Measurement of Hydroperoxides in Edible Oils Using the Ferrous Oxidation in Xylenol Orange Assay

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The application of the FOX2 (ferrous oxidation in xylenol orange, version 2) method to the measurement of hydroperoxides in edible oils is described. Sample preparation involves dissolving oil (10 mg) in 1 mL of propan-1-ol and mixing an aliquot (100 μL) with 900 μL of the FOX2 reagent in a 1.5 mL microcentrifuge tube. Absorbance of the supernatant is read at 560 nm following incubation for 30 min at room temperature and centrifugation at 12000g for 5 min. Analysis by the FOX2 assay of 21 varieties of freshly opened vegetable oils revealed that edible oils contained 0.96–6.74 mmol kg^{-1} of hydroperoxides. The intra-assay coefficient of variation for the FOX2 assay was generally less than 10%. There was a strong negative correlation (Spearman's rank correlation) between initial hydroperoxide levels and total tocopherols in the oil ($r_S = -0.48$; $p < 0.025$). Oils aged for 6 months at room temperature after opening showed an increase in hydroperoxide content which correlated negatively with total tocopherol content ($r_S = -0.39$; $p < 0.05$). Surprisingly, there was a significant negative correlation between initial thiobarbituric acid reactive material (TBARM) values and hydroperoxide content as well as a significant positive correlation between total tocopherols and initial TBARM values. These results suggest that TBARM values for edible vegetable oils may not accurately predict true peroxidation and confirm that the FOX2 assay is appropriate for the determination of "peroxide values".

Keywords: *Hydroperoxide; vegetable oils; xylenol orange; FOX assay*

INTRODUCTION

Reaction of oxygen with unsaturated lipids produces a wide range of compounds with hydroperoxide as the initial product (Frankel, 1983). Lipid peroxidation is responsible for the changes in taste and odor of fats and oils by the production of secondary low molecular weight aldehydes. In addition, occurrence of oxidized lipids in foods has been considered to be cytotoxic, atherogenic, and carcinogenic [for review see Addis and Warner (1991)].

Currently employed assays for food rancidity involve measurement of hydroperoxides and low molecular weight aldehydes. Hydroperoxides are commonly monitored by iodometric assay using the ability of hydroperoxides to oxidize iodide to iodine, which is then titrated with thiosulfate or trapped by excess iodide to yield the triodide anion (Hicks and Gebicki, 1979; Robards et al., 1988; Thomas et al., 1989). A major disadvantage of the iodometric assay, however, is its susceptibility to interference by molecular oxygen as well as the reaction of liberated iodine with other components in the system.

Aldehyde analysis is based on the assessment of thiobarbituric acid reactive material (TBARM) or p-anisidine reactive substances (Robards et al. 1988). TBA reacts most extensively (under the conditions used for its measure of lipid peroxidation) with malondialdehyde, which is a decomposition product of endoperoxides formed during peroxidation of polyunsaturated fatty acids possessing three or more double bonds (Porter, 1980). In edible oils, malondialdehyde is thought to be mainly derived from linolenic acid which accounts, variably, for less than 1% of total fatty acid composition (White, 1992).

In response to the need for a simple and sensitive global test for authentic hydroperoxides, we have developed a method based upon the oxidation of ferrous to ferric ions by hydroperoxides under acidic conditions in the presence of the ferric ion indicator, xylenol orange (Jiang et al., 1991, 1992). Two versions of the assay have currently been developed (Wolff, 1994). FOX1 is suitable for the determination of very low (<1 μM) levels of hydrogen peroxide in aqueous buffers. The FOX2 assay is appropriate for the measurement of lipid hydroperoxides and has been used, *inter alia*, for the measurement of hydroperoxides accumulating during peroxidation of low-density lipoprotein (Jiang et al., 1991, 1992) and present in plasma (Nourooz-Zadeh et al., 1994). We now describe the use of the FOX2 assay to measure hydroperoxides in edible vegetable oils. In addition, the quantitative relationships between FOX2-detectable hydroperoxides, TBARM assays, and tocopherols are examined.

MATERIALS AND METHODS

Samples. Dietary oils were purchased from local supermarkets in London. The samples were immediately analyzed after opening of bottles which were then kept at room temperature until further analysis. Samples were withdrawn from the upper 1 cm of oil bottles, and bottles were not shaken during storage.

