USE OF A CELITE–POTASSIUM *tert.*-BUTOXIDE COLUMN FOR RAPID SAPONIFICATION OF MICROGRAM AMOUNTS OF GLYCERIDES AT ROOM TEMPERATURE

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SUMMARY

In a simple, two-phase procedure, a capillary tube containing a bed of potassium *tert.*-butoxide supported on Celite 545 is used to saponify microgram amounts of glycerides applied to it in *n*-hexane. The reaction is carried out at room temperature and is complete within 5 min. The fatty acids are recovered from their soaps by acidification of the column with hydrogen chloride and extraction with hexane or carbon disulfide. They can be recovered in 91–97% of theory, if desired, and analyzed by gas-liquid chromatography. As little as 1 μg of peanut oil was successfully saponified and qualitatively analyzed for its constituent fatty acids.

INTRODUCTION

A previous paper[1] described a rapid method for quantitatively transmethylating microgram amounts of glycerides (fats, oil, synthetic triglycerides) at room temperature with a hydrocarbon solvent and a capillary containing a bed of potassium methylate supported on Hyflo Super-Cel. In attempts to prepare *n*-butyl esters with a sodium *n*-butoxide–Celite column in an otherwise identical procedure, practically no transesterification occurred, but instead the glycerides were nearly quantitatively saponified. Potassium *tert.*-butoxide behaved similarly. Advantage has been taken of these observations to develop a simple, rapid saponification method conducted at room temperature and applicable to microgram amounts of glycerides. Details are described, together with a simple technique for recovering the fatty acids from their soaps for analysis by gas–liquid chromatography (GLC).

Existing methods for saponification at the microgram level have been confined to carrying out the reaction on thin-layer chromatographic (TLC) plates[2] and paper chromatograms[3]. Both of these techniques require heating and neither allow for the separation of the liberated fatty acids. A cold saponification method has been reported[4] but it has been applied only at the milligram level.

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MATERIALS

Celite 545 was obtained through Fisher Scientific* (King of Prussia, Pa., U.S.A.). Potassium *tert.*-butoxide (t-BuOK) was from Aldrich (Milwaukee, Wisc., U.S.A.). Sodium *n*-butoxide was purchased from Research Organic Chemicals (Belleville, N.J., U.S.A.). Melting-point capillaries open at both ends (1.6–1.8 mm × 100 mm), and Critoseal (a vinyl plastic putty) were from A. H. Thomas (Philadelphia, Pa., U.S.A.); TLC plates coated with silica gel G (250 μm) were from Analtech (Newark, Del., U.S.A.). Synthetic homogeneous triglycerides were Nu Chek Prep's (Elysian, Minn., U.S.A.). Mixed synthetic triglycerides were a gift from Prof. R. G. Jensen, University of Connecticut, Storrs, Conn., U.S.A. Solvents were ACS grade or better and were used without purification.

EXPERIMENTAL

Purification of Celite 545

Celite 545 contained fatty acid salts in sufficient amounts to interfere in the analysis of the saponified glycerides. It was purified as follows: 70 g were transferred to a chromatography tube fitted into the pouring spout of a bottle of conc. hydrochloric acid. A wad of glass wool was placed on the surface of the bed and a vacuum was drawn on the column for 10 min. The acidified Celite was extracted continuously for 8 h with dichloromethane in a Soxhlet apparatus, then dried for 24 h at 100°.

Preparation of saponification powder

Celite 545 (1.0 g) and t-BuOK (0.3 g)** were rapidly weighed and ground together vigorously for 30 sec in an 8.2-cm mortar. The resultant powder was transferred to a dry 9-ml screw-cap vial and stoppered by pulling the sleeve portion of a rubber septum (sleeve 7 × 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 vial was stored at room temperature in a desiccator over P_2O_5.

