	ex	c	107.9	e
7.51 (d, 2 H)		b	117.0	b
7.57 (dd, 1 H)	J = 13.2 Hz	e	127.6	a
	with D_2O		139.8	l
	appears more like a t		141.8	l
7.82 (d, 2 H)		a	157.5	d/f
8.15 (d, 1 H)		d	162.4	j
8.77 (dd or t, 1 H)	J = 13.2 Hz	f	177.3	i
11.8 (s, 2 H)	ex	g		

[a] s, singlet; d, doublet; dd, doublet of doublets; t, triplet; br, broad peak; ex, exchangeable; var, variable peak; J, coupling constants. For assignment letters, refer to Figure 1.

of the aromatic ring of SA. Excitation and emission spectra of this molecule were characteristic of 1-amino-3-iminopropene derivatives produced from the reaction of MA with amino compounds (Chio and Tappel, 1969; Arya et al., 1974). The isolated adduct in this study gave yellow powderlike crystals with a melting point of 203 °C. Its UV–vis spectrum in 0.1 M HCl showed absorption bands at λ 396 ($\epsilon \approx$ 10 500), 332 (ϵ = 28 000), and 256 nm (ϵ = 14 000), thus confirming that the adduct was of a highly conjugated nature. The absorption spectrum of SMS was identical to that of the SA–MA system in 0.1 M HCl. However, SMS was not stable in the acidic medium as a decrease in the intensity of the 396-nm band was observed. Formation of the adduct is probably initiated by attack of the nucleophilic amine group of SA onto carbon 1 of MA followed by dehydration, thus forming an enamine/imine compound. This reversible reaction is likely followed by a similar reaction of the intermediate 1:1 complex with a second molecule of SA forming a N,N′-disubstituted 1-amino-3-iminopropene adduct. Furthermore, when a 0.1 M $AgNO_3$ solution was added to dissolved SMS in deionized H_2O, no precipitate was formed, which implies that the adduct did not exist as a hydrochloride salt. The proposed chemical structure of this adduct is presented in Figure 1.

The FTIR spectrum of SMS exhibited bands representative of group frequencies associated with the proposed molecule (Table I). Vibrational bands of the aromatic C=C and C=N conjugated system were observed at 1600, 1579, 1491, and 1411 cm^{-1}. The N–H bending of the sulfonated amine group of SA was detected at 1637 cm^{-1}. Characteristic asymmetric and symmetric stretching bands of SO_2 moieties were noticed at 1336 and 1152 cm^{-1}, respectively. Group frequencies in the FTIR spectrum of SMS were almost identical to those found in the SA parent molecule; however, SMS vibrational bands were generally

broader. Strong asymmetric and symmetric stretching of primary amine bands of SA at 3478 and 3376 cm^{-1}, respectively, were absent in SMS. In addition, asymmetric and symmetric signals of the sulfonated primary amine moiety of SA at a 3360-cm^{-1} shoulder and at 3266 cm^{-1}, respectively, were obscured by two strong IR absorption signals at 3168 and 3060 cm^{-1}. These signals may possibly be due to =C—H stretching of the extended conjugation of the aromatic system of SMS.

The 300-MHz ^1H spectrum of the SMS adduct in DMSO-d_6 displayed five resonances. Two AA'BB' doublets centered at δ 7.57 and 7.92 were diagnostic of the para-disubstituted aromatic protons of SA moieties. Integration of the AA'BB' doublets, relative to the δ 7.44 signal, indicated a total of eight protons, suggesting that the proposed adduct included two SA molecules. A singlet at δ 7.44, with a relative integration of four protons, was assigned to the amino protons of the sulfonated groups of SA. Further evidence of this assignment was available from the $^1H/D_2O$ exchange/DMSO-d_6 NMR spectrum which showed that the intensity of the signal at δ 7.44 decreased substantially after addition of D_2O. Furthermore, assignments for the aromatic and sulfonated amino protons made above are supported with those determined for the SA parent molecule. However, protons in the adduct were shifted by 0.5–1.0 ppm downfield compared to SA itself. A singlet from the ^1H spectrum of SA at δ 5.82, with a relative integration denoting two protons which were exchangeable, was assigned to the primary amine group. The NMR signal for these amino protons was not observed in SMS, possibly indicating the point of cross-linkage between the carbonyl moieties of MA with the amine groups of SA. Further ^1H NMR data of SMS showed a doublet centered at δ 8.92 and a triplet at δ 6.56, with a relative integration equivalent to two and one protons, respectively, and a coupling constant of 11.3 Hz, as being characteristic of the *trans*-vinyl protons of MA.

