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Effect of Antioxidants on Oxidative Stability of Edible Fats and Oils: Thermogravimetric Analysis

MARLEEN VAN AARDT,*,† SUSAN E. DUNCAN,† TIMOTHY E. LONG,‡ SEAN F. O'KEEFE,† JOSEPH E. MARCY,† AND SUSAN R. SIMS§

Department of Food Science and Technology, Virginia Polytechnic Institute and State University, Duck Pond Drive, Blacksburg, Virginia 24061, Department of Chemistry, Virginia Polytechnic Institute and State University, West Campus Drive, Blacksburg, Virginia 24061, and Eastman Chemical Co., Kingsport, Tennessee 37662-5125

Thermogravimetric analysis was used to determine the oxidative stability of various edible oils (olive oil, milkfat) and triacylglycerides (triolein, trilinolein), while the effect of natural (α-tocopherol, ascorbic acid) and synthetic antioxidants (butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), and tertiary butyl hydroquinone were evaluated by addition to trilinolein. Oil resistance to oxidation was obtained by measuring the increase in sample weight due to the uptake of molecular oxygen, the temperature at maximum sample weight, and the temperature at the onset of oxidation. When comparing sample weight increase, trilinolein proved to be oxidatively less stable than triolein, olive oil, and milk fat, while triolein was less stable than olive oil and milk fat. Olive oil showed significantly higher stability than milkfat when comparing the temperature at the onset of oxidation. When comparing effectiveness of antioxidants, a combination of 0.01% BHA and 0.01% BHT increased trilinolein stability the most.

KEYWORDS: Oxidative stability; oils; milk fat; antioxidants; thermogravimetric analysis; TGA

INTRODUCTION

Oxidation of unsaturated lipids is one of the major causes of the development of off-flavor compounds and the reduction in nutritive value of food products (*1*). Although lipid oxidation can be induced by catalytic systems such as light, temperature, enzymes, metals, metalloproteins and microorganisms, the reactions involve free radical and/or active oxygen species (*2*, *3*). Triplet oxygen lipid oxidation, a free radical process, has been extensively studied during the past 70 years. However, triplet oxygen oxidation does not fully explain the initiation step of lipid oxidation. Singlet oxygen is involved in the initiation of triplet oxygen lipid oxidation, because singlet oxygen can react directly with double bonds without the formation of free radicals (*4*). During the last 30 years, increased attention has been given to singlet oxygen oxidation of foods, because (i) the rate of singlet oxygen oxidation is much greater than that of triplet oxygen oxidation, and (ii) singlet oxygen oxidation produces compounds absent in triplet oxygen oxidation due to the different reaction mechanisms (*4*). Interaction with light, sensitizers, and oxygen is mainly responsible for singlet oxygen formation in food (*5*).

In food systems, naturally occurring antioxidants, such as tocopherols and ascorbic acid, protect lipids against oxidation by either quenching free radical reactions or by scavenging oxygen. However, natural antioxidants are often lost during processing or storage, necessitating the addition of exogenous antioxidants that will effectively retard the onset of lipid oxidation (*6*, *7*). Butylated hydroxyanisole (BHA), butylated hydroxytolune (BHT), and tertiary butyl hydroquinone (TBHQ) are synthetic antioxidants widely used in the food industry as direct food additives or as indirect additives through diffusion from plastic packaging (*2*, *8*).

Direct quantification of oxidation, by measuring oxygen consumption, is often difficult in food or biological systems. Alternative measures for determining extent of oxidation include chemical methods (peroxide value, thiobarbituric acid test, anisidine value, and carbonyl value), spectrophotometric (UV absorption, electron spin resonance spectroscopy, and chemiluminescence), chromatographic, and sensory methods. Gas chromatography combined with mass spectroscopy (GC-MS) is most widely used for the measurement of flavor compounds resulting from oxidation either by headspace analysis or by direct injection of the product. High-performance liquid chromatography (HPLC) is a very useful technique to measure peroxides, hydroperoxides and secondary oxidation products (*9*).

