Determination of TBA Number by High Performance Liquid Chromatography

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ABSTRACT

A high performance liquid chromatographic (HPLC) method for the quantitation of malonaldehyde in aqueous distillates was developed. Compared with the standard TBA test, the HPLC method was faster, and less affected by side reactions. A total of 5 min was necessary to assay each distillate and only malonaldehyde was detected. The standard curves were reproducible and standards were stable for up to 6 days. The HPLC method could detect malonaldehyde levels ranging from 1×10^{-11} to 4×10^{-11} mol/10 μL and either peak height or peak area could be used to quantitate the malonaldehyde concentration. The coefficient of determination between absorbance values determined by the TBA test and peak heights determined by HPLC was 0.946. Twenty-one freeze-dried chicken samples with TBA numbers ranging from 3.93 to 16.6 were used for this correlation.

INTRODUCTION

One of the commonly used methods for assessing the stability or rancidity of fats or fatty foods has been the thiobarbituric acid (TBA) test. The extent of oxidative rancidity is usually expressed in terms of TBA number (mg malonaldehyde/kg sample) by comparing the optical density of the TBA-malonaldehyde colored complex with that of standards prepared from 1,1,3,3-tetraethoxypropane (TEP). In acid solution, the acetal is quantitatively hydrolyzed to malonaldehyde.

Sinnhuber et al. (1) established that the colored complex responsible for the absorbance maximum at 532-535 nm was due to the condensation of two molecules of TBA with one molecule of malonaldehyde. Their findings were later confirmed by Schmidt (2). Malonaldehyde is produced during the autoxidation of polyunsaturated fatty acids and is a highly reactive dicarbonyl. Because of its reactivity, most of the malonaldehyde present in fatty foods exists bound to other food constituents and very little of it exists in the free form (3,4).

The TBA test has been performed in various ways, but the two most common are: (a) the TBA reagent in strong acid is added to the food product and the whole mixture heated in a water bath until maximal color is developed. The color complex is extracted with a suitable solvent and measured spectrophotometrically (5-7); and (b) the food product is first steam-distilled from an acid solution and a portion of the distillate is mixed with the TBA reagent. This mixture is heated and the resulting color complex directly measured in a spectrophotometer (8-10). Both methods require the presence of acid (pH 0.9-1.5) to liberate malonaldehyde from some precursors (11,12) as well as catalyze the condensation of malonaldehyde with the TBA reagent.

The advantages of the distillation method are numerous: the distillate is obtained as a clear aqueous solution obviating the extraction step; it is distilled rapidly, minimizing oxidation during analysis; it only contains the steam-volatile constituents which reduce the possibility of interfering compounds reacting with the TBA reagent and finally, the TBA reagent is diluted by half when mixed with the distillate, again lessening the possibility of side reactions occurring during the color-forming step.

Although the distillation procedure is preferable to the extraction method for many food products, it still requires the formation of the colored complex for the final determination of the TBA number. There are reports in the literature (13,14) indicating that the TBA reagent can react with a variety of compounds present in oxidized foods other than malonaldehyde and that impurities in the reagents themselves can also lead to the production of interfering colored products.

Because of these drawbacks with the colorimetric determination of malonaldehyde, work was undertaken to develop a high performance liquid chromatographic method for the direct quantitation of malonaldehyde in food products.

MATERIALS AND METHODS

Reagents

(a) TBA solution: 0.02 M 2-thiobarbituric acid (Eastman Organic Chemicals) in 90% glacial acetic acid. (b) TMP solution: 1,1,3,3-tetramethoxypropane, bp 178-179 (Fisher Scientific). (c) Freeze-dried chicken meat: obtained commercially. (d) Mobile phase: acetonitrile (850 mL, Fisher HPLC grade) mixed with 150 mL 1% acetic acid (Fisher reagent grade) and filtered through 0.45 μm Fluoropore filters (Millipore Inc.).

