The 2-Thiobarbituric Acid Reagent for Determination of Oxidative Rancidity in Fish Oils

A. J. de KONING and M. H. SILK, Fishing Industry Research Institute, University of Cape Town, Rondebosch, Cape Town, South Africa

Abstract

A method has been devised for the quantitative determination of malonaldehyde in oxidized fish oils by means of the 2-thiobarbituric acid reagent in alcoholic solution. TBA numbers and peroxide values have been determined at intervals during hydroperoxidation of pilchard oil. The curves follow the same general pattern but the numerical relationship between them depends upon the temperature of oxidation.

In alcoholic solution, the reaction between thiobarbituric acid and malonaldehyde is carried out in the dark, because sunlight causes a decrease in optical density of the TBA-malonaldehyde complex at 532 mμ with the appearance of a second maximum at 452 mμ. The nature of the compounds responsible for absorption at these two wave-lengths is discussed.

Introduction

In recent years the Kohn and Liversedge color reaction with 2-thiobarbituric acid (TBA) has been widely used to estimate oxidative rancidity in fatty materials (1,2,4,5,12,17,18,20,22).

The TBA reagent determines the amount of malonaldehyde arising from oxidative decomposition of unsaturated fatty acids with more than one double bond (9,14). Oxidative rancidity is frequently expressed in terms of TBA number or milligrams of malonaldehyde per kilogram of material, measured by comparing the optical density of the pink TBA-malonaldehyde reaction product with that of standards prepared from 1,1,3,3-tetraethoxypropane (TEP) which gives rise to malonaldehyde on acid hydrolysis.

The TBA-reaction has been successfully applied to the measurement of rancidity in dairy (2–4,12,20), fishery (1,13,22) and meat (17,18) products. Experimentally the procedure requires dispersion of the test material in a water medium containing the TBA reagent, but Schmidt (9) has pointed out that the existence of a two-phase system can lead to errors in the case of oils and fats. We have confirmed this in measuring the oxidative rancidity of pilchard oil by the method of Yu and Sinnhuber (22), which is eminently satisfactory for materials such as fish meal. Applied to pilchard oil, the method gave results which sometimes differed by as much as 100% on the same sample. Addition of a detergent to the two-phase system of water and oil as suggested by Biggs and Bryant (2) gave turbid solutions which could not be read in the spectrophotometer.

Removal of malonaldehyde from the oil by steam distillation was tried according to the method of Sidwell et al. (12) and Tarladgis et al. (17), but was found to be unreliable in application to pilchard oil. The method requires distillation of only a certain percentage of the malonaldehyde during an arbitrarily chosen time, but in the case of fish oils with high peroxide values the amount in the distillate was not proportionate to the total amount in the sample, and duplicate estimations sometimes differed by as much as 50%.

Since previous methods did not appear to be satisfactory, a modified experimental procedure was devised to determine the TBA numbers of oxidized fish oils with a standard deviation of 10%. It was found that difficulties arising from the existence of a two-phase system during condensation between malonaldehyde and TBA could be overcome by carrying out the reaction in a monophase system of ethyl alcohol. The standard calibration curve in aqueous-alcoholic medium was identical to that obtained by Sinnhuber and Yu (13) in aqueous medium alone.[1] TBA numbers for fish meal were identical in both aqueous and alcoholic solution, but in the case of fish oils only the alcoholic medium afforded reproducible results.

In aqueous-alcoholic solution there is a marked influence of light on the absorption spectrum of the TBA-malonaldehyde reaction product. The maximum at 532 mμ disappears with the simultaneous appearance of a peak at 452 mμ. This observation has been investigated, and a study has been made of the relationship between peroxide value and TBA number during oxidation of pilchard oils at room temperature and at 75C.

Experimental

Reagents and Apparatus

TBA Reagent. This was prepared in a black-painted vessel. Sodium citrate buffer (25 ml) according to Kohn and Liversedge (5) was added to a solution of thiobarbituric acid (0.5 g) in warm ethyl alcohol (50 ml). Crystals of sodium citrate deposited on standing but this did not affect the quality of the reagent which was used within 4 hr of preparation.

