19. Rouser, G., G. Kritchevsky and A. Yamamoto, in Lipid Chromatography Analysis, Edward Arnold, London, 1967, p. 99.
20. Christie, W.W., in Lipid Analysis, Pergamon, Oxford, 1973, p. 85.
21. Lee, F.A., and L.R. Mattick, J. Food Sci. 26:273 (1961).
22. Gill, C.O., M.J. Hall and C. Ratledge, Appl. Env. Microbiol. 33:231 (1977).
23. Uzuka, Y., T. Kanamori, T. Koga, K. Tanaka and T. Naganuma, J. Gen. Appl. Microbiol. 21:157 (1975).
24. Rattray, J.B.M., A. Schibeci and D.K. Kidby, Bacteriol. Rev. 39:197 (1975).
25. Kates, M., and R.M. Baxter, Can. J. Biochem. Physiol. 40: 1213 (1962).
26. Rhim, J.W., M.S. Thesis, Korea Adv. Inst. Sci., 1980.

[Received January 13, 1983]

Effect of Selected Antioxidants on the Stability of Virgin Olive Oil

A.K. KIRITSAKIS[1], **C.M. STINE** and **L.R. DUGAN JR.**, Department of Food Science and Human Nutrition, Michigan State University, East Lansing, MI 48824

ABSTRACT

Virgin unrefined olive oil was protected from oxidation with the antioxidants butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and tertiary butylhydroquinone (TBHQ) and in one case propyl gallate (PG). All the antioxidants improved the stability of olive oil under accelerated conditions (oven test) and storage conditions at 50 C. In the oven test, where the type of oil used was the same as that used in long-term storage studies (room temperature and 50 C), the relative inhibition effect of the antioxidants was in the following order: TBHQ = BHA > BHT. The combinations of BHA and BHT with TBHQ displayed better stabilizing qualities. Antioxidants did not prevent peroxide formation in olive oil stored at room temperature in daylight; these samples oxidized to a high degree, probably due to the catalytic action of chlorophyll. Citric acid (CA) used alone did not affect the oxidative stability of the oil in the oven test and at room temperature in the dark, but exhibited a negative effect at 50 C. The reduction in peroxide content with teritary butylhydroquinone (TBHQ) in the dark at 50 C was greater than anticipated from the oven studies. Potency of the antioxidants under these conditions (50 C) was in the following order: TBHQ > BHT > BHA. The combinations of BHA 0.01% or BHT 0.01% with TBHQ 0.005% used in the dark at 50 C were less effective than TBHQ 0.01%.

INTRODUCTION

Olive oil extracted from the fruits of the tree *Olea europaea* is one of the very few if not the only plant oil in the world which can be consumed in its natural state without being further treated or refined. The oil obtained from healthy mature olive fruits by mechanical means, without any chemical treatment, is called "virgin". This oil is of the highest quality, and is the type used in this study.

Like other vegetable oils, olive oil undergoes oxidative deterioration as a result of many factors. The autoxidation of olive oil results in development of off-flavors and odors and some of its physical properties may also be altered (1). The prevention of autoxidation in olive oil is of great importance from the standpoint of palatability and economy.

Polyphenols, which are natural inhibitors of oxidation in olive leaves, were found to favor the stability of olive oil (2, 3). Hirahara (4) observed an antioxidant effect when alcohol or ether extract from cloves were added to olive oil. The effect of some synthetic antioxidants on the stability of olive oil has been studied (5, 6).

The comparative effect of the antioxidants butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tertiary butylhydroquinone (TBHQ), and propyl gallate (PG) (Eastman Chemical Company, Kingsport, TN) with respect to retardation of autoxidation of virgin olive oil stored at different conditions, was studied. The effect of citric acid used alone was also investigated. Photooxidation of the oil exposed to daylight at room temperature storage was observed.

