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Effect of meat curing agents and phosphates on thiobarbituric acid (TBA) numbers of ground beef determined by the aqueous acid extraction TBA–C_{18} method

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The effects of ascorbic acid (0–0·06%), sodium nitrite (0–0·03%), and phosphates (0·5%) on thiobarbituric acid (TBA) numbers obtained by a new aqueous acid extraction TBA–C_{18} method were evaluated both in malonaldehyde containing aqueous systems and in ground beef samples. Sodium nitrite significantly ($P < 0·05$) decreased the formation of red coloured malonaldehyde–TBA complex and addition of sulphanilamide or ascorbic acid did not successfully overcome this problem. All the phosphates tested in this study did not interfere with the formation of the malonaldehyde–TBA complex in both aqueous solutions and ground beef. Therefore, the aqueous acid extraction TBA–C_{18} method can be used for malonaldehyde measurement in meat products containing phosphates, but interference with the malonaldehyde–TBA reaction by nitrite remained a problem.

INTRODUCTION

Sodium nitrite is added to cured meat products to fix their colour (Okayama *et al.*, 1991) and flavour (Noel *et al.*, 1990), to inhibit *Clostridium botulinum* growth (Sofos *et al.*, 1979*a*) and toxin formation (Sofos *et al.*, 1979*b*), and to stabilize lipids against oxidation (Zubillaga & Maerker, 1987). The thiobarbituric acid (TBA) method of Tarladgis *et al.* (1960) is the most frequently used test for measuring malonaldehyde, as one of the degradation products of lipid peroxidation, in muscle foods. However, this TBA method cannot be used in meat products containing sodium nitrite, because nitrite can also react with malonaldehyde (Zipser & Watts, 1962). During the TBA reaction the malonaldehyde–nitrite complex does not produce the same red pigment as the malonaldehyde–TBA complex (Kolodziejska *et al.*, 1990). Zipser and Watts (1962) modified the method by adding sulphanilamide to minimize the interference by nitrite. However, sulphanilamide itself decreased the TBA numbers of meat samples even in the absence of nitrite (Shahidi *et al.*, 1985). The reaction of malonaldehyde and sulphanilamide to produce a 1-amino-3-iminopropene derivative was assumed to be responsible for underestimation of TBA numbers (Shahidi *et al.*, 1991).

Ascorbic acid is also frequently used in meat curing. At levels higher than 40 ppm in minced meat, ascorbic acid has been reported to react with nitrite to form nitrogen monoxide (Norwitz & Keliher, 1986). This reaction has also been reported to be dependent on pH, being only weak or moderate in basic solutions and very strong under neutral or acidic conditions (Norwitz & Keliher, 1987). These findings may suggest that the presence of ascorbic acid in cured meat could cause either positive or negative interference during TBA analysis.

Polyphosphates are also widely used in meat products to improve water retention, increase binding and improved palatability (Sofos, 1986). Inclusion of phosphates in ham curing brine resulted in higher-quality products (Vollmar & Melton, 1981). However, Molins *et al.* (1987) suggested that phosphate in beef patties might interfere with lipid peroxidation analysis by the TBA method of Tarladgis *et al.* (1960), because it resulted in lower TBA number than the control at day 0.

Research in our laboratory has led to the development of a new aqueous acid extraction thiobarbituric acid-C_{18} (TBA–C_{18}) method (Raharjo, 1992). Although this method has better specificity and sensitivity than other spectrophotometric TBA methods, its application

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in cured meat and phosphate-containing meat products has not been evaluated. Therefore, the objective of this study was to evaluate the effects of curing agents and phosphates in raw and cooked ground beef on the TBA numbers obtained by the aqueous acid extraction TBA–C_{18} method.