Preparation of saponification column

The end of a melting-point capillary tube was sealed by pushing it into Critoseal. The tube was weighed***, the open end was inserted through the septum of the vial containing the saponification powder and the capillary was dabbed several times into the powder. Approx. 8–10 mg of powder was loaded into the capillary. If more was required, the powder was pushed into the capillary with a tamper made from a paper clip, and the above process was repeated. After the tube was properly charged, the powder was pushed into the capillary so that about 1.0–1.5 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

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* 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.

** Potassium *tert.*-butoxide was studied more extensively than sodium *n*-butoxide because it is more readily available from commercial sources.

*** In practice it may not be necessary to obtain a weight of the powder. Each millimeter of the compact bed is approx. equal to 1 mg.

was pressed into a compact column with another tamper. The capillary was then severed about 4 cm above the bed.

Saponification of sample

The glyceride was dissolved in hexane at concentrations up to 15 μg/~~ml~~ µl. A volume of the solution was applied to the column from a 10-μl syringe by placing the needle tip lightly on the bed and expelling the solution slowly onto the column. After application of the solution, some unwetted portion of the column bed should remain. Each microliter of solution will wet approx. 1 mg (1 mm) of the column material. After 5 min the column was eluted with 50 μl of hexane forced through the column by 3 p.s.i. nitrogen pressure. The effluent was spotted directly from the capillary onto the origin of a TLC plate in order to determine whether the saponification reaction was complete.

Isolation of fatty acids from soaps

The fatty acids retained on the column from the saponification step were liberated from their potassium soaps by gassing the capillary with hydrogen chloride (Fig. 1). The capillary was inserted through a septum fitted onto a vial containing conc. hydrochloric acid (fresh each day) and the vapors were drawn by vacuum through the capillary for 10 min*. The acids were then eluted in one of two ways.

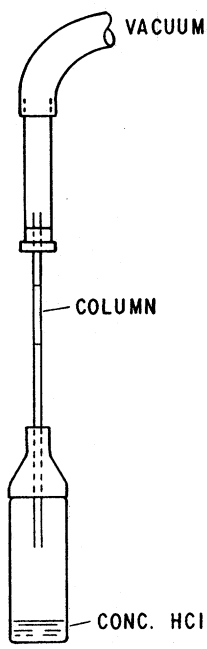

Fig. 1. Apparatus for acidifying microcolumn with hydrogen chloride.

* Alternatively, the capillary can be dabbed into a powder (made by grinding 1 ml of 0.1 *N* NaOH and 1–2 mg phenol red on 2 g Celite) so that about 1 mg is retained. This is pushed tightly against the bed. Complete acidification of the column is then indicated by a change from purple to pink.

Procedure A: If only qualitative information was needed, carbon disulfide was added to the capillary with a syringe and forced through the column by light nitrogen pressure. The first 8–9 μl (about 8–9 mm) of effluent emerging was taken up as thoroughly as possible with a 10-μl syringe and analyzed by gas–liquid chromatography (GLC) as described below. Procedure B: If a quantitative analysis was desired, the column was eluted with about 3 bed volumes of hexane, and the effluent was collected in a 2-ml vial. The solvent was evaporated under nitrogen at room temperature, and the residue was taken up in a definite volume of carbon disulfide for GLC analysis.

Gas–liquid chromatography

GLC analysis was conducted by use of a Hewlett-Packard 5750 A instrument. The column was 240 $\times$ 0.3 cm I.D. silanized stainless-steel packed with 90–100 mesh Anakrom ABS containing 7.5% stabilized ethylene glycol adipate plus 2% phosphoric acid. Helium was used as the carrier gas at 30 ml/min. Injection port and flame ionization detector temperatures were kept at 250° and the column temperature was maintained at 220°.