Reagents. Ammonium ferrous sulfate, hydrogen peroxide (H_2O_2), butylated hydroxytoluene (BHT), thiobarbituric acid (TBA), and catalase were obtained from Sigma Chemical Co. (Poole, Dorset, U.K). Xylenol orange [o-cresolsulfonphthalein-3,3-bis(methyliminodi-

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0021-8561/95/1443-0017$09.00/0 © 1995 American Chemical Society

acetic acid sodium salt)] and triphenylphosphine were purchased from Aldrich (Gillingham, Dorset, U.K.). All other chemicals and reagents used were of the highest purity available.

Preparation of FOX2 Reagent. FOX2 reagent was prepared by dissolving xylenol orange and ammonium ferrous sulfate in 250 mM H_2SO_4 to final concentrations of 1 and 2.5 mM, respectively. One volume of this concentrated reagent was added to 9 volumes of HPLC grade methanol containing 4.4 mM BHT to make the working reagent which comprised 250 μM ammonium ferrous sulfate, 100 μM xylenol orange, 25 mM H_2SO_4, and 4mM BHT in 90% v/v methanol. The working reagent which has an extinction coefficient for hydroperoxides of approximately 4.3×10^4 M^{-1} cm^{-1} was routinely calibrated against solutions of H_2O_2 of known concentration (Jiang et al., 1991). Reagent was also obtained as the commercial preparation (PeroXOquant Quantitative Peroxide Assay: lipid compatible formulation) from Pierce (Rockford, IL).

Liposome Preparation. One hundred milligram aliquots of oil were vortexed with 10 mL of phosphate-buffered saline (10 mM potassium phosphate, pH 7.4, 150 mM NaCl). The dispersion was ultrasonicated for 3 min while cooling on ice to make small liposomes. The resulting liposomes were incubated in the presence of 10 μM copper sulfate at 37 °C. Samples (100 μL) were removed at defined time intervals and assayed for hydroperoxides and TBARM.

Measurement of Lipid Peroxidation Products. *(A) Hydroperoxides.* Oil samples were prepared for assay by dissolving 10 mg of each oil in 1 mL of propan-1-ol. An aliqout of the solubilized oil (100 μL) was then mixed with FOX2 reagent (900 μL) in a 1.5 mL microcentrifuge tube. The assay mixture was incubated at room temperature for 30 min and was then centrifuged at 12000*g* for 5 min to remove micelles. The supernatant was carefully decanted, and absorbance was read at 560 nm.

For iodometric assay of hydroperoxides, an aliquot of propanol-solubilized oil (25 μL) was injected into a Warburg cuvette containing 1.5 mL of degassed methanol/glacial acetic acid (1:1 v/v) and 1 mL of degassed saturated solution of potassium iodide 20% in methanol. Absorbance at 290 nm was monitored for 3 min. A standard curve generated with hydrogen peroxide gave an extinction coefficient for tri-iodide at 290 nm of 4.7×10^4 M^{-1} cm^{-1}, which is in good agreement with the value published previously (Hicks and Gebicki, 1979).

(B) Thiobarbituric Acid Reactive Material (TBARM). For the measurement of TBARM, an aliqout of propanol-solubilized oil (100 μL) was mixed with 6.7% TBA (1 mL) and 20% trichloroacetic acid (0.5 mL) followed by incubation at 95 °C for 20 min. Absorbance was monitored at 532 nm after centrifugation at 12000*g* for 5 min. The concentration of lipid peroxidation products was calculated as malondialdehyde equivalents using the extinction coefficient for the malondialdehyde–thiobarbituric acid complex of 1.56×10^5 M^{-1} cm^{-1} at 532 nm (Slater and Sawyer, 1971).

Effect of Catalase on Hydroperoxides in Oil. To determine whether FOX2-generated signal in oils might be related to the presence of hydrogen peroxide, aliquots (90 μL) of propanol-solubilized oil were placed in microcentrifuge tubes to which 10 μL of catalase (1000 units/mL) or water was added and which was then vortexed and incubated at room temperature for 30 min. FOX2 reagent (900 μL) was added and incubated for another 30 min. After centrifugation at 12000*g* for 5 min, absorbance of the supernatant was monitored at 560 nm.

