Journal of Agricultural and Food Chemistry

OCTOBER 1997
VOLUME 45, NUMBER 10

Effect of Dietary Thyme on the Oxidative Stability of Egg Yolk

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The effect of dietary thyme on the oxidative stability of shell eggs over a 60-day refrigerated storage has been evaluated. Also, the influence of dietary thyme and storage time on the oxidative stability of liquid yolks adjusted to various pH values and agitated in the absence or presence of light has been investigated. Yolk lipid oxidation was determined by monitoring malonaldehyde formation through use of third-order derivative spectrophotometry. Results showed that although malonaldehyde is not produced during storage of shell eggs, it is present in the yolk of fresh eggs. It is also evident that thyme treatment reduced oxidation of liquid yolk, which significantly increased with light and increased acidity at pH 3 and declined thereafter as acidity increased to pH 2. A comparative examination of the antioxidant activity of various synthetic and natural antioxidants added to yolk suggested that thymol, the main antioxidant constituent of thyme, cannot be considered totally responsible for the oxidative resistance of the thyme-treated yolk. There may be additional thyme components with antioxidant activity that pass into egg yolk providing antioxidant properties.

Keywords: *Dietary thyme; yolk stability; lipid oxidation; egg storage; malonaldehyde*

INTRODUCTION

Lipid oxidation during food processing and storage is of major importance. As the polyunsaturated lipids oxidize, they form hydroperoxides, which are susceptible to further oxidation or decomposition to secondary reaction products such as short-chain aldehydes, ketones, and other oxygenated compounds that may adversely affect flavor, taste, nutritional value, and overall quality of foods (Vercellotti et al., 1992). Lipid oxidation during food storage is influenced by several parameters such as storage temperature, oxygen availability, exposure to light, and water activity (Lingnert, 1992). Apart from these parameters, the rate of lipid oxidation is also markedly affected by pH (Richardson and Korycka-Dahl, 1983). With increasing acidity, increases in lipid oxidation have been observed in pork meat (Yasosky et al., 1984), fish flesh (Fischer and Deng, 1977), and eggs (Pike and Peng, 1988b).

The oxidative stability of shell eggs in storage has not been a major problem normally. Eggs seemed to have built-in antioxidant characteristics that maintain the flavor in extended storage. Constituents such as phosvitins appear to be very effective in preventing oxidation of yolk lipids. Recently, however, dietary modified eggs have been produced, and there is extensive interest in their sales potential. Such eggs are highly unsaturated and, therefore, they may be more prone to oxidation during storage, particularly, at low pH as in yolk-containing acidic foodstuff preparations. Studies on the oxidative stability of shell eggs in storage from hens fed unsaturated lipids have shown that such eggs contain oxidation products that may lower their nutritive value (Marshall et al., 1994). Therefore, as the emerging egg technology produces more dietary modified eggs, there may be an increasing interest in the oxidative deterioration of marketed eggs.

A major preventive measure of lipid oxidation is the use of natural and synthetic antioxidants that function either by scavenging chain-carrying peroxyl radicals or by diminishing the formation of initiating lipid radicals (Yamamoto and Niki, 1990). Synthetic antioxidants such as butylated hydroxytoluene and butylated hydroxyanisole are currently approved to control lipid oxidation in foods, but recent concern over their use (Imaida et al., 1983; Okada et al., 1990) has created a

S0021-8561(97)00300-2 CCC: $14.00

need and prompted research for alternative antioxidants. In the past few years, interest in the use of extracts of natural herbs as a substitute for synthetic antioxidants to stabilize fat-containing foodstuffs has been shown. Extracts of rosemary and sage have played an important role, but other herbs of the Labiatae family, such as thyme, have also exhibited substantial antioxidant activity (Economou et al., 1991; Schwartz et al., 1996). Studies on the stabilizing activity of the essential oil of thyme in lipid systems have shown that carvacrol and particularly thymol are the phenolic components which are primarily responsible for its antioxidative activity (Farag et al., 1989; Deighton et al., 1993). Other studies which focused on the composition of antioxidant constituents in thyme leaves have further shown that, apart from carvacrol and thymol, other phenolics such as caffeic acid, *p*-cymene-2,3-diol, and several biphenylic and flavonoid compounds also exhibit antioxidative activity which, for some of them, is higher than that of α-tocopherol (Schulz and Herrmann, 1980; Miura and Nakatani, 1989a,b).