EXPERIMENTAL PROCEDURE

The olive oil used in this study was derived from the fruits of the olive tree cultivar *Tsounati*, grown on the island of Crete, and was extracted by hydraulic pressing. Three samples of virgin unrefined olive oil from different harvesting periods and having different initial stability and free fatty acid (FFA) content were obtained. They were numbered as olive oil no. 1, no. 2, and no. 3 and had a peroxide value (PV) of 16, 35 and 12 and FFA content expressed as oleic acid of 0.5%, 2.1% and 1.0%, respectively.

The fatty acid composition of the oil was determined by gas liquid chromatography (GLC) in a Beckman GC-4 gas chromatograph. Boron trifluoride-methanol (Sigma Chemical Company, St. Louis, MO) was used for preparing methyl esters (7). The percentage fatty acid composition of the examined samples varied from 7.6 to 13.6 palmitic, 3.0 to 3.3 stearic, 75.6 to 83.9 oleic, and 5.3 to 7.6 linoleic. Traces of palmitoleic and linolenic acids were detected.

The antioxidants were evaluated under accelerated oxidative conditions (oven test at 65 C and 100 C) and under less stressful conditions (room temperature and 50 C). The oil was heated to 60 C and after the addition of antioxidant was held at room temperature for 24 hr with occasional stirring to ensure complete solution of the antioxidants. Citric acid was added to the oil as a solution in a mixture of ethanol and distilled water 1:1 (v/v).

For the oven test (usually referred to as the Schaal oven test), the oil was placed in petri dishes (5.5" in diameter and 3/4" in height) and kept in a constant temperature oven. Samples were removed periodically for peroxide determination. The oven stability of the oil was taken as the number of days needed for the peroxide content of the

[1]Current address: Higher Technical Educational School (Katee), Department of Food Technology, Salonika, Greece.

oil to reach 120 meq/kg oil.

For the long-term storage studies, 100 g of oil was placed in clear jars (2" in diameter and 4.5" in height), loosely capped. Jars were stored at room temperature (20–28 C) and at 50 C. At one week intervals during the first month, and at two weeks for the rest of the storage period, a jar was removed from storage. The oil was mixed and a sample removed for analysis of peroxide value, diene conjugation and TBA absorption values. Five jars of each oil sample were stored in order to reduce the number of samplings taken from a given jar in a short time span.

Peroxide values were determined by a modified Wheeler method (8) and were reported as meq/kg oil. For diene conjugation studies, 10 mg of oil was weighed accurately into small petticups and placed into 30 mL test tubes. Ten mL of purified isooctane (2,2,4-trimethyl pentane) was added and the mixture was shaken on a Fisher minishaker. The mixture was then filtered through a Whatman No. 1 filter paper and the absorbance measured on a Beckman DU spectrophotometer. The 2-thiobarbituric acid (TBA) test employed in this study, was a modification of the method of Dunkley and Jennings (9). The TBA reagent consisted of 0.025 M 2-thiobarbituric acid in 1 M phosphoric acid. Four mL of oil was pipetted into a 55 mL test tube and an equal volume of TBA solution was added. For the color extraction, chloroform distilled in glass (Burdick & Jackson Laboratories, Inc., Muskegon, MI) was added to the TBA reaction solution and the absorbance was measured at 532 nm and 535 nm on a Beckman DU spectrophotometer using TBA solution as a blank.

RESULTS AND DISCUSSION

Oven Studies

Oven stability results for olive oil nos. 1 and 2 are shown in Table I. All antioxidants used had an improving effect on the oven stability of the olive oil. The degree of effectiveness, however, was found to vary in the three different samples of olive oil used and was dependent on the concentration used. The difference in stability between the samples may be due to the fact that the amount of natural antioxidants (tocopherols and phenols) present in olive oil differed from one sample of oil to another. It might also be due to the percentage variation of unsaturated fatty acids of the three samples used. The variation in the unsaturation in turn depend on the area where the trees are grown (10) and/or the degree of maturity of the fruits (11).