Ethyl Alcohol and Methyl Alcohol. These solvents were purified according to the method of Stout and Schuette (16).

Alcoholic-HCl (0.6 N). Dry hydrogen chloride gas was passed through ethyl alcohol until the required weight increase was achieved.

1,1,3,3-Tetraethoxypropane (TEP). The TEP was supplied by K & K Laboratories, Jamaica, N. Y. and was used as an approximately 2×10^{-4} molar solution in 40% ethyl alcohol.

Epihydrin Aldehyde and Glyceraldehyde. These chemicals were supplied by Stuart Patton of the Pennsylvania State University.

All other chemicals were analytical reagent grade.

[1] The calibration curve given by Sinnhuber and Yu (13) refers to "molar concentration TEP (x 10^6)" and not "molar concentration TEP (x 10^7)" as printed.

TABLE I

Recovery of TEP Added to Commercial Sunflower Oil

% TEP added	% TEP recovered	% Recovery
0.1044	0.1039	99.5
0.1117	0.1137	101.8
0.0583	0.0545	93.5

Apparatus. The 250 ml. flat-bottom reaction flasks were painted black and all operations were carried out in subdued light. Optical densities were determined with a Unicam Model S.P. 500 spectrophotometer.

Procedure

Alcoholic-HCl (15 ml of 0.6 N) and TBA reagent (6 ml) were pipetted into a black-painted 250 ml flask in which the air had been replaced by nitrogen.

A 100–200 mg sample of the fish oil was accurately weighed in a glass capsule and added to the reagent. The flask was then connected to a reflux condenser and heated on a boiling water bath with intermittent shaking. After 30 min, 79 ml of aqueous 0.6 N HCl was added through the condenser and the refluxing continued for a further 10 min. The flask was then disconnected and cooled in running water for approximately 2 hr.

A 15 ml portion of the solution was then shaken vigorously for 30 sec with 15 ml petroleum ether (B. Pt. 40–60C) to extract residual fish oil. The upper layer was removed with a pipette and the solution shaken once more with 15 ml petroleum ether. After standing for 16 hr in the dark, the upper layer was again discarded and the optical density determined at 532 mμ against a reagent blank in a 1 cm cuvette. The TBA number in milligram malonaldehyde per kilogram of oil was obtained by multiplying the $E^{1\%}_{1cm}$ value by 46.5 [Solutions with a high optical density were diluted with 0.01 N HCl (19)].

Standardization. The calibration equation was obtained by carrying out the above procedure with appropriate quantities of standard TEP solution in place of fish oil. In each determination the volume was adjusted to 100 ml by adding the necessary amount of aqueous HCl through the condenser. The extraction with petroleum ether was omitted.

Determination of Peroxide Value. Peroxide values were determined according to the method of Wheeler (21) with the modifications suggested by Stansby (15), except that 25 ml of solvent were used instead of 10 ml as recommended. Peroxide values were expressed as milligram atoms of active oxygen per kilogram of oil.

Results

Standardization. The relationship between the concentration of malonaldehyde in terms of TEP and the optical density of the TBA-malonaldehyde complex at 532 mμ, is given by the expression

$$\text{Moles TEP/litre} = 6.49 \times 10^{-6} \times OD_{532\,m\mu}$$

This equation gives a calibration curve identical to that obtained by Sinnhuber and Yu (13) in aqueous medium.

Recovery of Malonaldehyde. The method showed approximately 100% recovery of TEP added to a commercial sunflower cooking oil of peroxide value 5.1 (Table I). Optical densities were measured against a blank prepared from the cooking oil without TEP.

Reproducibility of Results. Table II shows the results of TBA number determinations carried out on 4 different samples of oxidized pilchard oil with different peroxide values. In each case the standard deviation is less than 10% of the arithmetic mean shown for 7 to 9 determinations.

