Use of Cyclic Anhydrides to Remove Cholesterol and Other Hydroxy Compounds from Fats and Oils

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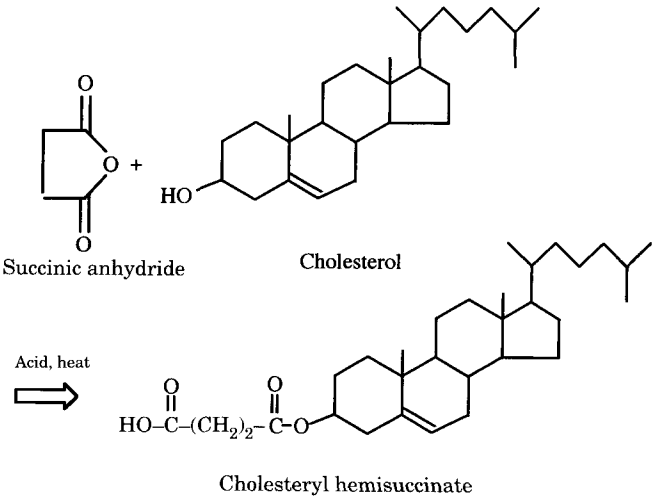
A method for removing cholesterol from animal fats has been developed based on the reaction between the hydroxyl group of cholesterol and cyclic anhydrides. The reaction forms monoesters with acyl chains having a terminal free acid group. The conversion of cholesterol into an acid derivative makes it possible to remove the cholesterol from fats by extraction with aqueous alkali. A study of the reaction in model systems showed that optimal conditions were a molar ratio of cholesterol to succinic anhydride of 1:3 at 135°C for several hours. Acid catalysts increased the rate of the reaction, and acetic acid was selected not only because of its catalytic power but also because its reflux prevented the distillation of the cyclic anhydride from the reaction mixture. If all the cyclic anhydride was added at the beginning of the reaction, animal fats such as lard, tallow and milk fats were reduced in their cholesterol content by about 40%. A study of the reaction mechanism showed that the cholesterol reduction could be increased to 60–70% by altering the amount and the addition sequence of cyclic anhydride. The effectiveness of acid catalysts was related inversely to the negative logarithm of their acid dissociation constant (pK), but as their effectiveness increased, so did their tendency to form other, unwanted esters of cholesterol. A mixture of monoethyl fumarate and acetic acid sped the reaction, compared with acetic acid alone, with minimal formation of cholesteryl acetate. Studies with ^{14}C-cholesterol showed little formation of products other than cholesteryl hemisuccinate during the reaction. The procedure removed some of the tocopherol from the fat but had no other detectable effect on fat stability. The procedure also can be used to concentrate the lactone precursors from milk fat.

KEY WORDS: Animal fats, cholesterol removal, cyclic anhydrides, hydroxyfatty acids, tocopherol.

Coronary heart disease (CHD) is one of the most significant diet-related health problems in the United States, accounting for nearly 40% of deaths annually (1). CHD is multifaceted, and the course of its pathology is influenced by both dietary and nondietary risk factors: genes, age, exercise, obesity, diabetes, stress, cigarette smoking, hypertension and hyperlipidemia. Among these, hyperlipidemia has been identified as a major risk factor for atherosclerosis and is associated with elevated circulatory levels of cholesterol, cholesteryl esters and triglycerides. Diet generally is accepted as the most important controllable factor in the development of hyperlipidemia, and reduction in dietary lipids containing saturated fatty acids and cholesterol is recommended for the prevention, arrest and reversal of atherosclerosis. As a result, several processes have been developed to reduce the cholesterol content of food products. These processes include solvent (2,3) and supercritical extraction (4–7), treatments with cyclodextrin (8) or activated carbon (9), steam stripping (10) and reduction of cholesterol by enzymes (11,12).