MATERIALS AND METHODS

Aqueous acid extraction TBA–C_{18} method

Ground beef samples (10 g) were homogenized with 40 ml of 5% (w/v) aqueous trichloroacetic acid (TCA) (Mallinckrodt, Paris, KY) in an Osterizer Blender (Sunbeam Corp., Milwaukee, WI) for 1 min, with addition of 0·15% butylated hydroxytoluene (BHT) (Sigma Chemical Co., St Louis, MO) based on fat content prior to homogenization. The meat homogenate was centrifuged at 10 000 *g* for 5 min and the supernatant was filtered through a Whatman micro fibre glass filter grade C (Whatman, Hillsboro, OR) into a 50 ml volumetric flask. The filtrate volume was adjusted to 50 ml using 5% (w/v) aqueous TCA, and a portion (5 ml) was reacted with 5 ml of 80 mM thiobarbituric acid (TBA) (Sigma Chemical Co.) in a test tube with a screw cap while heating in a water bath (National Appliance, Portland, OR) of 94 ± 1°C for 5 min. The pH of the red pigment formed was adjusted to approximately 7 with 5 N NaOH (Mallinckrodt) and 0·2 ml of 3% (w/v) phosphate buffer of pH 7·2 (Becton Dickinson and Co., Cockeysville, MD) prior to passage through a solid phase extraction Sep-Pak™ C_{18} cartridge (Waters, Milford, MA).

Prior to its use, the C_{18} cartridge was washed with 10 ml of absolute methanol (Mallinckrodt) followed by 10 ml of distilled water at a flow rate of approximately 20 ml/min. The flow rate was determined by loading the solution into a 12-ml syringe (Becton Dickinson and Co.), which was connected with a C_{18} cartridge, then manually passed through the cartridge with a plunger for a specified period of time. The sample containing red coloured adduct was loaded to the syringe and passed through the predeveloped C_{18} cartridge at a flow rate of approximately 5 ml/min to allow adequate time for the C_{18} matrix to bind the red coloured adduct. The solution that came out of the cartridge was discarded. Unreacted TBA solution and other unwanted components in the cartridge were removed by eluting the loaded sample with 10 ml of distilled water at a flow rate of approximately 10 ml/min. The eluent coming out of the cartridge was discarded. The malonaldehyde–TBA complex was recovered and separated from other TBA-reactive substances (TBARS) by eluting the cartridge with 10 ml of absolute methanol at a flow rate of approximately 10 ml/min. Absorbance of the methanol eluent (10 ml) containing the malonaldehyde–TBA complex was scanned from 400 to 600 nm at 5 nm intervals using a spectrophotometer (Bausch and Lomb, Rochester, NY) and the wavelength of maximum absorbance (λ_{MAX}) was found to be 525 nm. The TBA numbers (mg malonaldehyde equivalents/kg meat) were calculated according to the procedure of Pikul *et al.* (1989).

Effects of curing agents of TBA reaction in model systems

A factorial experiment (4 × 4 × 2) was designed to evaluate the effects of four levels of sodium nitrite (0, 0·01, 0·02 and 0·03%) (Sigma Chemical Co.), four levels of ascorbic acid (0, 0·02, 0·04 and 0·06%) (Pfizer Inc., New York, NY), and two levels of sulphanilamide (0 and 0·02%) (Sigma Chemical Co.) on the reaction of malonaldehyde and TBA. All of these ingredients were dissolved in distilled water. For the reactions, portions of the sodium nitrite solutions (1 ml) were pipetted into 8 ml -size test tubes with screw caps, followed by addition of 1 ml of the ascorbic acid solutions. After 5 min at room temperature, 1 ml of 16 μM malonaldehyde prepared from 1,1,3,3-tetraethoxypropane (Sigma Chemical Co.) in 5% (w/v) aqueous TCA was added, and the mixture was shaken in a Vortex–Genie mixer (Scientific Industries, Springfield, MA) for 1 s. Portions (1 ml) of 0 or 0·02% aqueous sulphanilamide solution were subsequently added to the mixture, followed by addition of 1 ml of 80 mM thiobarbituric acid (Sigma Chemical Co.) in distilled water. Finally the mixtures were heated in a water bath (National Appliance, Portland, OR) at 94 ± 1°C for 30 min. After cooling, the reaction mixture was subjected to solid phase extraction using a Sep-Pak™ C_{18} cartridge (Waters, Milford, MA) as described above. The absorbance of the red coloured malonaldehyde-TBA complex was measured at 525 nm using a spectrophotometer.