In a study conducted in our laboratory on the performance of laying hens fed dietary thyme (Tserveni-Goussi et al., 1994), eggs that might incorporate thyme constituents were collected. Possible incorporation of the antioxidant constituents of thyme into egg yolk through feeding may prolong storage time and reduce or eliminate the need for additional oxidative stabilization. In this paper, the effect of dietary thyme on the oxidative stability of liquid yolk at various pH, storage, and lighting conditions is investigated and comparison of the modified eggs is made with control eggs spiked with different synthetic and natural antioxidants.

MATERIALS AND METHODS

Instrumentation. Spectrophotometric measurements were carried out with a Shimadzu UV-160A double-beam spectrophotometer in 1-cm quartz cells. Normal spectra were obtained between 350 and 460 nm at a scanning speed of 1500 nm/min. Third-order derivative spectra were produced by digital differentiation ($d^3A/d\lambda^3$, where A = absorbance and λ = wavelength) of the conventional spectra obtained at scanning speed of 480 nm/min, using a derivative wavelength difference ($\Delta\lambda$) setting of 21 nm.

Liquid chromatography was carried out with a Gilson system (Gilson Medical Electronics, Villiers-le-Bel, France) consisting of two Model 305 piston pumps, a Model 805 manometric module, a Model 811 C dynamic mixer, a Model 117 UV detector set at 283 nm, and a Model TC 831 column heater. Injections were made in a Hichrom (Reading, U.K.), 250 × 4.6 mm i.d., column packed with Nucleosil 120, C_{18}, 5 μm, through a Rheodyne Model 7161 injection valve (Cotati, CA) equipped with 100-μL sample loop. The mobile phase consisted of acetonitrile and water (40/60, v/v), delivered in the system at a rate of 1 mL/min.

A vortex mixer (Heidolph, Germany), a Centra-MP4 IEC centrifuge (Needman Heights, MA), and a pH meter (WTW, Model 522, Weilheim, Germany) were used for sample preparation.

Reagents. Analytical grade butylated hydroxytoluene (BHT), ethylenediaminetetraacetic acid (EDTA), 2-thiobarbituric acid, the malonaldehyde precursor 1,1,3,3-tetraethoxypropane, sodium azide, ascorbic acid, and thymol were purchased from Sigma Chemical Co. (St. Louis, MO), whereas trichloroacetic acid, petroleum ether, hexane, acetonitrile, hydrochloric acid, and sodium hydroxide were obtained from Merck (Darmstadt, Germany).

Extraction of Thyme. Air-dried *Thymus vulgaris* plant material ground to pass a 0.5 mm screen was extracted in a Soxhlet apparatus for 6 h using hexane as extractant. Following extraction, remaining solvent in thyme extract was removed by heating at 50 °C. The obtained thyme extract was diluted with methanol and submitted, without further manipulation, to liquid chromatographic analysis (Solinas et al., 1981) for the determination of its thymol concentration. This thyme extract, which was found to contain 98.7 mg of thymol/g, was employed as a tested natural antioxidant.

Hens and Diets. Twenty 35-week-old Lohman laying hens were randomly assigned to two test diets (n = 10 per treatment) that were based on a typical corn−soybean layer ration with or without 3.0% ground *T. vulgaris* plant material. Diets were formulated to meet the requirements for nutrient and energy content for laying hens (National Research Council, 1984), stored in airtight containers, and given to hens *ad libitum* daily. Egg sample collection commenced 4 weeks after feeding of the dietary treatment began and lasted for 2 weeks. All eggs collected during that period were stored at 4 °C, pending further experimentation.

Egg Experimentation. Twelve eggs from each dietary treatment collected during the last 2 days of the feeding trial were used to investigate the effect of diet on yolk oxidation of shell eggs in storage. At the start of this investigation, three eggs freshly collected from each dietary treatment were directly submitted to analysis for yolk malonaldehyde content. The remaining eggs were put in a refrigerator, stored at 4 °C, and analyzed in sets of three eggs for yolk malonaldehyde content after 15, 30, and 60 days of storage.

