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Measurement of malonaldehyde in apple juice using GC–MS and a comparison to the thiobarbituric acid assay

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Abstract

Malonaldehyde (MA) in gamma-irradiated apple juice was measured using a GC–MS method and in comparison to the thiobarbituric acid (TBA) assay. The profiles of MA content as a function of radiation dose were similar using both methods, however, MA content was higher when measured with the TBA assay compared to the GC–MS method. The overestimation of MA using the TBA assay increased as the amount of MA decreased. Use of the GC–MS method demonstrated MA content in irradiated juice declined rapidly during storage at 5 °C. MA content appeared to decrease much less when the TBA assay was used. The GC–MS method also allowed measurement of formaldehyde and acetaldehyde in addition to MA. Published by Elsevier Science Ltd.

Keywords: GC–MS; Irradiation; Juice; Malonaldehyde; Thiobarbituric acid assay

1. Introduction

Malonaldehyde (MA) has been extensively studied in biological and medical sciences due to its reactivity with biological macromolecules and possible connection to cancer and other diseases (Janero, 1990). MA is also widely applied as an index of lipid oxidation and rancidity of various foods, especially meats and meat products (Guillen-Sans & Guzman-Chozas, 1998; Raharjo & Sofos, 1993). The traditional method for MA determination has been the thiobarbituric acid (TBA) assay owing to its simplicity. In this method, MA reacts with TBA via an acid-catalyzed nucleophilic-addition reaction yielding a pinkish-red chromophore with an absorbance maximum at 532 nm. However, TBA is not specific to MA, and many other compounds may give colored products that also absorb at 532 nm. Simple carbohydrates and pigments are present in many foods of plant origin, such as juice, and these compounds are known to interfere with the TBA assay (Baumgartner, Baker, Hill, & Wright, 1975). To avoid the interferences conferred by carbohydrates and pigments, improved methods have been developed (Du & Bramlage, 1992; Hodges, DeLong, Forney, & Prange, 1999). However, heating (95 °C) and the strong acidity used in these and all TBA-based methods may increase oxidation and MA formation (Yeo, Helbock, Chyu, & Ames, 1994). Many HPLC and GC based methods have been used for more accurate and sensitive analysis (Bird, Silas, Hung, Hadley, & Draper, 1983; Botsoglou, Fletouris, Papageorgiou, Vassilopoulos, Mantis, & Trakatellis, 1994; Li & Chow, 1994; Raharjo, Sofoa, & Schmidt, 1992; Spanier & Trayer, 1991). Most of these methods, however, were still based on color reaction of MA with TBA under elevated temperature and high acidity.

New derivatization methods have been developed using more reactive compounds, such as pentafluorophenylhydrazine (PFPH) or 2,4-dinitrophenylhydrazine without the strong acidity and heating (Tomita, Okuyama, Hatta, & Kawai, 1990; Wong, Yeo, & Shibamoto, 1991; Yeo, Liu, Helbock, & Ames, 1999). These derivatives could be separated by GC–MS or HPLC. These methods have been used to assay MA in urine, sperm and blood samples (Tomita et al., 1990; Wong et al., 1991; Yeo et al., 1999). In the present report, the GC–MS method (Tomita et al., 1990; Yeo et al., 1999) was modified for analysis of MA in apple juice, a carbohydrate-rich food and compared with the TBA assay. The GC–MS method was also able to simultaneously measure acetaldehyde (ACT) and formaldehyde (FA), two compounds possibly important in juice flavor.

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2. Materials and methods

2.1. Chemicals and samples

2,6-Di-tert-butyl-4-methyphenol (BHT), malonaldehyde bis(diethyl acetal) (TEP), pentafluorophenylhydrazine (PFPH) and trichloroacetic acid were purchased from Aldrich[1] (Milwaukee, WI). Pasteurized juice was purchased from a local supermarket while fresh apple juice was prepared from 'Fuji' apples (*Malus* × *domestica* Borkh) using a Champion MAR-48C juicer (Plastaket Manufacturing Co., Lodi, CA).