Studies have indicated that the TBA test cannot be used for measuring lipid peroxidation in cured meat products (Zipser & Watts, 1962; Shahidi *et al.*, 1985), because these cured meat products may contain approximately 0·008 –0·012% sodium nitrite (Hotchkiss & Cassens, 1987). Therefore, the question raised is whether low levels of nitrite also interfere with the new TBA–C_{18} method. Another factorial (4 × 2 × 2) experiment was designed to evaluate the effects of four low levels of sodium nitrite (0, 0·0006, 0·0012 and 0·0018%), two levels of ascorbic acid (0 and 0·02%), and two levels of sulphanilamide (0 and 0·02%) on the reaction between malonaldehyde and TBA. The reactions and subsequent C_{18} treatments were performed as described above.

Effects of curing agents on TBA numbers of ground beef

Ground beef (18·2–24·6% fat) was divided into four batches of 200 g each. Each of these four batches was mixed, using a Kitchen-Aid mixer (KitchenAid, Inc., St Joseph, MI) at speed 2 for 1 min, with each of the following sets of ingredients which were predissolved in 20 ml of distilled water: (a) 0·0156% $NaNO_2$ and 0·05% ascorbic acid, (b) 0·05% ascorbic acid only, (c) 0·0156%

$NaNO_2$ only, and (d) no ingredients or distilled water (20 ml) only, which was used as a control. Each of these treated aliquots (approximately 220 g each) was divided into two smaller aliquots of 100 g and 120 g. Each of the 100 g portions was divided into five smaller portions of 20 g each, placed in plastic cups (Solo Cups Co., Urbana, IL) with caps and stored aerobically at 4°C. Each of the 120 g meat portions was placed in a 200 ml glass beaker, covered with aluminium foil, and heated in a water bath at 94 ± 1°C for 20 min until an internal temperature of 71°C was reached. These cooked ground beef samples were then divided into 20 g portions, placed in plastic cups with caps, and stored aerobically at 4°C. The TBA numbers of these raw and cooked ground beef samples were analysed by the aqueous acid extraction TBA–C_{18} method as described above after 0, 2, 4, 6 and 8 days of storage.

At each sampling time, the control (meat without additives) samples were divided into two portions of 10 g each. One portion was analysed in the same way as the other treated samples, while the other portion was mixed with 1·56 mg sodium nitrite (0·0156% w/w) prior to aqueous acid extraction TBA–C_{18} analysis. This particular experiment was designed to determine whether the sodium nitrite interfered with malonaldehyde detection in the meat samples during the TBA-C_{18} analysis or not. The TBA numbers were calculated according to the procedure mentioned above.

Effects of phosphates on TBA reaction in model systems

A factorial (5 × 3) experiment was designed to evaluate the effects of five different phosphates dissolved in distilled water (0·5% w/w) and three levels of malonaldehyde standard (2, 4 and 6 μM) prepared in 5% (w/v) aqueous trichloroacetic acid on the TBA reaction. These phosphates were sodium hexametaphosphate (SHMP), sodium tripolyphosphate (STPP), tetrasodium pyrophosphate (TSPP), sodium acid pyrophosphate (SAPP), and potassium tripolyphosphate (KTPP) provided by the FMC Corporation (Philadelphia, PA). The malonaldehyde solutions (2 ml) were reacted with 1 ml of 0·5% phosphate solutions in test tubes with screw caps. After shaking for 1 s using a Vortex-Genie mixer, 2 ml of 80 mM TBA solution was added to each mixture, which was subsequently heated in a water bath at 94 ± 1°C for 30 min. The reaction mixture was subsequently subjected to solid phase extraction using a Sep-Pak™ C_{18} cartridge as described above.