TBA Number vs. Peroxide Value of a Pilchard Oil During Oxidation at 20–25C. A fresh pilchard oil of peroxide value 5.1 and TBA number 48.1 was oxidized at 20–25C over a period of 6 months. Air was blown through the sample at the rate of 5 ml per sec, but after 640 hr the oil became too viscous to blow, and was therefore transferred to a glass dish where it was exposed to air in the form of a thin layer. Peroxide values and TBA numbers were determined at intervals with the results shown in Figure 1. Each point on the TBA curve represents the average of 7 to 9 determinations. Measurements were made after storing the reaction medium for the standard time of 16 hr in the dark. In the case of fish oil the value of the absorption maximum at 532 mμ was found to increase by about 14% after storage for an additional 80 hr in the dark.

TBA Number vs. Peroxide Value of a Pilchard Oil Oxidized at 75C. A further sample of fresh pilchard oil with initial peroxide value 5.1 and TBA number 48.1 was oxidized by air-blowing (5 ml per sec) at 75C. Figure 2 shows the changes in TBA number and peroxide value over a period of 130 hr.

Influence of Sunlight on the Nature of the TBA-Malonaldehyde Reaction Product. A) When the reac-

TABLE II

Reproducibility of TBA Numbers Determined on Pilchard Oils with Different Peroxide Values

	1	2	3	4
	119.8	327.5	1214	1876
	138.6	343.1	1245	1645
	122.5	350.1	1356	1351
	131.7	279.5	1508	1768
	108.4	336.1	1646	1638
	117.2	295.2	1524	1714
	115.3	312.4	1454	1638
	140.5	277.3	1443	.....
	.....	.....	1335	.....
Average TBA No.	124 ± 11	315 ± 27	1414 ± 131	1661 ± 150
Peroxide Value	21.4	92.4	464.0	600.7

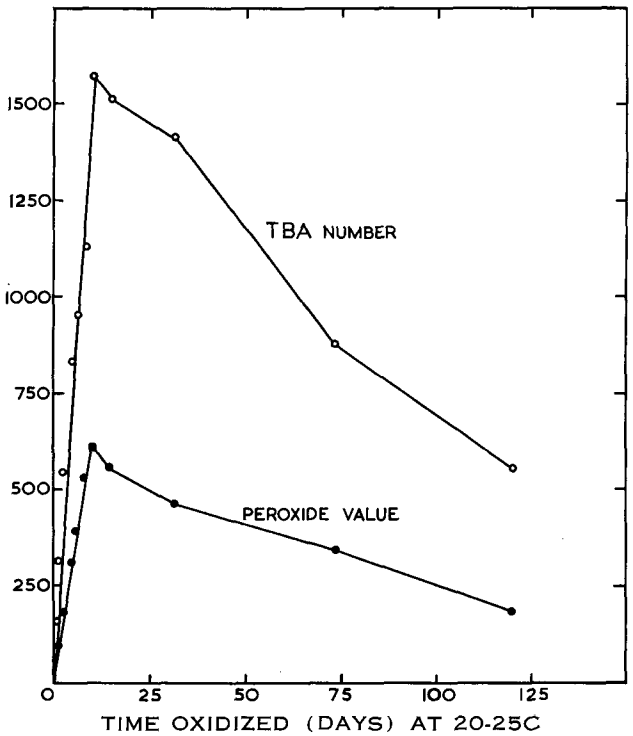


FIG. 1. Change in TBA number and peroxide value during oxidation of pilchard oil at 20–25C.

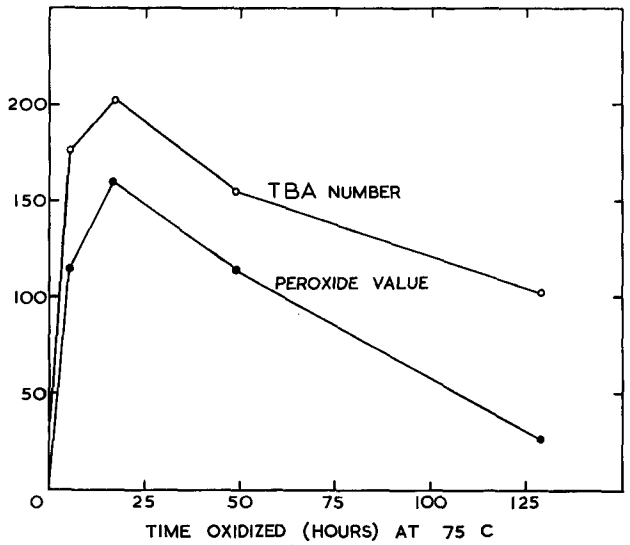


FIG. 2. Change in TBA number and peroxide value during oxidation of pilchard oil at 75C.