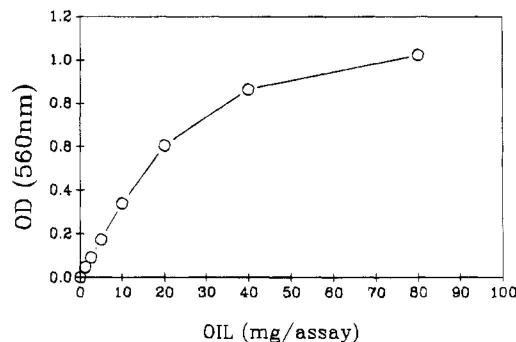

Figure 1. Effect of increasing oil concentration on the performance of the FOX2 assay. Sample preparation involved dissolving increasing amounts of virgin olive oil in 1 mL of propan-1-ol followed by mixing a 100 μL aliquot of the sample with 900 μL of the FOX2 reagent. After incubation for 30 min, the sample was centrifuged at 12000*g* for 5 min, and absorbance of the supernatant was monitored at 560 nm. Data represent mean ± SD of quadruplicate measurements. Error bars lie within the mean data points. Similar data were obtained for other oils that contained less hydroperoxide.

Effect of Triphenylphosphine (TPP). Propanol-solubilized oils samples (90 μL) were transferred to 1.5 mL microcentrifuge tubes to which 10 μL of TPP (10 mM in methanol) or methanol alone was added. After incubation at room temperature for 30 min, FOX2 reagent (900 μL) was added and incubated for another 30 min at room temperature. Absorbance of the supernatant was monitored at 560 nm following centrifugation at 12000*g* for 5 min.

Vitamin E Analysis. Oil samples (100 mg) were dissolved in 1 mL of propan-1-ol containing 20 μg of vitamin E acetate as internal standard. HPLC separation was performed on a Hypersil-ODS column (10 cm × 5 mm, particle size 5 μM; Chrompack, the Netherlands) using dichloromethane–acetonitrile–methanol–water (15:90:10:30 by volume) at a flow rate of 0.8 mL/min. Signal was monitored at 280 nm (Applied Biosystem). Data presented are the sums of individual tocopherols including alpha-, delta-, and gamma-tocopherols.

RESULTS AND DISCUSSION

Evaluation of the Effect of Oil Content on the Performance of the FOX2 Assay. Figure 1 shows the effect of increasing oil content on absorbance measurement in the FOX2 assay. The analysis revealed that there was a linear relationship between oil content and absorbance measured at 560 nm up to 20 mg in the assay. Thereafter, absorbance measurement started to deviate from linearity due to immiscibility of excess oil when present in large amounts in the methanolic FOX2 reagent. We chose to conduct the assay at a final oil content of 1 mg in the assay to achieve a near-complete solubilization of the oil in the methanolic FOX2 reagent.

Confirmation of Oil Hydroperoxide Content. To confirm authenticity of hydroperoxides assessed by the FOX2 assay, oil samples were pretreated with catalase and triphenylphosphine, respectively. Catalase was used to destroy any hydrogen peroxide present. Triphenylphosphine was used to reduce hydroperoxides to their corresponding alcohols (Barnard and Wong, 1976; Nakumura and Maeda, 1991). As shown in Figure 2, catalase had no effect on the signal monitored at 560

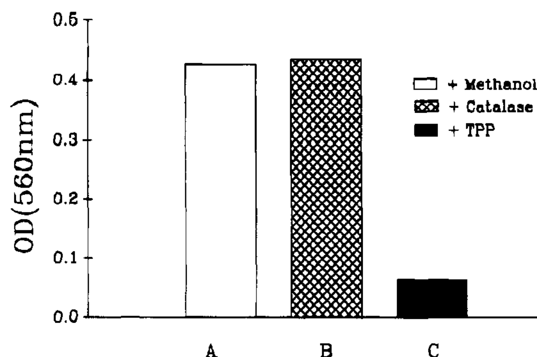

Figure 2. Effect of catalase and triphenylphosphine on hydroperoxides in olive oil using the FOX2 assay. Samples were prepared by dissolving 10 mg of oil in 1 mL of propan-1-ol. A 90 μL aliquot of the sample was then incubated either (A) with 10 μL of methanol (as a control incubation), (B) with 10 μL of catalase (1000 U/mL), or (C) with 10 μL of triphenylphosphine (10 mM in methanol) for 30 min at room temperature. FOX2 reagent (900 μL) was then added, and the sample was incubated for another 30 min. The sample was centrifuged at 12000g for 5 min, and absorbance of the supernatant was measured at 560 nm. Data represent the means of quadruplicate measurements. Coefficients of variation were less than 2.5%.