2.2. Preparation of MA standard solution

The hydrolysis of TEP to produce MA was by the method of Csallany, Der, Manwaring, and Addis (1984) with modification. TEP (0.1 μmol) was dissolved in 10 ml of 0.01 N HCl and incubated at 50 °C for 90 min. The concentration of the standard solution was determined by measuring absorbance at 245 nm ($\varepsilon = 13{,}700$).

2.3. Measurement of MA using the TBA-based assay

The method of Hodges et al. (1999) was followed. Briefly, after proper dilution, apple juice was added to a test tube containing 1.6 ml of either (1)−TBA solution: 20% (w/v) trichloroacetic acid and 0.01% butylated hydroxytoluene or (2) +TBA solution: containing the above plus 0.65% TBA. Samples were heated at 95 °C in a water bath for 25 min, cooled and centrifuged at 1300 ***g*** for 10 min at 5 °C. Absorbance at 440, 532, and 600 nm was then measured. MDA was calculated using the formulas developed by Hodges et al. (1999).

$$[(\mathrm{Abs532}_{+\mathrm{TBA}} - \mathrm{Abs600}_{+\mathrm{TBA}}) - (\mathrm{Abs532}_{-\mathrm{TBA}} - \mathrm{Abs600}_{-\mathrm{TBA}})] = A. \quad (1)$$

$$[(\mathrm{Abs440}_{+\mathrm{TBA}} - \mathrm{Abs600}_{+\mathrm{TBA}})0.0571] + B. \quad (2)$$

$$\mathrm{MDA}\left(\mathrm{nmol\ \ ml^{-1}}\right) = [(A - B)/157000]10^6. \quad (3)$$

2.4. Standard procedures for measurement of MA, FA and ACT using the GC–MS method

Juice (250 μl) and 150 μl 0.5 M KH_2PO_4 buffer (pH 3.0) were added to a 2-ml Eppendorf tube. BHT (20 μM final concentration) was also added to prevent oxidation, then 20 μl of 5 mg ml^{-1} PFPH was added to derivatize MA and other carbonyl compounds. After vortexing, the mixture was incubated at ambient (23 °C) temperature for 30 min, then 250 μl hexane was added. After votexing for 30 s, a 2 μl aliquot of the hexane layer was injected into a Hewlett-Packard 5890/5971 GC–MSD (Agilent Technologies, Palo Alto, CA) equipped with a HP-5 trace analysis column (30 m 0.32 mm i.d., 0.25 μm film thickness). The GC oven temperature was held at 50 °C for 1 min, increased at 20 °C min^{-1} to 280 °C, then held for 1 min. The temperatures of the injector and transfer line were 250 and 280 °C, respectively. The electron multiplier voltage was raised by 506 V between 5 and 7 min to increase sensitivity. Helium was the carrier gas at a linear flow rate of 20.7 cm^{-1}. Amounts of MA, FA and ACT were calculated from standard curves.

2.5. Standard curves

Various amounts of MA, FA or ACT were added to non-irradiated pasteurized juice or fresh juice. MA in the samples was then measured using the standard procedures of GC–MS method. For fresh juice, after incubation at ambient temperature for 30 min (for derivatization), the mixture was centrifuged at 1300 ***g*** for 10 min, and then the derivatized MA, FA and ACT in the supernatant were partitioned into hexane, and analyzed by GC–MS.

2.6. Optimization of PFPH concentration

After addition of BHT and phosphate buffer, different amounts of PFPH (5 mg ml^{-1}) were added to apple juice spiked with MA (4 nmol ml^{-1}). The mixture was incubated at ambient temperature for 30 min, then the derivatives were partitioned into hexane and analyzed by GC–MS.

2.7. Effect of incubation duration

Stability of the MA-spiked juice was determined using the standard procedures of GC–MS method except that various incubation durations for the PFPH derivatization were used.

2.8. Effect of pH

The pH of mixtures containing 5 ml MA-spiked juice plus 3 ml 0.5 M NaH_2PO_4 was adjusted in 0.5 increments from 1.5 to 6.5 using 1 N NaOH or 1 N HCl. The final volume of the mixture was adjusted to 10 ml with deionized water. MA in the solution was measured using the GC–MS method.

