et al. (4). Weigh the fat into an Evelyn cuvette. Add 10 ml of solvent (80 volumes ethyl alcohol; 20 volumes benzene) to dissolve the fat. Add 1 drop of ammonium thiocyanate solution and read the absorbancy of the fat blank. Add 1 drop of ferrous chloride solution and measure the absorbancy of the red color with the 515 mμ filter.

REFERENCES

1. Hills, G. L., and C. C. Thiel, J. Dairy Research, *14*, 340 (1946).
2. Sumner, R. J., Ind. Eng. Chem. Anal. Ed., *15*, 14 (1943).
3. Koch, R. B., B. Stern, and C. G. Ferrari, Arch. Biochem. Biophys., *78*, 165 (1958).
4. Stine, C. M., H. A. Harland, S. T. Coulter, and R. Jenness, J. Dairy Sci., *37*, 202 (1954).

MARGARET G. DRIVER
Armed Forces Food and Container Institute
Chicago, Ill.

R. B. KOCH
Minneapolis-Honeywell Regulator Co.
Hopkins, Minn.

HAROLD SALWIN
Food and Drug Administration
Washington, D. C.

[Received January 23, 1962—Accepted May 13, 1962]

Preparation of 9-*trans*, 12-*trans* - Octadecadienoic Acid[1]

DURING CURRENT INVESTIGATIONS of the metabolism of *trans* fatty acids, it became necessary to prepare large quantities of 9-*trans*, 12-*trans*-octadecadienoic acid, the all-*trans* isomer of linoleic acid. This material is most conveniently made by *cis-trans* isomerizing natural linoleic acid and then purifying by recrystallization.

Jackson, et al. (1) and Kass and Burr (2) have reported methods for preparing 9-*trans*,12-*trans*-octadecadienoic acid using a Se catalyst. However, Se *cis-trans* isomerization has two major disadvantages. First of all, complete removal of toxic Se is essential for biological investigations. The above workers used vacuum distillation to accomplish Se removal after isomerization, but they did not report any analysis of residual Se in their final product. Teeter, et al. (3) have reported that Se was not completely removed by standard vacuum distillation nor by several other chemical treatments, but was only removable by repeated molecular distillations. Secondly, Se isomerization of linoleic acid is known to produce *trans* monoenes as a by-product (4,5). If these *trans* monoenes are present in sufficient quantity (as when recrystallization filtrates are re-isomerized), they cannot be removed by recrystallization.

Kass and Burr (2) have used a HNO_2 catalyst to prepare 9-*trans*,12-*trans*-octadecadienoic acid from linoleic acid, but reported extensive nitrogenous by-product formation and a very poor yield. We have found that by-product formation can be minimized by shortening reaction time, and that those nitrogenous by-products formed can be completely and easily removed on a column of silicic acid. Furthermore, Litchfield, et al. (5) have demonstrated that HNO_2 isomerization of linoleate does not produce the *trans* monoene by-products that Se does. This allows recrystallization filtrates to be re-isomerized to improve yields. Therefore, HNO_2 appeared to be the better isomerization catalyst for our purposes.

A procedure for making 9-*trans*,12-*trans*-octadecadienoic acid has been developed using readily available safflower oil as a starting material. A linoleic acid concentrate was prepared from safflower fatty acids by urea adduct formation. After HNO_2 isomerization, nitrogenous reaction by-products were removed on a column of silicic acid. Recrystallization from acetone yielded 4.5 g of 9-*trans*,12-*trans*-octadecadienoic acid per 100 g of safflower oil.

Preparation of Linoleic Acid Concentrate. A solution of 215 g of NaOH in 430 ml of distilled water was prepared and cooled to room temperature. This solution was slowly added with stirring to 1 kg of alkali-refined safflower oil and one liter of 95% ethanol in a 6 liter reaction flask. The mixture was stirred until homogeneous and then let stand for one hour with occasional stirring. One liter of distilled water and enough 3 M H_2SO_4 (about 850 ml) were added to bring the solution to a pH of 3. The solution temperature was not allowed to rise above 50C during acid addition. The fatty acid layer was allowed to separate, and the lower aqueous layer was siphoned off and discarded. The fatty acid was then washed with distilled water (NaCl was added to the first washings to hinder emulsion formation) until the wash water was neutral. The fatty acid layer was then dried under vacuum.