The Warburg manometer is an example of a method that does direct quantification of oxidation by measuring the uptake of atmospheric oxygen as an increase in sample weight. Thermo-

* To whom correspondence should be addressed. Tel.: (540) 231-6806. Fax: (540) 231-9293. E-mail: mvanaard@vt.edu.
† Department of Food Science and Technology, Virginia Polytechnic Institute and State University.
‡ Department of Chemistry, Virginia Polytechnic Institute and State University.
§ Eastman Chemical Co.

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gravimetric analysis (TGA) is another oxygen absorption method used extensively in the polymer chemistry industry for the measurement of oxidative stability of polymers. TGA continuously monitors changes in sample weight while the sample is subjected to controlled increases in temperature in a modified atmosphere environment. An estimation of oil resistance to oxidation is obtained by measuring percent weight gain due to oxidation (*9*). The use of TGA for food applications has received limited attention. Gennaro et al. (*10*) used TGA for evaluating the effect of antioxidants on oxidative stability of virgin olive oil in an oxygen environment, while Rudnik et al. (*11*) and Biswas and Staff (*12*) studied the oxidative stability of linseed oil and distilled grains, respectively. TGA shows high reproducibility. It requires a small sample amount (~10 mg) and it takes a relatively short time of analysis (~20 min per sample).

The first objective of this study was to determine the sensitivity of TGA in measuring oxidative stability of various edible oils, fats, and triacylglycerides. The second objective was to measure the effect of various antioxidants on trilinolein stability. Since the Code of Federal Regulations specifies the maximum addition of antioxidants such as BHT, BHA, and TBHQ to fats and oils as 200 ppm (*13*), this level was not exceeded in our study. Natural antioxidants such as tocopherols and ascorbic acid are generally recognized as safe when used in accordance with good manufacturing practice, and therefore are not limited (*9*). Because literature shows prooxidant effect of tocopherol at high concentrations (*14*, *15*), α-tocopherol was tested at 100 ppm (0.01%) and 200 ppm (0.02%).

MATERIALS AND METHODS

Milk fat, olive oil, trilinolein, triolein, and tristearin were evaluated for oxidative stability using TGA with a temperature program where initial temperature was constant at 100 °C for 5 min. In a separate experiment, trilinolein stability was evaluated in the presence of various antioxidants: (i) 0.01% α-tocopherol, (ii) 0.02% α-tocopherol, (iii) 0.01% ascorbic acid and 0.01% α-tocopherol, (iv) 0.01% BHA and 0.01% BHT, and (v) 0.01% TBHQ, where initial temperature was constant at 70 °C for 5 min. The different temperature programs are a result of either having to evaporate water or solvent from the system.

Standard Materials and Sample Preparation. Trilinolein (C18:2 Δ9cis, Δ12cis)(50 mg in amber glass ampules), triolein (C18:1 Δ9cis) (100 mg in amber glass ampules), and tristearin (C18:0) (1 g in amber glass ampules) were purchased from Sigma (Saint Louis, MO), and stored at −5 °C. Virgin olive oil was purchased in a 250-mL clear glass container from a local grocery store in Blacksburg, VA. Olive oil aliquots (5-mL) were stored under nitrogen gas at 4 °C in a dark environment until further use. Fresh raw milk was obtained locally from the Virginia Tech dairy farm within approximately 2 h of milking. Fat was extracted within 12 h according to the Bligh and Dyer lipid extraction procedure (16). Milk fat was then stored under nitrogen at 4 °C in a dark environment until further use. BHA, BHT, TBHQ, α-tocopherol, and ascorbic acid were purchased from Aldrich (Milwaukee, WI). Weighed quantities of antioxidants were dissolved in a 1:9 (v/v) mixture of methanol/chloroform, and then added to weighed trilinolein samples.