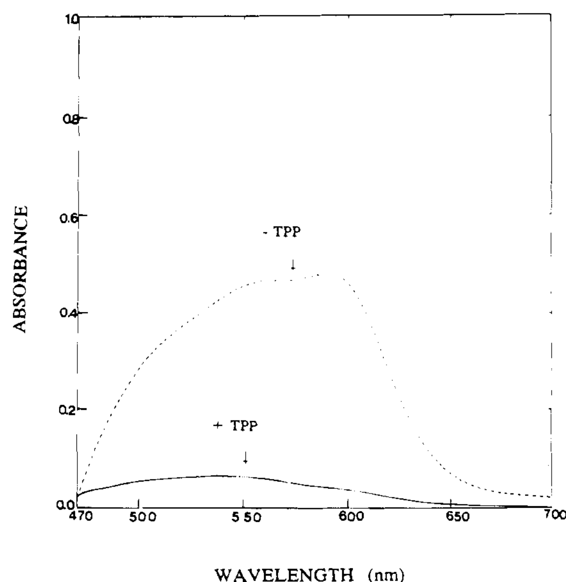

Figure 3. Spectral characteristic of oil hydroperoxides before and after TPP treatment in the FOX2 assay. Sample was prepared by dissolving 10 mg of oil in 1 mL of propan-1-ol. An aliqout (90 μL) was incubated with 10 μL of triphenylphosphine (10 mM in methanol) or 10μL of methanol alone for 30 min at room temperature. FOX2 reagent (900 μL) was added, and the sample was incubated for another 30 min. The sample was centrifuged at 12000g for 5 min, and absorbance was scanned from 470 to 700 nm.

nm. By contrast, the signal was greatly reduced when the sample was pretreated with triphenylphosphine. Representative difference spectra of olive oil samples in the FOX2 assay with and without triphenylphosphine pretreatment are presented in Figure 3. These data allow the conclusion that oil components which react with the FOX2 reagent were authentic hydroperoxides present in the samples.

Assessment of Lipid Peroxidation Products in Edible Oils. The applicability of the FOX2 assay to measure hydroperoxides in oils was tested by analyzing 21 edible oils. Using the FOX2 assay, hydroperoxide values in the oil samples ranged from 0.96 to 6.41 mmol kg^{-1} with an intra-assay coefficient of variation for quadruplicate measurements generally less than 10% (Table 1). By contrast, hydroperoxide levels found using the iodometric method were lower than those seen with the FOX2 assay (Table 1), in part because there seemed to be a "threshold" effect below which hydroperoxide levels could not be measured, even with standard hydroperoxides (data not shown). One explanation for this observation may be the very rapid changes in absorbance at 290 nm which occurred after injection of the solubilized oil sample, and which could not be recorded accurately, in combination with the high and mobile baselines generally associated with this version of the iodometric assay. In addition, in our hands, the intra-assay coefficients of variation for quadruplicate analyses in the freshly opened oils using the iodometric assay varied from 30 to over 100%. TBARM values in the oil samples ranged from 0.12 to 0.46 mmol kg^{-1} and were much lower than the hydroperoxide values. Table 1 also gives the values for total tocopherols (alpha-, delta-, and gamma-tocopherols) in the oils which ranged from being undetectably low (i.e. less than 10 μmol kg^{-1}) in one brand of refined olive oil to almost 10 mmol kg^{-1} in wheat germ oil.

Storage Effect. FOX2 assay of the oil samples after storage for 2 months at room temperature revealed only small differences in hydroperoxide content when compared to the newly opened bottles. However, a 2-fold increase in the level of the hydroperoxides was observed in the oil samples after storage for 6 months after opening (Table 2). Several of the oils contained levels of hydroperoxides, determined by the FOX2 assay, which approached or exceeded 10 mmol kg^{-1}.