The $^{13}C\{^1H\}$ NMR spectrum revealed five resonances for the 15-carbon adduct, suggesting that the compound had considerable symmetry. Assignment for the ^{13}C NMR spectrum was aided by APT data which showed the presence of one or two quaternary carbons (δ 141.1 and 141.2) and four tertiary carbons (δ 100.2, 117.7, 127.7, and 159.6). The vinyl protons of MA at δ 6.56 and 8.92 correlated with ^{13}C resonances at δ 100.2 and 159.6, respectively. Carbon atoms bonded to the aromatic protons at δ 7.57 and 7.92 were assigned to ^{13}C resonances at δ 117.7 and 127.7, respectively. The ^{13}C NMR spectrum of the parent SA molecule assisted in these assignments. The two equivalent tertiary carbons of SA adjacent to the sulfonamide group displayed a signal at δ 127.5 identical to that in SMS, while the other two tertiary carbons adjacent to the primary amine group showed a signal at δ 112.5. Furthermore, weak NMR signals produced by the sulfonamide- and amino-ipso carbons of SA at δ 152.0 and 130.0, respectively, were not observed in the spectrum of the adduct. A ^{13}C signal at δ 152.0 was anticipated since cross-linking of SA with MA at the amino group was not expected to have changed the chemical environment, and hence the NMR signal, of this ipso carbon to any great extent. However, one or two quaternary carbon signals were detected at δ 141.1 and 141.2, and ipso carbon assignments were not clear. It is conceivable that the ^{13}C NMR signal at δ 141.1/141.2 is representative of the sulfonamide-ipso carbon and that the signal from the amino/imino-ipso carbon was obscured by ^{14}N quadrupole broadening in the 1-amino-3-iminopropene adduct. A summary of the ^1H and ^{13}C NMR assignments for SMS is presented in Table II.

The normalized EI mass spectrum of the SMS adduct ($C_{15}H_{16}O_4N_2S_2$) revealed that the base peak had m/z 172, which is characteristic of the molecular mass of SA. The molecular ion was detected at m/z 380 with a modest intensity of 1%. Major fragment ions included m/z 156 (75%), 108 (33%), 93 (24%), 92 (53%), 66 (12%), 65 (44%), 64 (40%), 48 (21%), and 43 (15%).

Characterization of the SMT Adduct. The reaction of MA with TBA and SA formed an orange—rather than the typical pink—chromogen of TBA–MA. The isolated pigment gave orange powderlike crystals which had no definite melting point up to 350 °C. Its UV–vis spectrum in 0.1 M HCl/DMSO solvent revealed absorption bands at λ 532, 472, 372, and 278 nm due to $n-\pi^*$ and $\pi-\pi^*$ transitions, which are diagnostic of a highly conjugated system. The absorption characteristics of SMT were identical to those of the SA–MA–TBA model system in 0.1 M HCl except that a bathochromic shift of the 278-nm band to 266 nm had occurred, which is probably due to a concentration effect. Furthermore, a greater absorbance of the 372-nm band was noted, suggesting that the SMT adduct was partially hydrolyzing at the amine junction forming a molecule of the 1:1 TBA–MA intermediate and a molecule of SA. Although molar extinction coefficients for the absorption bands in SMT could not be determined since the adduct in solution degraded with time, the 472- and 532-nm bands were observed even after weeks of storage. Furthermore, when a 0.1 M $AgNO_3$ solution was added to dissolved SMT in deionized H_2O, no precipitate was formed, thus suggesting that the adduct did not exist as a hydrochloride salt.

Addition of TBA reagent to the SA–MA model system induced a spectral shift with loss of the previously observed 256- and 332-nm UV bands and the 396-nm visible band. New absorption maxima at 372, 472, and 532 nm were noted. Moreover, SA addition to the TBA–MA system did not shift the already observed 532-nm absorption band but produced new maxima at 372 and 472 nm. Absorption characteristics of the SA–MA system to which TBA was introduced, and of the TBA–MA system to which SA was added, as well as of the SA–MA–TBA system itself were identical. Furthermore, absorption of UV or visible radiation of a heated TBA–SA system was not observed above 320 nm. UV absorption below 320 nm is probably due to the aromaticity of phenyl groups of SA and the pyrimidine residue of TBA. Hence, lack of absorption in the visible range further suggests that absorption at 472 nm is due to some multiple interaction(s) between MA with both SA and TBA molecules. The proposed chemical structure of the SMT product is presented in Figure 1.