RESULTS AND DISCUSSION

The ability of the Celite–t-BuOK powder to saponify glycerides almost quantitatively was demonstrated by TLC analysis of the effluent from the saponification column and is depicted in Fig. 2 for redfish, herring, palm, peanut and corn oils, and for beef tallow and milkfat. In addition to these glycerides, cottonseed, beef, coconut, olive, sperm, tall, linseed, soybean, rapeseed, and dogfish oils, and cocoa butter, SSS (in cyclohexane), PPP, PPO, POP, POO, PSS, LLL, and EEE* were also saponified in near quantitative yields. Both crude and refined plant and marine oils behaved similarly. Most fats and oils analyzed showed no triglyceride spot by TLC analysis after a 30-sec reaction time, but traces of unsaponified glycerides were detected from some samples. However, when the reaction time was extended to 5 min no unsaponified glycerides could be detected on the TLC plates from any of the oils saponified by this technique.

All glycerides also underwent partial transesterification to yield traces ($<2\%$) of *tert.*-butyl esters of the fatty acids. These esters are indicated by the arrow on the TLC plate shown in Fig. 2. The extent of *tert.*-butyl ester formation was established by saponifying synthetic triglycerides and analyzing the effluent from the saponification column by GLC by use of standard curves prepared from synthetic *tert.*-butyl esters of fatty acids.

A level of 15 μg of glyceride per mg of column packing was the maximum practical ratio for columns made in the size capillaries specified. Larger ratios were impractical because of the limitation on flow-rate imposed by the larger amount of long-chain fatty acid soaps formed. The smallest amount of glyceride applied to a saponification column was 1 μg. The GLC pattern of the fatty acids obtained from saponification of 1 μg of peanut oil applied to a 4-mg column in 3 μl of hexane is

* Abbreviations: P = palmitic; S = stearic; O = oleic; L = linoleic; E = elaidic.