825 g of urea was dissolved in 2.55 liters of methanol by heating in a six liter reaction flask. 940 g of safflower fatty acids was heated to 80C under nitrogen and added to the methanol solution under nitrogen. If the solution became cloudy, it was heated until clear. The solution was cooled overnight under nitrogen to room temperature. The urea adduct crystals were then filtered and discarded. Another 825 g of urea was dissolved in the filtrate by heating under nitrogen, and the solution was cooled overnight and filtered as before. 4 liters of distilled water and 500 ml of petroleum ether (30–60C boiling range) were added to the filtrate with mild stirring, and the solvent layer was allowed to separate. The solvent layer was removed, washed twice with distilled water, once with 100 ml of 1% HCl, and then with distilled water until the wash water was neutral. The solvent and any traces of water were removed from the fatty acids under vacuum. The yield was about 340 g of fatty acid showing a purity of 98–99% linoleic acid by gas-liquid chromatography (GLC) analysis. (Linoleic acid purity at this point was probably less than GLC results indicated, since non-volatile safflower unsaponifiables were probably concentrated by the urea crystallizations.) Yield and purity depended somewhat on the room temperature used for the urea crystallizations. Lower room temperatures gave a lower yield and a higher purity. If GLC analysis indicated less than 98% linoleic acid at this point, another urea adduct crystallization was performed. If the final acetone recrystallization filtrates were to be re-isomerized to increase yields, 99+% linoleic acid was required.

Isomerization and Purification Procedure. 340 g of linoleic acid concentrate and 135 ml of 6 M HNO_3 were placed in a 2 liter reaction flask fitted with a dropping funnel, a stirring shaft, an outlet to a bubble trap, and a connection to a source of nitrogen. The flask was purged with nitrogen. 205 ml of freshly prepared 2 M $NaNO_2$ was added over a period

[1] Presented at the AOCS meeting in Atlanta, 1963.

of 3 min with very vigorous stirring to achieve intimate mixing of the aqueous and fatty layers. As $NaNO_2$ solution was added, gas escaped through the bubble trap and the temperature rose to about 60C. The formation of nitrogenous reaction by-products will gradually change the color of such a reaction mixture from yellow to orange to reddish-brown as *cis-trans* isomerization proceeds. For maximum yield of *trans* isomers, the reaction was stopped as soon as an orange color was observed or 18 min after starting to add the $NaNO_2$ solution, whichever was sooner. The products were *immediately* transferred to a separatory funnel containing 500 ml of distilled water and 1.5 liters of petroleum ether. Gentle shaking dissolved most of the isomerization catalyst in the aqueous layer, which was quickly drawn off and discarded. The solvent layer was washed 5–10 times with a 5% (w/v) solution of NaCl in distilled water to remove as many of the colored reaction by-products as possible. Any red gummy material appearing at the water-solvent interface was discarded with the aqueous layer. After washing twice with distilled water, the solvent solution of isomerized acids was left overnight at 0C and then decanted from the red gummy precipitate which formed. The solution was dried over anhydrous Na_2SO_4 to remove traces of water and then filtered.

At this point, the isomerized linoleic acid usually contained about 45–55% 9-*trans*,12-*trans*-octadecadienoic acid as determined by capillary GLC (6,7) or about 90–120% isolated *trans* bonds as measured by infrared spectroscopy (8) using methyl elaidate as a calibration standard. If *trans* isomer content fell below these ranges, the isomerization reaction was repeated.

300 g of reagent grade, powdered silicic acid was activated by heating overnight at 110C. A chromatography column was prepared using a slurry of the silicic acid in dry petroleum ether. The solvent head was allowed to drop almost to the top of the silicic acid. Then the linoleic acid solution was added to the column, and the eluate was collected. When the solvent head had once more reached the top of the silicic acid, the column was flushed with 2.2 liters of petroleum ether. The solvent was then evaporated from the eluate under reduced pressure. After one pass through the column, 116 g of isomerized linoleic acid having a very slight yellowish tint was obtained. If any of the brownish-red color remained, the above procedure was repeated with a fresh column of silicic acid.

116 g of the isomerized fatty acid was dissolved in 580 ml of acetone and left overnight at −20C in a freezer. The crystals formed were filtered using a Buchner funnel cooled to −20C and were washed with 20 ml of acetone also cooled to −20C. This recrystallization procedure was repeated twice more using 5 ml of acetone per gram of fatty acid. Filtrates were saved if desired for re-isomerization to improve yields. The third recrystallization yielded 45 g of white 9-*trans*,12-*trans*-octadecadienoic acid crystals with the properties shown in Table I.

