Improved method for determination of the position of double bonds in polyenoic fatty acid esters

J. N. Roehm and O. S. Privett

University of Minnesota, The Hormel Institute, Austin, Minnesota 55912

SUMMARY An improved method is described for determination of the position of double bonds in polyenoic fatty acid methyl esters. Partial reduction with hydrazine is employed without prior hydrolysis to the free fatty acid, and the resulting monoenes are identified by reductive ozonolysis.

SUPPLEMENTARY KEY WORDS hydrazine reduction · ozonolysis · reductive gas-liquid chromatography

Determination of the position of *cis* and *trans* double bonds in polyenoic fatty acids via hydrazine reduction and ozonolysis has been reported by Privett and Nickell (1). This method employs partial reduction of the acid with hydrazine to yield monoenes, isolation of the *cis* and *trans* monoenes by argentation chromatography, and identification via reductive ozonolysis. Since each of the monoenoic acids formed in this manner represents one position of unsaturation in the original acid, the structure can be readily determined.

Because fatty esters form hydrazides in the presence of hydrazine hydrate (2), the described method started from the free acid, which was converted to its hydrazine salt and reduced. The salt then had to be acidified and the resulting acid extracted and esterified prior to analysis. Furthermore, it was usually necessary to convert the original sample to acids through saponification prior to reduction. We now find that the methyl ester can be partially reduced with hydrazine under certain conditions, and the method can therefore be greatly simplified.

Partial Reduction with Hydrazine. A small amount, preferably about 5 mg, of the methyl ester sample, stabilized against autoxidation by the addition of 0.01% propyl gallate or nordihydroguaiaretic acid, is dissolved in 0.5 ml of a 10% (v/v) methanol solution of hydrazine hydrate (Eastman Organic Chemicals, Rochester, N. Y.). This solution is heated at 40°C with vigorous stirring until a maximum concentration of monoenes (tested by GLC, see below) is obtained. Since hydrazide formation and reduction are competing reactions in this case, it is necessary to stir the reaction vigorously in order to incorporate as much oxygen as possible into the solution so that reduction will be favored. (Reduction with hydrazine requires oxygen; water and nitrogen are by-products.)

The reduction is monitored by GLC: 100 µl of the reaction mixture is taken at intervals and shaken with 500 µl of petroleum ether and 400 µl of distilled water. An aliquot of the upper phase containing the esters is then analyzed directly by GLC. When the maximum

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Abbreviation: GLC, gas-liquid chromatography.

concentration of monoenes is obtained (see, for example, Fig. 1), the reaction is stopped by the addition of water and the esters are extracted with petroleum ether.

The hydrazides formed during reduction are only slightly soluble in petroleum ether and remain at the origin during thin-layer chromatography. They will not interfere with GLC analysis, but if desired they can be easily separated from the esters by thin-layer or column chromatography.

Isolation of Monoenes. The *cis* and *trans* monoenoic methyl esters are isolated by thin-layer chromatography on Silica Gel G (Merck, A. G., Darmstadt, Germany) impregnated with silver nitrate as described by Barrett, Dallas, and Padley (3). The plate is sprayed with 2′,7′-dichlorofluorescein and viewed under UV radiation, which makes the ester bands visible. The monoenoic esters (those with R_f corresponding to methyl oleate) are then recovered from the adsorbent by extraction with diethyl ether–petroleum ether 1:1 in a separatory funnel in the presence of 1% aqueous HCl. The upper phase is washed with distilled water, dried over sodium sulfate, evaporated to dryness, and redissolved in pentane.

Reductive Ozonolysis. The solution of esters is introduced into 10 ml of pentane that has been saturated with ozone at −65°C as previously described (4). The solution is allowed to stand for 1 min and the excess ozone is then removed under reduced pressure. The ozonides thus formed are then thermally reduced by the procedure of Nickell and Privett (5). This reduction and simultaneous GLC analysis was carried out in a Packard gas chromatograph equipped with dual columns and hydrogen flame detectors. Coiled glass columns (6 ft. × ¼ inch I.D.) packed with 30% silicone grease on Chromosorb W were employed, and the column temperature was programmed from 50 to 190°C at 5°C/min with a nitrogen flow rate of 60 ml/min.

Results and Discussion. A sample of purified methyl linolenate was analyzed by the method described. Fig. 1 shows the course of its reduction, and the production of partially reduced and completely reduced esters. Since hydrazine simultaneously reacts with the ester group to form hydrazides, an internal standard of methyl pentadecanoate was used to permit determination of the absolute amount of monoene formed. Although the maximum percentage of monoenes (47%) was reached in 5 hr, the maximum yield in milligrams was reached at approximately 4 hr (Fig. 1). During this 5 hr period, the total weight of esters was reduced to the extent of about 50%. Because the yield of monoenes was maximal over a period of about 2 hr, precise monitoring of the reaction was not required.

Reductive ozonolysis and subsequent GLC analysis of the *cis* monoenes isolated by thin-layer chromatography yielded the expected fragments (C_3-aldehyde and C_{15}-aldehyde ester; C_6-aldehyde and C_{12}-aldehyde ester; C_9-aldehyde and C_9-aldehyde ester). The *cis* monoenes were thus shown to be a mixture with double bonds in the 9, 12, and 15 positions. The identification of these fragments, coupled with the fact that no other fragments were detected, showed that no migration of double bonds occurred during the procedure. Quantitative analysis of the fragments showed that these three esters were produced in approximately equal proportions, although with less symmetrical molecules double bonds in different positions may not be reduced at the same rates. The lack of a *trans* monoene band in the thin-layer chromatogram demonstrated that no *cis–trans* isomerization occurs during reduction.

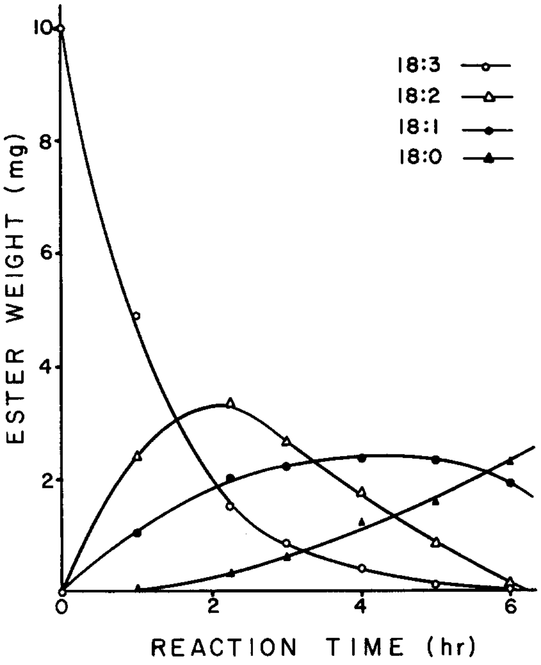

FIG. 1. Partial reduction of methyl linolenate with hydrazine, as monitored by GLC with an internal standard of methyl pentadecanoate.

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