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# Countercurrent distribution of soybean fatty acid methyl esters biosynthetically labeled with $H^3$ and $C^{14}$

H. J. Dutton, E. P. Jones, C. R. Scholfield, W. Chorney, and N. J. Scully*

*Northern Regional Research Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Peoria, Illinois; and Argonne National Laboratory, Lemont, Illinois*

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## SUMMARY

Fatty acids randomly labeled with $C^{14}$ and with $H^3$ were prepared by culturing soybean plants (*a*) in the presence of $C^{14}O_2$, and (*b*) with $H^3OH$ in the nutrient medium. After maturation of the beans, extraction of the oils, and transesterification of the glycerides with methanol, the methyl esters were readily separated by countercurrent distribution to yield fractions of pure linoleate (7 g and 469 $\mu$c of $C^{14}$-labeled esters), 97% linolenate, a 2:1 mixture of oleate and palmitate, 83% stearate, and concentrates of $C_{20}$ and higher acid esters. Over-all recoveries of 90% may be expected from countercurrent distribution.

Biosynthetically labeled lipids have been prepared by the culture of soybeans (1), *Chlorella pyrenoidosa* (2), and *Phycomyces blakesleeanus* (3). Small amounts of individual fatty acids have been isolated in the last two investigations. For the production of labeled linoleic acid, the use of soybeans has an advantage in the large amount of oil produced, in its relatively simple fatty acid system, and in the preponderance of linoleic acid (> 50%) found in its glycerides.

Recently a countercurrent distribution procedure was described for the preparation of individual fatty acid methyl esters,[1] which employs hydrocarbon and acetonitrile solvents. The present paper describes the application of this procedure to the isolation of soybean fatty acid methyl esters which had been biosynthetically labeled during growth with tritium and $C^{14}$.[2]

## MATERIALS AND METHODS

The culture of soybeans from seedlings to various stages of maturity in three Argonne-type plant growth isotope chambers (1, 4), (*a*) in the presence of $C^{14}O_2$, (*b*) with $H^3OH$ in the nutrient solution, and (*c*) under control conditions will be described in another publication, together with observations of the efficiency of production of isotopically labeled soybean oil and the effects of radiation on plant growth and composition.[3] After harvesting the seed in various maturity groups, the beans were dried, ground, and extracted with pentane-hexane. Analyses for fatty acid compositions of these maturity groups were made by gas-liquid chromatographic (GLC) methods after formation of the methyl esters by transesterification with methanol. The $C^{14}$-labeled esters were then combined to yield 16 g, and the tritium-labeled esters to total 5 g. These samples served as starting materials for the preparative countercurrent distribution to be described.

*Gas-Liquid Chromatography.* A 5-foot column of

* Dutton, Jones, and Scholfield performed this work at the Northern Regional Research Laboratory; Chorney and Scully performed this work, in part, at the Argonne National Laboratory under the auspices of the U.S. Atomic Energy Commission.

[1] C. R. Scholfield and H. J. Dutton. Presented at the American Oil Chemists' Society meeting, New Orleans, La., April, 1959.

[2] W. Chorney, N. J. Scully, L. H. Mason, and H. J. Dutton. In preparation.

[3] See footnote 2.

Resoflex 296[4] (adipate polyester of diethylene glycol) on Celite® 545, operated at 200° with a helium flow rate of 50 ml per minute, was used to analyze the methyl esters in the Aerograph gas chromatograph. Approximately 500 theoretical plates are calculated for this column.

*Radioactivity.* Activity of both tritium and $C^{14}$-labeled esters was determined by use of liquid scintillation procedures (5). Toluene containing 4 g POP (2,5-Diphenyloxazole) and 30 mg POPOP (1,4-*Bis*-2-[5-Phenyloxazolyl] Benzene) per liter was used as the scintillation solution. Radioactivity of fatty acids issuing from the GLC equipment was determined by condensing the esters from the gas stream in the scintillation solution contained in individual vials for 1-minute periods and by assaying the vials with a Packard Automatic Tri-Carb Scintillation Spectrometer.

*Countercurrent Distribution.* Separation of the methyl esters was performed in a 200-tube automatic countercurrent distribution instrument. Acetonitrile (40 ml) and pentane-hexane (10 ml) were used as the solvent pair. In successive experiments the $C^{14}$ esters and the $H^3$ esters were introduced into the first ten and the first four tubes of the instrument, respectively. After 200 transfers, the petroleum ether layers were collected by the withdrawal technique, four 10-ml aliquots being combined in each collection tube; distribution was continued until 640 transfers had been applied. At this stage, the slowest moving ester, methyl linolenate, had been "eluted" from the instrument. Radioactivity of every other collection tube was determined by introducing 0.1-ml aliquots into 15 ml of scintillation solution and by assaying the activity with the scintillation spectrometer. Based on this distribution curve for radioactivity, combinations of tubes were made, solvents were evaporated, compositions determined by GLC, and the preparations sealed in ampules *in vacuo*.

