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A Rapid Spectrophotometric Method for Determining the Linoleic and Linolenic Acid Components of Soybean Oil[1]

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THE A.O.C.S. tentative method CD-7-48 (1), when used for analyzing soybean oil, can be simplified in the type of glassware used, in the procedure of isomerization, and in the method of obtaining the specific absorption of ultraviolet light. The Spectroscopy Committee (2) recommended simplification of the analysis of soybean oil by measuring light absorption at 233 mμ and 268 mμ only. The work of Brice *et al.* (4) and of Herb and Riemenschneider (5) showed that an isomerization period of 45 min. in air with 11% KOH-glycerol reagent resulted in very satisfactory analysis of several vegetable oils. Dutton, Northern Utilization Research Branch, Peoria, Illinois, suggested that light absorption at 268 mμ and 233 mμ might be measured on the original 100-ml. solutions of isomerized material in two special test cells with quartz windows spaced to provide solution depths of about 1 mm. and 0.1 mm. to 0.15 mm., respectively. For the purpose of perfecting the method of analysis as presented here, a sample of soybean oil analyzed in 1948 by eight different laboratories to test A.O.C.S. method CD-7-48 (1, 2) was used. Our analyses of this soybean oil, which was in storage at zero degrees F. continuously since 1948, indicate no change in fatty acid composition of the oil during this relatively long period of storage.

Three different simplified spectrophotometric methods of analysis of soybean oil were tested. The simplest of these methods and the most reliable as indicated by reproducibility of analyses requires the following described equipment, reagents, and procedure:

Apparatus

1. Beckman DU Spectrophotometer with a holder for four 1-cm. square quartz cells.
2. Two special cells with quartz windows spaced 1 mm. and 0.1 to 0.15 mm.
3. 100 or more lime or Pyrex glass bottles, 125 ml., wide mouth, fitted with screw caps lined with Teflon[3] gaskets. (We use clear lime glass bottles 1¾ in. square and 4¼ in. in height.) The caps must close the bottles with a vapor-tight seal. Teflon gaskets withstand the solvent and chemical action of hot Methanol-KOH-glycerol solutions.
4. Several metal trays to hold 12 or more 125-ml. bottles tightly enough to permit vigorous shaking without dislodging the bottles. Our bottle trays were made of a long loaf bread pan and pieces of 2 in. x 4 in.-mesh welded wire fencing.
5. A 100-ml. burette, graduated to 1 ml., rapid refill and automatic zero type for dispensing a measured volume of methanol.
6. A 10-ml. and a 5-ml. hypodermic syringe.
7. Electric heating mantle and flask to heat 3 to 5 liters of KOH-glycerol reagent to 200°C.
8. A forced draft air oven thermostatically controlled to maintain 180°C. ± 3 degrees. (We use Precision Scientific Company Type A oven on full heat, 3900 watts.)
9. Three C. thermometers, 0 to 100, 0 to 200, 0 to 220.
10. Shallow, glass 1-ml. cups of large diameter to hold samples.

Reagents

1. Methanol, synthetic absolute, to pass the test of optical density in A.O.C.S. method CD-7-48 (1).
2. 11% KOH-glycerol reagent containing 17.5 g. 85% KOH (ACS reagent), per 100 ml. of glycerol prepared as described by Brice (3).
3. Several hundred grams of soybean oil of known fatty acid composition to be analyzed daily as a check sample and to provide a means of calculating the length of light paths in the special cells.

Procedure

1. Weigh .1000 to .1250 g. ± 0.2 mg. of a well mixed sample of soybean oil into a 1-ml. cup.
2. Heat a portion of the KOH-glycerol reagent to 100°C. in a large beaker to lower the viscosity of this reagent. Use a 10-ml. hypodermic syringe to transfer 8.6 ml. (11 g.) of reagent to each 125-ml. bottle. A large number of bottles may be prepared at one time since capped bottles of reagent can be stored for several weeks without change.
3. A cool or preheated air oven may be used. Place several trays of uncapped bottles containing reagent in the oven. Set the heat control for 180°C. and continue the heating until a thermometer in one bottle with the bulb immersed in the reagent reads 180°C.
4. Wear asbestos gloves to remove these racks of bottles at 180°C. from the oven. Quickly add one small cup of oil to each bottle of hot reagent in rack except the two reagent blanks. Immediately shake the bottles in this rack vigorously for three to five seconds. Proceed, likewise, to add cups of oil to each rack of bottles followed by three to five seconds of vigorous shaking. Finally shake all of the bottles in the racks for two minutes. By placing the bottle racks on the slippery surface of a laboratory bench, one technician can do a thorough job of shaking 48 samples (four trays) at one time. Inade-

