

## Esterification of fatty acids at room temperature by chloroform-methanolic HCl-cupric acetate

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**Summary** A new procedure for the preparation of methyl esters from free fatty acids under mild conditions was investigated. Free fatty acids are dissolved in a mixture of chloroform-methanolic HCl-cupric acetate and kept at room temperature for 30 min for complete esterification. The method is suitable for esterification of long-chain acids, such as 18:0, and for very long chain acids, such as 24:0. Fatty acids from brain glycerophosphatides, which included a high concentration of polyenes such as 20:4 (n - 6), 22:4 (n - 6), and 22:6 (n - 3), were also esterified by the same procedure, and neither artifact formation nor loss of unsaturated acids was observed.

**Supplementary key words** methyl ester · polyunsaturated fatty acids

The most frequently used methods for preparation of methyl esters from free fatty acids are: heating with methanolic HCl or methanolic boron trihalide (1); reacting with diazomethane (2); or reacting with a mixture of 2,2-dimethoxypropane-methanol-HCl (3). However, artifact formation or loss of polyunsaturated fatty acids occurs during the course of these esterification procedures (4-7).

Formation of methyl esters of 2-hydroxy acids occurs at room temperature when copper chelates of 2-hydroxy fatty acids (8) are dissolved in anhydrous methanolic HCl (9). This observation prompted an examination of the esterification of nonhydroxy fatty acids under similar mild conditions. In this communication, a new procedure is presented which allows nearly complete esterification of nonhydroxy fatty acids at room temperature in a short period of time. Reagents necessary for this procedure are more economical than those for most other methods and are, unlike boron trihalides, dimethoxypropane, and diazomethane, relatively nontoxic and stable.

**Materials.** 15% methanolic HCl was purchased from Supelco, Inc. (Bellefonte, Pa). It was diluted to 0.5 N with redistilled methanol and stored in a refrigerator (10). Unisil, 100-200 mesh, was obtained from Clarkson Chemical Co. (Williamsport, Pa.).

[1-<sup>14</sup>C]Lignoceric acid (tetracosanoic acid, 24:0) and [1-<sup>14</sup>C]cerebronic acid (2-hydroxytetracosanoic acid, 24h:0) were synthesized in this laboratory (9). [1-<sup>14</sup>C]Stearic acid (octadecanoic acid, 18:0) was obtained from New England Nuclear Corp. (Boston, Mass.). These

radioactive fatty acids were mixed with corresponding nonradioactive acids and purified by preparative thin-layer chromatography immediately before use.

**Procedures.** Approximately 1 mg of stearic or lignoceric acid was dissolved in 0.2 ml of chloroform (with slight warming if necessary). 0.2 ml of 20 mM cupric acetate monohydrate in methanol and 1 ml of 0.5 N HCl in methanol were added, and the mixture was left for the specified time (10-60 min) at room temperature or at 37°C. The reaction mixture was extracted either three times with 2 ml of hexane after addition of 0.4 ml of water or with 2 ml of chloroform after addition of 2 ml of water. The pooled extracts (hexane or chloroform) were washed with water and then evaporated to dryness under a flow of nitrogen.

**Results and Discussion.** In order to determine the degree of esterification under various conditions, 1 mg each of 18:0, 24:0, or 24h:0 containing approximately 10,000 cpm each was esterified as specified in Figs. 1 and 2 and Table 1. Chloroform was used to extract the reaction product, and all added radioactivity was recovered in the chloroform. The chloroform-soluble material was then fractionated on a column containing 0.2 g of Unisil by elution with 5 ml of hexane-benzene 6:4 to obtain methyl esters of 18:0 or 24:0 and then with 2.5 ml of ether to recover unreacted free acids. When 24h:0 was used as the starting material, benzene was used instead of hexane-benzene to elute the esters. The solvents were evaporated from both fractions, the residues were dissolved in a toluene-based scintillation mixture, and the radioactivity was measured. The degree of esterification was calculated by dividing the radioactivity found in the methyl ester fraction by the radioactivity obtained in the methyl ester and free acid fractions. Further elution of the column with methanol did not yield a significant amount of radioactivity.

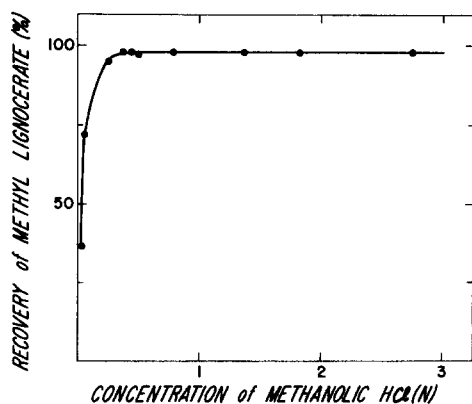
The formation of the methyl esters from lignoceric acid increased with increasing concentrations of methanolic HCl; at a concentration of 0.37 M the reaction was nearly complete (Fig. 1). Further increase of HCl concentration did not alter the yield of methyl lignocerate.