All other collected eggs were broken, yolks were separated, and adhering albumen was removed by rolling on a paper towel. Yolk pools were prepared from each dietary treatment, and the contents were mixed with a wire whisk. Part of these pools was used to examine the effect of diet on the oxidative stability of the liquid egg yolk under different pH, lighting, and storage conditions. Another part was used to evaluate the antioxidant activity of various synthetic and natural antioxidants added to control egg yolk to compare it with that exhibited by the modified eggs.

For studying the effect of diet on the oxidative stability of liquid egg yolk under different pH, lighting, and storage conditions, three replicates of six 9-g portions were taken from each pool and transferred into 100-mL flasks, to which 10 mL of water was also added. Water addition was indispensable for decreasing yolk viscosity and providing rapid dispersion of other solutions added thereafter. The pH of the first four flasks per replicate from each pool was adjusted at values of 2.0, 3.0, 4.0, and 5.0, respectively, by addition, under vigorous vortexing, of 2 N hydrochloric acid. The pH of the fifth flask was left at its initial value of 6.2, whereas the pH of the remaining flask was adjusted at pH 9.0 using 1 N sodium hydroxide solution. To prevent microbial growth, a volume of 50 μL of sodium azide solution (60 mg/mL) was also added in each flask (Pike and Peng, 1988b). Following pH adjustment, additional water was pipetted in each flask to adjust the volume of total liquids added to 16 mL. All flasks were then covered with air-permeable film to retain moisture, yet not to exclude air, and submitted to medium agitation on a temperature-controlled shaker bath (GFL, GmbH, Hannover, Germany) at 20 °C in the dark or on a wrist action shaker (GallenKamp, Germany) at 25 °C in daylight. For ensuring even exposure of samples to air during the 22-day period of agitation, all flasks were inspected daily for film cracking.

For evaluating the antioxidant activity of synthetic and natural antioxidants, additional flasks with control egg yolk were prepared at pH 3 and 4, each flask being spiked with each one of the following compounds: 0.3 g of EDTA, 2.5 mg of BHT, 0.3 g of EDTA plus 2.5 mg of BHT, 2.5 mg of ascorbic acid, 0.25 mg of thymol, 2.5 mg of thyme extract that contained 0.25 mg of thymol, 2.5 mg of thymol, and 25 mg of thyme extract that contained 2.5 mg of thymol. Agitation, lighting, and storage conditions were as above.

At the start of the above experiments and at stated time intervals thereafter, 2-g samples were removed from each flask and directly analyzed for malonaldehyde concentration.

Malonaldehyde Analysis. Determination of malonaldehyde was carried out by a third-order derivative method (Botsoglou et al., 1994) slightly modified to suit egg yolk analysis. In this method, yolk sample (2 g) was mixed with 8

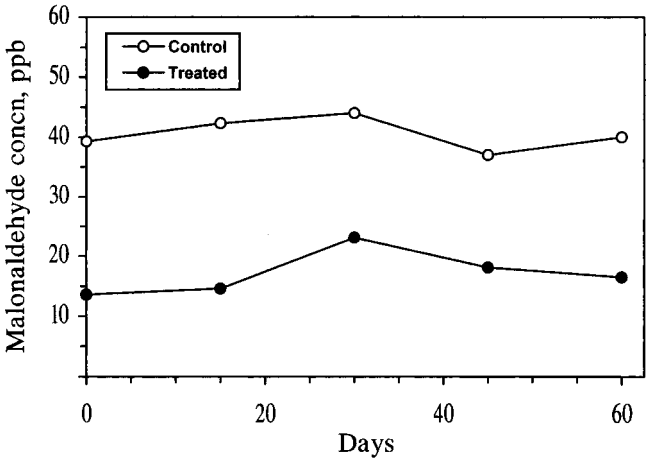

Figure 1. Effect of diet and storage time on malonaldehyde concentration of yolk of shell eggs.

