JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Liquid Chromatography−Mass Spectrometry (LC-MS) Investigation of the Thiobarbituric Acid Reactive Substances (TBARS) Reaction

DANIEL JARDINE,† MICHAEL ANTOLOVICH,‡ PAUL D. PRENZLER,‡ AND KEVIN ROBARDS*,‡

School of Science and Technology, Charles Sturt University, Locked Bag 588, Wagga Wagga 2678, Australia, and Department of Chemistry, Macquarie University, Sydney 2109, Australia

The thiobarbituric acid reactive substances (TBARS) assay is a commonly used method for the detection of lipid peroxidation. Malondialdehyde is formed as a result of lipid peroxidation and reacts with thiobarbituric acid to form a pink pigment that has an absorption maximum at 532 nm. Other compounds also react with thiobarbituric acid to form colored species that can interfere with this assay, but little is known about these interfering species. This is the first investigation using LC-MS and MS-MS to study the structures of the pink adduct as well as a common unstable yellow interference compound, which absorbs at 455 nm. Also, the presence of barbituric acid impurities in the thiobarbituric acid reagent was found to produce 1:1:1 thiobarbituric acid/malondialdehyde/barbituric acid and 2:1 barbituric acid/malondialdehyde adducts that absorbed at 513 and 490 nm, respectively, indicating that thiobarbituric acid should be purified before use.

KEYWORDS: TBARS; mass spectrometry; interferences

INTRODUCTION

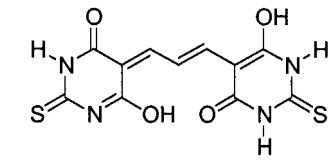
Lipid peroxidation is considered to be responsible for the development of rancidity in stored foods and related to heart disease, cancer, and aging in animals. The detection of lipid peroxidation is therefore of importance in many situations. The thiobarbituric acid reactive substances (TBARS) assay was proposed over 40 years ago and is now the most commonly used method for this purpose (*1*). In this assay malondialdehyde that is formed as a result of lipid peroxidation (*2*) is reacted with thiobarbituric acid to form a pink pigment that has an absorption maximum at 532−535 nm. Many other substances, including other alkanals, protein, sucrose, and urea, may react (*3*, *4*) with thiobarbituric acid to yield colored species and thus contribute to overestimation of the extent of lipid peroxidation. Some specificity is provided (*5*) by the choice of analytical wavelength, as alk-2,4-dienals and, to a lesser extent, alk-2-enals produce the red pigment absorbing at 530 nm, whereas alkanals in general produce a yellow chromogen absorbing at 450 nm. Further enhancement in specificity has been achieved (*6*) by the high-performance liquid chromatographic separation of the complex prior to measurement. Other approaches to improving specificity and sensitivity include extraction of the MDA prior to formation of the chromogen and/or derivative spectrophotometry (*7*).

The pigment absorbing at 532 nm is considered to be a condensation product of thiobarbituric acid and malondialdehyde in a 2:1 molar ratio. However, the nature of the interfering substances has not been systematically investigated despite the fact that the reaction is assuming increased importance because of its extensive use. For instance, lipid peroxidation during spinach senescence was measured by the TBARS procedure (*8*), HPLC separation, and photometric measurement of the product. The authors reported the elution of a sharp peak corresponding to the 2:1 condensation product. In this case, measurement of the isolated compound probably improved the reliability of this empirical procedure. However, in cases involving direct spectrophotometric measurement (*9*), the reliability of the procedure is less assured. The most detailed study of the reaction was reported by Guzmán-Chozas et al. (*10*), who reported a yellow pigment absorbing at 450 nm arising from alkanals and an orange pigment (λ_{max} = 494 nm) arising from 2,4-dienals.

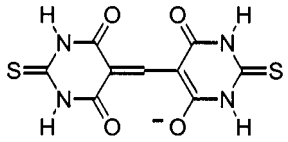
Kosugi et al. (*11*) have investigated the reaction of 2-thiobarbituric acid with 2-enals and found that a colorless 1:1 adduct was initially formed. This compound then reacted with another molecule of 2-thiobarbituric acid to produce the 2:1 adduct; however, this reaction proceeded only in the presence of oxygen. They did not determine the structure of the 2:1 adduct. The colored compound produced from three different 2-enals had the same absorbance (532 nm) and HPLC retention time, indicating that the formation of this compound is independent of the structure of the 2-enal. In a study of the reactions of sulfanilamide and 2-thiobarbituric acid with malondialdehyde,

* Corresponding author (telephone 02 6933 2540; fax 02 6933 2737; e-mail krobards@csu.edu.au).
† Macquarie University.
‡ Charles Sturt University.