*Oxidation of Methyl Oleate.* The D Fractions were saponified in 95% ethanol with potassium hydroxide at reflux temperature for 45 minutes, acidified with hydrochloric acid, and the extracted acids oxidized as soaps at room temperature with a mixture of metaperiodate and permanganate (6). The mixture of monobasic and dibasic acids was recovered by extraction with ethyl ether. Conditions of oxidation as described (6) do not cause a shift in double-bond position.

[4] Cambridge Industries, Cambridge, Mass. Mention of commercial equipment or products does not constitute endorsement by the U.S. Department of Agriculture over those of other manufacturers.

*Liquid-Liquid Partition Chromatography.* Aliquots of the cleaved acids were applied to a silicic-acid column (7) for the separation of all monobasic acids as one peak, and of individual dibasic acids as separate peaks. Subsequently, fractions of the monobasic-acid peak were recombined and these acids were applied to a different silicic-acid column (8) to separate individual monobasic acids.

## RESULTS AND DISCUSSION

A gas-liquid chromatogram for the tritium-labeled esters from mixed mature and immature beans is given in Figure 1. The curve for radioactivity parallels the

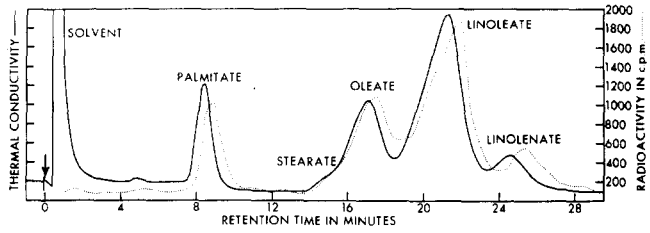


FIG. 1. Gas-liquid chromatogram of tritium-labeled methyl esters. Solid line=thermal conductivity; dotted line=radioactivity.

thermal conductivity curve, as would be anticipated if radioactive atoms were evenly distributed among the fatty acids. The radioactivity curve lags slightly behind because of the time required for the gaseous stream to flow from the conductivity detector into the scintillation solution. By integrating areas under thermal conductivity curves, the compositions of the $C^{14}$- and $H^3$-labeled samples of esters used for countercurrent distribution were calculated and are given in Table 1.

The radioactivity curve for countercurrent distribution of the $C^{14}$-labeled methyl esters is shown in Figure 2. Fractions A and B differed in content of $C_{20}$ and $C_{22}$ saturated acids, but detailed analyses of these minor fractions were not made. Fraction C, as calculated from GLC data, is 85.2% methyl stearate. Similarly, Fraction D was composed of 0.3% stearate, 32.7% palmitate, and 67.1% "oleate." (The GLC analyses would not have distinguished $C_{18}$ monoenes with double bonds in the 8, 9, and 10 positions.) In this pentane-hexane acetonitrile system, the effect of introducing a double bond into methyl stearate, i.e., methyl oleate, is approximately equal to the effect of reducing the chain length by 2 carbons, i.e., methyl palmitate. If separation of this binary system were

TABLE 1. COMPOSITION OF $H^3$- AND $C^{14}$-LABELED METHYL ESTERS AND FRACTIONS SEPARATED BY COUNTERCURRENT DISTRIBUTION

| Fractions | Transfer No. | Approximate Weight Recovered | Per Cent of Total Recovered Weight | Fatty Acid Compositions | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Stearic | Palmitic | Oleic | Linoleic | Linolenic | $C_{20}$ Higher |
| | $H^3$-labeled esters | *mg* | | 2.5 | 11.0 | 23.6 | 53.5 | 9.3 | |
| A | 200–231 | 37.3 | 1.14 | 1.2 | | | | | 98.9 |
| B | 232–263 | 50.5 | 1.54 | 2.1 | | | | | 97.9 |
| C | 264–287 | 154.3 | 4.70 | 83 | 12 | | | | 5.0 |
| D | 288–366 | 1,054.0 | 32.15 | 0.5 | 32.8 | 66.7 | | | |
| E | 367–494 | 1,760.0 | 53.7 | | | | 99+ | | |
| F | 495–681 | 222.0 | 6.8 | | | | 3.1 | 96.9 | |
| | | 3,278.1 | | | | | | | |
| | $C^{14}$-labeled esters | | | 2.0 | 11.05 | 23.3 | 53.1 | 10.6 | |
| A | 201–224 | 70.2 | .5 | | | | | | 100 |
| B | 225–252 | 202 | 1.5 | .8 | | | | | 99.2 |
| C | 253–288 | 595 | 4.4 | 85.2 | 9.6 | | | | 5.2 |
| D | 289–368 | 4,250 | 31.7 | .3 | 32.7 | 67.1 | | | |
| E | 369–492 | 7,040 | 52.6 | | | | 99+ | | |
| F | 493–640 | 1,230 | 9.2 | | | | 2.1 | 97.9 | |
| | | 13,387.2 | | | | | | | |

required, a urea crystallization or micro distillation prior to distribution would readily remove the palmitate.