Fatty Acid Profiles. Fatty acid contents of milk fat, olive oil, trilinolein, and triolein were determined. Extracted milk fat and oils were methylated by in situ transesterification with 0.5 N NaOH in methanol followed by 14% boron trifluoride in methanol. Undecenoate (Nu-Check Prep, Elysian, MN) was used as the internal standard. Samples were injected by using an auto-sampler into a Hewlett-Packard 5890A gas chromatograph equipped with a flame ionization detector (Hewlett-Packard, Sunnyvale, CA). Methyl esters were separated on a 100-m × 0.25-mm i.d. fused silica capillary column (CP-Sil 88, Chrompack, Middleburg, The Netherlands). Pure methyl ester standards (Nu-Check Prep, Elysian, MN; Supelco Inc., Bellefonte, PA) were used to identify peaks and determine response factors for individual fatty acids. An 80 to 1 split ratio was used for injection of 0.5 μL of hexane containing methyl esters of all samples. The carrier gas was ultrapure hydrogen, and inlet pressure was maintained at 23.1 psi linear flow. Injector temperature was maintained at 250 °C, and detector temperature was maintained at 255 °C. The initial oven temperature was 70 °C (held for 1 min), increased 5 °C/min to 100 °C, (held for 2 min), increased 0 °C/min to 175 °C (held for 40 min), and increased 5 °C/min to 225 °C (held for 15 min) (17).

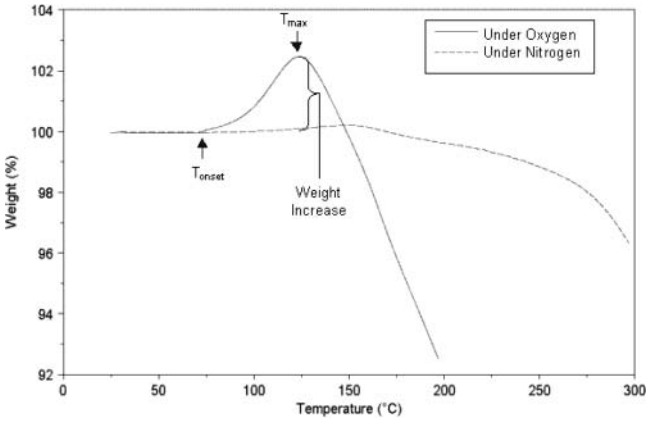

Figure 1. Typical TGA graph of weight loss* and oxidation** of trilinolein under nitrogen and oxygen, respectively (Temperature Program: * 10 °C/min to 70 °C, 70 °C for 5 min, 2 °C/min to 200 °C; ** 10 °C/min to 300 °C).

Thermogravimetric Analysis. A thermobalance (TGA 2950, TA Instruments, New Castle, DE) was used. The TGA was calibrated with "alumel" alloy and nickel for temperature settings and with a 100-mg standard for weight accuracy. Approximately 9 mg of sample was placed on a tared aluminum balance pan. The pan was placed in a room-temperature furnace, and the exact sample weight was determined. The temperature program for oil samples without antioxidants consisted of raising the temperature at a rate of 10 °C/min to 100 °C, then holding this temperature constant for 5 min to remove all traces of moisture. The sample was then heated to 250 °C at a rate of 2 °C/min to detect the oxidation peak. Because trilinolein samples containing added antioxidants also contained trace amounts of solvent, the temperature program differed in that the temperature was held constant at 70 °C for 5 min, rather than at 100 °C as previously described. Another reason for holding temperature constant at 70 °C and not at 100 °C was due to the fact that oxidation of trilinolein was observed as early as 70 °C. O_2 was used as the purge gas to establish a suitable environment for the oxidation process (flow of 50 cm^3/min) (*10*), while N_2 was used throughout as control. All samples were analyzed in triplicate.

Statistical Analysis. Analysis of variance tested the null hypothesis that sample means were equal, and the alternative hypothesis that means were not equal. Fisher's protected least significant difference (LSD) was the mean separation method used. A significance level of $p < 0.05$ was established to detect statistical differences. Analysis was performed using SAS Version 7, (SAS Institute, Inc., Cary, NC) (*18*).

