A Gas Chromatographic Method for Testing Antioxidants

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ABSTRACT AND SUMMARY

A gas chromatographic method for evaluating the effect of antioxidants is described. Emulsions of linoleic acid both with and without antioxidant are oxidized enzymatically. Concentrations of unreacted linoleic acid are measured at varying incubation times and various concentrations of both lipoxidase and antioxidant. Two antioxidants have been tested. The method is simple, precise, and reproducible. The inhibition mechanism and experimental conditions are discussed.

INTRODUCTION

Antioxidants are widely used in food processing to prevent undesirable decomposition processes. The efficiency of most antioxidants varies with the system to which they are added. They are most frequently evaluated by their ability to increase the stability of fats. Evaluations of the antioxidant activity require well-defined conditions and should be rapid and simple to perform. Various approaches have been used to determine the antioxidant activity.

Some of the conventional methods like the active oxygen method (AOM) (1), oxygen absorption method (2), the modified ASTM bomb method (3), and Shaal oven method (4) are rather time-consuming, but give reasonable results. Lately, more rapid methods have been developed (5,6). Hamilton and Tappel (7) described a polarographic method for determining the antioxidant efficiency based on hemoglobin catalysis of lipids and measurement of oxygen uptake. Furthermore, a method reported by Kendrick and Watts (8) employed heme compounds to catalyze the lipid oxidation. Quite recently a rapid oxygen uptake technique based upon the acceleration of the lipid oxidation by hemin has been described (9,10). All the abovementioned studies have been based upon measurements of the oxygen uptake in dynamic lipid emulsion systems. Because of the complexity of the oxidation reactions and the difficulties in standardizing the lipid systems these results were not reproducible (9). The evaluation of the antioxidants has, therefore, been based on the induction period (5,6,8,9), or the time needed to utilize 90% of the dissolved oxygen in the emulsion (7).

Other research workers have tested the antioxidant activity by measuring the peroxide value in the oxidative test system (11,12). The oxidation products, however, are susceptible to decomposition during accomplishment of the analysis and the peroxide value is less reliable as test parameter.

In this paper we present a gas liquid chromatographic (GLC) method for determination of the antioxidant activity. A well-defined system based upon the lipoxidase catalyzed oxidation of unsaturated fatty acids was applied. Gas chromatographic measurements of unreacted fatty acid in emulsions containing different concentrations of antioxidant were performed. The incubation time was also varied. A measure of the inhibitory effect of the system was obtained by comparing the data to corresponding data obtained from emulsions without antioxidant. The data were tested statistically.

EXPERIMENTAL PROCEDURES

Instrumental

A Carlo Erba model 2100 gas chromatograph equipped with a flame ionization detector was used. The column was a 1.5 m x 2 mm ID glass tube packed with 10% SP-2340 on acid washed Chromosorb W, 100/120 mesh (Supelco, Bellefonte, PA). Operating conditions were: column temperature 195 C, injector-detector temperature 270 C; carrier gas (nitrogen) flow rate 25 ml/min, hydrogen flow rate 30 ml/min, and air flow rate 300 ml/min. 2 µl of the sample solutions were injected.

Chemicals

Linoleic acid (99%), heptadecanoic acid (99%), lipoxidase from soybean (type II, lot 75c-5030, activity = 48000 units/mg), and Tween 20 were obtained from Sigma Chemical Co. (St. Louis, MO). Ethoxyquin (EMQ) and tertiary butylated hydroxyanisole (BHA) were supplied by Koch-Light Laboratories Ltd. (Colnbrook, England) and Fluka AG (Buchs, Switzerland), respectively. EMQ and BHA were used without further purification. All solvents, anhydrous Na_2SO_4, $NaH_2PO_4 \cdot 2H_2O$, and $Na_2HPO_4 \cdot 12H_2O$ were of analytical grade and obtained from E. Merck (Darmstadt, Germany) and BDH Chemicals Ltd. (Poole, England). The n-hexane was redistilled before use.