Effects of phosphates on TBA numbers of ground beef

Raw ground beef (18·2–24·6% fat) was divided into six batches of 130 g each. Each of these batches was further divided into two (60 g and 70 g) portions. Each of the 60 g portions was divided into 20 g samples, placed in plastic cups with caps, and stored at 4°C for up to 8 days. The other (70 g) portions were placed into 140 ml glass beakers, covered with aluminium foil and heated in a water bath at 94 ± 1°C for 20 min until an internal temperature of 71°C was reached. Each of these cooked ground beef portions was further divided into three samples of 20 g each. These samples were placed in plastic cups with caps and stored under the same conditions as the raw samples. Each of the above phosphates (SHMP, STPP, TSPP, SAPP and KTPP) was individually mixed in dry form with a meat sample, at a level of 0·5% (w/w), prior to aqueous acid extraction TBA–C_{18} analysis. The TBA numbers of these raw and cooked ground beef samples were determined after 0, 4 and 8 days of storage at 4°C

Statistical analyses

The data obtained from the factorial experiments in model systems were analysed using one-way analysis of variance to evaluate the main effects from each factor and their interactions. The effects of curing ingredients (ascorbic acid and sodium nitrite) on the TBA numbers of raw and cooked ground beef were evaluated using linear regression analysis. The data of the experiments examining the possibility of interference in the TBA reaction by sodium nitrite or phosphates were analysed by comparing the absorbance or TBA numbers of treated samples with that of control samples using a *t*-test (Steel & Torrie, 1980). Each experiment in this study was replicated four times.

RESULTS AND DISCUSSION

Effects of curing agents on TBA reaction in model systems

In this study, the wavelength of maximum absorbance for the pure malonaldehyde–TBA complex was determined to be 525 nm (data not shown). Other studies on the measurement of lipid peroxidation using various versions of the TBA method have reported several different wavelengths of maximum absorbance such as 530 nm (Tarladgis *et al.*, 1964), 531 nm (Salih *et al.*, 1987), 532 nm (Tomas & Funes, 1987; Pikul *et al.*, 1989), 535 nm (Rhee *et al.*, 1984) and 538 nm (Ke *et al.*, 1984; Hoyland & Taylor, 1989). The wavelength at which the absorbance of the malonaldehyde–TBA complex is measured should be that wavelength at which a given spectrophotometer results in maximum absorbance in the 400–600 nm region. Therefore, the wavelength of 525 nm was used for spectrophotometric measurements in this study.

Each of the three factors in this experiment (sodium nitrite, ascorbic acid and sulphanilamide) and their combinations had significant ($P < 0·01$) effects on the formation of red coloured malonaldehyde–TBA complex (Fig. 1). In the absence of ascorbic acid, sodium nitrite at levels of 0·02% or higher completely inhibited the formation of red coloured malonaldehyde–TBA complex. The addition of ascorbic acid at levels of 0·04% or higher could partially overcome the inhibitory effect of 0·01 and 0·02% sodium nitrite on

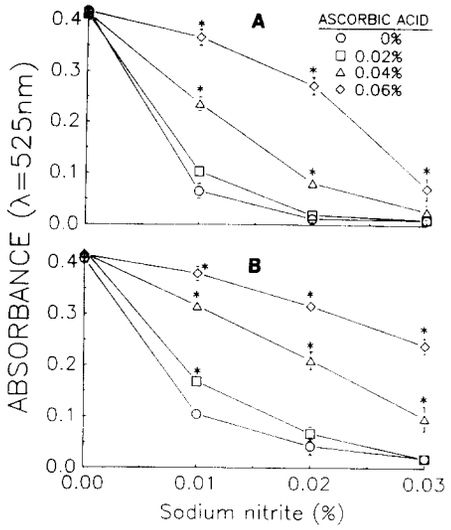

Fig. 1. Effects of ascorbic acid (0–0·06%) and sulphanilamide (**A**: 0% and **B**: 0·02%) on the formation of red coloured malonaldehyde–thiobarbituric acid complex in aqueous solutions containing sodium nitrite (0–0·03%). The asterisk (*) indicates that the value is significantly ($P < 0.05$) different from the corresponding value of 0% ascorbic acid.