[1] Publication No. 274 of the U. S. Regional Soybean Laboratory.
[2] Chemist and Agent (Physical Science Aid), respectively, Field Crops Research Branch, Agricultural Research Service, U. S. Department of Agriculture.
[3] E. I. du Pont de Nemours and Company, Chicago, Ill., has a listing of concerns supplying Teflon in sheet form.

quate shaking of the bottles results in incomplete saponification, erratic and low values of light absorption, and extreme difficulty in dissolving the last traces of material in the small oil cups. Two technicians can process about 60 samples and return the trays of bottles to the 180°C. temperature oven in about 4 min. The bottles and contents will cool to about 150°C. during this 4-min. period.

5. Time the isomerization period for 45 min. after the thermometer, which was placed in the bottle of reagent, reads 175°C. About 30 min. of the 45 min. of isomerization will be at 180°C.
6. Remove these trays of bottles from the oven and place them in a draft of air. When they have cooled to about 60°C., add 93.5 ml. of methanol to each bottle. Immediately cap the bottle (vapor-tight) and set it in a partially filled tank of water warmed to 65° to 70°C. A metal tank, heated by an electric hot plate, large enough to hold 60 bottles, is satisfactory. When the bottles and contents are warmed to 65° to 70°C., a minimum of shaking is required to obtain complete solution of the isomerized material in the methanol.
7. Place these warm solutions (in tightly capped bottles) in a draft of air near the Beckman instrument and allow about 1 hr. for them to come to instrument room temperature. At room temperature these bottles of solution should contain 100 ml. ± 1 ml. To avoid differences in optical density due to changes in volume, the reading on the Beckman should be taken at the same temperature. Corrections of the data for differences in volume due to temperature changes are also satisfactory. Daily tests on one or two bottles of solution made by draining the contents into 100-ml. volumetric flasks confirm the volumetric accuracy of this procedure. Other laboratories using different conditions should determine the volume of methanol necessary to make 100 ml. of solution.
8. If 1-cm. cells are to be used for measurement of the absorption of light at 268 mμ and 233 mμ, it is necessary to prepare dilutions with methanol of 10/1 and 100/1 in volumetric flasks of all of these bottles of solution. With the diluted solution the analyses can be completed as described in A.O.C.S. method CD-7-48 (1).
9. If two special continuous flow cells with light paths 1 mm. and 0.1 to 0.15 mm. are available, absorption of light at 268 mμ and 233 mμ can be measured very rapidly on a few ml. taken from these original bottles of solutions.

A. For linolenic acid:

Place the special cell with 1-mm. light path in the Beckman DU Spectrophotometer. With the wavelength set at 268 mμ, force about 5 ml. of the reagent blank through the cell with a 5-ml. syringe. With the cell filled with blank reagent, set the instrument to read zero optical density, using the narrowest possible slit width. Avoid introducing air bubbles into the cells during filling. Consecutively feed about 5 ml. of solution from each test sample through the cell. Read the optical density of each solution while it is within the cell. Refill the test cell with blank reagent as often as seems necessary to keep the instrument in proper adjustment.

B. For linoleic acid:

Place the special cell with an approximate light path of 0.1 mm. in the Beckman instrument and follow essentially the same procedure as described above for the 1-mm. cell to obtain optical density measurements at 233 mμ on all of these same samples.

The depth of solution between the quartz windows of these special cells becomes greater with prolonged use of the cells because KOH dissolves some of the quartz. The light path in our 0.139-mm. cell is now 0.142 after being used to analyze about 700 samples. The length of the light path also changes appreciably with several degrees difference in temperature. The light path of a cell can be measured very easily as described here, and the light path of each cell should be checked regularly, depending upon the amount of use and the temperature at the time of use. Prepare 10 to 1 and 100 to 1 dilutions from two bottles of 100 ml. of reagent blank and also from two bottles of 100 ml. of the check sample of soybean oil. Measure the optical density of the 10 to 1 dilutions at 268 mμ and the 100 to 1 dilutions at 233 mμ in 1-cm. cells. Measure the optical density at 268 mμ and 233 mμ in the special cells on a few ml. taken from these same bottles of solution.