Analytical Procedure

Linoleic acid, stock solution: Ca. 150 mg of linoleic acid was emulsified in 0.2 M phosphate buffer (pH = 7.0) under nitrogen atmosphere according to known procedure (13). The solution was stored at 5 C.

Lipoxidase, stock solution: 10 mg of lipoxidase was dissolved in 10 ml ice cold 0.2 M phosphate buffer. The solution was stored in the freezer and kept in an ice-water bath while in use. The diluted solution (0.2 mg/ml) was prepared from the stock solution. The activity of the stock solution was tested as follows: 5 µl enzyme, 250 µl linoleic acid (0.25 mg/ml), and 2.75 ml borate buffer pH 9 were mixed in the cuvette and the absorbance was measured at 234 nm. The activity = 46000 units/mg was calculated from the increase in optical density per minute and was constant in all experiments.

Internal standard, stock solution: Heptadecanoic acid was used as internal standard for the gas chromatographic analyses. The acid was dissolved in methanol at a concentration of 1 mg/ml. The solution was stored at 5 C.

Antioxidant, stock solution: The antioxidants were dissolved in acetone at the concentration of 20 mg/ml. Diluted solutions of 0.5 mg/ml and 5 mg/ml were prepared. All solutions were stored in the freezer (-18 C) due to the reported instability of EMQ in polar solvents (14) and kept in an ice-water bath while in use. BHA was added to the reaction mixture, while the EMQ solution was emulsified in the stock solution of fatty acid at appropriate concentrations (1 mg/ml, 0.25 mg/ml, and 0.025 mg/ml in the final solution), due to the low solubility of EMQ.

Enzyme reaction: To 2 ml sample solution was added 1 ml phosphate buffer and 0.1 ml acetone or 0.1 ml antioxidant solution (BHA). The mixture was incubated at 20 C (water bath) after addition of ca. 0.2 ml enzyme solution. The amount of the enzyme was calculated from a constant weight ratio linoleic acid-lipoxidase. Several ratios were applied in our experiments. In this paper we present those of 30:1 and 150:1. Air was passed continuously through the reaction mixture at flow rate 30 ml/min. After the appropriate incubation time the oxidation reaction was terminated by acidifying the mixture with 1N H_2SO_4 and

TABLE I

Mean Values of Weight Ratio Unreacted Linoleic Acid-Initial Amount Linoleic Acid (Percentage) at Varying Incubation Time and Antioxidant Level[a]

Incubation time (min)	Concentration of BHA[b]		Concentration of EMQ[c]		Without antioxidant
	5 mg/ml	0.5 mg/ml	5 mg/ml	0.5 mg/ml	
1	100.2	102.6	100.6	99.3	97.4
3	99.2	98.7	100.7	97.7	94.1
5	97.1	98.2	97.4	95.5	93.1
7	95.4	94.2	95.6	94.3	90.7
10	93.4	90.1	90.1	90.5	88.4
12	90.6	88.1	88.8	87.4	86.6
15	88.7	87.3	85.8	81.9	82.2

[a]Weight ratio linoleic acid-lipoxidase–150:1.
[b]BHA = butylated hydroxyanisole.
[c]EMQ = ethoxyquin.

TABLE II

Mean Values of Weight Ratio Unreacted Linoleic Acid-Initial Amount Linoleic Acid (Percentage) at Varying Incubation Time and Antioxidant Level[a]

Incubation time (min)	Concentration of BHA[b]			Concentration of EMQ[c]			Without antioxidant
	20 mg/ml	5 mg/ml	0.5 mg/ml	20 mg/ml	5 mg/ml	0.5 ml/ml	
1	99.3	95.9	97.2	98.2	98.0	98.5	96.0
3	94.5	92.0	90.6	93.5	92.3	93.1	86.4
5	85.4	87.6	83.0	91.1	86.9	88.2	77.7
7	82.2	81.0	76.2	84.9	82.3	82.2	71.0
10	76.7	71.4	67.9	77.1	76.8	74.3	60.8
12			61.8	72.5	71.4	68.8	53.0
15	65.2	58.0	56.2	63.2	62.4	59.0	45.1

[a]Weight ratio linoleic acid-lipoxidase-30:1.
[b]BHA = butylated hydroxyanisole.
[c]EMQ = ethoxyquin.

addition of 2 ml methanol. Unreacted linoleic acid was extracted and converted to the methyl ester according to the following procedures. The incubation time varied from 1 to 15 min.

