

Methods for the Isolation and Characterization of Constituents of Natural Products

XXI. Use of a Celite-Potassium Methylate Column for Rapid Preparation of Methyl Esters from Microgram Amounts of Glycerides

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INTRODUCTION

A number of publications from this laboratory have described column reactions that are useful in the analysis of microgram and sometimes submicrogram amounts of lipid constituents (3-6). In continuing our efforts in this direction, a method is detailed below which utilizes a very small column of potassium methylate mixed with diatomaceous earth to transmethyrate microgram amounts of glycerides. The procedure is rapid, simple, and convenient, and is executed without the use of methanol *per se*.

MATERIALS²

Hyflo Super-Cel (Fisher Scientific Co., King of Prussia, Pa.) was dried at 110°C for 48 hr; powdered potassium methylate (CH₃OK) was purchased from the Ventron Corp., Danvers, Mass.; melting-point capillaries open at both ends 1.6-1.8 × 100 mm and Critoseal (a vinyl plastic putty) were from A. H. Thomas Co., Philadelphia, Pa.; 100-μl volumetric capillaries sealed at one end and graduated in 10 μl increments were obtained from Friedrich and Dimmock, Inc., Millville, N.J.; solvents were ACS grade or better and used as received.

EXPERIMENTAL

Preparation of transmethyating powder. Hyflo Super-Cel (1.0 g) was ground thoroughly³ in a mortar (8.2 cm o.d., 60 ml capacity) for 1 min with 0.3 g CH₃OK. The powder was transferred to a dry 5-ml screw cap vial and stoppered by pulling the sleeve portion of a rubber septum (sleeve 7 ×

¹ Agricultural Research Service, U.S. Department of Agriculture.

² Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

³ As much pressure should be applied to the pestle as possible, and the powder should be scraped from the sides about midway through the grinding period.

11 mm) over the threads of the vial. The septum was punctured with a hypodermic needle and wire until a glass capillary could be inserted smoothly. The powder was kept at room temperature during the working day and at -18°C at other times.

Preparation of Microcolumn. A melting-point capillary was sealed at one end by pushing it into Critoseal. It was weighed,⁴ the open end was inserted through the septum of the vial containing the transmethylating powder, and the capillary was dabbed into the powder several times. Approximately 8–10 mg was loaded into the capillary in this manner. If more powder was needed, the capillary was withdrawn, the powder pushed into the capillary with a tamper (paper clip), and the process repeated. The powder was finally pushed into the capillary so that 1–1.5 cm of space remained between the end of the capillary and the bed. The capillary was cut below the Critoseal plug, and, while the end portion of the bed was retained with a tamper, the powder was pressed into a compact column with another tamper. The capillary was then cut 4–5 cm above the bed.

Transmethylation of sample. The glyceride, fat, or oil was dissolved in petroleum ether, *n*-hexane, cyclohexane, or benzene at a concentration not exceeding $7\ \mu\text{g}/\mu\text{l}$. A volume⁵ of the solution was applied to the column from a $10\text{-}\mu\text{l}$ syringe by placing the needle tip lightly on the bed and expelling the solution slowly into the column. The column was then eluted with any of the solvents mentioned above or with CS_2 or CH_2Cl_2 using N_2 pressure.⁶ One of three procedures was used for collecting the effluent for subsequent analysis of the methyl esters: (a) if only a few micrograms of lipid had been applied to the column or when quantitative recovery of the methyl esters was not necessary, the column was eluted with 1 bed volume of solvent. The first 9–10 μl (about 9–10 mm) emerging was taken up as thoroughly as possible in a $10\text{-}\mu\text{l}$ syringe and analyzed; (b) when quantitative recovery of the methyl esters was desired and the expected esters were long chain ($\geq \text{C}_{14}$), the column was eluted with 3 bed volumes of solvent, the effluent was collected in a 2-ml vial, the solvent was removed at room temperature under a stream of N_2 , and the residue was taken up in a definite volume of CS_2 for analysis; and (c) as in (b) except that the effluent was continuously removed from the capillary with a hypodermic syringe and transferred to a volumetric capillary. The

⁴ In practice it may not always be necessary to obtain the weight of powder. Each millimeter of the compact bed is approximately equal to 1 mg.

⁵ A volume of the solution was applied so that not more than $2\ \mu\text{g}$ of lipid/mg of packing was present. Some unwetted portion of packing must remain after application. One microliter of solution will wet approximately 1 mg of packing.