Data in Table I demonstrate that samples from olive oil no. 1 containing 0.005% TBHQ exhibited better stability than those containing 0.01% BHT which in turn exhibited better stability than those containing 0.01% BHA. It is interesting to note that the order of effectiveness of the antioxidants was reversed when olive oil no. 2, with higher initial peroxide value (35), was used. In this case, samples containing 0.005% TBHQ showed the lowest stability whereas those containing 0.01% BHA showed the highest (Table I). BHA performed better than BHT in sample no. 2 but not in sample no. 1. Antioxidants BHA and BHT used in combination with TBHQ gave better results than when used alone. The combination of 0.01% BHA and 0.01% TBHQ was found to be the most effective of those evaluated in the oven studies. The use of 0.005% citric acid alone had no effect on the oven stability of olive oil no. 2.

Results obtained from the oven test in which olive oil no. 3 was used are shown in Table II. These results indicate that this sample which had the lowest degree of oxidative degradation (as determined by the initial peroxide value), showed lower stability than nos. 1 and 2. BHA and PG were quite effective in increasing the stability of olive oil no. 3, but TBHQ and BHT were only slightly effective.

TABLE II

Stability Studies with Olive Oil Treated with Different Antioxidants (Oven Test at 65 C)

Antioxidant (wt %)	Stability of olive oil no. 3[a] Oven days	Protective factor[b]
None (control)	16	1.00
0.02 BHA	27	1.68
0.02 BHT	20	1.25
0.02 TBHQ	20	1.25
0.02 PG	26	1.62

[a]Initial peroxide value 12.

[b]Protective factor is expressed as stability of the sample containing antioxidant/stability of the control sample.

TABLE I

Stability Studies with Olive Oil Treated with Different Antioxidants (Oven Test at 65 C)

Antioxidant (wt %)	Stability of olive oil					
	No. 1[a]				No. 2[b]	
	100 g sample		50 g sample			
	Oven days	PF[c]	Oven days	PF[c]	Oven days	PF[c]
None (control)	32	1.00	28	1.00	20	1.00
0.01 BHA	36	1.12	30	1.07	31	1.55
0.01 BHT	38	1.18	31	1.10	25	1.25
0.005 TBHQ	41	1.28	36	1.28	24	1.20
0.01 TBHQ	–	–	–	–	31	1.55
0.01 BHA + 0.005 TBHQ	–	–	–	–	34	1.70
0.01 BHT + 0.005 TBHQ	–	–	–	–	28	1.40
0.01 BHA + 0.01 TBHQ	51	1.59	41	1.46	40	2.00
0.01 BHT + 0.01 TBHQ	51	1.59	41	1.46	33	1.65
0.005 Citric acid	–	–	–	–	20	1.00

[a]Initial peroxide value 16.

[b]Initial peroxide value 35.

[c]PF = protective factor and is expressed as: stability of the sample containing antioxidants/stability of the control sample.

In an oven test of 100 C, a decrease in peroxide value was observed after one week. The decrease might result from hydroperoxide decomposition at a rate higher than that at which they were formed at 100 C. It has been reported (12) that hydroperoxides are unstable products, especially at temperatures higher than 100 C.

A significant increase in the rate of oxidation was observed when a smaller sample size was used (Table I). This was probably related to the difference in surface/volume ratio between the two samples. All further tests were made on 100 g samples to provide consistency of results.

Room Temperature Storage

Peroxide values of samples stored at room temperature, ranging from 20 to 28 C, in the dark were essentially unchanged at the end of 22 weeks. This may be due to the fact that the virgin oil used contained chlorophyll which has been reported to act as an antioxidant in the dark (13, 14). The ultraviolet absorption of the oil sample at 233 nm, changed only slightly in 22 weeks. Control samples (containing no antioxidant) gave slightly higher ultraviolet absorption values than the samples treated with antioxidants.

