notes on methodology

Rapid quantitative procedure for removing cholesterol from butter fat

D. P. Schwartz, C. R. Brewington, and L. H. Burgwald

Dairy Products Laboratory, Eastern Utilization Research and Development Division, Agricultural Research Service, U.S. Department of Agriculture, Washington, D.C.

SUMMARY Cholesterol is rapidly and quantitatively removed from butter fat by passage of the fat in hexane or benzene solution over a column of Celite impregnated with an aqueous solution of digitonin.

KEY WORDS cholesterol · removal · small amounts · column · impregnated · digitonin

The quantitative analysis of butter fat for hydroxylated constituents other than cholesterol is complicated by the presence of relatively high concentrations of this sterol (nearly 50 mole % of the hydroxyl-containing components of butter fat). A method for the quantitative removal of cholesterol from butter fat under very mild conditions has therefore been developed. The sterol is removed without contamination of the fat with another alcohol, which might occur if an alcoholic solvent were employed in the procedure.

The most obvious approach to the problem was the removal of cholesterol as the digitonide. However, the quantitative precipitation of cholesterol from butter fat with digitonin in solution is hindered because of a lack of a suitable mutual solvent for the fat and digitonin, especially when alcohols are to be avoided.

We observed that an aqueous solution of digitonin adsorbed onto an inert support rapidly and quantitatively removes cholesterol from benzene or hexane solution. Subsequent trials with hexane or benzene solutions of butter fat showed that a procedure based on this observation removes cholesterol from the fat.

Procedure. Digitonin is first extracted continuously in a Soxhlet apparatus for 8 hr with redistilled benzene (Fisher Scientific Company, Silver Spring, Md., crystallizable grade),[1] then air-dried overnight. The extraction procedure was arbitrarily adopted to diminish the possibility of contamination of the fat by benzene-soluble impurities in the digitonin. A stock solution of the glycoside is then made by dissolving (with heat) 60 mg (49 μmoles) of it per ml of distilled water. The digitonin remains in solution for approximately 1 hr after cooling before visible precipitation occurs. If the solution is not used before precipitation commences, the suspension should be rewarmed to effect solution. A slight turbidity always prevails in the solution, but this has no effect on the results.

One milliliter of the solution is pipetted into a 10 cm mortar and ground onto 2 g of Celite 545 (Johns-Manville, Baltimore, Md.). The grinding should be thorough, so that the digitonin is distributed homogeneously onto the Celite. After grinding has been continued for a few minutes, the mortar should be scraped with a spoon so that all of the Celite is removed from the bottom and sides of the mortar and piled into the center. The Celite is then reground, removed from the mortar, and transferred to a chromatography column (1.8 × 13 cm) that contains about 5 ml of redistilled hexane (High Purity Grade, Phillips Chemicals Co., Bartlesville, Okla.). The Celite is packed down with a tamping rod under moderate pressure. The excess hexane is drained off and 10 ml of a 30% (w/v) solution of butter fat in benzene (or hexane) is permitted to flow through the column at a rate not exceeding 0.75 ml/min. Nitrogen pressure may be used to attain the desired flow rate. When the last of the solution has just entered the column, the pressure (if used) is interrupted and the sides of the tube are washed down with three 1-ml portions of the solvent. Ten milliliters of solvent is used to elute the residual fat from the column, under pressure if necessary.

Tests. The efficiency of removal of cholesterol from butter fat by the digitonin column was checked by allowing the effluent from the column to react with pyruvyl chloride 2,6-dinitrophenylhydrazone, a colored acid chloride which quantitatively acylates all hydroxylated constituents of the fat (Schwartz, D. P., and C. R. Brewington, unpublished data). The mixture was applied to a column of magnesia, and the unhydroxylated components were eluted with $CHCl_3$. The column was then eluted with a gradient of methanol in chloroform, which separated the derivatives (all of which remained on the column after the washing with $CHCl_3$) into hydroxyglycerides, cholesterol, mono- and diglycerides, etc. Each class obtained was subjected to thin-layer chromatography (TLC) on Aluminum Oxide G, with methyl cyclohexane–diethylamine 7:3 as solvent. No band was observed in the position normally occupied by the cholesterol derivative in the class separation on the column, and no spot for the cholesterol derivative was observed when the bands from that column were subjected to TLC (Fig. 1). Details of these methods will be published later.

As a further check on the efficiency of the digitonin column, cholesterol (purified via the dibromide) was added to tricaprylin in amounts simulating those found

Abbreviation: TLC, thin-layer chromatography.

[1] Reference to certain products or companies does not imply an endorsement by the Department over others not mentioned.

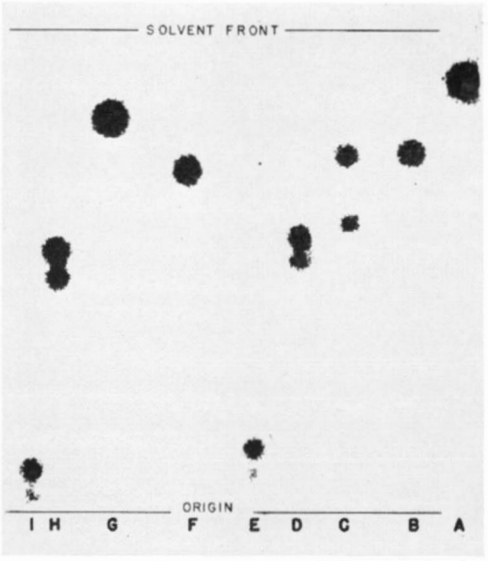

FIG. 1. Thin-layer chromatogram of bands obtained from column chromatography of the 2,6-dinitrophenylhydrazone derivatives of pyruvate esters of hydroxylated constituents of butter fat. A, B, C, D, and E represent successive fractions eluted from fat not treated with digitonin. Spot F is authentic cholesterol pyruvate 2,6-dinitrophenylhydrazone. Spots G, H, and I are successive fractions obtained from fat applied to the digitonin column.

in butter fat (i.e., 0.25–0.35%) and a volume of the solution taken so that 24 μmoles of cholesterol would pass over the column. The fat obtained from the effluent was saponified by the method of Schwartz, Burgwald, and Brewington (1). Cholesterol was determined in the entire nonsaponifiable matter by the method of Abell, Levy, Brodie, and Kendall (2). No cholesterol was detectable in it.

Complete removal of cholesterol by the digitonin column from hexane or benzene solutions of cholesterol, or from butter fat or tricaprylin–benzene solutions of cholesterol was found to occur at a molar ratio of digitonin to cholesterol of 2:1, but not at a ratio of 1.5:1 (at the recommended flow rate). It is possible that the ratio might be narrowed if slower flow rates were used. The usual ratio of digitonin to cholesterol utilized in quantitative precipitation studies is 10:1 (3).

Attempts to assay cholesterol, in butter fat that had been freed from cholesterol ester by silicic acid chromatography, by the Liebermann-Burchard method after passage of the fat over the digitonin column and saponification were unsuccessful because of interference from other nonsaponifiable constituents.

Manuscript received 13 May 1966; accepted 9 September 1966.

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