tion between alcoholic-TBA and malonaldehyde was carried out in the dark, the product had a pink color with an absorption maximum at 532 mμ. However, if the reaction was carried out in daylight, an orange-brown product was obtained with two absorption maxima, one at 532 mμ and the other at 452 mμ. The orange-brown solution became pink after storage in the dark for 70 hr and the 452 mμ maximum disappeared with a slight increase in absorption at 532 mμ. These effects are illustrated in Figure 3.

B) TBA undergoes a condensation to yield a colored compound without addition of malonaldehyde. Thus a solution of 0.04% TBA in a mixture of 4 parts 0.6 N HCl and 1 part 0.6 N alcoholic-HCl, turned yellow in sunlight with development of an absorption peak at 452 mμ. This maximum disappeared after 48 hr storage in the dark and a slight increase in absorption was observed at 532 mμ (Fig. 4).

The same phenomenon was observed when methyl

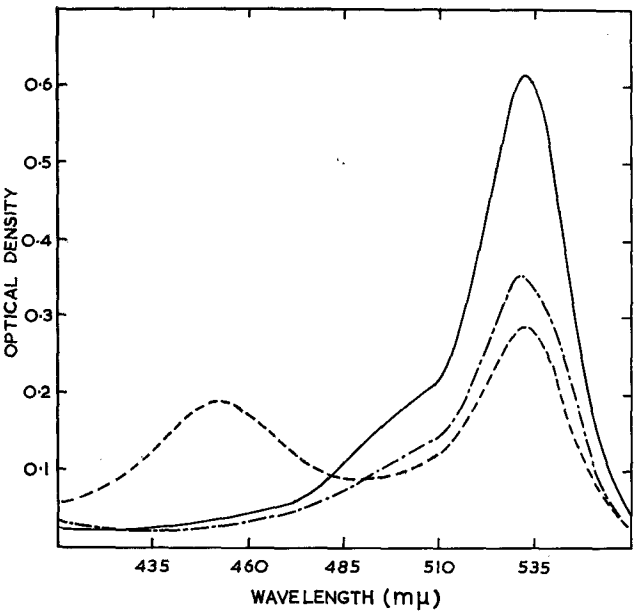


FIG. 3. A) Absorption of the derivative obtained by reaction between alcoholic-TBA and malonaldehyde in the dark (———). B) Absorption of the derivative obtained by reaction between alcoholic-TBA and malonaldehyde in sunlight (— — —). Absorption after storage of product B for 70 hr in the dark(—·—·—·). The maximum at 452 mμ disappears with an increase in absorption at 532 mμ.

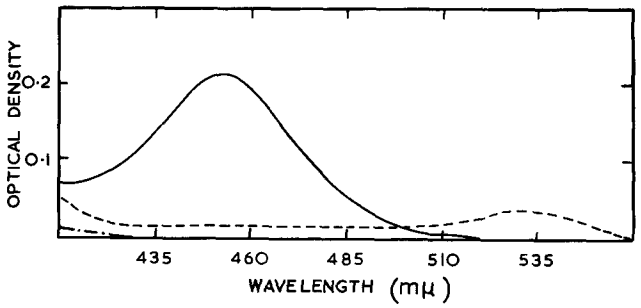


FIG. 4. A) Absorption of 0.04% TBA in a solution of 1 part 0.6 N alcoholic-HCl and 4 parts 0.6 N HCl (—·—·—). B) Absorption of solution A after exposure to sunlight (———). C) Absorption of solution B after 48 hr storage in the dark (— — —).

alcohol was used in place of ethyl alcohol, but no color was produced when TBA in aqueous solution was exposed to sunlight.