SCHEME 1

We have developed an effective and economic method to remove cholesterol from animal fats, based on the reaction between cyclic anhydrides and the hydroxyl group on cholesterol. The principle of the reaction is illustrated in Scheme 1. Cholesteryl hemisuccinate, the final product of the reaction, can be extracted easily from fats with aqueous alkali, a step commonly used in industry to remove free fatty acids.

MATERIALS AND METHODS

Lard, suet and unsalted butter were obtained from local groceries. For storage tests, lard was produced by applying a wet-rendering procedure to fatty tissues obtained from the Meat Laboratory, Iowa State University (Ames, IA). Tallow was wet-rendered from beef suet, and unsalted butter was melted and centrifuged to produce milk fat. All fats were stored at 4.4°C.

Cholesterol, 5-cholestane, 1-monooleyl-*rac*-glycerol and succinic anhydride were purchased from Sigma Chemical (St. Louis, MO). The 4-^{14}C cholesterol (50 μCi) in toluene solution was purchased from Amersham International (Amersham, United Kingdom). Methyl-*tert*-butyl ether, monoethyl fumarate and chloroacetic acid were purchased from Aldrich Chemical Company (Milwaukee, WI). Acetic acid, acetonitrile, chloroform, hexane, methanol and 2-propanol were high-performance liquid chromatography (HPLC)-grade and were purchased from Fisher Scientific (Fair Lawn, NJ). Alumina absorption (80–200 mesh) and other common chemicals were also purchased from Fisher Scientific. Cholesteryl hemisuccinate was synthesized according to Klein *et al.* (13).

Cholesterol (1.29 mMol) and 1 to 4 mMol succinic anhydride in 33 mL of solvent (dodecane, pyridine or xylene)

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were heated at 30–140°C in the model systems. Acid catalysts tested at 0.833 mol/L were acetic, propionic, succinic, fumaric, choloracetic, cholesteryl hemisuccinate, monomethyl succinate and monoethyl fumarate.

To test extraction of the cholesteryl hemisuccinate with aqueous alkali, 5 to 15 mL of various alkalis were mixed with 5 g of the reaction product of the xylene model system, and the mixtures were centrifuged to separate the oil and aqueous layers. The following aqueous alkalis were tested: 11% NaOH, 20% and 5% Na_2CO_3, and 5% $NaHCO_3$, $KHPO_4$ and K_2HPO_4.

In a typical reaction with animal fats, 50 g of fat and 2.5 g of acetic acid with various amounts of succinic anhydride or glutaric anhydride were used at 135°C. The succinic anhydride was added not only at the beginning of the reaction, but also in the following three reaction sequences:

Schedule A: 0.16 g of succinic anhydride was divided into five equal portions (0.032 g each); one of them was added at the beginning of the reaction and the others on every hour for 4 h.

Schedule B 0.16 g of succinic anhydride was split into six portions (0.0753, 0.0376, 0.0188, 0.0094, 0.0094 and 0.0094 g) according to the relative ratios of 1, 1:2, 1:4, 1:8, 1:8 and 1:8. They were added in sequence to the reactor on every hour for 6 h.

Schedule C: 0.16 g of succinic anhydride was split into six portions (0.0359, 0.0314, 0.0299, 0.0269, 0.0179 and 0.0179 g) according to the relative ratios of 1, 7:8, 5:6, 3:4, 1:2 and 1:2. Each portion was further divided into three equal fractions. They were added to the reactor every 20 min in sequence.

Lard triglycerides were purified by passage through an alumina column according to Jensen (14).

Hydroxyl values were determined by the AOCS Official Method Cd 13-60 (15). Succinic anhydride was determined according to Goddu *et al.* (16). For tocopherol analysis, saponification was according to Manz and Philpp (17) and separation by HPLC according to Barnett *et al.* (18). For stability tests, fats were stored in 50-mL beakers in a 60°C oven. Peroxide values (PVs) were determined by the Stamn method (19).