10.1021/jf011336a CCC: $22.00
Published on Web 02/12/2002



1



2

Figure 1. Structures of the pink 2:1 thiobarbituric acid/malondialdehyde condensation product (**1**) and the yellow compound (**2**).

Pegg et al. (*12*) confirmed the structure of the 2:1 thiobarbituric acid/malondialdehyde adduct as structure **1** (**Figure 1**).

This paper reports the use of LC-MS and MS-MS to examine the nature of the reaction products formed from the reaction of thiobarbituric acid with propanal, formic acid, and oxidation products of linoleic acid.

MATERIALS AND METHODS

Chemicals. Propanal and 2-thiobarbituric acid were obtained from Merck (Darmstadt, Germany), linoleic acid was obtained from Eastman (Rochester, NY), acetic acid was from Mallinckrodt (Paris, KY), and HPLC grade methanol was from BDH (Poole, U.K.). Chemicals were used without further purification.

TBARS Reaction. TBARS were produced using an adaptation of the method of Kishida et al. (*1*). Linoleic acid was used as a model lipid, and oxidation was induced by Cu(II) ions. Thus, linoleic acid (100 μL) and copper(II) chloride (0.05 mM, 300 μL) were added to a test tube that was vortexed and heated in a Certomat shaking water bath at 40 °C for 20 h. Butylhydroxytoluene (BHT) (10 mM; 20 μL) was added to terminate the reaction. Thiobarbituric acid solution (freshly prepared; 0.67% w/v in 0.1 M HCl, 3 mL) was added, and the tube was vortexed, heated in a boiling water bath for 10 min, and then cooled. The aqueous layer was removed and added to a separate tube containing 2.5 mL of butanol. The butanol was removed under nitrogen and the sample dissolved in water for LC-MS analysis.

The reaction of propanal was performed by mixing equal volumes of 10 mM solutions (in 10% aqueous acetic acid) of propanal and thiobarbituric acid. Reactions were performed at 100 and at 40 °C for 30 min.

Mass Spectrometry. Mass spectrometric analyses were performed using a Quattro II triple-quadrupole mass spectrometer (Micromass, Altrincham, Cheshire, U.K.) in the flow injection analysis (FIA) mode using electrospray ionization (ESI). The solvent flow rate was 10 μL/min. The solvent was methanol. For collision-induced dissociation (CID) spectra, argon was used as the collision gas at a pressure of 2.5×10^{-3} mbar. Unit mass resolution was used for both quadrupoles.

Liquid Chromatography—Mass Spectrometry (LC-MS). Samples were analyzed using a Beckman liquid chromatograph, consisting of a model 126 pump and a model 168 diode array detector. The Quattro II was as used for the mass spectrometry analysis. Two different HPLC columns were employed: an SGE (Ringwood, Australia) Wakosil, C18 column (150 × 2 mm i.d., 5 μm) and an SGE C18 (250 mm × 4.6 mm i.d., 5 μm). These were used with gradient elution with the solvents being 0.1% aqueous acetic acid (solvent A) and methanol (solvent B). Data were acquired by both the Masslynx data system for the mass spectrometer and the Beckman data system for the diode array; two wavelengths from the diode array were also recorded by the Masslynx system to allow alignment of the data.

During LC-MS, scans were performed for both positive and negative ions. Cone voltage fragmentation was also used in both ion modes. The same LC gradient was used for both columns, from 5% solvent B to 95% solvent B in 30 min, then 10 min at 95% solvent B, and then from 95% solvent B to 5% solvent B in 10 min. The flow rate for the 2 mm column was 0.2 mL/min and that for the 4.6 mm column 1 mL/min. A splitter system was used on the solvent flow from the HPLC that allowed ~50 μL of the flow to the electrospray source.