Extraction: Unreacted linoleic acid was extracted from the reaction mixture by a methanol-chloroform solvent mixture made of 2 ml methanol, 1 ml internal standard solution, and 3 ml chloroform. The chloroform layer was separated off, and the extraction was repeated twice with 3 ml chloroform (15).

Derivatization: The chloroform extracts were evaporated to dryness by highly purified nitrogen and the methyl esters of the fatty acids were prepared by the boron trifluoride method (16). The dry residue was dissolved in 0.5 ml 10% BF_3 in methanol and refluxed for 2 min. After addition of 1 ml hexane through the condenser the solution was refluxed 1 min more. Saturated NaCl solution was added to the cooled reaction mixture to float the hexane solution of the ester into the neck of the flask. The organic solution was dried (with anhydrous Na_2SO_4) and injected directly into the gas chromatograph.

Standard curve: Solutions containing from 1 to 9 mg of linoleic acid were prepared from the stock solution and treated according to the above-mentioned extraction and derivatization procedures. From the gas chromatograms the ratio of the peak height of methylated linoleic acid and C17-acid was calculated and plotted against the concentrations of linoleate. A least squares linear regression was performed to obtain a representation of the data. The procedure was carried out for each lot of stock solution.

Analysis of the data: Parallel samples of linoleic acid were oxidized enzymatically, and unreacted acid was analyzed according to the above-mentioned procedure. Based on the standard solution the amount of unreacted linoleic acid was calculated. The percentage of unreacted acid in the reaction mixture was calculated and plotted against the reaction time.

RESULTS AND DISCUSSION

When applying the extraction and derivatization procedures as described, 100% recovery of linoleic acid was obtained. The percentage of unreacted linoleic acid in the oxidative reaction system was calculated at varying incubation times and at various concentrations of antioxidant. We have tested two antioxidants, BHA and EMQ, at two levels of lipoxidase. The mean values of the experimental data on the linoleic acid-lipoxidase weight ratios 150:1 and 30:1 are presented in Tables I and II, respectively. Gross errors were eliminated prior to the mean value calculations by the method outlined by Gottschalk and Dehmel (17). The standard deviation of the method was calculated from the parallels of experimental data, $s_d = \pm 0.86\%$.

When comparing the data it is clear that the lowest ratio linoleic acid-lipoxidase exhibits more pronounced oxidation profiles and consequently higher sensitivity for testing antioxidants. The longest incubation times accentuated the differences between systems with and without antioxidant. However, a sufficiently rapid procedure was required, and reasonable sensitivity was obtained within 15 min. Table I shows that the highest ratio of fatty acid-enzyme in the emulsions with antioxidant had induction time varying from 1 to 3 min. The length of this period may be used as a parameter for the inhibitive effect of the antioxidants and will be studied further in our laboratory. The advantage of the latter system is that the degree of oxidation is more similar to that of a food system in contrast to the 30:1 acid-enzyme ratio.

It is worth noticing the differences between BHA and EMQ under our experimental conditions (Table II). At the two lowest antioxidant concentrations EMQ appeared more efficient than BHA, whereas at the highest antioxidant level

TABLE III

The Effect of the Concentrations of the Antioxidants BHA[a] and EMQ[b] on the Protective Indices (PI)[c]

	Concentration of antioxidant		
	20 mg/ml	5 mg/ml	0.5 mg/ml
BHA	1.75	1.42	1.28
EMQ	1.76	1.72	1.58

[a]BHA = butylated hydroxyanisole

[b]EMQ = ethoxyquin.

[c]30:1 weight ratio linoleic acid-lipoxidase.