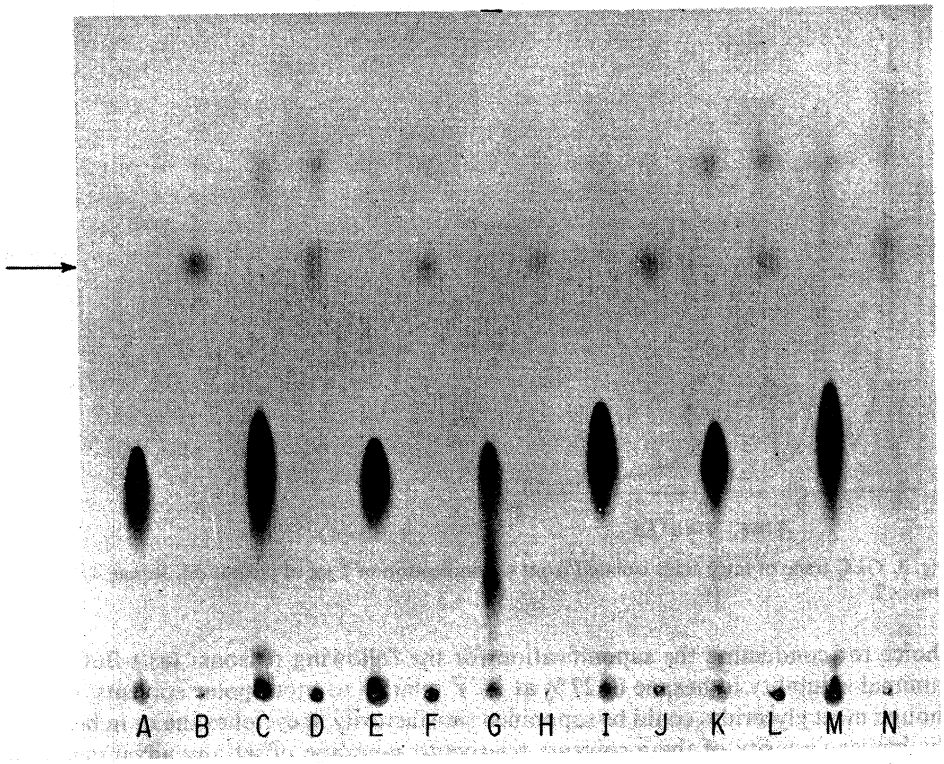

Fig. 2. TLC plate showing efficiency of a t–BuOK-Celite column for saponifying glycerides: (A) 115 μg beef tallow; (B) beef tallow effluent; (C) 140 μg crude redfish oil; (D) crude redfish oil effluent; (E) 107 μg palm oil; (F) palm oil effluent; (G) 98 μg milkfat; (H) milkfat effluent; (I) 150 μg crude peanut oil; (J) crude peanut oil effluent; (K) 87 μg crude corn oil; (L) crude corn oil effluent; (M) 142 μg crude herring oil; (N) crude herring oil effluent. Solvent: benzene; detection: 8.5% H_3PO_4 + 3% $Cu(OAc)_2$, followed by heating at 200°; arrow denotes *tert.*-butyl ester spots.

shown in Fig. 3. The fatty acids were obtained by use of elution Procedure A the effluent being injected directly into the instrument.

The quantitative aspects of the recovery of fatty acids from their soaps formed on the column were studied. For these determinations synthetic homogeneous and mixed triglycerides containing palmitic, stearic, oleic, and elaidic acids were employed. Approximately 100–150 μg of the glycerides were applied to 10–12 mg columns. After acidification with hydrogen chloride the columns were eluted by Procedure B, and the fatty acids were quantitated from standard curves. Recoveries ranged from 91 to 97%.

The Celite–t-BuOK powder retained its ability to thoroughly saponify triglycerides over at least a 7-week period. During this interval about 80 punctures of the septum were made by capillary tubes. When other Celites (Hyflo Super-Cel, Analytical Grade Celite, and Filter Cel) were substituted for Celite 545 in mixtures with t-BuOK, just as effective saponification occurred, but these supports were less satisfactory in that flow-rates were reduced relative to Celite 545.

Among the solvents investigated in these studies, *n*-hexane was the solvent of

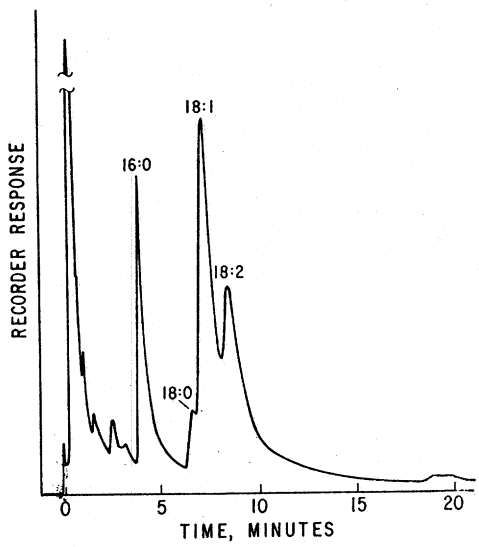

Fig. 3. GLC trace of fatty acids isolated from saponification of 1 μg of peanut oil. Range 4, attenuation $\times 2$.

choice for conducting the saponification for the following reasons: (a) t-BuOK has minimal solubility in hexane (0.27% at 25°)[5] relative to more polar solvents. (b) Although most glycerides could be saponified satisfactorily in cyclohexane or in benzene, the higher viscosity of these solvents relative to *n*-hexane offset any advantage they may have had as superior lipid solvents. Tristearin, however, was poorly soluble in *n*-hexane but was sufficiently soluble in cyclohexane and was saponified in the latter. (c) Dichloromethane, carbon tetrachloride and carbon disulfide caused the column to become yellow, presumably because of reaction with the base.

Finally, studies were carried out in which sodium *n*-butoxide was compared to t-BuOK in its ability to saponify glycerides. Although the sodium *n*-butoxide–Celite powder saponified glycerides as readily as did the t-BuOK–Celite powder, it had a lower capacity for glycerides. *n*-Butyl esters were also formed and to about the same extent ($<2\%$) as the *tert.*-butyl esters. It is interesting that a solution of sodium *n*-butoxide in *n*-butanol readily transesterifies milkfat[6], whereas in this study sodium *n*-butoxide on Celite 545 results in almost complete saponification.

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