RESULTS AND DISCUSSION

The TBARS reaction involving linoleic acid gave several products that were resolved by HPLC and detected by absorption in the ultraviolet and visible regions. From three-dimensional contour plots, the dominant peak absorbing in the visible region showed a maximum at 532 nm, at which it is usually quantified. A yellow product exhibiting strong absorption at 455 nm was also observed. These two products were separated on a Waters Sep-Pak Vac C18 cartridge. The yellow product was not retained, whereas the pink product was eluted with methanol. Following this fractionation to remove the yellow product, analysis of the pink TBARS reaction product by HPLC on the 4 mm × 250 mm C18 column, using a diode array detector, showed a major peak at 7.2 min and two smaller peaks at 5.9 and 4.8 min (**Figure 2A**). There were also a number of UV-absorbing peaks observed, but further study was restricted to elucidating the nature of the colored pigments.

The UV−vis spectrum and the negative-ion ESI spectrum of the peak at 7.2 min are shown in **Figure 2B**. The corresponding data for the peak at 5.9 min are shown in **Figure 2C**. The intensity of the peak at 4.8 min was not sufficient to produce a mass spectrum, but the UV spectrum is shown in **Figure 2D**. The mass spectrum in **Figure 2C** shows similar fragments to those in **Figure 2B**, but in addition to the pseudomolecular ion being 16 amu less than the thiobarbituric acid product, there is a difference of 16 amu in the major fragments. It is proposed that this peak is formed from a barbituric acid impurity in the thiobarbituric acid giving rise to a 1:1:1 thiobarbituric acid/barbituric acid/malondialdehyde condensation product. The third peak in the chromatogram of **Figure 2A** (t_R = 4.8 min) would then correspond to the 2:1 condensation product of barbituric acid and malondialdehyde. The substitution of sulfur for oxygen was accompanied by a progressive shift in the absorption maximum from 532 to 513 nm and to 490 nm (**Figure 2B−D**). Confirmation of the structure of these compounds was obtained by reacting the mixture with hydrogen peroxide in glacial acetic acid (*13*), one of the products of this reaction involving replacement of sulfur by oxygen. The LC-MS analysis of this reaction mixture showed a peak with the same retention time and UV spectrum as the peak in **Figure 2D**. The mass spectrum showed a molecular weight of 292. The observation of the reaction of lipid oxidation products with barbituric acid impurities suggests that the thiobarbituric acid requires purification before use in the TBARS assay. It would appear that this precaution is not generally adopted (*7*, *10*).

For the analysis of the yellow product absorbing at ~455 nm the SGE Wakosil C18 column was used as this column retained this compound, whereas the SGE C18 column did not. The chromatogram monitored at 455 nm and the mass chromatogram at *m*/*z* 297 taken from the lipid oxidation reaction are shown in parts A and B, respectively, of **Figure 3**, together with the UV−vis spectrum (**Figure 3C**) and the negative-ion electrospray mass spectrum (**Figure 3D**). This indicates that the yellow compound (t_R = 14.7 min) has a molecular weight of 298. The isotopic pattern of the molecular ion peak indicates that there are two sulfur atoms present, so the compound is