Approximately 80% esterification of lignoceric acid occurred without the addition of cupric acetate when the reaction was performed at room temperature (Fig. 2). The yield of methyl lignocerate increased with increasing cupric acetate concentrations, and nearly quantitative esterification was achieved when the concentration of added methanolic cupric acetate was 8 mM or higher. On the other hand, the concentration of cupric acetate had no ef-

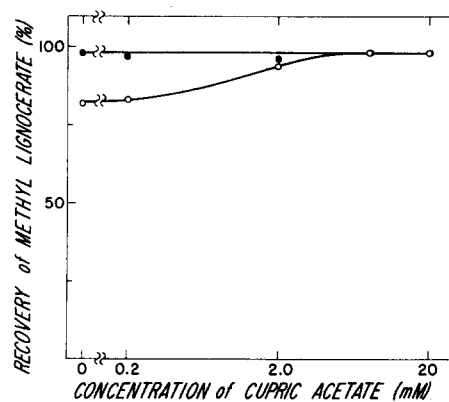
Abbreviations: Fatty acids are identified by carbon number and number of double bonds; h represents a 2-hydroxy group.

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**Fig. 1.** Recovery of  $[1-^{14}\text{C}]$ lignoceric acid as its methyl ester as a function of HCl concentration. 1 mg of lignoceric acid containing 10,000 cpm was dissolved in 0.2 ml of  $\text{CHCl}_3$  and 0.2 ml of 20 mM cupric acetate in methanol. To this was added 1 ml of methanolic HCl, and the mixture was left at  $37^\circ\text{C}$  for 30 min. The methyl ester formed was separated from unreacted free acid by silicic acid chromatography, and the degree of esterification was assayed as described in the text. Essentially the same results were obtained by performing the esterification at room temperature.



**Fig. 2.** Recovery of  $[1-^{14}\text{C}]$ lignoceric acid as its methyl ester as a function of cupric acetate concentration. The reaction conditions were similar to those described in Fig. 1, except that different concentrations of methanolic cupric acetate were used. Open circles indicate the recovery when the reaction was performed at room temperature and closed circles when performed at  $37^\circ\text{C}$ .

fect on the esterification when the reaction was performed at  $37^\circ\text{C}$ . Dehydration of cupric acetate to the anhydrous form did not improve the yield of the methyl lignocerate.

The yields of methyl esters from different fatty acids by the present procedure were determined at two reaction temperatures and various reaction times (Table 1). Except when the reaction was performed at  $25^\circ\text{C}$  for 10 min, 18:0 appeared to be esterified almost completely under all conditions examined. On the other hand, the effect of cupric acetate was more apparent when the longer-chain acid 24:0 was esterified at  $25^\circ\text{C}$ . The yield of methyl esters of 24h:0 was lower, approximately 85%, even at  $37^\circ\text{C}$ . The yield did not improve by extending the reaction time to 16 hr. In this case, a higher temperature may be required for complete esterification.

The suitability of the procedure for polyunsaturated

acids was examined by esterification of fatty acids from brain glycerolipids, which contain large amounts of polyenoic acids. A mixture of methyl esters that was prepared by mild alkaline methanolysis (11) from total rat brain lipids was saponified (12) under nitrogen. The free fatty acids obtained were reesterified by the present procedure and compared with the starting material by gas-liquid chromatography on both DEGS and OV-1 columns (13). It was found that the methyl ester composition was essentially identical. Methyl esters of 20:4 ( $n - 6$ ), 22:4 ( $n - 6$ ), and 22:6 ( $n - 3$ ) were 8.8, 8.8, and 16.2% of the total methyl esters, respectively, before the saponification and 8.7, 8.9, and 15.9%, respectively, after the reesterification. Examination of the methyl esters by a gas-liquid chromatography-mass spectroscopy system (DuPont model 21-491) indicated that there was no structural alteration during the course of esterification. This observation proved that the present method is suitable to use for the esterification of polyunsaturated fatty acids.

**TABLE 1.** Effect of reaction time and temperature on esterification

Fatty Acid	Reaction Time	Recovery of Methyl Esters	
		$25^\circ\text{C}$	$37^\circ\text{C}$
	<i>min</i>	%	
18:0	10	94.2 (87.4)	97.2 (97.4)
	25	97.3 (97.0)	96.5 (96.6)
	45	97.2 (96.9)	97.2 (96.9)
	60	97.3 (96.8)	97.5 (96.9)
24:0	10	97.2 (60.6)	99.3 (98.7)
	25	98.8 (79.4)	99.1 (97.7)
	45	99.2 (87.0)	99.0 (99.0)
	60	98.9 (92.6)	98.9 (98.4)
24h:0	25	77.5 (68.7)	82.0 (75.0)
	45	83.0 (72.9)	85.5 (73.4)

1 mg of each fatty acid containing 10,000 cpm was esterified with a chloroform-methanolic HCl-cupric acetate mixture at either 25 or  $37^\circ\text{C}$  for the length of time specified; the yield of methyl esters was calculated as described in the text. Numbers in parentheses indicate yields of methyl esters when cupric acetate was omitted from the reaction mixture.

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