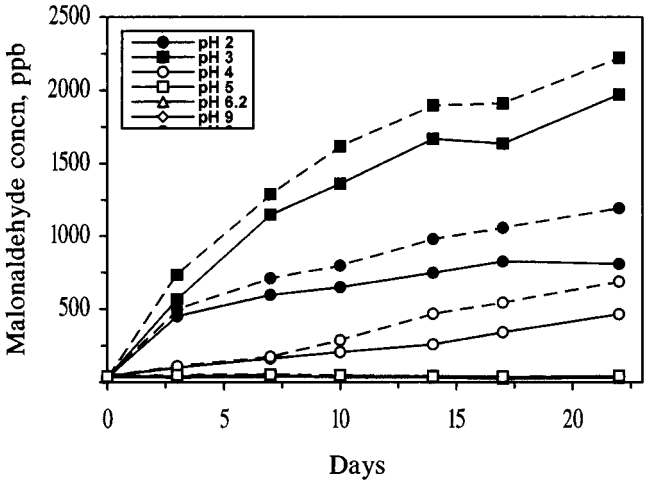

Figure 2. Effect of storage time on malonaldehyde concentration of control (dashed lines) and dietary thyme (solid lines) egg yolk adjusted to various pH values and agitated in the absence of light.

mL of 5% aqueous trichloroacetic acid and 5 mL of hexane containing 0.8% BHT. The mixture was vortexed for 30 s, the top hexane layer was discarded, and the bottom aqueous layer was collected to be filtered through Whatman No. 541 filter paper. Following filtration, a 2.5-mL aliquot was transferred to another tube and mixed with 1.5 mL of 0.8% aqueous 2-thiobarbituric acid, and the mixture was incubated for 30 min at 70 °C. After incubation, the mixture was allowed to cool under tap water and submitted to third-order derivative spectrophotometry at the instrumental conditions mentioned before. The height of the third-order derivative peak that appeared at 521.5 nm was used for calculation of the concentration of malonaldehyde in the examined extract on the basis of slope and intercept data of the computed least-squares fit of a freshly prepared standard curve.

Statistical Analysis. Data were analyzed using analysis of variance, and significant differences among treatments were tested using Duncan's test (Duncan, 1955).

RESULTS AND DISCUSSION

The effect of dietary treatment with thyme on yolk oxidation upon storage of shell eggs is shown in Figure 1. The extent of lipid oxidation, as measured by malonaldehyde formation, differed ($P < 0.01$) between treatments but did not change with storage time. Although it is apparent that malonaldehyde could not be produced during egg shell storage, its presence was evident in all yolks. The malonaldehyde found in the yolk of fresh eggs might be due to either the consumption and subsequent deposition of malonaldehyde that was already present in the diets or the *in vivo* production of malonaldehyde by the hens fed the diets. The former possibility appears unlikely because in that case the levels of malonaldehyde should have been equal among treatments. The latter seems to reasonably explain the lower concentrations of malonaldehyde found in eggs from hens fed thyme as compared to controls (Figure 1). Possible transfer of the antioxidant constituents of thyme into the hen through feeding might inhibit the chain reaction involved in oxidation of the consumed lipids, thus decreasing the oxidation products transferred into the yolk.

Consistent with our results, other workers (Marshall et al., 1994; Aymond and Van Elswyk, 1995) have also reported that although yolk malonaldehyde cannot be produced during shell egg storage, it can be found in fresh eggs. These workers reported, however, much higher malonaldehyde concentrations than those found in the present study (100−180 vs 40 ng/g of control yolk). This discrepancy is most likely to reflect a difference in the methods applied to detect lipid oxidation. A traditional distillation method (Tarladgis et al., 1964) that enhances the degradation of lipids during the distillation stage even in presence of phenolic antioxidants (Raharjo et al., 1993) has been extensively used in previous research.

Since the yolks from both dietary treatments were found to be inherently resistant to oxidative deterioration upon storage of shell eggs, additional experiments were carried out to evaluate yolk stability under various pH and lighting conditions that could promote lipid oxidation. The effect of dietary treatment on the oxidative stability of yolk adjusted at pH 2, 3, 4, 5, and 9, or left unadjusted at pH 6.2, and agitated for 22 days in the absence of daylight is shown in Figure 2. The unadjusted yolks from both treatments did not show any substantial malonaldehyde increase, as expected. Resistance to lipid oxidation was also shown by the yolks adjusted at pH 9 and 5. In contrast, a pH-dependent increase of lipid oxidation over time was observed in the more acidic samples. As yolk pH decreased from 5 to 3, oxidation was markedly increased over time and declined as pH further decreased to 2. This pH- and time-dependent oxidation, although following a similar pattern for both treatments, was clearly less intense for the thyme treatment yolks as compared to the controls. After 22 days of agitation, the malonaldehyde detected in the thyme treatment yolks accounted for 89% and 32% of that detected in the controls at pH 3 and at both pH 2 and 4, respectively. This may be due to the presence of antioxidant compounds presumably coming from the thyme fed to the hens.