2.9. Stability of MA

The pH of MA-spiked juice was adjusted as mentioned above. The mixtures were incubated at ambient

[1] Mention of brand or firm names does not constitute an endorsement by the US Department of Agriculture above others of a similar nature not mentioned.

temperature for 6 h before MA in the mixture was analyzed using the standard procedures.

2.10. Effect of irradiation and thermal processing

Juice in 5-ml glass vials sealed with Teflon-lined septa and screw caps was irradiated at 5 °C (Fan & Gates, 2001). For pasteurization, glass vials (5 ml) containing fresh juice were submerged in 95 °C water for 90 s, and then the juice was cooled by placing the vials into ice water.

3. Results and discussion

A typical gas chromatogram of PFPH derivatives in juice irradiated to 4.7 kGy shows the retention time of MA and FA derivatives were 5.31, and 5.65 min respectively (Fig. 1). The ACT derivatives exhibited two closely separated peaks with retention times of 5.90 and 5.97 min, suggesting that there were two isomers (*syn* and *anti*; Shara, Dickson, Dagchi, & Stohs, 1992).

The mass spectra of PFPH derivatives in juice irradiated with 4.7 kGy showed molecular ions of 234, 210, and 224 for MA, FA and ACT, respectively (Fig. 2). The same molecular ions were observed for PFPH derivatives of MA, FA and ACT standards.

A series of experiments were conducted to find out the suitable conditions for the derivatization reaction in apple juice. The amount of PFPH required for the derivatization of MA in MA-spiked pasteurized juice was at least 10 μl 5 mg mL^{-1} (Fig. 3A). This amount was 4 times of that required for medical samples (Yeo et al., 1994, 1999), and may reflect the presence of relatively high concentrations of PFPH-reactive compounds in apple juice, such as ACT and FA (see below). Based on this result, 20 μl PFPH (5 mg ml^{-1}) was used in the standard method to ensure complete derivatization.

The minimum incubation time for the derivatization process at ambient temperature was 15 min, and the optimum pH was 3–4 (Fig. 3B, C). Earlier studies have

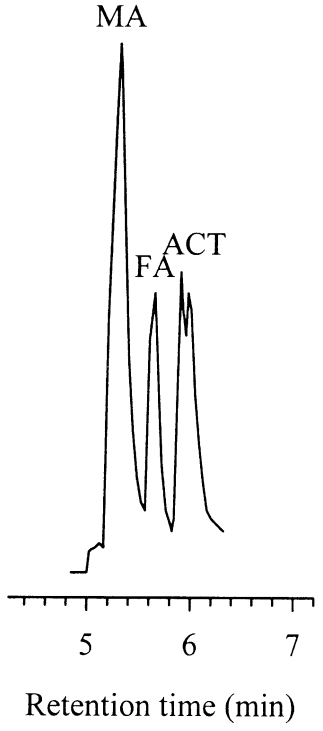

Fig. 1. Chromatogram of PFPH derivatives of malonaldehyde (MA), formaldehyde (FA) and acetaldehyde (ACT) in juice irradiated to an absorbed dose of 4.7 kGy.