C) *Reaction of TBA with Epihydrin Aldehyde and Glyceraldehyde.* Patton (8) has shown that epihydrin aldehyde and glyceraldehyde react with TBA in aqueous solution to give a yellow product which has an absorption maximum at 456 mμ.

This reaction was compared with the development of an absorption peak at 452 mμ on exposure of an alcoholic solution of TBA to sunlight (see B above).

Epihydrin aldehyde and glyceraldehyde were condensed in the dark with TBA in aqueous-alcoholic solution to give a yellow compound with an absorption maximum at 455 mμ (Fig. 5). On continued storage in the dark the absorption maximum shifted to 485 mμ (Fig. 5).

D) *Reaction of TBA with Formic Acid.* Schmidt (10) has shown that formic acid reacts with TBA in aqueous solution to give a yellow derivative with an absorption maximum at 452 mμ.

A compound with the same absorption maximum at 452 mμ is formed by reacting TBA and formic acid in a mixture of ethyl alcohol and water (1:4) away from the influence of light (Fig. 6). A decrease in absorption at 452 mμ with a corresponding increase of

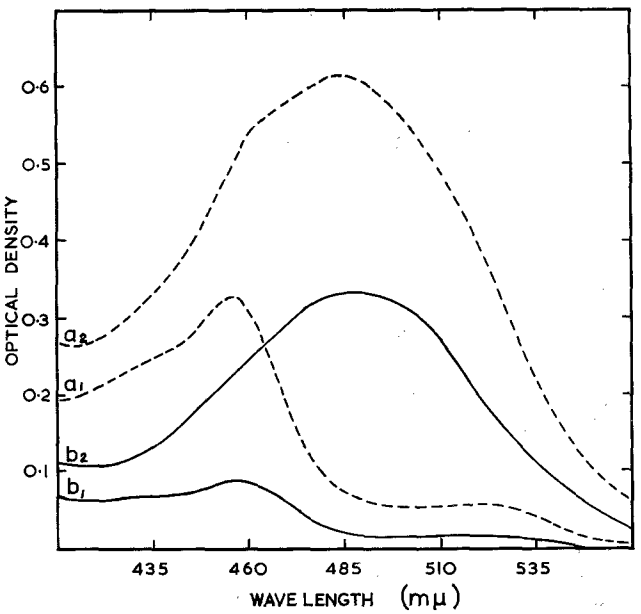


FIG. 5. a.1. Absorption of the derivative obtained by reaction between TBA and glyceraldehyde. a.2. Absorption of the TBA-glyceraldhyde derivative after 48 hr storage in the dark. b.1. Absorption of the derivative obtained by reaction between TBA and epihydrin aldehyde. b.2. Absorption of the TBA-epihydrin aldhyde derivative after 48 hr storage in the dark.