TABLE I

Reproducibility of Analysis of Soybean Oil for Linolenic Acid on Eight Different Samples of Soybean Oil by Two Operators

	1st day % acid linolenic	2nd day % acid linolenic	3rd day % acid linolenic	4th day % acid linolenic	Averages % acid linolenic
Soybean oil 1	8.72 8.73	8.70 8.48	9.13 8.97	8.77 8.78	8.79[a]
Soybean oil 2	6.79 6.67	6.58 6.44	6.91 7.01	6.73 6.72	6.73
Soybean oil 3	7.12 7.08	6.89 7.11	7.41 7.65	7.24 7.57	7.25
Soybean oil 4	6.30 6.28	6.48 6.38	6.95 7.00	6.50 6.50	6.55
Soybean oil 5	7.12 7.22	6.89 6.93	7.63 7.61	7.06 7.28	7.22
Soybean oil 6	6.28 6.13	6.01 6.15	6.85 6.27	6.41 6.67	6.35
Soybean oil 7	7.41 7.14	7.26 7.02	7.61 7.39	7.28 7.22	7.29
Soybean oil 8	7.56 7.39	7.13 7.34	7.86 7.88	7.67 7.65	7.56
Averages.....	7.12	6.99	7.51	7.25	=7.22

Arithmetic mean 64 analyses..... 7.22%
Standard deviation of a single determination[b]..... ±0.24%
95% confidence of single determination..... ±0.49%

[a] Sample No. 1 is the Spectroscopy Committee sample that was analyzed by eight laboratories with an average linolenic acid content of 8.81%.

[b] This s.d. applies to the variability among the eight determinations for each oil.

Calculation of the light path of the special cells:

$$\text{Specific extinction coefficient} = \frac{D}{bxc}.$$

Where D is the observed optical density, b is the length of the light path in cm., and c is the concentration per liter.

$$\text{Equate 1 cm. cell } \frac{D}{bxc} = \frac{D}{bxc} \text{ special cell.}$$

Solve for b, which is the length of light path of special cell.

10. Calculations — If iodine values of the soybean oil are available, much of the complex calculation of results can be eliminated with a nomograph. The method of construction of the nomograph as described by Narayan (6) except with natural acid constants is satisfactory, and the nomograph will save time in calculation of the fatty acid percentages.

Equations for the calculation of fatty acid composition, using natural acid constants, are:

$$\%\ \text{linolenic acid} = \frac{K268}{48.6} \times 100.$$

Where K268 is the observed extinction coefficient at 268 mμ and 48.6 is the standard extinction coefficient for pure linolenic acid (4).

$$K233 = KT233 - \frac{58.6}{48.6} \cdot K268.$$

Where K233 is the extinction coefficient due to linoleic acid and KT233 is the total observed extinction coefficient at 233 mμ (due to both linolenic and linoleic)

$$\%\ \text{linoleic} = \frac{K233}{93.9} \times 100.$$

93.9 is the extinction coefficient of 100% linoleic acid at 233 mμ.

58.6 is the extinction coefficient of 100% linolenic acid at 233 mμ.

No pure acids were available for standardization tests of this method. One sample of ethyl linoleate (prepared by debromination) which had an iodine

TABLE II

Reproducibility of Analysis of Soybean Oil for Linoleic Acid on Eight Different Samples of Soybean Oil by Two Operators

	1st day % acid linoleic	2nd day % acid linoleic	3rd day % acid linoleic	4th day % acid linoleic	Average % acid linoleic
Soybean oil 1	51.32 51.52	51.40 50.86	51.58 51.29	51.37 51.69	51.38[a]
Soybean oil 2	51.42 51.21	50.40 50.17	50.84 52.27	51.75 51.66	51.22
Soybean oil 3	51.52 51.03	50.02 50.76	51.27 51.84	51.17 52.41	51.25
Soybean oil 4	50.90 51.73	51.28 51.29	50.90 51.05	51.18 51.47	51.22
Soybean oil 5	50.98 49.96	49.93 50.51	50.18 50.26	50.46 50.76	50.38
Soybean oil 6	47.48 47.36	47.38 47.36	47.46 48.20	46.94 47.52	47.46
Soybean oil 7	50.93 49.87	50.49 50.07	48.92 49.52	50.52 50.48	50.10
Soybean oil 8	52.91 52.69	52.46 53.45	52.94 52.82	52.95 53.12	52.91
Averages.....	50.80	50.48	50.70	50.97	= 50.74

Arithmetic mean of 64 analyses..... 50.74%
Standard deviation of a single determination[b]..... ±0.42%
95% confidence interval of a single determination..... ±0.98%

[a] Sample No. 1 is the Spectroscopy Committee sample that was analyzed by eight laboratories with an average linoleic acid content of 51.39%.