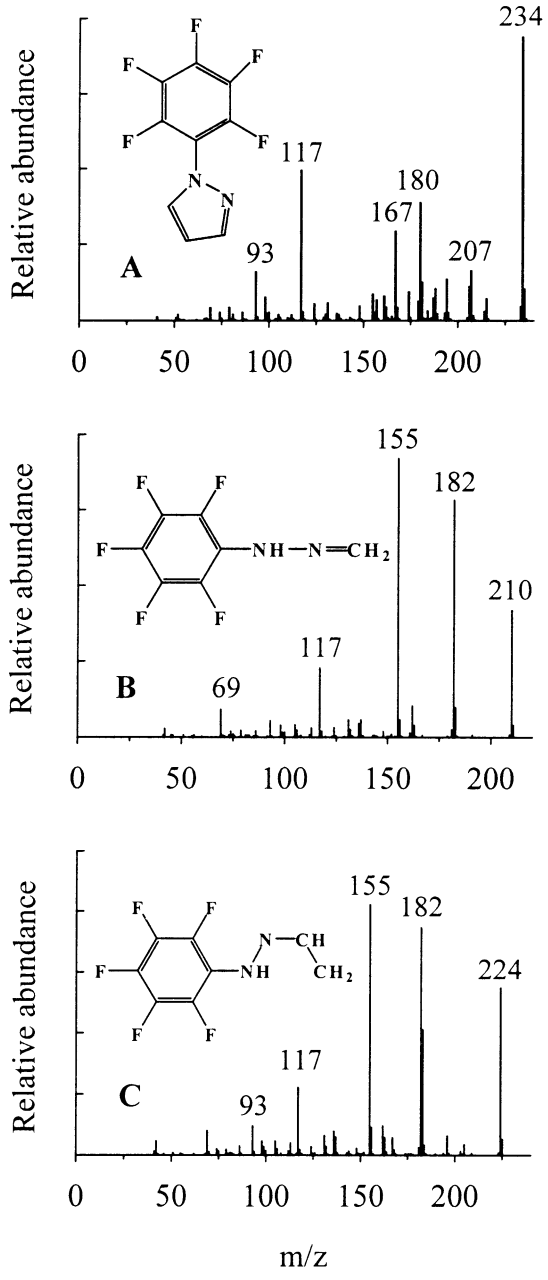

Fig. 2. Mass spectrum of PFPH derivative of malonaldehyde (A), formaldehyde (B) and acetaldehyde (C) in irradiated juice.

suggested that the optimum pH range for derivatization is 4–5 (Tomita et al., 1990; Yeo et al., 1994) and that, under these conditions, derivatization reaches a maximum between 30 and 120 min and then decreases (Tomita et al., 1990). At ambient temperature, MA was most stable at pH 3.0 (Fig. 4). The longer incubation time used in the earlier report (Tomita et al., 1990) may contribute to the degradation of MA and MA-PFPH at pH 4–5.

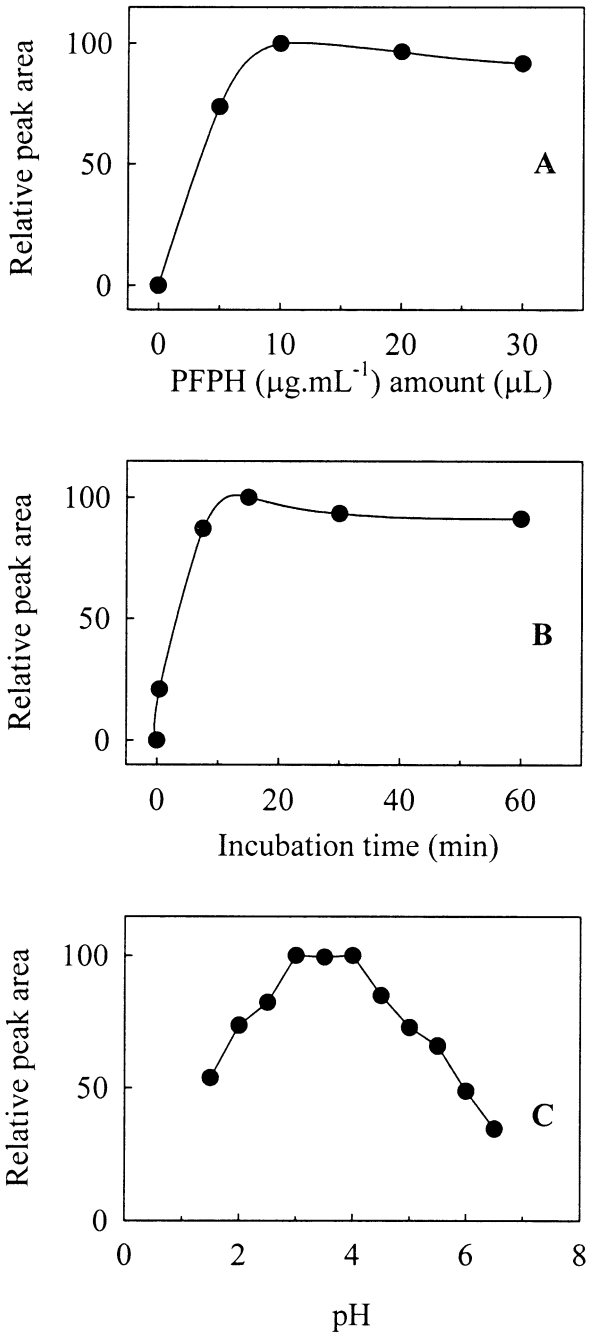

Fig. 3. Amount of PFPH (A), incubation time (B) and pH (C) effect on the condensation reaction of malonaldehyde with PFPH.