The FTIR spectrum of the SMT adduct exhibited vibrational bands diagnostic of group frequencies associated with both TMT and SMS (Table I). The three vibrational bands characteristic of the secondary amide group in the substituted pyrimidine were observed at 1638, 1489 (1510, shoulder), and 1301 cm^{-1}. The C=S stretching of thioamide moieties was noted at 1130 cm^{-1}. No vibrational band at 2550 cm^{-1} due to sulfhydryl groups was observed. Asymmetric and symmetric stretching bands of SO_2 moieties of SA were observed at 1337 and 1130 cm^{-1}, respectively. Strong asymmetric and symmetric stretching of primary amine bands of SA at 3478 and 3376 cm^{-1}, respectively, were again absent in the adduct as was the case for SMS, perhaps signifying the point of cross-linking between SA and MA molecules. Moreover, the asymmetric stretching band of the sulfonated primary

amine of SA was observed at 3367 cm^{-1}; however, its symmetrical counterpart was obscured by broad signals at 3209 and 3087 cm^{-1}. These are possibly due to a =C—H stretching rock of the extended conjugation of the aromatic system of SMT.

The 300-MHz ^1H NMR spectrum of SMT in DMSO-d_6 revealed eight resonances. Two AA'BB' doublets centered at δ 7.82 and 7.51 with a relative integration equivalent to four protons were diagnostic of the two sets of equivalent aromatic protons of SA. A singlet at δ 7.34 with a relative integration equivalent to two protons, and whose signal disappeared by D_2O addition, was characteristic of the sulfonamide protons of SA. Furthermore, assignments for the aromatic and sulfonated amino protons made above are supported by those determined for SMS as well as the SA parent molecule. A broad peak at δ 3.44, which varied slightly depending on the concentration and temperature during the NMR experiment, was assigned to the amide NH protons of TBA. A singlet at δ 11.8 with a relative integration equivalent to two protons was assigned to the protonated OH group in the TBA moiety prepared in an acidic medium. The *trans*-vinyl protons from MA in the SMT adduct were more difficult to assign. The time-average C_{2v} symmetry of TMT and SMS adducts is inappropriate for SMT. Three chemical shifts for the vinyl protons, each with a relative integration of one proton and with a coupling constant of 13.2 Hz, were detected at δ 7.57, 8.15, and 8.77. The doublet centered at δ 8.15 was assigned to the vinyl proton adjacent to the substituted pyrimidine moiety. The ^1H signal at δ 7.57 was partially obscured by an aromatic proton signal of SA at δ 7.51. The δ 7.57 signal appeared to be a doublet of a doublet which would be characteristic of the central proton in the MA moiety. However, in the $^1H/D_2O$ exchange/DMSO-d_6 NMR spectrum this resonance appeared more as a triplet than as a doublet of doublets. The δ 8.77 signal in the ^1H spectrum appeared as a triplet but may have been an overlapping doublet of doublets. In the $^1H/D_2O$ exchange/DMSO-d_6 NMR experiments, this signal appeared as a doublet, indicating that exchange between a neighboring proton had occurred. Possibly the cross-linking N atom of SA was protonated and had an interaction with the adjacent proton; however, when D_2O was added, this interaction was lost and a doublet emerged.

The $^{13}C\{^1H\}$ NMR spectrum revealed nine resonances for the proposed 13-carbon adduct indicative of decreased symmetry for SMT compared with TMT and SMS. The APT spectrum showed that five of the nine nonequivalent carbons in the molecule were quaternary in nature, while the other four were tertiary. Signals of the quaternary carbons at δ 177.3, 162.4, and 103.5 were assigned to the positions found in their respective TBA counterpart. Aromatic protons at δ 7.51 and 7.82 correlated with ^{13}C resonances at δ 117.0 and 127.6, respectively, which are similar to assignments for SMS. Quaternary carbons detected at δ 139.8 and 141.8 may actually be tautomers of one signal representative of the ipso carbon of the sulfonated moiety. As was with SMS, the signal from the amino/imino-ipso carbon was obscured by ^{14}N quadrupole broadening. Only two ^{13}C NMR signals were detected for the three carbon atoms of the MA group in SMT. The resonance at δ 107.9 was assigned to the central carbon atom of MA, while the tertiary carbon signal at δ 157.5 was assigned as accidently degenerate. Alternatively, the δ 157.5 signal can be assigned to ^{14}N quadrupole broadening obscuring the ^1H NMR signal from its adjacent carbon atom. A summary of the ^1H and ^{13}C NMR assignments is presented in Table II.