Storage at 50 C

Peroxide values of the control and samples containing antioxidants are presented in Figures 1 and 2. After the 10th week, the control samples showed a continuous and rapid increase in peroxide value, whereas the peroxide value of the samples with 0.01% BHA and those with 0.01% BHT decreased continuously until this study was terminated (Fig. 1). Peroxide values of the samples containing TBHQ started to decrease after the 2nd week. Under these conditions, the order of effectiveness of antioxidants was found to be TBHQ > BHT > BHA. When 0.01% TBHQ was used, the results were almost the same throughout the storage period whether it was combined with BHA or BHT. When TBHQ was used at 0.005%, however, the combination of TBHQ with BHT was more effective than the combination of TBHQ with BHA (Fig. 2). This superiority of TBHQ over

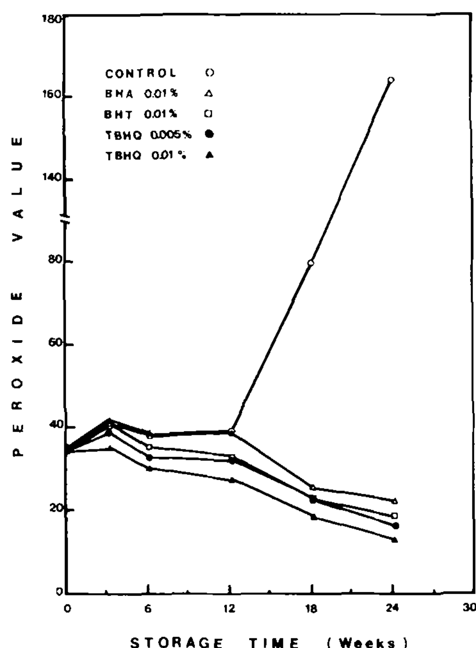


FIG. 1. Peroxide formation in olive oil no. 2 containing antioxidants and stored at 50 C.

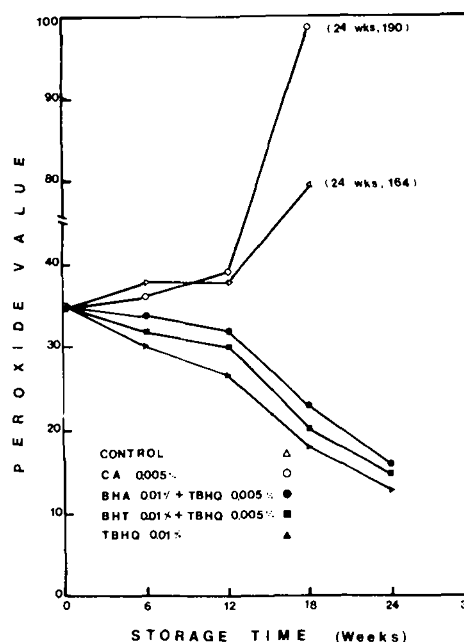


FIG. 2. Peroxide formation in olive oil no. 2 containing antioxidants and stored at 50 C.

BHT or BHA agrees with findings in other oils (15, 16).

Citric acid did not affect the oxidative stability of the oil stored at room temperature but it did exhibit a negative effect at 50 C (Fig. 2). This is an unusual phenomenon, which may be due to the fact that naturally occurring trace metals in olive oil are not readily available for chelation prior to deodorization or heat treatment (17).

The peroxide value of all samples containing antioxidants stored at 50 C, was decreased during the storage period. This indicated a peroxide decomposer (18) role for antioxidants.