The rapid increase of oxidation seen in yolk at pH 3 is difficult to explain. To determine the possible involvement of metal ions, the effect of adding 0.3 g of EDTA and/or 0.07 g of BHT to pH 3 adjusted yolk was evaluated in the absence of daylight. Figure 3 shows that the addition of EDTA resulted in much lower malonaldehyde concentrations; nevertheless, the oxidation was not entirely eliminated. On the other hand, the addition of BHT further inhibited malonaldehyde production, which was totally eliminated in the case of both compounds added. These results suggest that yolk oxidation can be partially inhibited by the addition of EDTA, which by forming chelates with metal ions prevents them from acting as prooxidants in the oxidative degradation of egg lipids.

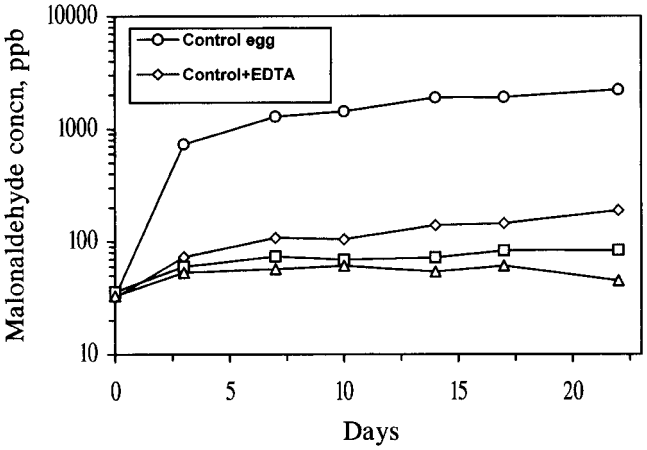

Figure 3. Effect of added synthetic antioxidants on malonaldehyde concentration of control yolk adjusted to pH 3.

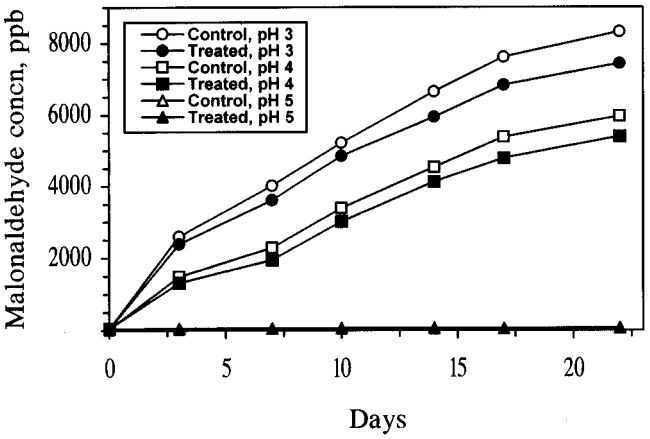

Figure 4. Effect of diet and storage time on malonaldehyde concentration of liquid yolk acidified at different pH values and agitated in the presence of light (the pH 5 control curve is overlapped by the pH 5 treated curve).

The ability of phosvitins, the major phosphoprotein components of egg yolk, to chelate metal ions may indicate that the observed oxidation profile might be due to denaturation of phosvitins by the low pH. Phosvitins have an exceptionally high serine content and most, or even all, of the serine residues are esterified to phosphate. This unique structure provides for numerous highly efficient metal-binding sites in clusters, exploiting the chelating properties of the phosphoryl groups. Phosvitin-bound iron may be assumed to account for most, if not all, of the iron present in egg (Allerton and Perlmann, 1965; Taborsky, 1982). In alkaline solutions (pH >8), the phosphate groups of phosvitins are diaionic and the net electronic charge density on the protein is about one negative charge per amino acid residue. Even in distinctly acidic solution (pH 3−5), the phosphate is singly but fully charged and the net negative charge density is a relatively high one-third charge per residue. It is only below about pH 2 that this protein becomes electrically neutral or positively charged. Nevertheless, the ferric phosvitin complex is strong enough to withstand pH variations. The ferric complex, once formed at neutral pH, will retain its iron even if the pH is subsequently lowered to 2 (Taborsky, 1980). On the basis of the above, phosvitins are expected to exert their protective action in the whole pH range examined.