RESULTS AND DISCUSSION

TGA makes it possible to (*i*) estimate a product's resistance to oxidation by measuring weight gain percent as a function of oxygen uptake by a sample and (*ii*) determine the temperature at maximum oxygen uptake (**Figure 1**). **Table 1** shows the weight increases in edible oil samples due to oxidation, as well as the temperature at maximum oxidation (T_{max}), while **Table 2** reports on the effect of various antioxidants on trilinolein stability.

When comparing the oxidative stability of edible oils, compounds were subjected to an oxygen environment in the absence of exogenous antioxidants, trilinolein proved to be significantly more unstable than triolein, tristearin, milk fat, and olive oil. The respective sample weight increases were 2.3250%

Table 1. Thermogravimetric Analysis of Various Edible Oils: Oxidative Stability as a Function of Weight Increase Due to Oxygen Consumption

edible oils	weight increase (%) ± *S*	T_{onset}[a] (deg C ± *S*)	T_{max}[b] (deg C) ± *S*
olive oil	0.23[f] ± 0.003	149.4[d] ± 0.80	162.1[d] ± 0.24
milk fat	0.23[f] ± 0.019	100.0[ce] ± 0.00	161.9[d] ± 2.58
triolein	0.89[e] ± 0.015	101.1[e] ± 0.60	151.4[e] ± 1.63
trilinolein	2.33[d] ± 0.069	70.4[f] ± 1.56	126.4[f] ± 0.66
tristearin	0.00[g] ± 0.000	0.0[g] ± 0.00	0.0[g] ± 0.00

[a] T_{onset}: Temperature at which sample weight increase is first detected. [b] T_{max}: Temperature at which sample weight increase is at its maximum. [c] Onset of oxidation occurred within the 5-min period at a constant temperature of 100 °C. [d–g] Means within a column with different superscript letters indicate significant differences at $p < 0.05$.

Table 2. Thermogravimetric Analysis of Trilinolein in the Presence of Natural and Synthetic Antioxidants: Oxidative Stability as a Function of Weight Increase Due to Oxygen Consumption

antioxidants added to pure trilinolein	weight incr (%) ± *S*	T_{max}[e] (deg C) ± *S*
no antioxidants (control)	2.21[b] ± 0.222	125.1[ab] ± 4.27
0.01% α-tocopherol	2.28[b] ± 0.080	117.3[c] ± 1.28
0.02% α-tocopherol	2.34[b] ± 0.196	119.9[bc] ± 6.79
0.01% α-tocopherol + 0.01% ascorbic acid	2.24[b] ± 0.075	121.2[bc] ± 3.21
0.01% BHA + 0.01% BHT	1.88[c] ± 0.108	131.8[a] ± 5.81
0.02% TBHQ	2.68[a] ± 0.056	116.5[c] ± 1.82

[a–c] Means within a column with different superscript letters indicate significant differences at $p < 0.05$. [e] T_{max}: Temperature at which sample weight increase is at its maximum.

Table 3. Fatty Acid Content (% of total fatty acid) of Various Edible Oils Determined by Gas Chromatography (GC)

sample	4:0	6:0	10:0	12:0	14:0	15:0	16:0	16:1	18:0	18:1	18:2
milk fat[a]	2.2	1.3	2.6	2.7	11.1	1.1	33.0	2.3	12.5	24.6	2.8
olive oil[a]							11.4		2.9	71.8	11.9
trilinolein											99.9
triolein										99.9	

[a] GC results obtained for extracted milk fat and virgin olive oil used in this study.

(trilinolein), 0.00% (tristearin), 0.8870% (triolein), 0.2337% (milk fat), and 0.2260% (olive oil) (**Table 1**). Fatty acid composition explains to some degree why trilinolein was oxidatively the least stable. Trilinolein consists of nearly 100% linoleic acid (C18:2) (**Table 3**), which provides the highest number of potential sites for oxidation (6 double bonds per molecule). However, when comparing that with triolein, which consists of approximately 100% oleic acid (C18:1), one would expect triolein to oxidize at half the rate of trilinolein, due to half the amount of potential oxidation sites, which is not the case.