The gas-liquid chromatogram for methyl linoleate of Fraction E is shown in Figure 3. Greatly overloading the column with Fraction E revealed only traces of impurities in this preparation. Because of current interest in the essential fatty acid function of linoleic acid, this ester is of particular value for biological experiments. Fortunately, as shown in Figure 3, it is isolated in high purity. Because a tail of the E fraction entered the F fraction, 2.1% of linoleate contaminated the linolenate. Comparable data for tritium-labeled soybean esters were obtained (Table 1).

In the countercurrent distributions for $H^3$- and $C^{14}$-labeled esters, 70% and 90%, respectively, of the esters introduced could be accounted for. Furthermore, the percentages of the recovered weight in the individual fractions closely approximate the composition of the starting materials and are indicative of no selective losses during countercurrent distribution. This isola-

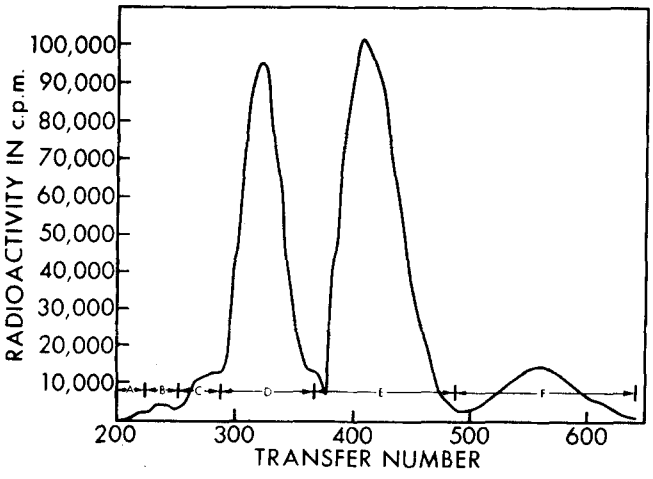


FIG. 2. Countercurrent distribution of $C^{14}$-labeled soybean methyl esters.

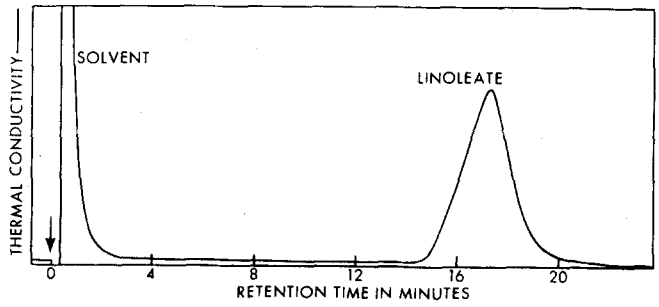


FIG. 3. Gas-liquid chromatogram of Fraction E.

tion of methyl esters can be readily carried out by laboratories possessing the requisite equipment. The extraction of ground beans, and countercurrent distribution operations described, were carried out by two people working over a period of two weeks. The amounts isolated are indeed practical for conducting subsequent experiments.

Although the specific activities of these preparations are low for these fatty acid esters, 0.067 $\mu$c per mg for $C^{14}$ and 0.026 $\mu$c per mg for $H^3$, preliminary observations[5] indicate that at least a tenfold increase in specific activity might be expected by alteration of cultural methods and labeling; in addition, the total yield of seed might be increased manyfold.

Since the soybean plants were grown from seedling stage to maturity in the presence of isotopes, random labeling of constituents would seem to be obligatory. In agreement with this inference, no selectivity for a given isotope between individual fatty acids is evident from data such as given in Figure 1 in which the ratio of radioactivity to thermal conductivity current appears to be constant for all fatty acids.