Work with ground pork meat has indicated that pH values below approximately 6.1 resulted in rapid increases in lipid oxidation, and it was postulated that histidine ionization ($pK = 5.8-6.5$) might reduce the chelation of metal catalysts by protein (Yasosky et al., 1984). Because of the complexity of egg yolk, it is difficult to determine a single cause for the increased oxidation at pH 3. It cannot be also excluded that denaturation of the protein surrounding low-density lipoprotein (LDL) occurs, which may result in disruption of its structure (Burley and Vadehra, 1989) and subsequent exposure of the long-chain polyunsaturated fatty acids within the LDL particles of yolks to prooxidants in the surrounding environment (Pike and Peng, 1988a).

Figure 4 shows the effect of dietary treatment on the oxidative stability of yolks adjusted to pH 2, 3, 4, 5, and 9 and agitated for 22 days in the presence of daylight. This pH- and time-dependent oxidation profile shows a pattern similar to that recorded in the absence of daylight (Figure 2) except that oxidation is now much more intense. In the dark, the malonaldehyde produced in the pH 3 adjusted control yolks could not surpass the level of 2.3 μg/g of yolk, while it exceeded the level of 8.3 μg/g of yolk in the presence of daylight. This indicates that lighting conditions, besides pH, constitute major oxidative factors. Figure 4 further shows that the thyme treatment yolks continue to present some resistance to oxidation even though the surrounding environment favors oxidation. The malonaldehyde detected in those yolks during the experimentation was consistently lower than that detected in the controls at pH 3 or 4. This suggests that antioxidant compounds presumably coming from the dietary thyme had been deposited into the yolks.

To better evaluate the antioxidant power of the compounds *in vivo* deposited in yolk, a comparison was made with added synthetic antioxidants such as BHT, which acts by interrupting the chain of free radicals, and ascorbic acid, which is a known oxygen consumer. Comparisons were further made with thymol, the main component of the essential oil of thyme, and a hexane extract of thyme plant that contained an equal amount of thymol. The patterns of the malonaldehyde production per treatment over an agitation period of 22 days in the presence of daylight at pH 3 and 4 are presented in Figure 5. The BHT agent showed an outstanding antioxidant activity, whereas ascorbic acid exhibited a definite prooxidant action.

The univalent reduction of metal ions by ascorbic acid followed by reduction of hydroperoxides to yield hydroxyl radical may provide one basis for the prooxidant function of this agent (Kanner et al., 1977; Kanner, 1994). Nevertheless, combinations of ascorbic acid and metal ions can be prooxidant or antioxidant depending upon their relative concentrations (Kanner et al., 1977). A number of possible mechanisms have been suggested for this apparent paradox, including reductive activation of metal ions, increased levels of the supposed prooxidant ascorbyl radical, and formation of unspecified ascorbate−metal complexes that may differently affect propagation and termination reactions of lipid oxidation (Richardson and Korycka-Dahl, 1983; Kanner, 1994). To date, the complex behavior of ascorbic acid in the oxidative stability of lipids has not been clearly defined.

Figure 5 further shows that thymol could not exert any antioxidant action when 0.25 mg of the compound was added in the 25-mL samples, an addition that corresponds to 27.8 μg of thymol/g of yolk. Despite that, the addition of a thyme extract that contained an equal amount of thymol resulted in marked inhibition of lipid oxidation. The addition, on the other hand, of a 10-fold amount (2.5 mg) of thymol resulted in antioxidant