Previous studies measuring MA in sperm and liver samples used an addition of concentrated H_2SO_4 to the reaction mixture to liberate bound MA from protein (Yeo et al., 1994, 1999). We found that addition of H_2SO_4 did not have any effect on the amount of total MA in juice (data not shown), possibly due to the low pH and relatively low amount of protein present in juice.

Standard curves of MA were linear when MA was spiked into either pasteurized, fresh, or irradiated juice in the range from 0 to 600 ng ml^{-1}, and the slopes of these curves were similar (data not shown). This suggests that chemical changes induced by pasteurization or irradiation do not affect the conversion of MA to MA-PFPH or the partition of MA-PFPH into hexane.

Irradiation increased the MA concentration measured using both analysis methods, and the profiles of MA concentration as the function of radiation dose were very similar (Fig. 5). Both showed a slow increase in the low dose range (0–2.7 kGy), and a sharp increase in the high dose range (2.7–8.9 kGy). However, the amount of MA was much higher using the TBA assay compared to the GC–MS method, and the overestimation decreased with the increase in MA concentration (Fig. 6). The TBA method was specifically developed for analysis of MA in carbohydrate-rich plant originated foods (Du & Bramlage, 1992; Hodges et al., 1999), the interference of carbohydrates and pigments has been considered and subtracted. Our results suggest that other interfering compounds in the juice samples may be present which may contribute to the overestimation.

During storage, MA in irradiated juice decreased when assayed using the GC–MS method but the decrease was less using the TBA assay (Fig. 7). After 2 weeks storage, MA assayed with the GC–MS declined by 89.3% but only 41.5% by the TBA method. After an additional 2 weeks at 5 °C, MA measured with GC–MS continued to decline when measured using GC–MS but

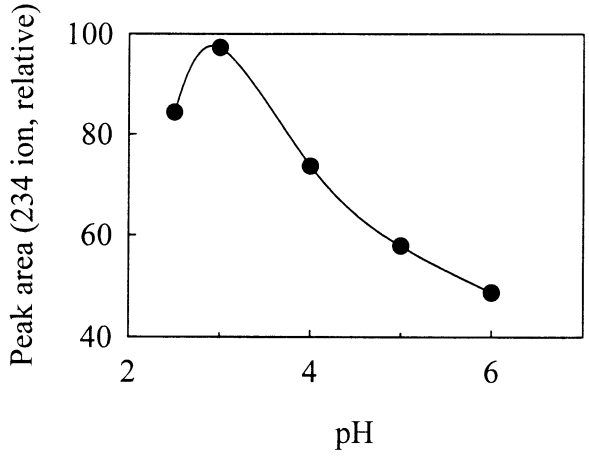

Fig. 4. Effect of pH on the stability of malonaldehyde in pasteurized juice.

not if the TBA assay was used. The poor performance of the TBA assay under these conditions may be due to interference from other compounds, including FA and ACT. These two compounds are very stable and do not change much during storage (Fan & Thayer, 2001). Our results suggest that although MA measured by the TBA assay can reveal the increase in MA due to irradiation, the amounts were seriously overestimated, and the TBA assay did not accurately reveal the change in MA levels that occurs during storage.