Doleiden et al. (*19*) reported reaction rates of singlet oxygen with oleic, linoleic, and linolenic acids of 0.74, 1.3, and 1.9 × 10^5 M^{-1} s^{-1}, respectively, which is relatively proportional to the number of double bonds in the molecules. On the other hand, triplet oxygen reacts with unsaturated fatty acids by abstracting allylic hydrogens. Once a hydrogen is removed, a pentadienyl radical intermediate is formed. The energy required for the removal of hydrogens at different carbons is quite different. Min and Akoh (*4*) reported that the relative reaction ratio of triplet oxygen with oleic, linoleic, and linolenic acid for hydroperoxide formation is 1:12:25, which is dependent on the relative

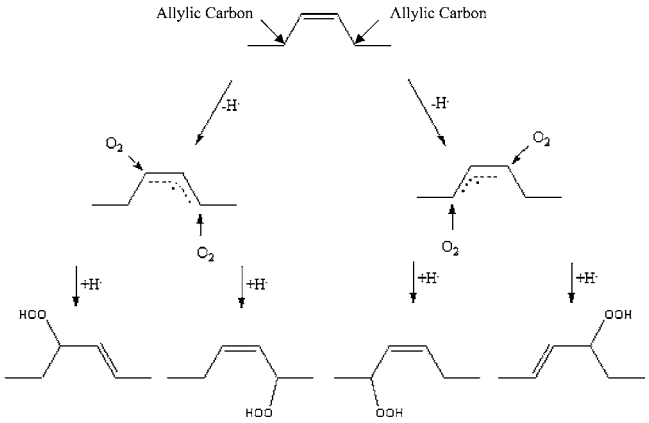

Figure 2. Hydrogen abstraction at the allylic carbons of unsaturated bonds in free radical oxidation of fatty acids (modified from 17).

difficulty for the radical formation in the molecule. The reaction rate of triplet oxygen with linolenic acid is approximately twice as fast as that of linoleic acid because linolenic acid has two pentadienyl groups in the molecule, compared with the linoleic acid with one pentadienyl group (*4*). The classic mechanism for free radical oxidation of unsaturated fatty acids involves hydrogen abstraction at the allylic carbons to produce delocalized three-carbon allylic radicals (*17*) (**Figure 2**). Various other proposed mechanisms also exist that explain the role of allylic hydrogens in free radical oxidation (*4*).

Although tristearin was not evaluated for fatty acid content, it is expected to contain approximately 100% stearic acid (C18:0) (Sigma, Saint Louis, MO), which correlates well with the fact that no oxidation was observed. The estimation of values such as (i) the average number of double bonds per molecule, (ii) the average number of allylic hydrogens per molecule, and (iii) the energy required for the removal of hydrogens at different carbons are difficult for complex fats such as milk fat and olive oil.

Subsequently, triolein showed lower oxidative stability than milk fat and olive oil. As mentioned before, triolein consists of approximately 100% oleic acid, while milk fat and olive oil consist of lower percentages of unsaturated fatty acids (**Table 3**). The major differences in fatty acid composition between milk fat and olive oil is that milk fat contains a larger amount of short chain saturated fatty acids and that olive oil contains approximately 71.7% oleic acid in comparison to 24.6% in milk fat. Sample weight increases did not indicate differences in olive oil and milk fat stability. However, the temperature at which a weight increase first was detected (T_{onset}, **Table 1**) indicated that olive oil oxidized at a higher temperature than milk fat. In a similar study done by Gennaro et al. (*10*) on the stability of olive oil, similar values were observed for increases in olive oil sample weight (0.22 ± 0.02) when exposed to oxygen. More research might clarify why olive oil oxidized at higher temperatures than milk fat.