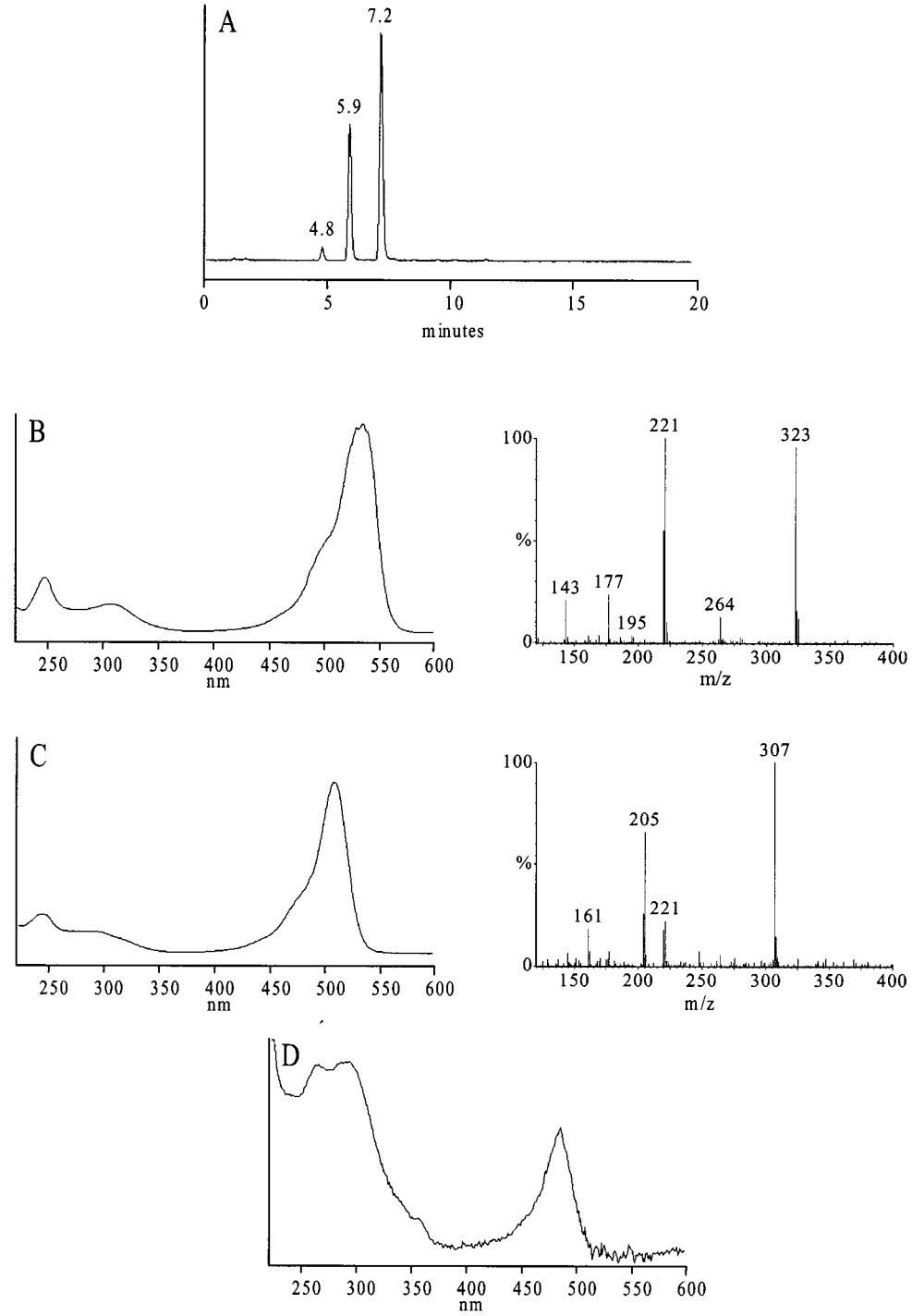


Figure 2. (A) HPLC chromatogram at 488 nm of the reaction mixture from the copper(II)-mediated oxidation of linoleic acid with thiobarbituric acid. (B) UV spectrum and negative-ion mass spectrum of the peak at 7.2 min. (C) UV spectrum and negative-ion mass spectrum of the peak at 5.9 min. (D) UV spectrum of the peak at 4.8 min.

probably a condensation product involving two thiobarbituric acid molecules. There was no corresponding positive ion observed.

Kosugi and Kikugawa (*14*, *15*) have reported that the reaction of saturated aldehydes with thiobarbituric acid also produces a yellow compound; therefore, the reaction of propanal with thiobarbituric acid was studied to further elucidate its structure. The chromatogram at 455 nm, as well as the UV and mass chromatograms from the LC-MS analysis of the reaction of propanal and thiobarbituric acid, was identical with that of **Figure 3**. This indicates that the compound formed in the lipid oxidation reaction is formed from a saturated aldehyde.

The reaction with propanal produced a yellow color after 30 min at 40 °C. After 30 h at 40 °C, the yellow color was still present but a pink compound had also appeared. This compound had the same retention time and molecular weight as the thiobarbituric acid/malondialdehyde 2:1 condensation product. The yellow compound was also produced in the propanal reaction at 100 °C for 0.5 h. It could also be produced in ∼30 s by the addition of propanal to a hot (80 °C) solution of