Analytical System

(a) Chromatograph: Waters Associates Model ALC/GPC 204 liquid chromatograph equipped with Model 440 UV absorbance detector, U6K septumless injector and Model 6000A pump. (b) HPLC column: μ-Bondapak C-18, 4 mm x 30 cm (Waters Associates). (c) Recorder and integrator: Omniscribe recorder (Houston Instruments) and Central Processor Model SP4000, Data Interface, Model SP40402 and Printer/Plotter Model SP4050 (Spectra Physics). (d) HPLC operating conditions: the eluate was monitored at 254 nm with a flow rate of 2.5 mL/min and sensitivity of 0.02 AUFS (absorbance units full scale). All injection volumes were 10 μL unless indicated otherwise. The experiments were conducted at ambient temperature and the retention time for malonaldehyde was 1.4 min. Typical chromatograms for the malonaldehyde standard and a chicken meat sample are shown in Figure 1.

Preparation of Malonaldehyde Standards

(a) HPLC: 10 μL of TMP solution was accurately diluted to 10 mL with 0.1 N HCl. This solution was decanted into a screw-capped test tube and immersed into a boiling water bath for 5 min, then quickly cooled in tap water. A working stock solution of malonaldehyde was prepared by pipetting 1.0 mL of the hydrolyzed acetal into a 100-mL volumetric flask and diluting to volume with water. The working stock solution was 6.07×10^{-5} M acetal or 4.37 μg/mL malonaldehyde. A 1:10 dilution of the working stock solution was made before preparing the actual standard curves. (b) TBA: two standard solutions of TMP (1×10^{-5} M and 1×10^{-4} M) were prepared in water.

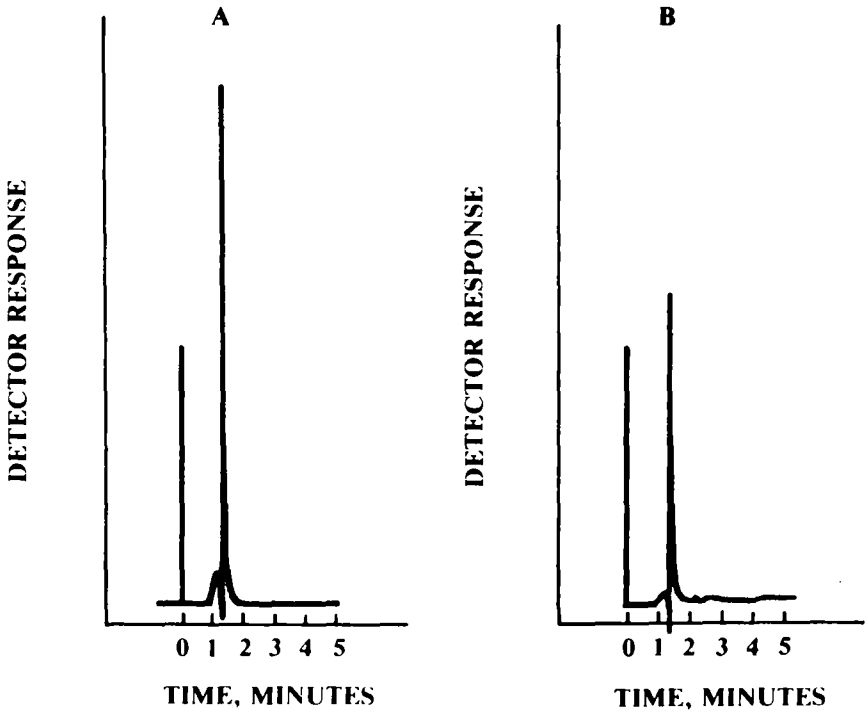

FIG. 1. High performance liquid chromatograms showing (A) the malonaldehyde standard and (B) the steam distillate from chicken meat.