[b] This s.d. applies to the variability among the eight determinations for each oil.

value of 164.3 (theoretical 164.7) was isomerized by this method. The specific extinction coefficient at 233 mμ adjusted to 100% linoleic acid was 90.3. The adopted specific coefficient for linoleic acid by debromination by Brice (4) was also 90.3. When analyses of fatty acids of the soybean oil in the Spectroscopy Committee (2) report of 1949 are calculated with natural acid constants, the values for linolenic acid are changed from 8.40% to 8.81% and for linoleic 53.7% to 51.39%. Our analysis of this special sample of soybean oil with the special cells at 268 mμ and 233 mμ calculated with the standards published by Brice (4) give percentage values in satisfactory agreement with 8.81% and 51.39%; thus, based on our data, the choice of the standard extinction coefficients published by Brice seems satisfactory. As an indication of the reproducibility of analysis by this method, eight samples of soybean oil were analyzed by two technicians on four different occasions as shown in Tables I and II.

The good agreement with the analysis of the Spectroscopy sample of soybean oil, sample 1 in the tables, shows that the adopted extinction coefficients as published by Brice (4) are satisfactory constants for this method of analysis.

Statistical evaluation of the data in Tables I and II shows good reproducibility of analysis by this method. The 95% confidence intervals based on single determinations for linolenic acid ± 0.49% and linoleic acid ± 0.98% are satisfactory. Duplicate analyses reduce these values to about ± 0.35% and ± 0.69%, respectively. Since significant differences in percentages of fatty acids in a sample of oil were found on successive days, these duplicate analyses should not be determined side by side.

The day-to-day variability of analyses of a single oil was probably the result of differences in the total time of heating the sample in the oven during the isomerization period. A longer period of heating is required if the bottles of reagent are allowed to cool for a longer period because of delay in the addition of the oil cups. Extension of the time of heating by 20 to 30 min. during the isomerization period may result in 0.3 to 0.5% lower linolenic acid than will be obtained in a more normal time of heating. This effect of longer time of heating agrees with the information published by Brice (4) in Figures 2 and 3.

The reproducibility of these analyses show that satisfactory 100-ml. volumetric solutions were prepared by addition of a measured volume of methanol to isomerization bottles. Variability in volumes was a known cause of error in Tables I and II which might be eliminated. However volumetric solutions prepared as described avoid errors of spillage and incomplete transfer of isomerized material to volumetric flasks. The saving in time, labor, apparatus, and cleaning of glassware is very great if 100-ml. solutions are prepared in these capped bottles.

If an analysis of soybean oil is started at 8:00 a.m. with the oven and bottles of reagent at room temperature, about 1 hr. of heating time is necessary to bring the temperature to 180°C. After the oil cups are added and the 2 minutes of vigorous shaking are completed, it may require 30 min. to reheat the somewhat cooled bottles to 175°C. It requires only 5 to 10 min. longer in the oven to process 60 samples than to process 12 samples. The isomerization of 60 samples can be completed by 10:30 a.m. By 12 noon these 60 samples can be cooled and made up to 100-ml. solutions of isomerized material. By 1 p.m. these solutions may be cooled to room temperature and ready for the Beckman.

This air-oven method of isomerizing soybean oil with 11% KOH-glycerol results in methanol solutions of the isomerized material which are very light colored. These solutions are only a little more yellow in color than the reagent blanks.

Much of the rapidity of analysis of soybean oil depends upon two special continuous flow cells with

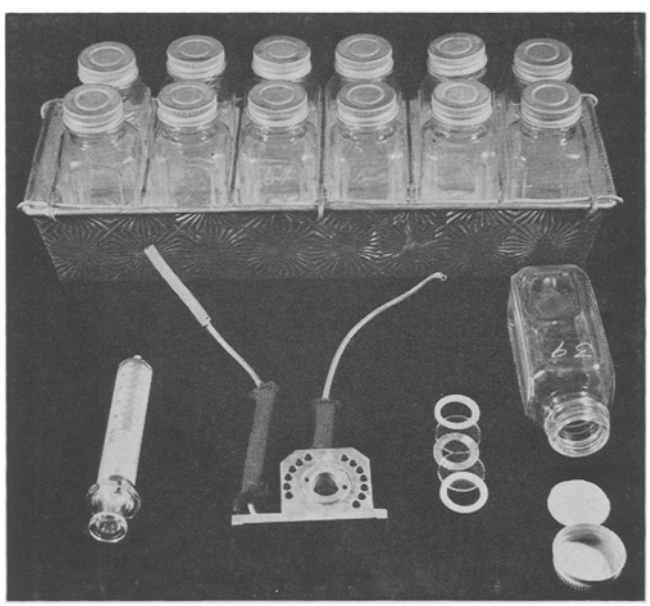

FIG. 1. The assembled special cell, its disassembled parts, and a hypodermic syringe; the rack of bottles with screw type cap with a "Teflon" gasket.