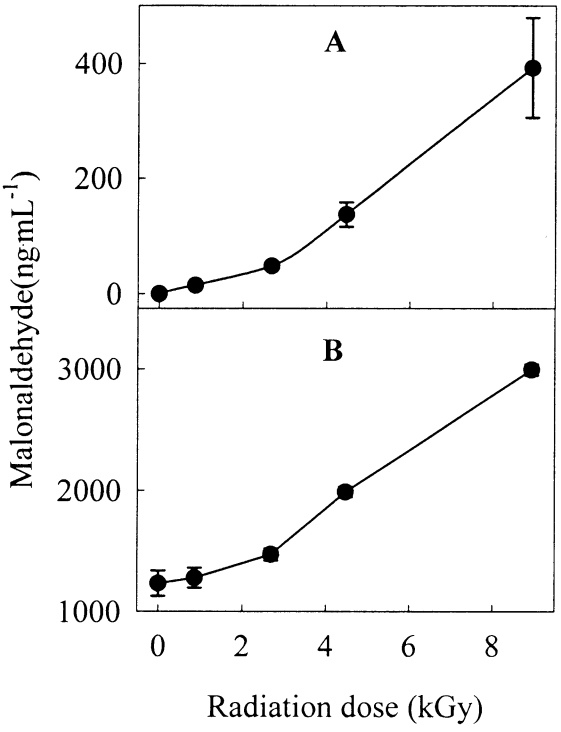

Fig. 5. Effect of radiation dose on juice malonaldehyde (MA) levels. MA was measured using either the GC–MS method (A) or the TBA assay (B).

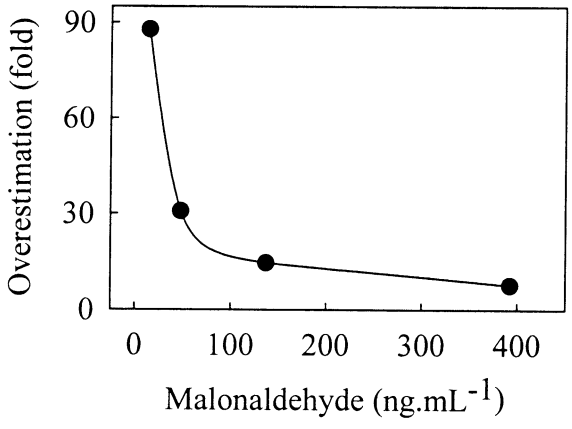

Fig. 6. Comparison of GC–MS and the TBA method for the determination of MA. Overestimation was calculated by dividing MA levels measured using the TBA assay by those measured using the GC–MS method.

Thermal processing increases MA concentration (18.3 ± 4.4 ng ml^{-1} for fresh juice vs 32.4 ± 2.5 ng ml^{-1} for the thermally processed juice) measured using the GC–MS method. Many earlier results have shown that cooking increases MA content when measured using the TBA assay (Newburg & Concon, 1980; Siu & Draper, 1978). Fresh juice contained much higher MA (18.3 ± 4.4 ng ml^{-1}) than the pasteurized juice (0.7 ± 0.6 ng ml^{-1}) purchased from a local supermarket. The low MA levels in the purchased juice may be due to the decrease in MA levels during transportation, storage and/or display. Our results also show that thermally processed juice has much lower MA levels than irradiated juice. This may be due, in part, to the instability of MA at high temperature. During thermal processing, MA is produced due to the increased oxidation of lipid and carbohydrates, but MA is not stable, it degrades or binds to other compounds at high temperatures (Aubourg, 1993) while irradiation was conducted at 5 °C where the reactivity of MA is lower.

The GC–MS method was also capable of analyzing FA and ACT in apple juice. An earlier GC–MS method could not separate FA from MA (Shara et al., 1992). The concentrations of FA and ACT in our samples were in the linear range of standard curves (Fig. 8), and irradiation increases FA and ACT formation (Fan & Thayer, 2001).

Our results show that MA levels in apple juice estimated by the TBA assay were higher than those measured using the GC–MS method, suggesting that the