Effect of Incubation under Pro-oxidant Conditions. We extended the above experiment in which hydroperoxide values were measured as a function of time after opening of the bottles by preparing liposomes from selected oils and incubating them with copper ions at 37 °C at pH 7.4 (Table 3). Changes in hydroperoxide levels and TBARM were measured at various time intervals up to 72 h. In the case of unrefined sunflower oil and corn oils (oils 8 and 11) incubation with copper ions caused a 10-fold increase in hydroperoxide level but a smaller increase in TBARM. By contrast, treatment of a virgin olive oil (oil 1) under the same conditions led to only a 6-fold increase in hydroperoxide level but a 30-fold increase in TBARM. Different oils, on peroxidation, thus seem to possess differing relationships between hydroperoxide and TBARM values.

Relationships between Hydroperoxides, Other Indices of Peroxidation, and Total Tocopherol. We examined the relationships (Spearman's rank correlation) between hydroperoxides in freshly opened oils, increases in hydroperoxides on aging of oils, TBARM, and total tocopherols (Table 4). We found, as expected, that the initial concentration of hydroperoxide in an oil was an important predictor of the increase in hydroperoxide content of the same oil on aging. We also found, as expected, that there was a significant inverse relationship between the initial level of hydroperoxide, the increase in hydroperoxide level on aging and total tocopherol content of the oil. However, we found a surprising negative relationship between initial hydroperoxide levels and TBARM ($r_S = -0.48$; $p = 0.015$) as well as a strong positive relationship between tocopherol levels and TBARM (Table 4). These relationships were only slightly altered (not shown) when the data pertaining to wheat germ oil (which contained very high levels of tocopherol) were omitted from the statistical analysis.

Table 1. Content of Lipid Peroxidation Products and Tocopherols in Freshly Opened Vegetable Oils

oil	ROOH (FOX2), mmol kg^{-1}	ROOH (IODO$^-$), mmol, kg^{-1}	TBARM, mmol kg^{-1}	tocopherol, μmol kg^{-1}
1 virgin olive	6.41 ± 0.04	1.24 ± 0.58	0.30 ± 0.04	312
2 virgin olive	5.43 ± 0.12	1.65 ± 0.47	0.36 ± 0.04	530
3 virgin olive	6.36 ± 0.2	1.63 ± 0.43	0.18 ± 0.06	367
4 virgin olive	5.01 ± 0.01	1.72 ± 0.46	0.17 ± 0.05	505
5 refined olive	2.97 ± 0.09	4.62 ± 5.37	0.15 ± 0.02	<10
6 refined olive	3.72 ± 0.10	1.89 ± 0.66	0.12 ± 0.02	289
7 refined olive	2.73 ± 0.12	0.80 ± 0.08	0.15 ± 0.02	247
8 crude sunflower	2.58 ± 0.13	0.80 ± 0.46	0.34 ± 0.05	2220
9 refined sunflower	2.39 ± 0.08	1.11 ± 0.41	0.46 ± 0.02	1336
10 refined sunflower	2.47 ± 0.14	0.90 ± 0.36	0.43 ± 0.03	1323
11 refined corn	0.96 ± 0.36	0.45 ± 0.32	0.43 ± 0.03	2091
12 refined corn	1.89 ± 0.56	0.86 ± 0.43	0.43 ± 0.02	1889
13 evening primrose	2.20 ± 0.02	2.03 ± 0.73	0.34 ± 0.12	1385
14 grapeseed	1.88 ± 0.05	1.45 ± 1.35	0.46 ± 0.06	1800
15 peanut	1.13 ± 0.13	2.28 ± 0.74	0.29 ± 0.03	743
16 peanut	2.02 ± 0.02	1.11 ± 0.37	0.44 ± 0.00	812
17 hazelnut	3.53 ± 0.07	1.70 ± 0.56	0.20 ± 0.07	575
18 safflower	2.89 ± 0.18	1.29 ± 0.48	0.56 ± 0.06	980
19 sesame	1.55 ± 0.11	0.57 ± 0.31	0.23 ± 0.10	1270
20 sesame	1.43 ± 0.09	1.75 ± 0.88	0.40 ± 0.03	410
21 wheat germ	3.07 ± 0.21	2.00 ± 0.48	0.25 ± 0.03	9950