TBA Test

The TBA test and distillation procedure described next were based on the method of Tarladgis et al. (8). Five-mL samples were mixed with 5 mL of TBA reagent. These mixtures were heated for exactly 30 min in a boiling water bath. The colored solutions were cooled in tap water and the absorbance measured at 535 nm.

Distillation of Meat Samples

Approximately 3 g of freeze-dried chicken meat was accurately weighed and slurried in a beaker with 80 mL of water. The pH was adjusted to 1.5 with 4 N HCl and the contents of the beaker poured into an 800-mL Kjeldahl flask. An additional 20 mL of water was used to rinse out the beaker. A small amount of Dow Antifoam A was applied to the neck of the flask and a few boiling chips added to prevent bumping. These flasks were connected to a standard macroKjeldahl unit and distilled. The distillation was conducted as quickly as possible using the maximal heater setting and terminated when 50 mL of distillate was collected in a 50-mL volumetric flask (ca. 10 min). The distillate was mixed well and 5 mL was used for the TBA test or 10 μL was used for HPLC analysis.

RESULTS AND DISCUSSION

Reproducibility and Stability of the Standard

Standards were prepared in duplicate and immediately assayed by HPLC. On two succeeding days, new standard solutions were prepared and assayed. After analysis, the standards were stored at 4 C for 6 days and then the standards were reanalyzed by HPLC. Both peak height and peak area were measured.

Figure 2 shows a plot of malonaldehyde concentration vs peak height for standards assayed on day zero. A total of 36 observations is plotted (6 levels in duplicate on 3 separate days). The slope, intercept and r^2 values were 4.01×10^8, 0.006×10^{-2} and 0.998, respectively. After 6 days of storage, the analysis was repeated on the same standards. The results for slope, intercept and r^2 were 4.28×10^8, 0.0014×10^{-2} and 0.997, respectively. There was no difference between the two slope values at the 5% level of significance.

A similar plot of malonaldehyde concentration vs peak area gave the following results. The slope, intercept and r^2 were 1.50×10^{15}, 0.26×10^4 and 0.996 for day zero, respectively. After 6 days of storage at 4 C, the results for slope, intercept and r^2 were 1.54×10^{15}, 0.285×10^4 and 0.995, respectively. As with peak height, the results showed no significant differences. The preparation of the standard malonaldehyde was very reproducible on a day-to-day basis and if stored at 4 C for up to 6 days, little decomposition occurred. Either peak height or peak area can be used to quantitate malonaldehyde over the concentration range studied.

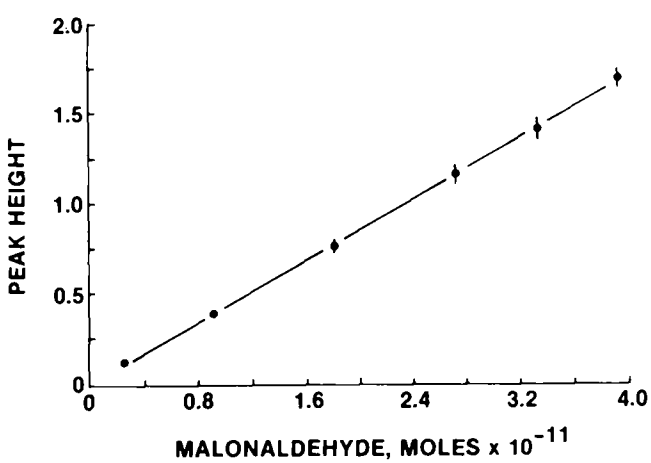

FIG. 2. HPLC standard curve (peak height vs concentration) based on 36 injections of 6 concentrations on 3 separate days. The vertical bars represent one standard deviation.