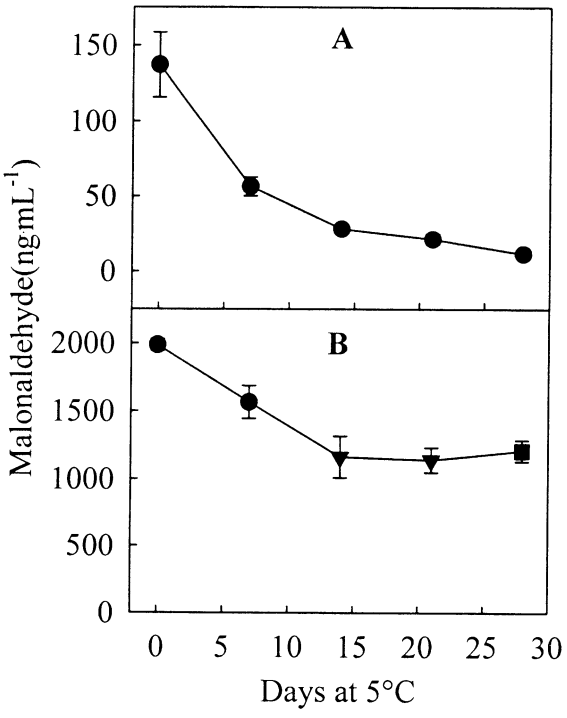

Fig. 7. Changes in MA levels of irradiated juice during storage at 5 °C. MA was measured using the GC–MS method (A) or the TBA assay (B).

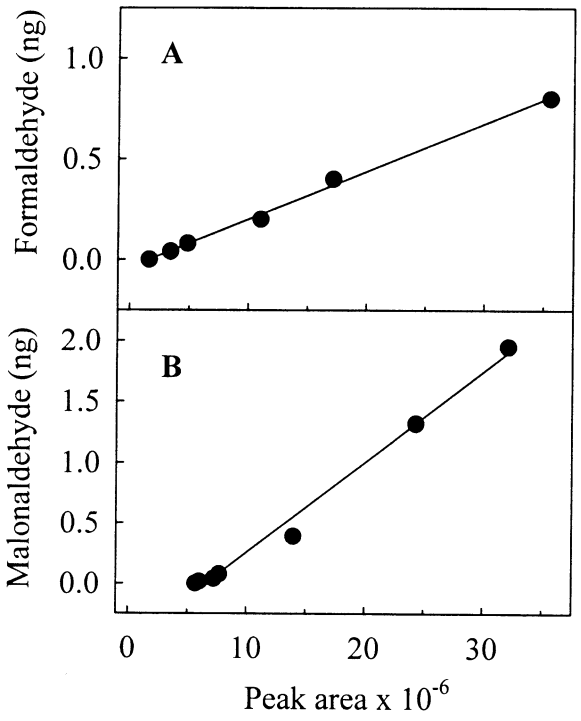

Fig. 8. Standard curves of formaldehyde (A) and acetaldehyde (B) in pasteurized apple juice.

acid-heating conditions of the TBA assay may promote artificial formation of MA, or the interfering compounds may be present in juice samples. Besides carbohydrates and pigments, there are many other compounds which may interfere in the TBA assay, including aldehydes (Careche & Tejada, 1988; Guillen-Sans & Guzman-Chozas, 1998; Guzman-Chozas, Vicario, & Guillen-Sans, 1997). MA reacts with TBA to form a pink chromophore with maximal absorbance at 532 nm. Aldehydes other than MA may also react with TBA to form yellow, orange or pink compounds (Kosugi, Keto, & Kikugawa, 1987; Guzman-Chozas et al., 1997; Sun et al., 2001) depending on incubation temperature and duration. Apple juice contains a much higher concentration of FA and ACT compared to MA (Fan & Thayer, 2001). At similar concentrations, most aldehydes are not as reactive as MA in term of the pink pigment formation (Guzman-Chozas et al., 1997), but the presence of high amounts of aldehydes in juice may contribute significantly to the TBA assay, especially at low MA levels.

FA and ACT are naturally occurring compounds in many fruits and vegetables. Concentration of these aldehydes changes during maturation and development. ACT is an intermediate product in the respiration of higher plants, an intermediate product of alcohol fermentation and a sugar metabolite (Davies, 1980). The widespread presence of these aldehydes suggests that MA measured by the TBA assay is not a reliable index for lipid oxidation in foods of plant origin. Cherif, Nodet, and Hagege (1996) show a strong discrepancy between TBA assay as a lipid oxidation and other indices, and concluded that TBA assay for lipid oxidation in plant tissues can lead to misinterpretations, and requires extreme caution, discretion, and correlative data from other indices.

In summary, a GC–MS method was developed for measurement of MA in apple juice. The TBA assay seriously overestimated MA levels in apple juice, compared to amounts measured using the GC–MS method. The GC–MS method can be used to simultaneously determine MA, FA, and ACT in apple juice and perhaps other carbohydrate-rich foods.

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