⁶ The capillary was inserted in a capillary holder such as those supplied with Microcaps or one made out of a septum fitted on a piece of glass tubing. A pressure of 3–4 lb/in.² was used.

contents of the capillary were mixed by drawing up and expelling the solution with a hypodermic syringe prior to analysis. This procedure was used with lipids containing short chain acids such as milkfat.

Gas-liquid chromatography (GLC). GLC was performed on a Hewlett-Packard 5750 instrument with a flame ionization detector. The column was 8 ft \times $\frac{1}{8}$ -in. silanized stainless steel packed with 7.5% stabilized ethylene glycol adipate plus 2% H_3PO_4 on 90-100 mesh Anakrom ABS. Helium was the carrier gas emerging from the column at 30 ml/min. Peak areas were determined with a Supergrator-2 (Columbia Scientific Industries, Wilmington, Del.), and quantitation was done by establishing standard curves prepared from pure methyl esters (NUCHEK Prep, Inc., Elysian, Minn.).

Thin-layer chromatography (TLC). TLC was carried out on precoated 250- μ m layers of Silica Gel G (Analtech, Inc., Newark, Del.) with benzene as solvent. Spots were visualized by spraying with 8.5% aqueous H_3PO_4 containing 3% cupric acetate and heating at about 200°C.

RESULTS AND DISCUSSION

The method was applied to the following synthetic glycerides⁷: P-S-S, O-O-O, P-P-O, P-O-S, P-P-P, S-S-S, M-M-M, and L-L-L, and to milkfat, beef tallow, and corn, cottonseed, olive, safflower, and soybean oils.

The effect of the solvent used to apply the lipid to the column on the completeness of transmethylation is shown in Fig. 1. Transmethylation was complete in hydrocarbon solvents but incomplete in CCl_4 , CH_2Cl_2 , and CS_2 . The reason for this is unclear. Regardless of the solvent used, however, the correct (within 1% of theory) ratio of fatty acids was always obtained when mixed synthetic glycerides were tested.

Recovery of methyl esters from synthetic triglycerides was between 92-95% of theory when the glycerides (26-39 μ g) were applied to the column in hydrocarbon solvents and when procedure (b) or (c) was used to collect the effluent from the column for analysis. With the chlorinated solvents or CS_2 , yields were only 42-70%.

Transmethylation apparently took place instantly upon contact of the glyceride with the powder. Longer contact times did not increase yields of methyl esters in those solvents in which incomplete transmethylation occurred. Consequently, it was possible to apply the glyceride in hydrocarbon solvents and elute with chlorinated solvents or CS_2 and obtain maximal yields of methyl esters.

Figure 2 shows a gas chromatogram of the methyl esters obtained from application of 2 μ g of cottonseed oil to a 4 mg transmethylating column. The results (in area percent) agreed well with those obtained by the al-

⁷ Abbreviations: L = linoleyl, M = myristoyl, O = oleoyl, P = palmitoyl, S = stearoyl.

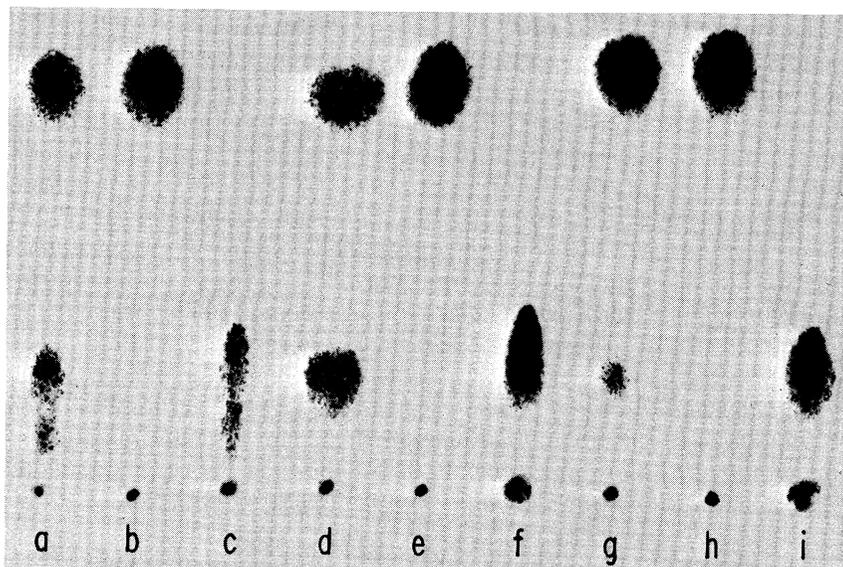


FIG. 1. Transmethylation of several fats and oils showing effect of solvent on completeness of reaction: (a) 31- μg milkfat in CS_2 ; (b) 31- μg milkfat in hexane; (c) milkfat control; (d) 32- μg corn oil in CH_2Cl_2 ; (e) 32- μg corn oil in benzene; (f) corn oil control; (g) 28- μg olive oil in CCl_4 ; (h) 28- μg olive oil in cyclohexane; (i) olive oil control.

kaline catalyzed procedures of Luddy *et al.* (2) and Christopherson and Glass (1).