Higher TBA absorption values were obtained on oil treated with only citric acid when compared to corresponding controls, which in turn showed higher values than those containing antioxidants. Both the control and the citric acid-treated samples reached a maximum in TBA absorption values and then declined until the end of the storage period (Fig. 3). Malonaldehyde production apparently declines after reaching a peak. Tarladgis and Watts (20) concluded that the malonaldehyde precursor is not stable. Possibly the TBA color-forming reactants are oxidized further and produce more unstable products or disappear by reacting with themselves or with other components of the system. The use of BHA gave higher TBA absorption values than did BHT, which in turn gave higher values than TBHQ when those antioxidants were used in the same concentration. The use of TBHQ at 0.005% resulted in higher TBA absorption values than when it was used at 0.01%. Passing the TBA reaction solution through a chromatographic column (19) gave no significant difference in TBA absorption values, indicating no interference from formation of yellow pigments in the TBA reaction.

The formation of pink color in olive oil, despite the fact that GLC analysis showed that it contained only traces of linolenic acid, may be derived from linoleic acid, since malonaldehyde responsible for the color formation in TBA test (21) may arise from the decomposition of endoperoxides which have been postulated to be formed in a diene system (22).

Ultraviolet absorption values of the control and the citric acid treated samples were in good agreement with the

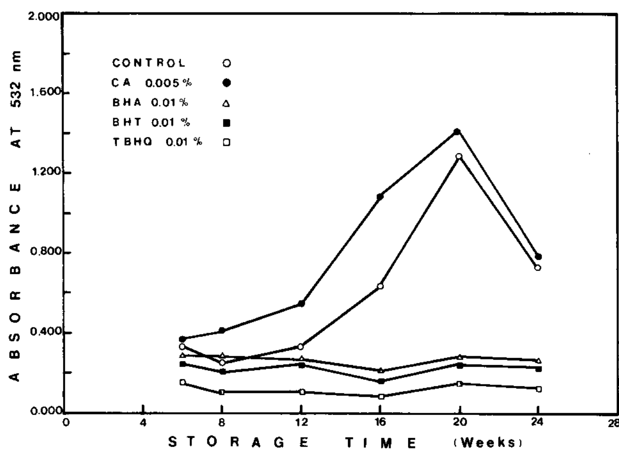


FIG. 3. TBA absorption values of olive oil no. 2 containing antioxidants and stored at 50 C.

peroxide values, which is in accordance with the findings of other workers who have reported a correlation in ultraviolet absorption in olive oil with the change in peroxide value (23, 24). Samples containing antioxidants either alone or in combination showed a reverse relation in peroxide value with ultraviolet absorption from the 10th week on, when a continuous decrease in peroxide values started, probably due to a decomposition effect of the antioxidants (18). The addition of 0.01% BHA, which exhibited the least effect in the decomposition of hydroperoxides, resulted in higher ultraviolet absorption values. The combination of 0.01% TBHQ with 0.01% BHA or 0.01% BHT which had shown a similarity in peroxide values exhibited almost the same ultraviolet absorption values at 233 nm.

Photooxidation of Olive Oil

Hydroperoxide data are shown in Figures 4 and 5. Figure 4 illustrates a significant difference in peroxide formation between samples containing no antioxidant and being exposed to the light and those stored in the dark. This is probably due to the photocatalytic oxidation of the olive

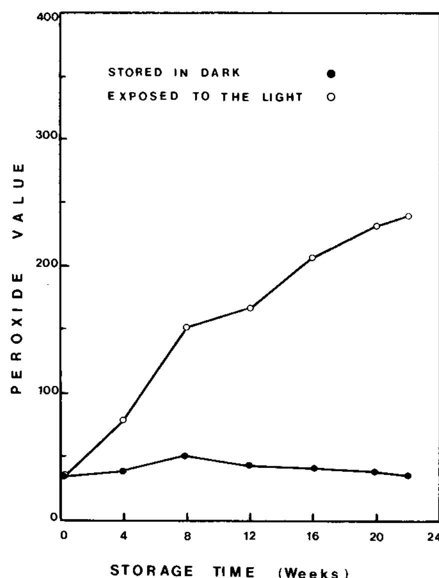


FIG. 4. The effect of light on peroxide formation in olive oil no. 2 (room temperature storage).