Random labeling among the carbon atoms of the fatty acid is also indicated by comparing the activities of palmitic acid and of the oxidative cleavage products of oleic acid in Table 2. Thus the activities per carbon atom for palmitic acid (1225), azelaic acid (1160), and pelargonic acid (1276) after the periodate-permanganate oxidation of $C^{14}$-labeled Fraction D are equal within limits of error. In Figure 4 a liquid-liquid partition chromatogram for oxidative scission acids is given for a column designed to separate dibasic from monobasic acids. The curve for dibasic acids was obtained by collecting, titrating, and counting alternate 2-ml

TABLE 2. RADIOACTIVITY (CPM/1000 PER MILLIMOLE) OF OXIDATIVE CLEAVAGE PRODUCTS FROM $H^3$- AND $C^{14}$-LABELED ESTERS OF FRACTION D

| Trial No. | Dibasic Chromatograms | | | | Monobasic Chromatogram of Monobasic Mixture* | | |
|---|---|---|---|---|---|---|---|
| | Monobasic* | Dibasic | | Recovery | $C_{16}$ | $C_9$ | Recovery |
| | | $C_{16}$ | $C_9$ | | | | |
| | | | | *per cent* | | | *per cent* |
| | | | $H^3$ Fraction | | | | |
| 1 | 1388 | 1460 | 1016 | 97.8 | 2052 | 1433 | 93.4 |
| 2 | 1712 | — | 1054 | 108.4 | 1992 | 1313 | 90.5 |
| Av. | 1550 | 1460 | 1035 | 103.1 | 2022 | 1373 | 92.0 |
| Activity per H atom | | 91 | 74 | | 65 | 81 | |
| | | | $C^{14}$ Fraction | | | | |
| 1 | 13380 | 13850 | 10260 | 89.1 | 19860 | 11650 | 90.2 |
| 2 | 15260 | 15050 | 10630 | 93.4 | 19380 | 11350 | 88.4 |
| Av. | 14320 | 14450 | 10445 | 91.3 | 19620 | 11500 | 89.3 |
| Activity per C atom | | 1445 | 1160 | | 1225 | 1276 | |

* This includes the palmitic acid present before oxidation as well as those monobasic acids produced upon oxidation.

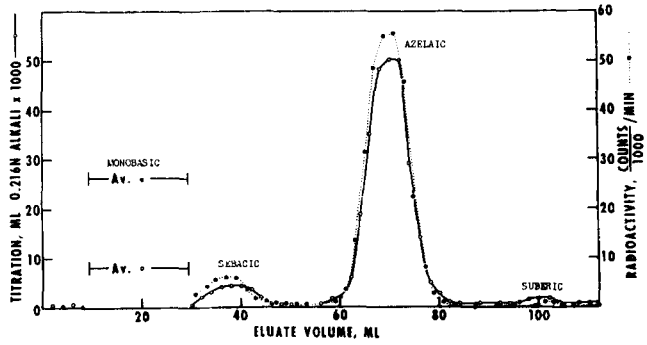


FIG. 4. Liquid-liquid partition chromatogram of dibasic acids from the periodate-permanganate oxidized Fraction D. Titration and activity of the mixed monobasic acid group are indicated as average values. (See Fig. 5.)

[5] Unpublished data from the Argonne National Laboratory.

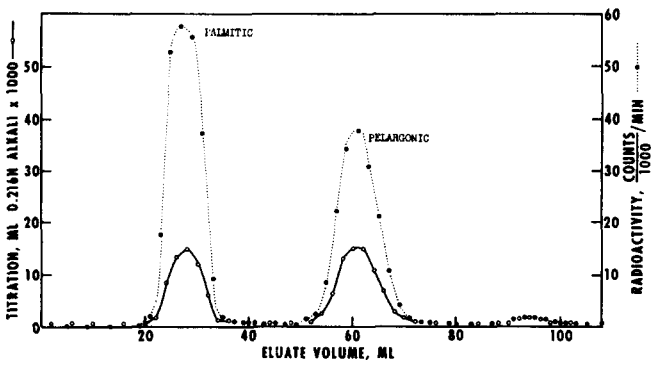


FIG. 5. Liquid-liquid partition chromatogram of the mixed monobasic acid group shown in Figure 4.

portions of eluate. The monobasic fraction was collected in one tube and aliquots were titrated and counted to give the average values of Figure 4. The rest was concentrated, reassayed for radioactivity, and applied to a second column designed to separate monobasic acids (Fig. 5). Alternate fractions in each of the chromatograms were assayed for radioactivity by liquid scintillation methods, and were titrated with 0.216 N NaOH using a Gilmont microburette. The data for $C^{14}$-labeled esters given in Figures 4 and 5, and from a duplicate oxidation and chromatograms (not shown), are presented in Table 2. Similar data of $H^3$-labeled esters were obtained and are also summarized in Table 2. The presence of sebacic and suberic acids (Fig. 4) is not unexpected. Jones and Stolp (6) have shown that these acids may be found when "pure" oleic acid is cleaved by the same procedure.

The constancy of $C^{14}$ activity per carbon atom for these scission acids is indicative of random labeling in the intact fatty acids. The slight deviation from constancy of $H^3$ activity per hydrogen atom for oleic acid cleavage products may be explained in part by the exchange of tritium atoms in labile positions during saponification and oxidation cleavage reactions.

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