A substantial difference in fatty acid composition and the presence of natural antioxidants in olive oil and milk might also contribute to the differences in T_{onset} (**Table 1**). In general, vegetable oils are high in tocopherols. While the tocopherol content of olive oil used in this study is unknown, **Table 4** shows the variety of naturally occurring tocopherols in olive and other food oils (*20*). Milk naturally contains low concentrations of α-tocopherol (25−35 $\mu g/g^{-1}$ fat) and ascorbic acid (<20 mg/L^{-1}) (*21*, *22*). Tocopherol levels in milk vary according to the diet of the cows, while pasteurization and skimming results in a reduction in the tocopherol content of milk (*23*, *24*).

Table 4. Natural Tocopherol Content of Various Food Oils (mg/kg)[a]

oil	α-tocopherol (mg/kg)	β-tocopherol (mg/kg)	γ-tocopherol (mg/kg)	δ-tocopherol
canola	210.0	1.0	42.0	0.4
coconut	5.0			6.0
corn	112.0	50.0	602.0	18.9
cottonseed	389.0		387.0	
olive	119.0		7.0	
palm	256.0		316.0	70.0
peanut	130.0		214.0	21.0
safflower	342.0		71.0	
sesame	136.0		290.0	
soybean	75.0	15.0	797.0	266.0
sunflower	487.0		51.0	8.0
walnut	563.0		595.0	450.0

[a] Adapted from Gunstone et al. (*19*). Blank entries = no value, or trace amounts.

Phospholipids in milk have also shown to be an effective antioxidant, either alone or in synergism with α-tocopherol (*25*, *26*). The phospholipids of the milk fat globule membrane consists of approximately 40−60% unsaturated fatty acids, of which one-third are polyunsaturated, which might indicate a high susceptibility to oxidation. It therefore appears that the role of milk phospholipids in milk fat oxidation is complex and depends on the nature of the medium and the oxidation condition (*26*).

It is well known that the mechanism of antioxidation varies from antioxidant to antioxidant. Primary or chain-breaking antioxidants such as BHA, BHT, TBHQ, and tocopherols inhibit or retard lipid oxidation by interfering with either chain propagation or initiation by readily donating hydrogen atoms to lipid peroxyl radicals. These antioxidants are effective because (i) they produce stable and relatively unreactive antioxidant radicals and (ii) they are able to compete with the lipid substrate (*27*). Ascorbic acid is known for its multifunctional effects. In foods, it can scavenge oxygen, shift the redox potential of food systems to the reducing range, act synergistically with chelators, and regenerate primary antioxidants. The synergistic effect between a free radical acceptor such as α-tocopherol and ascorbic acid is well recognized. The effect is explained by the regeneration and recycling of the tocopheroxyl radical intermediate ($TOC^{\bullet}$) to the parent phenol, α-tocopherol (*27*).

$$TOC^{\bullet} + ASC^{-} = TOC = ASC^{\bullet}$$

When comparing the effect of antioxidants on the oxidative stability of trilinolein, only a combination of 0.01% BHA and 0.01% BHT significantly retarded oxidation, whereas tocopherol alone or in combination with ascorbic acid did not significantly improve trilinolein stability (**Table 2**). Phenolic antioxidants such as BHA, BHT, TBHQ, and α-tocopherol can stop the reaction of two chain-carrying peroxyl radicals and thus break two kinetic chains per molecule. The equivalent efficiency of these antioxidants might play a role in antioxidant efficacy. BHA, BHT, and α-tocopherol each have one hydroxyl group that participates in the donation of protons to free radicals to convert them to more stable products. However, the molecular weight of α-tocopherol (430.72 g/mol^{-1}) is approximately double that of BHA (180.2 g/mol^{-1}) and BHT (220.4 g/mol^{-1}). By addition of similar weights of each of these antioxidants, approximately double the amount of hydroxyl groups is added in the case of BHA and BHT. This might partially explain the reduced efficacy of α-tocopherol versus BHA and BHT, but does not explain why differences have been seen in the antioxidant activity of the various tocopherol isomers, which have similar molecular weights. Antioxidant activity of tocopherols increase from the α through the δ-isomers, while vitamin activity decreases from the α through the δ-isomers (*28*). Gennaro and co-workers (*10*) found that 100 mg/kg^{-1} BHT was as effective in stabilizing olive oil against oxidation as was 50 mg/kg^{-1} of natural polyphenols such as 3,4-dihydroxyphenylethanol. BHA and BHT are fairly volatile antioxidants, which also makes them useful in dried food applications. BHA has a melting point of 48−65 °C while BHT has a melting point of 69 °C (Aldrich Chemicals, Milwaukee, WI).