the formation of red coloured adduct (Fig. 1). The results also indicated that ascorbic acid alone, up to 0·06%, did not interfere with malonaldehyde detection. Levels of sodium nitrite and ascorbic acid tested in this experiment are in the range of concentrations used in cured meat products (Hotchkiss & Cassens, 1987). The ascorbic acid added could reduce nitrite into nitrogen monoxide (Norwitz & Keliher, 1986) and this compound may be less reactive with malonaldehyde. Consequently, more malonaldehyde is available for the TBA reaction to produce red coloured malonaldehyde–TBA complex. Addition of sulphanilamide (0·02%) together with ascorbic acid (0·02–0·06%) showed a significant effect ($P < 0.01$) on overcoming the inhibitory effect of sodium nitrite on the TBA reaction. However, addition of 0·02% sulphanilamide alone did not successfully overcome the interference by sodium nitrite. Zipser and Watts (1962) suggested that the addition of sulphanilamide (0·02%) minimized the interference by nitrite. However, the sulphanilamide

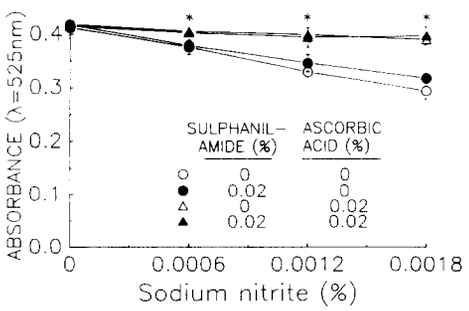

Fig. 2. Effects of ascorbic acid (0 and 0·02%), sulphanilamide (0 and 0·02%), and their combination on the formation of red coloured malonaldehyde–thiobarbituric acid complex in aqueous solutions containing sodium nitrite (0, 0·0006, 0·0012 and 0·0018%. The asterisk (*) indicates that the value is significantly ($P < 0.05$) different from the corresponding value of the control.

itself decreased the TBA number of meat samples even in the absence of nitrite (Shahidi *et al.*, 1985). The reaction of malonaldehyde and sulphanilamide to produce a 1-amino-3-iminopropene derivative was assumed to be responsible for underestimation of TBA numbers (Shahidi *et al.*, 1991).

At low levels of sodium nitrite (0·0006–0·0018%), the addition of 0·02% ascorbic acid fully suppressed the inhibitory effect exerted by nitrite on the formation of red coloured malonaldehyde–TBA complex (Fig. 2). This full suppression, however, could not be achieved by addition of sulphanilamide (0·02%) alone. This means that ascorbic acid could reverse the inhibitory effect of low levels of sodium nitrite on the malonaldehyde–TBA reaction, while sulphanilamide had no effect.

Effects of curing agents on TBA numbers of ground beef

Raw and cooked ground beef, with 0·0156% sodium nitrite, had very low levels of TBA numbers (less than 0·4) at day 0 which did not change significantly ($P > 0.05$) during storage (Fig. 3). This could indicate two possibilities. First, that sodium nitrite has strong antioxidative properties; or second, that sodium nitrite strongly interfered with the malonaldehyde–TBA reaction. These two mechanisms will be discussed later. The addition of ascorbic acid (0·05%) in combination with sodium nitrite (0·0156%) as curing ingredients did not ($P > 0.05$) change the interfering effect of nitrite in both raw and cooked ground beef. Addition of ascorbic acid (0·05%) in meat samples significantly ($P < 0.05$) lowered the TBA numbers as compared with the control, especially in cooked ground beef (Fig. 3). This confirms the well-known antioxidative activity of ascorbic acid (Dziezak, 1986; Ueda *et al.*, 1986).