Table 2. Alterations in Hydroperoxide Content on Aging of Opened Oils at Room Temperature

oil	hydroperoxides determined by the FOX2 assay, mmol kg^{-1}		
	fresh	open 8 weeks	open 26 weeks
1 virgin olive	6.41 ± 0.04	6.90 ± 0.26	14.87 ± 2.44
2 virgin olive	5.43 ± 0.12	6.70 ± 0.22	9.18 ± 1.33
3 virgin olive	6.36 ± 0.20	5.97 ± 0.15	9.20 ± 2.80
4 virgin olive	5.01 ± 0.01	5.95 ± 0.17	10.20 ± 0.88
5 refined olive	2.97 ± 0.09	3.84 ± 0.22	7.79 ± 0.44
6 refined olive	3.72 ± 0.10	2.26 ± 0.11	6.89 ± 0.22
7 refined olive	2.73 ± 0.12	2.45 ± 0.20	4.31 ± 0.44
8 crude sunflower	2.58 ± 0.13	3.21 ± 0.11	5.01 ± 0.22
9 refined sunflower	2.39 ± 0.08	2.83 ± 0.11	5.57 ± 0.44
10 refined sunflower	2.47 ± 0.14	3.22 ± 0.11	6.46 ± 1.33
11 refined corn	0.90 ± 0.36	1.52 ± 0.11	2.51 ± 0.22
12 refined corn	1.89 ± 0.56	1.90 ± 0.06	2.87 ± 0.66
13 evening primrose	2.20 ± 0.02	2.91 ± 0.13	8.00 ± 0.44
14 grapeseed	1.88 ± 0.05	2.45 ± 0.11	3.86 ± 0.00
15 peanut	1.13 ± 0.13	1.73 ± 0.15	3.86 ± 0.00
16 peanut	2.02 ± 0.02	2.12 ± 0.08	4.98 ± 1.11
17 hazelnut	3.53 ± 0.07	5.17 ± 0.08	10.30 ± 2.20
18 safflower	2.89 ± 0.18	4.58 ± 0.20	9.20 ± 0.00
19 sesame	1.55 ± 0.11	2.22 ± 0.15	3.80 ± 0.09
20 sesame	1.43 ± 0.09	2.84 ± 0.11	6.14 ± 0.44
21 wheat germ	3.07 ± 0.21	3.37 ± 0.20	5.99 ± 0.88

Table 3. Hydroperoxide Accumulation in Liposomes Oxidized *in Vitro*[a]

liposome type	ROOH, μM	TBARM, μM
native virgin olive	2.86	0.07
oxidized virgin olive	16.00	2.10
native corn	0.78	0.08
oxidized corn	8.82	0.38
native sunflower	1.00	0.06
oxidized sunflower	10.27	0.19

[a] Liposomes were prepared as described under Materials and Methods. Native liposome values refer to concentrations of hydroperoxides and TBARM prior to incubation. Oxidized liposome values refer to those obtained after the liposomes had been incubated with 10 μM copper ion in phosphate-buffered saline for 72 h. Concentrations given refer to the level of hydroperoxide and TBARM in the liposome incubation mixtures.

Table 4. Grid of Spearman's Rank Correlation Coefficients (and Their Probabilities) for Indices of Peroxidation and Tocopherols

	initial ROOH	delta ROOH	initial TBARM	tocopherols
initial ROOH		0.52 (0.008)	−0.47 (0.015)	−0.48 (0.013)
delta ROOH	0.52 (0.008)		−0.1 (0.07)	−0.39 (0.041)
initial TBARM	−0.47 (0.015)	−0.07 (0.38)		0.60 (0.002)
tocopherols	−0.48 (0.013)	−0.39 (0.041)	0.60 (0.002)	

[a] Spearman's rank correlations (and their probabilities) were obtained using a desktop computer statistical package (Unista Unistat Limited, London). Delta ROOH refers to the difference in hydroperoxide values from opening of bottles to their values after 26 weeks.

Since lipid peroxidation implies the accumulation of hydroperoxides, the lack of positive correlation between hydroperoxides and TBARM would seem to argue against the use of TBARM as a reliable measure of peroxidation. The positive relationship between TBARM and tocopherols (in the presence of a negative correlation between hydroperoxides and tocopherols) raises the possibility that TBARM and hydroperoxide values are measures of totally different aspects of oil rancidity.

ACKNOWLEDGMENT

We are grateful to the British Heart Foundation for financial support.

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Received for review May 10, 1994. Revised manuscript received September 27, 1994. Accepted October 4, 1994.[⊗]

JF940229W

[⊗] Abstract published in *Advance ACS Abstracts*, November 15, 1994.