Comments. Using the method described, 1 kg of safflower oil yielded 45 g of 9-*trans*,12-*trans*-octadecadienoic acid. With 1 kg of linoleic acid as a starting material, 135 g of product was obtained.

The properties of the final product are given in Table I. All analyses agree closely with expected values and the product is believed to be 99+% pure. Practically complete removal of the nitrogenous reaction by-products is indicated by less than 1 ppm nitrogen present in the final product. As a further check that no biologically toxic materials were present, weanling albino rats were fed a ration containing 5% methyl 9-*trans*,12-*trans*-octadecadienoate made by the method described here. After two weeks on this diet, visual inspection of the internal organs of the rats indicated no abnormal conditions present. The growth rates and liver weights of the test rats were equivalent to those of a control group.

TABLE I

Properties of 9-*trans*,12-*trans*-Octadecadienoic Acid Prepared by HNO_2 Isomerization

Analysis	Found	Expected
Melting point	27.5–28.1C	28–29C (1,2)
Melting point of mixture with authentic sample [a]	27.4–28.1C	28–29C (1,2)
%C	76.90	77.09
%H	11.39	11.50
%O	11.38	11.41
ppm N	<1	0
% 9-*trans*,12-*trans*-octadecadienoic acid by capillary GLC (6,7)	99+	100
Impurities determinable by TLC [b]	none	none
Absorptivity at 10.36 μ (8)	0.4391	0.4441 [a]

[a] Determined using an authentic sample of 9-*trans*,12-*trans*-octadecadienoic acid made by the Se catalyzed method of Kass and Burr (2).
[b] Thin layer chromatography on 0.250 mm thick Silica Gel G using petroleum ether-ethyl ether-acetic acid, 59/40/1, v/v/v, as the eluting solvent and dichlorofluorescein as the indicator.

The column chromatography conditions used here are considerably different from those used in analytical procedures. More fatty acid is applied to the column than the adsorption sites of the silicic acid can hold. Under these conditions, apparently the silicic acid adsorbs the more polar nitrogenous reaction by-products in preference to the ordinary fatty acids. The ratio of silicic acid to sample has been chosen so that all the nitrogenous by-products are adsorbed and a minimum amount of the normal fatty acids remains on the column. Test experiments have shown that with petroleum ether as an eluting solvent, this loss is approximately 150 mg of fatty acid per gram of silicic acid in that portion of the column where no nitrogenous by-products are present.

There are several possible alternative steps to the procedure described here. Another linoleic rich oil such as corn, sunflower, or poppyseed can be used in place of safflower oil as a starting material, but additional urea crystallizations would be necessary, thus lowering the yield. If 98+% linoleic acid is available, the initial saponification and urea crystallization steps may be omitted.

ACKNOWLEDGMENTS

This investigation was supported in part by grants from the National Institutes of Health (AH-6011), Cargill, Inc., and the Corn Products Co. Advice and assistance in preparing this manuscript by A. F. Isbell, Texas A & M Chemistry Department. Safflower oil supplied by the Pacific Vegetable Oil Corp.

REFERENCES

1. Jackson, J. E., R. F. Paschke, W. Tolberg, H. M. Boyd, and D. H. Wheeler, JAOCS, *29*, 229 (1952).
2. Kass, J. P. and G. O. Burr, J. Amer. Chem. Soc., *61*, 1062 (1939).
3. Teeter, H. M., E. W. Bell, J. L. O'Donnell, M. J. Danzig, and J. C. Cowan, JAOCS, *35*, 238 (1958).
4. Teeter, H. M., E. W. Bell, and M. J. Danzig, J. Org. Chem., *23*, 1156 (1958).
5. Litchfield, C., J. E. Lord, A. F. Isbell, and R. Reiser, JAOCS, in press.
6. Litchfield, C., A. F. Isbell, and R. Reiser, JAOCS, *39*, 330 (1962).
7. Litchfield, C., R. Reiser, A. F. Isbell, and G. L. Feldman, JAOCS, in press.
8. Method Cd-14-61, "Isolated *Trans* Isomers" in "Official and Tentative Methods of the AOCS," Chicago, 1961.

R. D. HARLOW, CARTER LITCHFIELD, AND RAYMOND REISER
Department of Biochemistry & Nutrition
Texas Agricultural Experiment Station
College Station, Texas

[Received June 5, 1963—Accepted June 18, 1963]