BHA and EMQ showed almost similar inhibitive effect in the oxidative system. Table II also shows that the inhibitive effect of BHA increased with concentration at the longest incubation times. For EMQ only a slight concentration effect appeared between the two highest antioxidant levels. The data show that the difference in effectiveness between the two antioxidants can be detected at the lowest antioxidant concentrations.

A Student's *t*-test (18) at 95% confidence level showed that the differences between the results with and without antioxidant were significant at both enzyme levels and all three antioxidant levels. The effect was more pronounced at the highest lipoxidase concentration. The above-mentioned differences between EMQ and BHA, and between the various antioxidant levels were also significant at 95% confidence level.

In order to facilitate the comparison of the antioxidant activities Hamilton and Tappel (8) defined a protective index (PI) as the ratio of the time required for reaction of 90% of the dissolved oxygen in the antioxidant system divided by the time for the control. Similarly, Berger (19) defined a protective factor (PF) as the induction period with additive divided by the initial induction period. Based on our results we propose a protective index (PI) as the time required to oxidize 30% of linoleic acid in the antioxidant system, divided by the time of the system without antioxidant. The PIs are listed in Table III and are calculated only from results obtained in the 30:1 acid-enzyme system.

The aromatic amine ethoxyquin has been found very efficient in biological systems (6,20-22). It is well known that EMQ decomposes to oxidation products of high antioxidative effect (23, J. Utne Skåre, personal communication). The phenolic compound butylated hydroxyanisole (BHA) shows varying activity (6,9,10). In most cases BHA and EMQ have been tested separately, particularly not in an enzyme catalyzed system, and the tests have been based on oils, fats, or other biological systems. In our oxidative system, however, the substrate is a simple unsaturated component. The pronounced difference between these two antioxidants found by Marco (6) were not obtained when tested by our method; the results are more in accordance with those of Cort et al. (10).

The inhibition mechanism by which BHA and EMQ operate is thought to be different. The mechanism of the lipoxidase catalyzed oxidation of linoleic acid is described by Siddiqi and Tappel (24). The inhibition reaction of BHA is explained as an abstraction of an electron or a hydrogen radical from the inhibitor to lipoxidase. This results in a decrease in the capacity of the enzyme for initiating linoleate oxidation (24). In autoxidation the hydrogen radical is abstracted by the peroxide radical ($R\dot{O}_2$). The high activity of amines in an autoxidative reaction may be due to a radical complex formed by the addition of the peroxide radical to the amine molecule through the lone electron pair on the nitrogen atom (25). A recent electron spin resonance (ESR) study on ethoxyquin suggests that the presence of a free radical is connected to the mechanism of the antioxidant (26). In order to explain that BHA and EMQ exhibit approximately the same activity when tested in our system, we may suggest that EMQ inhibits by a mechanism similar to that of BHA in the lipoxidase system. The active sites in the enzyme may be inactivated by abstraction of the amine hydrogen in the same way as the phenolic hydrogen. Complex formation between the free electron pair and the active site in the enzyme may also be a plausible mechanism for inactivating the enzyme. Still another possible explanation may be that EMQ inhibits by the traditional mechanism, preventing the formation of radicals in the chain reaction. Then the amine will strongly search for a $R\dot{O}_2$-radical to form a complex. The radical, however, may be partly protected by the enzyme, and the efficiency of the antioxidant will not be fully employed in the system.

In view of the complexity of the oxidative processes, we may conclude from our results that the antioxidant mechanism and efficiency are partly dependent on the catalyst present in the fatty system. Thus, it is important to consider the factors initiating the reaction and determining its pathways when choosing antioxidants.

The test system outlined here consists of a certain number of compounds that are easy to alter for modification of the system. Substrate, catalyst, antioxidant, and interacting substances can be changed and their concentrations varied. Also the physical factors, oxygen rate flow, and incubation time and temperature can be varied. Methods for determining the products of the oxidation reaction may be applied to the system. These modifications may give us information about the significance of the different factors on the mechanisms of the antioxidant inhibition.

Work is in progress in our laboratory for evaluating other antioxidants and modification of the system.

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