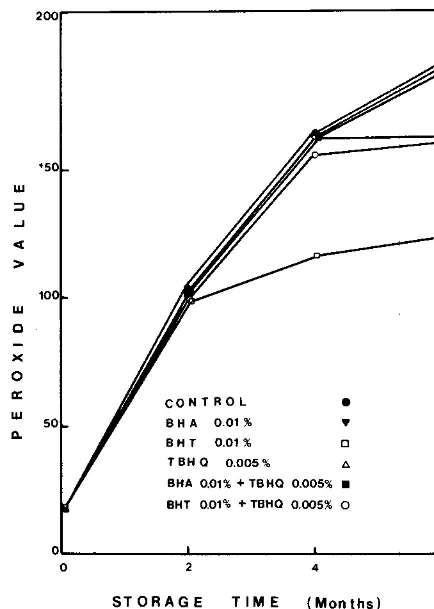
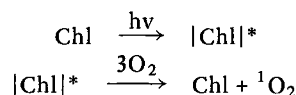


FIG. 5. The effect of light on peroxide formation in olive oil no. 1 containing antioxidants (room temperature storage).

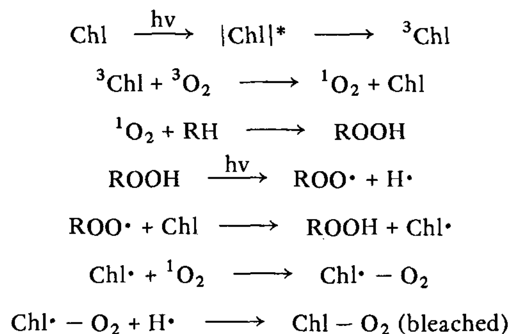
oil which contains natural pigments (chlorophyll, pheophytin). During photooxidation, liberation of nascent hydrogen from the photosensitizer (chlorophyll) may unite with molecular oxygen to form unstable hydroperoxides (25). This mechanism does not involve a free radical reaction but the action of singlet oxygen ($^1\Delta g$, $^1O_2$) which is produced by the transfer of excitation energy from chromophoric impurities to oxygen as shown by the reactions below (26, 27).



The activated singlet oxygen, which is more active than triplet oxygen, reacts more rapidly with unsaturated fatty acids and produces hydroperoxides at a rate of 1450 times faster than triplet O_2 (26). These hydroperoxides can decompose at room temperature, initiating the free radical mechanism of autoxidation (28, 29). Skinner (30) reported that singlet oxygen reacts in an electrophilic rather than in a free radical reaction. No numerical relation was found (31) between chlorophyll concentration and the rate of photooxidation in virgin olive oil.

The rate of hydroperoxide formation for the samples exposed to the daylight was almost unaffected by the presence of antioxidants (Fig. 5). Similar results have been reported by other workers (32, 33). As shown in Figure 5, only samples containing 0.01% BHT had a lower rate of peroxide formation from the second month to the end of the study. Carlsson (33) reported that the photooxidation of unsaturated oils is not prevented by known free radical scavengers, but is retarded by chelates which quench the singlet oxygen ($^1O_2$). During the photooxidation, the α-tocopherol present in oil exposed to the light undergoes rapid peroxidation and thus this natural antioxidant has no effect (33).

Antioxidants TBHQ and BHT presented color loss in the oil, whereas control samples and those containing BHA lost their color, due to the bleaching of chlorophyll as a result of photooxidation (34, 35). The mechanism of bleaching of chlorophyll was proposed by Sastry et al. (34) as follows:



Hydroperoxides are formed due to the action of singlet oxygen. These give rise to peroxy radicals on exposure to light. The peroxy radicals abstract hydrogen atoms from chlorophyll, thus disturbing its conjugated electron system. The resulting peroxy free radical of chlorophyll combines with a proton and is stabilized itself to a stable peroxide.

ACKNOWLEDGMENT

This is Michigan Agricultural Experiment Station Journal Article no. 10635.