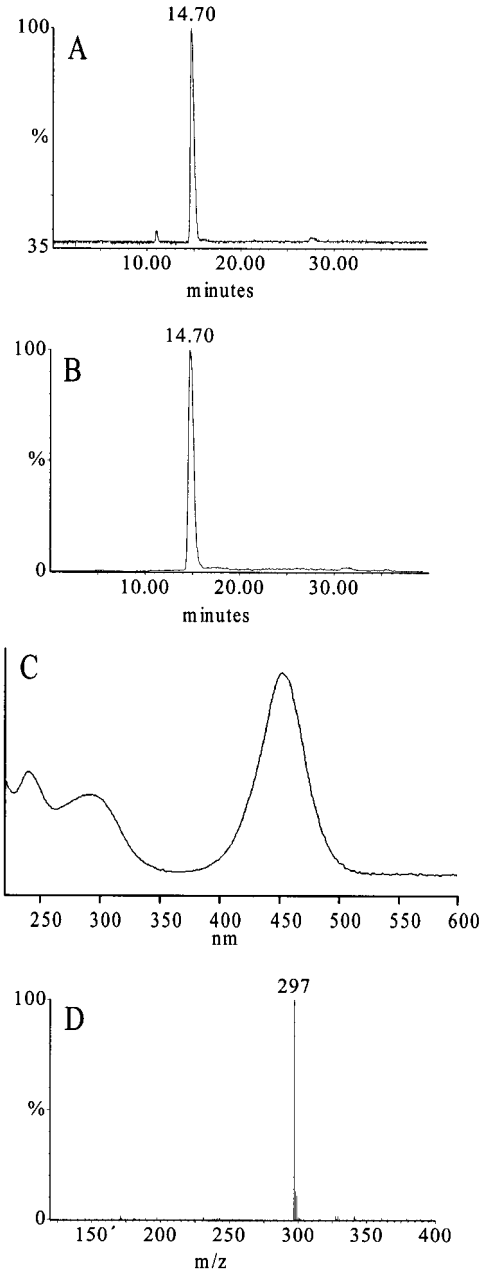


Figure 3. HPLC chromatograms of the yellow product resulting from the thiobarbituric acid reaction with the lipid oxidation products heated for 30 min at 100 °C: (A) chromatogram at 455 nm; (B) mass chromatogram at *m/z* 297; (C) UV–vis and (D) negative ion mass spectra of the peak at 14.7 min.

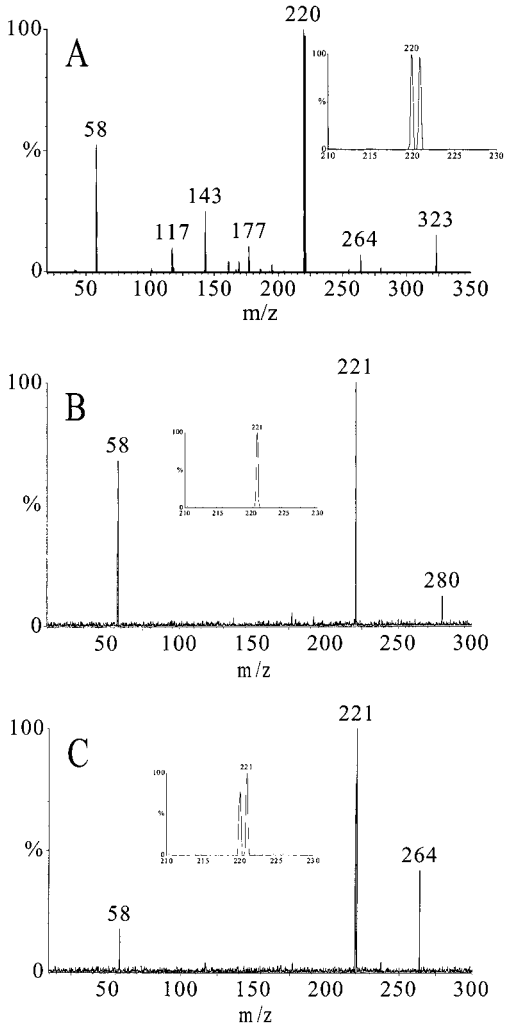


Figure 4. (A) Negative-ion MS-MS spectrum of *m/z* 323 (derived from the pink compound eluting at 7.2 min in **Figure 1A**). (Inset) Expansion of the region around *m/z* 220. (B) Negative-ion MS^3 spectrum of *m/z* 280. (Inset) Expansion of the region around *m/z* 220. (C) Negative-ion MS^3 spectrum of *m/z* 264. (Inset) Expansion of the region around *m/z* 220.

acidified thiobarbituric acid. Interference due to the formation of this yellow pigment has been reported (*7*) in the standard TBARS assay. Indeed, various authors (ref *7* and references cited therein) caution against the use of the assay when compounds producing this pigment are present. Attempts to purify the yellow compound by HPLC resulted in the color fading over time. It appeared that the compound was not stable in the absence of excess thiobarbituric acid. Guzmán-Chozas et al. (*10*) noted that formation of the yellow pigment was evident when alkanals reacted with *excess* thiobarbituric acid reagent.