All other oils and fats also gave satisfactory analyses at the 1–2 μg level. A larger (5 μg) sample of milkfat was required, however, and the methyl butyrate and methyl caproate peaks were under the solvent peak. This situation could be circumvented by transmethyating a larger amount (10 μg) in CS_2 ; in this case about half of the glycerides were transmethyated and some intact glycerides were injected into the instrument, although no adverse effects were apparent.

The powder retained its initial transmethyating characteristics for about 3 weeks. During this period approximately 70 punctures of the septum were made. There was a slight but gradual increase of methanol in the powder due to admittance of small amounts of moisture each time the septum was penetrated. There was also a slight and gradual accumulation of an unidentified component with a retention time close to methanol. Both peaks emerged under the solvent peak when transmethylation was conducted in any solvent other than CS_2 .

Attempts to prepare the transmethyating powder and methyl esters under strictly anhydrous conditions (i.e., all manipulations done in a dry box under N_2 and with chemically dried solvents) resulted in essentially

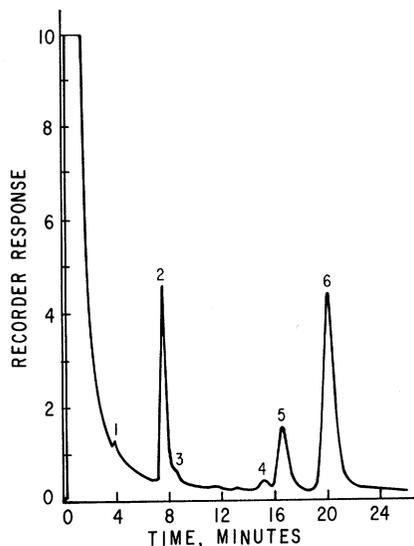


FIG. 2. Gas-liquid chromatogram of methyl esters obtained from application of 2 μg of cottonseed oil to 4 mg of transmethylating powder. Sample applied in 1- μl *n*-hexane and methyl esters were eluted with CS_2 . Temperature, 170°C; range 10; attenuation \times 4. Peak identification with area percent: (1) methyl myristate, 0.8; (2) methyl palmitate, 23.5; (3) methyl palmitoleate, 0.16; (4) methyl stearate, 2.2; (5) methyl oleate, 16.1; (6) methyl linoleate, 57.8. Values obtained by the method of Luddy *et al.* (2) were: (1) 0.8; (2) 23.9; (3) 0.14; (4) 2.1; (5) 16.0; (6) 57.2. Values obtained by the method of Christopherson and Glass (1) were: (1) 0.8; (2) 24.7; (3) 0.12; (4) 2.0; (5) 16.1; (6) 56.3.

no transmethylation. This is interpreted to mean that a certain minimum amount of moisture must be introduced into the powder initially to generate enough KOH to catalyze the reaction.

Hyflo Super-Cel was superior to some other types of diatomaceous earths for mixing with the CH_3OK . These included Celite 545, which never gave complete transmethylation with any of the lipids studied; analytical grade Celite, which gave variable results and some saponification; and Filter-Cel, which usually gave complete transesterification in all of the solvents studied but gave extensive (up to 40%) saponification. Powdered CH_3OK alone was incapable of causing any transmethylation.

Sodium methylate, when substituted for CH_3OK , was inferior to the latter, giving lower yields of methyl esters, but this could be due to the physical form of the powder and not necessarily to lower reactivity.

SUMMARY

In a convenient, rapid procedure, a very small column of potassium methylate-Hyflo Super-Cel is used to convert microgram amounts of glycerides to methyl esters. Transesterification is complete in hydrocarbon but not in chlorinated solvents or in CS_2 . The methyl

esters can be recovered in 92–95% yield if desired. Regardless of the solvent used, the recovered methyl esters are representative of the original fatty acid composition of the glycerides.

ACKNOWLEDGMENT

Mr. Francis Luddy of this laboratory provided the glycerides and some transmethylated fats and oils prepared by the Luddy *et al.* procedure.

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