REFERENCES

1. Suarez, J.M.M., Manual of Olive Oil Technology, edited by J.M.M. Moreno, Food and Agriculture Organization of the United Nations, Rome, 1975, pp. 6–16.
2. Cantarelli, C., and Montedoro, Riv. Ital. Sostanze Grasse 46: 115 (1969).
3. Notte, E., and N. Romito, Ind. Agric. 9:325 (1971).
4. Hirahara, F., V. Takai and H. Iwao, Jpn. J. Nutr. 32:1 (1974).
5. Gutierrez, R.G.Q., Grasa Aceites 13:69 (1962).
6. Gutierrez, R.G.Q., Ibid. 14:249 (1963).
7. Morrison, R., and L.M. Smith, J. Lipid Res. 5:600 (1964).
8. Wheeler, D.H., Oil Soap 8:89 (1932).
9. Dunkley, W.L., and W.G. Jennings, J. Dairy Sci. 34:1064 (1951).
10. Petruccioli, G., Olearia 20:24 (1966).
11. Swern, D., Bailey's Industrial Oil and Fat Products, edited by D. Swern, Interscience Publishers, Wiley & Sons, Inc., New York, 1964, pp. 81, 202.
12. Cooney, M.P., C.D. Evans, A.W. Schwab and J.C. Cowan, JAOCS 35:152 (1958).
13. Taufel, K., C. Franzke and G. Heder, Fette, Seifen, Anstrichm. 61:1225 (1959).
14. Interesse, F.S., P. Ruggiero and M. Vitagliano, Ind. Agric. 9: 318 (1971).
15. Chahine, M.H., and R.F. MacNeill, JAOCS 51:37 (1974).
16. Cort, W.M., J.M. Scott, M. Araufo, W.J. Mergens, M.A. Cannalonga, M. Osadca, H. Harley, D.R. Parrish and W.R. Pool, JAOCS 6:174 (1975).
17. Cooney, M.P., C.D. Evans, A.W. Schwab and J.C. Cowan, JAOCS 35:152 (1958).
18. Dugan, L.R., Jr., Food Technol. 15:10 (1961).
19. Yu, T.C., and R.O. Sinnhuber, Ibid. 16:115 (1962).
20. Tarladgis, B.G., and B.M. Watts, JAOCS 37:403 (1960).
21. Sinnhuber, R.O., T.C. Yu and T. Chang Yu, Food Res. 23: 626 (1958).
22. Pryor, W.A., J.P. Stanley and E. Blair, Lipids 11:370 (1976).
23. Ninnis, L.N., and M.L. Ninni, Rev. Fr. Corps. Gras. 15:441 (1968).
24. Bartolomeo, D., and R. Sergio, Riv. Ital. Sostanze Grasse 46: 467 (1969).
25. Coe, M.R., Oil Soap 15:230 (1938).
26. Rawls, H.R., and P. J. Van Santen, JAOCS 47:121 (1970).
27. Clements, A.H., R.H. Van Den Engh, D.J. Frost, K. Hoogenhout and J.R. Nooi, JAOCS 50:325 (1973).
28. Foote, C.S., Acc. Chem. Res. 1:104 (1968).
29. Kaplan, M.L., Chem. Technol. 1:621 (1971).
30. Skinner, K., Chem. Engin. News 54:25 (1976).
31. Fedeli, E., and A. Brillo, Riv. Ital. Sostanze Grasse 52:109 (1975).
32. Borbolla, J.M.R., Alcala and S.R.V. Larbon, Grasas Aceites 14:12 (1963).
33. Carlsson, D.J., T. Suprunchuk and D.M. Wiles, JAOCS 53:656 (1976).
34. Sastry, Y.S.R., P.V. Rao and G. Lakshminarayana, Oleagineux 28:467 (1973).
35. Ramunni, A., Ann. Foc. Sci. 30:1 (1964).

[Received December 2, 1982]