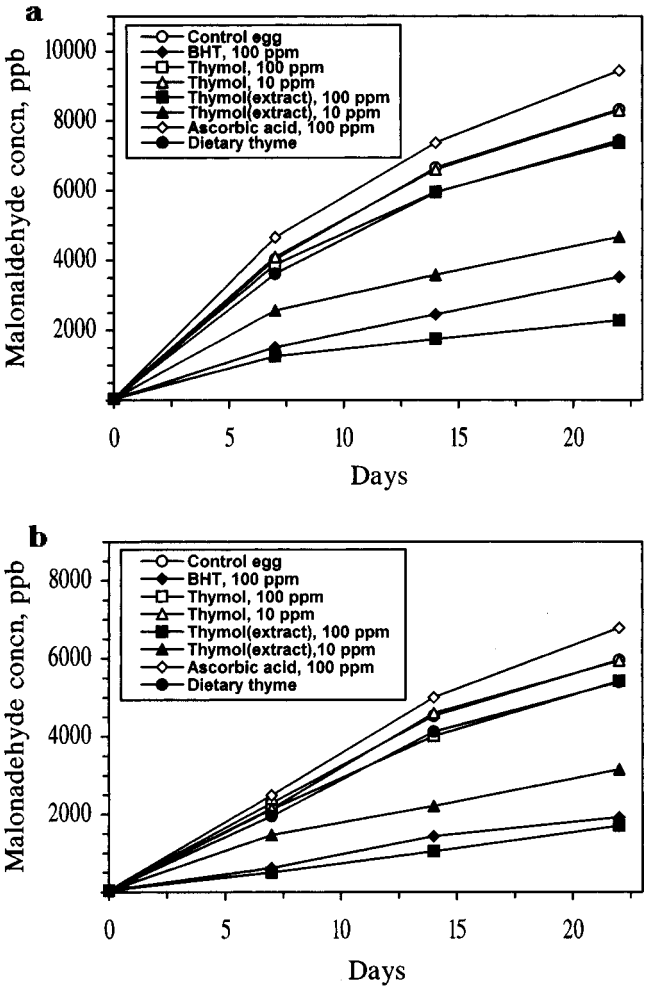

Figure 5. Effect of added synthetic/natural antioxidants and storage time on malonaldehyde concentration of liquid control yolk adjusted to pH 3 (a) and 4 (b) and agitated in the presence of light.

activity almost similar to that exhibited by the thyme treatment yolk itself, while the addition of a 10-fold amount of thyme extract showed a much more pronounced inhibition of lipid oxidation, almost equivalent to that presented by BHT. These results suggest that thymol was partially responsible for the antioxidant activity of the thyme extract. Other compounds with strong antioxidant activity should also occur in the thyme extract, which can account for its enhanced antioxidant activity.

One could argue that the measured malonaldehyde could have been an artifact of the analysis. The validity of malonaldehyde as an index of lipid peroxidation in biological materials has been clouded by controversy regarding its formation, its occurrence in various bound forms, and the specificity of the methods used for its measurement. The assay is based on the reaction between 2-thiobarbituric acid and malondiadehyde during heating at acid pH to produce a chromogenic complex. Malonaldehyde reactivity is influenced by several factors, and lipid peroxidation may even occur *in vitro* during the test itself (Gutteridge, 1982). Experienced investigators in the free radical field have cautioned that the assay, in general, may give misleading results (Halliwell, 1984). However, there are theoretical objections to all of the alternative methods that are currently available for investigating lipid peroxidation, and the malonaldehyde assay in all of its versions has the merit of simplicity. The new malonaldehyde assay used in the present study represents a substantial improvement over previous versions. Research previously conducted in our laboratory (Botsoglou et al., 1994) has shown that the efficient sample preparation procedure incorporated in the new method could suppress any lipid peroxidation arising during the acid-heating stage. Furthermore, any interference from other reactive compounds could be totally eliminated owing to the high discriminative power of the derivative processing.

Considering the extremely high thymol concentration (278 μg of thymol/g of yolk) needed to be added to a control yolk to display antioxidant activity equivalent to that of a thyme treatment yolk, one could say that additional thyme components should pass through feeding into egg yolk, thus providing its antioxidant properties. As there is no method available so far for the determination of thymol and other thyme phenolics in egg yolk, we could not analyze our samples. Additional research is needed toward developing such a method which could identify and quantify each of the main antioxidant constituents of thyme deposited into egg yolk.

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Received for review April 11, 1997. Accepted July 7, 1997.[⊗]

JF9703009

[⊗] Abstract published in *Advance ACS Abstracts,* September 1, 1997.