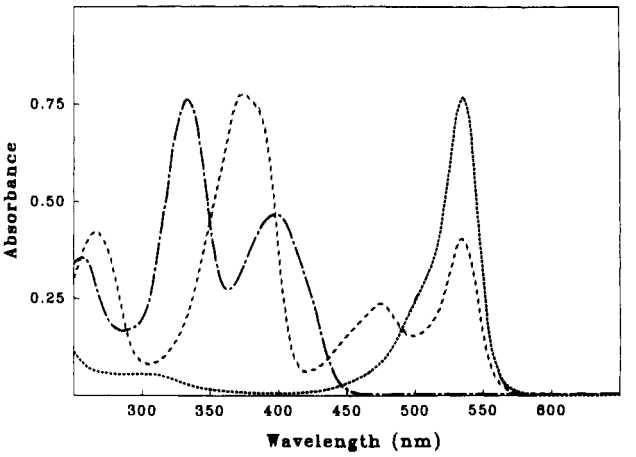

Figure 2. Absorption characteristics of TMT (- - -), SMS (– – –), and SMT (— —).

The normalized EI mass spectrum of the SMT adduct ($C_{13}H_{12}N_4O_4S_2$) showed that the base peak had m/z 65. This fragment ion also gave a major peak for both SA and SMS. The molecular ion was detected at m/z 352 with a modest intensity of 1%. Major fragment ions included m/z 180 (23%), 172 (80%), 156 (82%), 144 (54%), 128 (24%), 116 (28%), 108 (56%), 93 (21%), 92 (93%), 80 (18%), 69 (39%), 64 (22%), 63 (22%), 59 (54%), 44 (20%), 43 (54%), 42 (57%), and 41 (34%). Major fragment ions from TMT and SMS as well as their parent molecules TBA and SA, respectively, can be observed in the proposed SMT adduct.

Implications of Interaction of Sulfanilamide with Malonaldehyde in Determination of Oxidative State of Nitrite-Cured Meats. In determination of the oxidative state of cured meats by the commonly used TBA procedure, SA is generally added to the system to react with residual nitrite present. The SA addition would prevent the nitrosation of MA, which results in its underestimation in cured meats. Shahidi et al. (1991) showed that addition of as little as 50 ppm of nitrite to a MA solution prior to its distillation for the TBA test eliminated the formation of the TBA–MA complex, thus indicating complete nitrosation of MA. Results obtained by Kolodziejska et al. (1990) agreed with these findings. Addition of SA to the system prior to heating and steam distillation prevented this problem, however, the TBA values were still lower than when no nitrite or SA were present in the system. Similar observations were made for meat cured with at least 100 ppm of nitrite to which SA had been added. The latter results suffered from errors ranging from 6 to 20%. According to Kolodziejska et al. (1990), the reaction of MA with SA was reversible such that MA became available to react with the TBA reagent. These authors based their argument on the fact that the absorption intensities of the chromophore(s) of possible adducts of SA and TBA with MA and the typical TBA–MA system at the 532-nm band were equivalent. Although it is true that the absorption bands of the MA–SA model system in the UV region disappeared upon TBA addition and that the formation of Schiff bases is reversible, the above authors failed to acknowledge that the visible absorption spectrum of the TBA–MA–SA system was quite different from that of its counterpart devoid of SA. Likewise, SA addition to the prepared MA–TBA system did not shift the already observed absorption bands at 372 and 532 nm but produced a new band at 472 nm. Absorption characteristics of the MA–SA system to which

TBA was introduced, of the MA–TBA system to which SA was added, and of the TBA–SA–MA system itself were identical.

As mentioned previously, the reaction(s) of MA with TBA and SA resulted in the formation of an orange—rather than the typical pink—chromogen of TBA–MA. Addition of TBA reagent of increasing concentrations to a malonaldehyde solution containing a fixed amount of SA, prior to heating, resulted in an almost linear increase in the absorption intensity of the 532-nm band but did not seem to influence the absorbance at the 472-nm maximum. On the other hand, addition of increasing concentrations of SA to the system containing MA and a fixed amount of TBA reagent, prior to heating, caused an increase in the absorption at the 472-nm band yet did not appreciably change the absorption at 532 nm. Furthermore, differences in absorption values of the TBA–MA–SA systems were noted when the TBA reagent was added to heated MA–SA systems after additional heating. An increase in absorbance at the 532-nm band was observed, but no change in the absorption at 472 nm was evident. Absorbances at 532 nm were approximately half as intense as when TBA was added to the mixture before heating. Systems containing varying amounts of SA, but with a fixed amount of TBA reagent added after the initial heating, exhibited an increase in the absorbance at 532 nm. More interesting, however, is the fact that the absorption intensity at the 472-nm maximum was identical to that of systems prepared with MA, SA, and TBA added prior to heating. Thus, the compounds responsible for absorption at 472 nm appear to be independent of the time at which the TBA reagent was added to the system.

The appearance of the new band at 472 nm in the TBA–MA–SA system, as it has now been fully documented, suggests the presence of a second chromophore due to multiple interactions between MA with both SA and TBA reagent. The adduct is a condensation product of one molecule of each of TBA and SA cross-linked by the highly reactive three-carbon moiety of MA.

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