Tandem Mass Spectrometry of Yellow and Pink Compounds. The pink compound produced from either the lipid oxidation or the extended reaction of propanal could be isolated using a C18 Sep-Pak cartridge. This method could not be used for the yellow compound as it was not retained by the C18 cartridge, and it was therefore separated by preparative HPLC.

Tandem mass spectrometry of the pink compound is shown in **Figure 4A**. Most fragmentations proceed from retro-Diels–Alder type reactions similar to those described for thiopental by Spell et al. (*16*). The fragments at *m/z* 280 and 264 can be produced from either the ring containing the negative charge or the noncharged ring. A second retro-Diels–Alder type reaction from either the *m/z* 280 or 264 ions produces the ion at *m/z* 221. To determine the origin of the *m/z* 220 ion, MS-MS-MS was performed, where the *m/z* 323 ion was fragmented in the electrospray source and the *m/z* 280 or 264 ion was selected by the first quadrupole for subsequent MS-MS analysis. The results of these experiments are shown in **Figure 4B**,**C**. The *m/z* 280 ion does not produce the *m/z* 220 ion, only *m/z* 221 and 58; however, the *m/z* 264 ion does fragment to produce both the *m/z* 221 and 220 ions. The loss of mass 44 from the *m/z* 264 ion is attributed to the loss of CH_2NO. The data are consistent with structure **1**.

The MS-MS fragmentation of the yellow compound (**Figure 5**) is very similar to that for the pink compound. The main losses are the same, that is, losses of 59, 102, and 103 from the

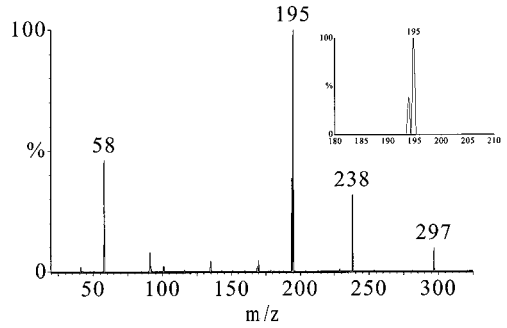


Figure 5. Negative-ion MS-MS spectrum of *m*/*z* 297 (derived from the yellow compound eluting at 14.7 min in **Figure 3**). (Inset) Expansion of the region around *m*/*z* 194.

molecular ion giving rise to the peaks at *m*/*z* 238, 195, and 194. The similarity of the fragmentations indicates that the structures of the two compounds must be similar. Bigwood et al. (*17*) have proposed that the yellow compound has the structure **2** (**Figure 1**) on the basis of an analogous reaction involving hexanal and *N*,*N*′-diethylbarbituric acid. The structure proposed by Bigwood et al. (*17*) is supported by the LC-MS and MS-MS data presented here. The unstable yellow compound has its origin in the reaction of saturated aldehydes, although it is also formed from the reaction of thiobarbituric acid with formic acid at 80 °C for 1 h. The latter does not proceed to form the pink chromogen over 4 weeks kept at ambient temperature. The subsequent formation of the pink compound from saturated aldehydes containing more than one carbon may arise from the reaction of the 2-enal produced from the aldol condensation of the saturated aldehyde.

This paper represents the first LC-MS investigation of the TBARS reaction and supports structure **1** as the pink chromogen typically quantified in this assay. The presence of barbituric acid impurities giving rise to other adducts with malondialdehyde was noted, and these side reactions will also influence quantification of lipid peroxidation. MS^n techniques are growing in importance in the quantification of analytes, and this study provides data useful for the development of an MS^n method for the quantification of MDA. Further investigation is required of the colorless intermediate products of the TBARS reaction.

Supporting Information Available: Data from the LC-MS analysis of the reaction mixture from the copper(II)-mediated oxidation of linoleic acid with thiobarbituric acid after reaction with hydrogen peroxide in glacial acetic acid. This material is available free of charge via the Internet at http://pubs.acs.org.

LITERATURE CITED

(1) Kishida, E.; Kamura, A.; Tokumaru, S.; Oribe, M.; Iguchi, H.; Kojo, S. Re-evaluation of malondialdehyde and thiobarbituric acid-reactive substances as indices of autoxidation based on oxygen consumption. *J. Agric. Food Chem.* **1993**, *41*, 1−4.