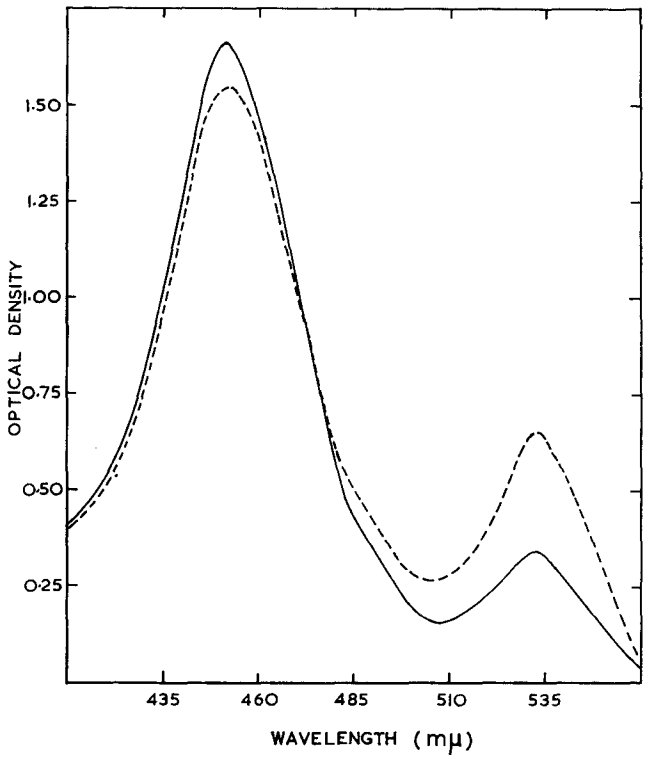


FIG. 6. A) Absorption of the TBA-formic acid derivative after 96 hr storage in the dark (———). B) Absorption of the TBA-formic acid derivative after 144 hr storage in the dark (— — —).

absorption at 532 mμ is shown after 96 hr and 144 hr storage in the dark (Fig. 6).

Discussion

The peroxide value of oils is a useful criterion of decomposition during the early stages of oxidation, but is of lesser value once the peak of the oxidation curve has been passed. The TBA number has been shown to be a useful quality index for assessment of rancidity in the early stages of oxidation (9), but no definite information could be found on the relationship between peroxide value and TBA number during continued hydroperoxidation of oils.

Several unsuccessful attempts were made to determine the TBA number of oxidized oils according to published methods. For example the procedure of Yu and Sinnhuber (22) and the distillation methods of Sidwell et al. (12) and Tarladgis et al. (17), all gave erratic results when applied to fish oil.

A new method was therefore devised to overcome errors inherent in the existence of a two-phase system between oil and water during reaction between malonaldehyde and TBA reagent. By carrying out the determination in a monophase system of ethyl alcohol it was found that the recovery of malonaldehyde added to sunflower oil was approximately 100% and that the standard deviation was less than 10% of the arithmetic mean for a series of 7 to 9 determinations on fish oils with different peroxide values (Table II).

TBA numbers determined in alcohol solution were compared with peroxide values at various intervals during oxidation of pilchard oil at 20–25C and at 75C. The curves followed the same general pattern at both temperatures (Figs. 1 and 2) but the maximum TBA number and peroxide value were less at 75C than at 20–25C. This would indicate that not only hydroperoxides, but also malonaldehyde or its precursors, are more rapidly decomposed at the higher temperature. Were it not for the fact that the malonaldehyde formed by oxidative degradation is itself labile, the TBA number might be expected to show a continued increase beyond the peak of the peroxide value curve. Since this is not the case [cf. Schmidt (9)], it would seem that the TBA number cannot be regarded as a more useful criterion of rancidity than peroxide value after the initial stages of oxidation.

Comparison of Figures 1 and 2 shows that the relationship between TBA number and peroxide value depends upon the temperature of oxidation. For example, an oil having a peroxide value of 160 may have a TBA number of 480 or 200 depending upon whether it was oxidized at 20–25C or at 75C.

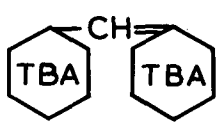
The measurement of TBA number in alcoholic medium introduces a complication which is not encountered in aqueous medium. The reaction must be carried out in the dark because daylight reduces the intensity of the TBA-malonaldehyde peak at 532 mμ and causes the appearance of a second maximum at 452 mμ (Fig. 3).

A product with an absorption maximum at 452 mμ can be obtained by the action of sunlight on an alcoholic solution of TBA alone. The yellow derivative absorbing at 452 mμ is converted to a pink compound with a peak at 532 mμ during storage in the dark (Fig. 4).

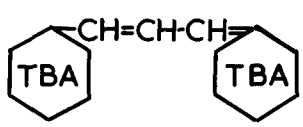
A yellow compound formed by reaction between TBA and epihydrin aldehyde or glyceraldehyde shows an absorption maximum at 455 mμ but is converted to a compound with a peak at 485 mμ after 48 hr storage in the dark (Fig. 5). The compound cannot therefore be identical with the reaction product of sunlight on TBA in alcoholic solution which is converted to a substance with a maximum at 532 mμ after storage in the dark (Fig. 4).