short light paths and fused quartz windows as shown in Figure 1. With these cells the original 100-ml. solutions need not be diluted and measures of optical density of the solutions at one wavelength may be determined on four or more samples per minute. When the cell windows are new, optical density readings may be read with slit widths as low as .2 to .3. Herb (7) showed that truest values of optical density were obtained with the narrowest slit widths. As the cells become etched in use from the action of KOH, wider slit widths become necessary. Etching of the quartz has not proved to be a serious problem. The windows may be good for several thousand analyses before they need to be replaced or removed and polished. Cells should be flushed daily with concentrated HCl and thoroughly rinsed with distilled water.

The cells are difficult to construct without leaks. A seepage leak past the cell gaskets will allow a film of isomerized oil to spread over the quartz window. Methanol-KOH-glycerol-soap solutions are especially difficult to contain. Excessive tightening of the compression ring to prevent leaks often breaks the quartz windows. Use of cements of any kind to prevent leaks caused cloudy unusable cells due to the solvent action of methanol. Rubber or cork gaskets are unsatisfactory because of the action of KOH.

Summary

A reagent of 11% KOH-glycerol was satisfactory for isomerizing soybean oil at 180°C. in open bottles in a forced-draft air-oven. A rapid means of analyzing soybean oil for linolenic and linoleic acid was developed. The optical densities of methanol solutions were measured at 268 mμ and 233 mμ in special cells with quartz windows and short light paths, and the percentages were calculated by means of a nomograph.

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Influence of High Energy Radiation on Oxidation of Oleic Acid and Methyl Oleate[1,2,3]

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NUMEROUS STUDIES have shown that oleic acid and its esters react with oxygen in a typical autoxidation process. The rate of reaction is quite slow at lower temperatures. At elevated temperatures the number of secondary reactions is usually increased so that the primary products, the hydroperoxides, are partially destroyed. Some workers (1, 6) have successfully used low temperatures and ultraviolet radiation for production of hydroperoxides from oleic acid and its esters. Khan (2) claimed that long oxidation periods at 0°C. in the dark quantitatively produce methyl hydroperoxidooleate.

The aim of this study is to learn how high-energy radiations, such as gamma rays and high-energy electron beams, affect oxidation of oleic acid and its esters.

Experimental

Oxidation under the influence of gamma radiation was accomplished in a large glass tube fitted with a stopper holding a fritted glass gas sparger, a small glass tube containing a thermocouple, and a U-tube of small diameter glass tubing carrying a short length of resistance wire to serve as a heater when needed. Oxygen was introduced into the sample through the sparger.

The gamma radiation was from a cylindrical source of Cobalt 60 (Co^{60}). This source was rated at 33r/sec. A stronger source rated at 118 r/sec. was used after February 1955.

Peroxide values were determined by a slight modification of the Wheeler (7) iodometric method. Total carbonyl values were determined colorimetrically by a modification of the method of Lappin and Clark (5). The $E^{1\%}_{1cm.}$ for α,β-unsaturated carbonyls was determined by ultraviolet spectrophotometric measurements at 224 mμ according to King (4).

The oleic acid used was Armour and Company Neo-Fat 92-04. The methyl oleate was prepared by esterification, a low temperature crystallization in acetone, and finally by distillation at less than 1 mm Hg pressure through a 20 cm. Vigreaux column.

Oxidation of Oleic Acid. Oleic acid was oxidized under the influence of gamma radiation from a Co^{60} source of 33 r/sec. at 20°C. Typical results are shown in Table I. Under these conditions a peroxide value of 1229 me/kg was obtained in 216 hrs. This value representing 19.3% conversion to peroxide as monohydroperoxidooleic acid is not the maximum attainable. However the rate of peroxide formation was greatly reduced at this point, and formation of secondary products was accelerated as shown by the marked increase in total carbonyl value. The $E^{1\%}_{1cm.}$ value at 224 mμ measures the amount of α,β-unsaturated carbonyls in oxidizing oleic acid and oleates according to King (4). After a small increase these values remained fairly constant throughout the oxidation.

For comparative purposes another sample of oleic acid was treated with oxygen in the dark at 20°C. After 355 hrs. there was only a trace of peroxide, a very low carbonyl value, and a low $E^{1\%}_{1cm.}$ value at 224 mμ. This clearly illustrates the magnitude of the

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