Sodium nitrite (0·0156%) definitely interfered with the aqueous acid extraction TBA–C_{18} analysis on both

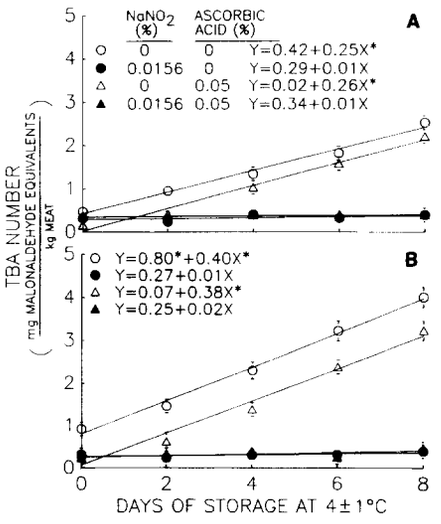

Fig. 3. Effects of ascorbic acid (0 and 0·05%), sodium nitrite (0 and 0·0156%), and their combinations on the thiobarbituric acid (TBA) numbers of raw (**A**) and cooked (**B**) ground beef during storage at 4 ± 1°C for up to 8 days. The asterisk (*) indicates that the intercept or slope is significantly ($P < 0.05$) higher than zero.

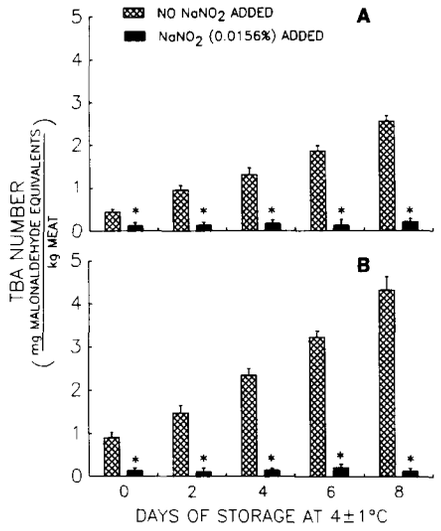

Fig. 4. Effect of sodium nitrite (0·0156%) addition prior to aqueous acid extraction thiobarbituric acid–C_{18} (TBA–C_{18}) analysis on the thiobarbituric acid (TBA) numbers of uncured raw (**A**) and cooked (**B**) ground beef which had been stored at 4 ± 1°C for up to 8 days. The asterisk (*) indicates a significant difference ($P < 0·01$) from the corresponding untreated meat sample.

raw and cooked ground beef (Fig. 4). This was indicated by constant low TBA numbers resulting from rancid meat samples analysed by the aqueous acid extraction TBA–C_{18} method with sodium nitrite (0·0156%) added prior to the analysis. On the other hand, the corresponding meat samples with no sodium nitrite added prior to the analysis had increasingly higher TBA numbers during storage at 4°C. Therefore, the antioxidative property of sodium nitrite in cured meat should be confirmed by a different methodology, such as the carotene bleaching method or gas chromatographic procedures (Zubillaga & Maerker, 1987).

Table 1. Effect of phosphates on the formation of red coloured malonaldehyde–TBA complex in aqueous solutions

Treatment	Malonaldehyde added (μM)		
	2	4	6
	Absorbance at 525 nm[a]		
No phosphate (control)	0·14 ± 0·02[b]	0·25 ± 0·03	0·36 ± 0·02
Sodium hexametaphosphate	0·15 ± 0·03	0·23 ± 0·03	0·34 ± 0·04
Sodium tripolyphosphate	0·15 ± 0·01	0·24 ± 0·02	0·34 ± 0·02
Tetrasodium pyrophosphate	0·15 ± 0·02	0·24 ± 0·01	0·33 ± 0·02
Sodium acid pyrophosphate	0·14 ± 0·01	0·24 ± 0·02	0·34 ± 0·03
Potassium tripolyphosphate	0·14 ± 0·02	0·24 ± 0·02	0·35 ± 0·02

[a] The absorbance of the malonaldehyde–thiobarbituric acid (TBA) complex was determined by the aqueous acid extraction TBA–C_{18} method. No significant difference ($P > 0·05$) was found between the control and phosphate-treated samples.
[b] Each entry in the table indicates the mean and its standard deviation from four replications.