Formic acid, on the other hand, reacts with TBA to give a product which has an absorption maximum at 452 mμ and is converted to a substance with a peak at 532 mμ after storage in the dark (Fig. 6). Reaction between TBA and formic acid therefore yields a compound which is similar to, if not identical with, that formed by the action of sunlight on TBA in alcoholic solution.

Schmidt (10) has proposed structure I (λmax 452 mμ) for the reaction product of TBA with formic acid, and structure II (λmax 532 mμ) for the condensation product of TBA with malonaldehyde.



I (λMAX 452 mμ)



II (λMAX 532 mμ)

Thus sunlight irradiation of an alcoholic solution of TBA (Figs. 4 and 6) probably gives rise to compound I. The reaction could possibly take place through a free radical mechanism.

Compound II may be identical with the compound formed when an alcoholic solution of TBA is exposed to sunlight and subsequently stored in the dark (Fig. 4). If this is the case, it is possible that compound II is formed from compound I in aqueous alcoholic solution when stored away from light (Fig. 4).

Methine dyes such as compounds I and II apparently undergo changes in structure when in solution.

This is evidenced by the TBA reaction products with epihydrin aldehyde or glyceraldehyde (Fig. 5) and with formic acid (Fig. 6), although the phenomenon has also been observed by Landucci et al. (6,7) in the case of various aldehydes present in gelatin. The mechanism of these reactions does not appear to be understood and requires further investigation.

Shepherd (11), Sinnhuber et al. (14) and Schmidt (9) have shown that certain pyrimidines are capable of forming the same pigment as malonaldehyde on reaction with TBA. The mechanism is thought to involve hydrolysis of the pyrimidine ring to produce an oxyacrolein intermediate (9) which is tautomeric with malonaldehyde and therefore undergoes condensation to produce compound II. Although TBA itself has a pyrimidine structure, it is substituted in the 4 and 6 positions and would therefore be unlikely to give rise to malonaldehyde on hydrolysis (9). No color is formed on exposure of an aqueous acid solution of TBA to sunlight, but if hydrolysis were to occur, the expected product would be malonic acid which would not give the same compound as malonaldehyde in any reaction with TBA.

REFERENCES

1. Andersson, K., and C. E. Danielson, Food Technol., *15*, 55 (1961).
2. Biggs, D. A., and L. R., Bryant, Can. J. Technol., *31*, 138 (1953).
3. Dunkley, W. L., Food Technol., *5*, 342 (1951).
4. Jennings, W. G., W. L. Dunkley, and H. G. Reiber, Food Research, *20*, 13 (1955).
5. Kohn, H. I., and M. Liversedge, J. Pharmacol. et Exp. Therap., *82*, 292 (1944).
6. Landucci, J. M., J. Pouradier, and M. Durante, Bull. Soc. Chim. France, *22*, 857 (1955).
7. Landucci, J. M., J. Pouradier, and M. Durante, Compt. Rend. Soc. Biol., *149*, 919 (1955).
8. Patton, S., Food Research, *25*, 554 (1960).
9. Schmidt, H., Fette-Seifen-Anstrichmittel, *61*, 127 (1959).
10. Schmidt, H., *Ibid*, *61*, 881 (1959).
11. Shepherd, R. G., Anal. Chem., *20*, 1150 (1948).
12. Sidwell, C. G., H. Salwin, and J. H. Mitchell, JAOCS, *32*, 13 (1955).
13. Sinnhuber, R. O., and T. C. Yu, Food Technol., *12*, 9 (1958).
14. Sinnhuber, R. O., T. C. Yu, and C. T. Yu, Food Research, *23*, 626 (1958).
15. Stansby, M. E., Ind. Eng. Chem. (Anal. Ed.), *13*, 627 (1941).
16. Stout, A. W., and H. A. Schuette, *Ibid*, *5*, 100 (1933).
17. Tarladgis, B. G., B. M. Watts, M. T. Younathan, and L. Dugan, Jr., JAOCS, *37*, 44 (1960).
18. Turner, E. W., W. D. Paynter, E. J. Montie, M. W. Bessert, G. M. Struck, and F. C. Olson, Food Technol., *8*, 326 (1954).
19. Waravdekar, V. S., and L. D. Saslaw, J. Biol. Chem., *234*, 1945 (1959).
20. Wertheim, J. H., and B. E. Proctor, J. Dairy Sci., *39*, 391 (1956).
21. Wheeler, D. H., Oil and Soap, *9*, 89 (1932).
22. Yu, T. C., and R. O. Sinnhuber, Food Technol., *11*, 104 (1957).