Table 5. Weight Loss Temperatures of Antioxidants under Nitrogen and Oxygen

antioxidants	T_d[a] (nitrogen) (deg C)	T_d[a] (oxygen) (deg C)	weight incr (oxygen) (%)
α-tocopherol	264.4	226.6	0.378
ascorbic acid	195.9	188.5	0.027
BHA	119.7	113.5	n/d[b]
BHT	119.5	105.5	n/d
TBHQ	157.5	131.1	n/d

[a] T_d: Weight loss temperature associated with a change in chemical structure. [b] n/d, not detected.

Table 2 does not indicate a significant difference between the effect of α-tocopherol alone or in combination with ascorbic acid on trilinolein stability. Even though ascorbic acid is mostly soluble in water, Frankel et al. (*29*) observed that hydrophilic ascorbic acid was a more effective antioxidant in bulk corn oil than in emulsified corn oil. In contrast, the lipophilic ascorbyl palmitate was a more effective antioxidant in a corn oil emulsion than in bulk corn oil, due to its orientation in the oil−water interface. The ability of ascorbic acid to scavenge oxygen is also a well-known effect. One ascorbic acid molecule reacts easily with atmospheric oxygen and behaves as a two-electron donor. The most probable reason for not observing an improved stability of trilinolein when ascorbic acid was present is that the TGA flow of pure oxygen (50 cm^3/min^{-1}) exceeded the theoretical maximum consumption level of headspace of ascorbic acid (3.3 mg/cm^{-1}) (*27*).

All antioxidants were evaluated alone in the presence of oxygen and nitrogen (**Table 5**) to ensure that when added to trilinolein, initial weight decreases was not attributed to antioxidant weight decrease but rather to the instability of the oil. T_d is the measure of initial weight loss of the sample and does not indicate that a sample is necessarily stable over the temperature range in which a sample maintains a consistent weight. A significant difference might have been observed between α-tocopherol alone and in combination with ascorbic acid if T_{onset} could have been measured. However, in this study, the combination of the temperature and solvent evaporation temperature caused all T_{onset} values to fall within 5 min of isothermal treatment at 70 °C. Rudnik and co-workers (*11*) compared T_{onset} of linseed oil in the presence of BHA and a combination of α-tocopherol, ascorbyl palmitate, ascorbic acid, and ethoxylated ethylene glycol. They found that a combination of natural antioxidants at 0.05% proved more effective as antioxidant than the addition of 0.01 or 0.02% BHA.

From **Table 2**, it seems that the addition of 0.02% TBHQ had a prooxidant effect. This might be partially due to the fact that TBHQ has two hydroxyl groups that are available to donate protons to reactive free radicals and a comparatively lower formula weight of approximately 166.22 g/mol^{-1}.

In conclusion, TGA is a valuable technique for evaluating oxidative stability differences between different oils and fats,

as well as evaluating small differences in oxidative stability that occur upon addition of antioxidants to oils. It is reproducible, requires a small sample weight, and takes a relatively short time to analyze each sample. However, further research needs to be done on factors such as the effect of load on activity, etc. Altogether, this technique may be a viable option for use as a quality control measure in the food industry.

ABBREVIATIONS USED

TGA, Thermogravimetric analysis; BHT, Butylated hydroxytoluene; BHA, Butylated hydroxyanisole; TBHQ, Tertiary butyl hydroquinone; ASC, Ascorbic acid; Toc, Tocopherol

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