Effects of phosphates on TBA reaction in model systems and ground beef

Phosphates, such as SHMP, STPP, TSPP, SAPP and KTPP, are approved for use in meat processing to improve water retention, particle binding and palatability of meat products. The total maximum concentration of phosphate allowed in finished cured meat products in the United States is 0·5% (Sofos, 1986). Therefore, all the phosphates used in this study were added at the maximum level of 0·5% (w/w) in both

Table 2. Effect of phosphate on TBA numbers (mg malonaldehyde equivalents/kg meat) of raw and cooked ground beef during storage at 4°C

Treatment	Days of storage at 4°C		
	0	4	8
	TBA numbers (mg malonaldehyde equivalents/kg meat)[a]		
Raw ground beef:			
Control (ground beef only)	0·47 ± 0·18[b]	1·66 ± 0·24	3·04 ± 0·35
Sodium hexametaphosphate	0·55 ± 0·16	1·50 ± 0·20	3·10 ± 0·28
Sodium tripolyphosphate	0·40 ± 0·10	1·74 ± 0·19	2·84 ± 0·26
Tetrasodium pyrophosphate	0·39 ± 0·23	1·58 ± 0·15	2·80 ± 0·22
Sodium acid pyrophosphate	0·48 ± 0·15	1·60 ± 0·08	2·98 ± 0·30
Potassium tripolyphosphate	0·56 ± 0·09	1·40 ± 0·25	3·02 ± 0·16
Cooked ground beef			
Control (ground beef only)	0·92 ± 0·12	2·70 ± 0·28	4·04 ± 0·30
Sodium hexametaphosphate	1·04 ± 0·22	2·65 ± 0·18	4·17 ± 0·26
Sodium tripolyphosphate	0·77 ± 0·17	2·58 ± 0·20	4·25 ± 0·38
Tetrasodium pyrophosphate	0·90 ± 0·11	2·85 ± 0·16	3·94 ± 0·24
Sodium acid pyrophosphate	1·10 ± 0·15	2·60 ± 0·26	3·80 ± 0·32
Potassium tripolyphosphate	0·88 ± 0·10	2·62 ± 0·20	4·10 ± 0·18

[a] The TBA numbers of the meat samples were determined by the aqueous acid extraction TBA–C_{18} method. No significant difference ($P > 0·05$) of TBA numbers was found between phosphate-treated ground beef and its corresponding control. The phosphates (0·5%) were added to the meat immediately before the TBA test was performed.
[b] Each entry in the table indicates the mean and its standard deviation from four replications.

model systems and ground beef. These phosphates were added immediately before the aqueous acid extraction TBA–C_{18} method was performed on raw or cooked ground beef. The results of this study indicated that all of the phosphates tested in aqueous solutions did not significantly ($P > 0.05$) interfere with the malonaldehyde–TBA reaction (Table 1). Similar results were also found in both raw and cooked ground beef (Table 2). Since the objective of this particular experiment was to evaluate the possibility of phosphate interference with the TBA reaction, the potential antioxidative effects of phosphates were not examined. The findings of this study are not in agreement with the conclusion of Molins *et al.* (1987) who indicated that phosphates may have interfered with the distillation TBA method. Their study, however, was not designed to evaluate the potential interference of phosphates with the TBA test. It should also be mentioned that the study of Molins *et al.* (1987) evaluated frozen beef patties. Witte *et al.* (1970) found that the distillation and unmodified aqueous acid extraction TBA tests were of limited use for frozen meat samples. Therefore, the low TBA numbers in phosphate-treated patties could have been caused by the frozen state of the meat samples.

CONCLUSIONS

The extent of sodium nitrite interference with the formation of the malonaldehyde–TBA complex in aqueous solutions was affected by the presence of ascorbic acid and sulphanilamide. It was confirmed that sodium nitrite significantly ($P < 0.05$) decreased the formation of red coloured malonaldehyde–TBA complex and that addition of sulphanilamide or ascorbic acid did not successfully overcome this problem. Therefore, the aqueous acid extraction TBA–C_{18} method does not eliminate the problem of existing TBA tests in not being able to overcome the interference of nitrite with the malonaldehyde–TBA reaction. All the phosphates tested in this study did not interfere with the formation of the malonaldehyde–TBA complex in both aqueous solutions and ground beef. Therefore, the aqueous acid extraction TBA–C_{18} method can be used for malonaldehyde measurement in meat product containing phosphates.

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