(2) Fernández, J.; Pérez-Álvarez, J. A.; Fernádez-López, J. A. Thiobarbituric acid test for monitoring lipid oxidation in meat. *Food Chem.* **1997**, *59*, 345−353.

(3) Almandos, M. E.; Giannini, D. H.; Ciarlo, A. S.; Boeri, R. L. Formaldehyde as an interference of the 2-thiobarbituric acid test. *J. Sci. Food Agric.* **1986**, *37*, 54−58.

(4) Buttkus, H.; Bose, R. J. Amine-malonaldehyde condensation products and their relative color contribution in the thiobarbituric acid test. *J. Am. Oil Chem. Soc.* **1972**, *49*, 440−443.

(5) Marcuse, R.; Johansson, L. TBA (thiobarbituric acid) test for rancidity grading. II. TBA reactivity of different aldehyde classes. *J. Am. Oil Chem. Soc.* **1973**, *50*, 387−391.

(6) Bird, R. P.; Hung, S. S. O.; Hadley, M.; Draper, H. H. Determination of malonaldehyde in biological materials by high-pressure liquid chromatography. *Anal. Biochem.* **1983**, *128*, 240−244.

(7) Botsoglou, N. A.; Fletouris, D. J.; Papageorgiou, G. E.; Vassilopoulos, V. N.; Mantis, A. J.; Trakatellis, A. G. Rapid, sensitive, and specific thiobarbituric acid method for measuring lipid peroxidation in animal tissue, food, and feedstuff samples. *J. Agric. Food Chem.* **1994**, *42*, 1931−1937.

(8) López-Ayerra, B.; Murcia, M. Antonia; Garcia-Carmona, F. Lipid peroxidation and chlorophyll levels in spinach during refrigerated storage and after industrial processing. *Food Chem.* **1998**, *61*, 113−118.

(9) van den Berg, R.; Haenen, G. R. M. M.; van den Berg, H.; van der Vijgh, W.; Bast, A. The predictive value of the antioxidant capacity of structurally related flavonoids using the Trolox equivalent antioxidant capacity (TEAC) assay. *Food Chem.* **2000**, *70*, 391−395.

(10) Guzmán-Chozas, M.; Vicario, I. M.; Guillén-Sans, R. Spectrophotometric profiles of off-flavor aldehydes by using their reactions with 2-thiobarbituric acid. *J. Agric. Food Chem.* **1997**, *45*, 2452−2457.

(11) Kosugi, H.; Kato, T.; Kikugawa, K. Formation of yellow, orange and red pigments in the reaction of alk-2-enals with 2-thiobarbituric acid. *Anal. Biochem.* **1987**, *165*, 456−464.

(12) Pegg, R. B.; Shahidi, F.; Jablonski, C. R. Interactions of sulfanilamide and 2-thiobarbituric acid with malonaldehyde: structure of adducts and implications in determination of oxidative state of nitrite-cured meats. *J. Agric. Food Chem.* **1992**, *40*, 1826−1832.

(13) Grivas, S.; Ronne, E. Facile desulfurization of cyclic thioureas by hydrogen peroxide in acetic acid. *Acta Chem. Scand.* **1995**, *49*, 225−229.

(14) Kosugi, H.; Kikugawa, K. Thiobarbituric acid reaction of aldehydes and oxidized lipids in glacial acetic acid. *Lipids* **1985**, *20*, 915−921.

(15) Kosugi, H.; Kikugawa, K. Reaction of thiobarbituric acid with saturated aldehydes. *Lipids* **1986**, *21*, 537−542.

(16) Spell, J. C.; Srinivasan, K.; Stewart, J. T.; Bartlett, M. G. Supercritical fluid extraction and negative ion electrospray liquid chromatography tandem mass spectrometry analysis of phenobarbital, butalbital, pentobarbital and thiopental in human serum. *Rapid Commun. Mass Spectrom.* **1998**, *12*, 890−894.

(17) Bigwood, T.; Delve, R.; Read, G. A novel iron(III) catalyzed degradation of aliphatic aldehydes to their lower homologs with implications for lipid peroxidation chemistry. *J. Chem. Soc., Chem. Commun.* **1990**, 776−778.

Received for review October 8, 2001. Revised manuscript received December 11, 2001. Accepted December 12, 2001. We gratefully acknowledge the provision of an ARC grant and a Macquarie University Research Grant.

JF011336A