[Received March 1, 1962—Accepted December 14, 1962]

Cost of Producing Linoleic Acid from Safflower Oil

V. E. SOHNS, R. E. BEAL, and E. L. GRIFFIN, JR., Northern Regional Research Laboratory,[1] Peoria, Illinois

Abstract

Linoleic acid of 97% purity can be made from safflower oil by liquid-liquid extraction at a "cost to make" of about 21 cents a lb. Calculations for the cost estimate were based on pilot-plant investigations. Fixed capital investment for a plant with an annual capacity of 20 million lb has been estimated at approximately $1,800,000. Such a plant could be converted readily to the production of a variety of other fatty acids.

Introduction

LINOLEIC ACID has properties suggesting that it would be a preferred raw material for manufacture of a variety of industrial products such as alkyd resins, plasticizers, coatings, elastomers and dimer acids. However, because relatively pure linoleic acid has been available only as an expensive laboratory chemical, there have been no intensive studies to determine its suitability for use in such applications.

Safflower oil is a logical raw material from which to produce linoleic acid since the oil is readily available and its fatty acids contain more than 70% of linoleic acid. Beal and Brekke (2) describe pilot-plant studies on the production of linoleic acid from safflower oil by liquid-liquid extraction. Their process utilizes a combination of techniques adaptable to commercial production. Industry has shown interest in the process if the production costs are not prohibitive. From a cursory examination, it appears that large-scale production of linoleic acid could be economically feasible.

This paper reports the results of a cost study on the production of linoleic acid from safflower oil by liquid-liquid extraction in a hypothetical plant which has a capacity for producing 20 million lb of linoleic acid of 97% purity annually.

[1] A laboratory of the No. Utiliz. Res. & Dev. Div. ARS, U.S.D.A.

Process and Equipment

A qualitative flow diagram and a simplified quantitative flowsheet of the process are shown in Figures 1 and 2. The first step in the process is the continuous hydrolysis of safflower oil to convert it to fatty acids. Commercial-grade refined safflower oil used in this hypothetical plant has a composition such that approximately 75% linoleic acid is contained in the fatty acids formed by hydrolysis. No pilot studies were conducted on this phase of the process, but the equipment and general procedure for continuous fat-splitting as described by Barnebey and Brown (1) should be satisfactory. A stainless-clad steel tower, 60 ft high, 2.5 ft in diameter, should have sufficient capacity for hydrolyzing the daily requirement of 96,200 lb of safflower oil. The oil and water for hydrolysis should be deaerated to minimize oxidation before being pumped to the hydrolyzer. It is estimated that a retention time for the oil of 2 to 2.5 hr at a temperature of approximately 500F and that a pressure around 700 psig should approach optimum operating conditions for hydrolysis.

Glycerin formed by splitting the fats is separated from the fatty acid fraction and collected in the sweetwater. The sweetwater, which contains 13–15% glycerin, is concentrated in a vacuum evaporator to yield an 80% crude glycerin concentrate. Although the crude glycerin could be concentrated further and purified by distillation, this operation is not included here.

The fatty acids, 91,872 lb per day, are fractionated in stainless steel Podbielniak twin-centrifugal extractors, with furfural containing 2.5% water as the selective solvent and hexane as the secondary solvent. The procedure for the extraction follows that reported by Beal and Brekke (2). Temperature for extraction is 100F. For every pound of fatty acid fed to the extractors, 15 lb of furfural containing 2.5% water and 3 lb of hexane are required. Total combined feed