TABLE I

Recovery Values for Malonaldehyde

TMP ($M \times 10^{-8}$)	Absorbance at 535 nm: Without distillation	Absorbance at 535 nm: After distillation	(%)
1	0.101	0.086	85.1
2	0.200	0.149	74.5
3	0.308	0.214	69.4
4	0.407	0.290	71.2
5	0.517	0.340	65.0

TABLE II

Absorbance Ratios for Standard Malonaldehyde and Distilled Chicken Meat Samples

Sample	Peak height[a]: 254 nm	Peak height[a]: 280 nm	254/280
Standard malonaldehyde	2.98	0.15	19.0
	4.76	0.24	19.8
	8.91	0.45	19.8
Distilled chicken meat	2.38	0.12	19.8
	2.00	0.10	20.0

[a]40 μL sample volume, 0.05 AUFS sensitivity.

Recoveries

The recoveries for the distillation procedure were determined by adding TMP standards (1-5 mL of 1×10^{-4} M) to distillation flasks containing the appropriate amounts of acid and water and distilling the solution as described previously. The absorbance reading (TBA test) on 5 mL of the distillate was compared to 1-5 mL of 1×10^{-5} M TMP standards added directly to the TBA reagent. The TBA assay using both methods (with and without distillation) gave a linear response when concentration vs absorbance at 535 nm was plotted (Table I). The r^2 was 0.999 for the TMP standard prepared without distillation and 0.997 for the distilled standard. The overall recovery for the distillation procedure was 73.2% which is close to the value reported by Tarladgis et al. (8).

Peak Identification

A check on the purity and identity of the chromatographic peak obtained from the chicken samples was determined by performing an absorbance ratio test. The peak heights at 254 and 280 nm were measured simultaneously on a single sample by using both channels on the 440 detector in series. Forty μL of standard malonaldehyde (3 concentrations) and the distillate from two chicken samples were injected into the HPLC and the absorbance ratio 254/280 determined.

Table II shows the results for the standards and samples. The average ratio was 19.9 for both the standards and the samples, indicating that no major UV absorbing compounds were coeluting with the malonaldehyde.

TBA vs HPLC

Freeze-dried chicken meat samples were made rancid by incubating small portions of the meat wrapped in aluminum foil for 1 to 3 days at 37 C. Twenty-one samples were prepared in this manner with TBA numbers ranging from 3.93 to 16.6. These samples were tested for malonaldehyde levels by the TBA and HPLC assay procedures.

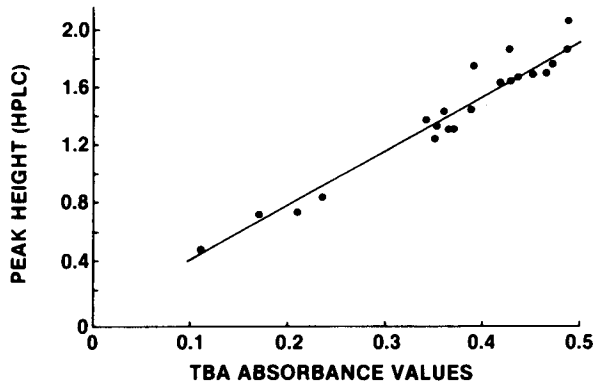

FIG. 3. Correlation between absorbance values determined by the standard TBA test and peak height HPLC measurements for freeze-dried chicken meat.

A plot of peak height (HPLC) vs absorbance (TBA test) was linear with an r^2 of 0.946 (Fig. 3). In terms of sensitivity, the TBA test could detect 1×10^{-8} mol of malonaldehyde/5 mL whereas the HPLC method was measuring levels that ranged from 1×10^{-11} to 4×10^{-11} mol of malonaldehyde/10 μL. The HPLC method has a number of important advantages over the standard TBA method. The HPLC procedure was faster because the 30-min incubation time was no longer needed. A total of 5 min/injection was required for the routine analysis for malonaldehyde by HPLC. The results were not affected by the presence of other color-forming constituents or side reactions as they might be with the TBA test. Finally, the levels of malonaldehyde can be more accurately determined as the results do not depend on the formation of a colored complex.

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