Cholesterol was concentrated from fats for analysis by saponification and extraction of the unsaponifiables and by the solid-phase extraction procedure derived from that of Hamilton and Comal (20). The latter procedure was used to concentrate cholesteryl hemisuccinate as well. Cholesterol was quantitated by gas chromatography (GC) and HPLC. GC was accomplished with a Varian Model 3700 instrument (Palo Alto, CA) and an SPB-1 capillary column (30 m × 0.28 mm). HPLC was carried out with a Shimadzu instrument (Tokyo, Japan) by using an evaporative light-scattering detector ELSD-2 (Varex Corporation, Burtonsville, MD) and a 25-cm LC-18 column (Supelco, Inc., Bellefonte, PA). The mobile phases consisted of 1.5:100 (vol/vol) acetic acid in acetonitrile (solvent A) and chloroform (solvent B); flow rate was 1 mL/min with a linear gradient from 86% A to 75% A in 20 min, 75% to 0% A in 20 min, and 100% B for another 20 min. For the experiment with ^{14}C-cholesterol, 30 μCi was added to a reaction with 25 g of lard. After 8 h, the lard was examined for radioactive products other than cholesterol and cholesteryl hemisuccinate with a Beckman (Fullerton, CA) HPLC and a radioisotope detector.

Milk fat (50 g) was reacted for 8 h with 2.5 g acetic acid and 0.16 g succinic anhydride at 135°C. Succinylated products were extracted with 5% aqueous sodium carbonate, centrifuged and collected from the emulsion layer. The emulsion layer was saponified, acidified with 6 N HCl and extracted by ethyl ether. The sample was injected into a Hewlett-Packard (Avondale, PA) GC 5890A mass spectrometer 5970 (GC/MS). A control sample was treated in the same way except no succinic anhydride was added.

RESULTS AND DISCUSSION

Optimization of the reaction conditions. The optimum reaction conditions in model systems were xylene solution refluxing at 130–140°C with a cholesterol/succinic anhydride ratio of 1:3. The reaction rate dropped quickly at temperatures below 120°C. Yields continued to increase for reaction times up to 8 h. When these conditions were applied to an animal fat in the absence of a refluxing solvent, considerable amounts of succinic anhydride distilled from the reaction mixture and crystallized on the cooler parts of the apparatus. This problem could be overcome by running the reaction in a sealed tube held at an appropriate temperature or by incorporating 5% by weight of acetic acid in the reaction mixture to provide reflux. The latter approach was the most convenient. Acetic acid was selected because it was not toxic and could be removed from the fat, along with the cholesteryl hemisuccinate, by washing with aqueous alkali. Acetic acid also catalyzed the reaction.

Of the aqueous bases used to extract the cholesteryl hemisuccinate, 5% aqueous sodium carbonate was the best and was capable of giving a complete extraction of the hemisuccinate. Sodium hydroxide caused some hydrolysis of the cholesteryl hemisuccinate. A volume of 5% aqueous sodium carbonate equal to that of the solvent or fat was used to extract the cholesteryl hemisuccinate from the reaction mixture.

Application of these reaction conditions to animal fats (lard, tallow, milk fat) resulted in a 35–45% reduction in their cholesterol content. Figure 1 illustrates the time course of a typical reaction in lard and indicates several changes in rate during the reaction. The results indicate that the decrease of cholesterol was fast at the beginning and then slowed down after 3 to 4 h; extending the reaction time beyond 8 h did not lead to more reduction in the cholesterol content. Similar results were obtained when glutaric anhydride was used instead of succinic anhydride.

To understand the kinetics of the reaction, the disappearance of succinic anhydride was determined when succinic acid was heated with lard (50 g) and acetic acid (2.5 g) for 8 h. Typical results are shown in Figure 2. Clearly, two different reaction kinetics contributed to the disappearane of succinic anhydride in the lard reaction. For the first 4 h, the concentration of succinic anhydride dropped rapidly, but it disappeared at a much slower rate afterward. The results indicate that most of the succinic anhydride was consumed in the early period of the reaction. It seems possible that the succinic anhydride could react not only with cholesterol but also with other compounds in the lard that have a free hydroxyl group; this can partly explain the retarded rate of cholesterol disappearance indicated in Figure 1.

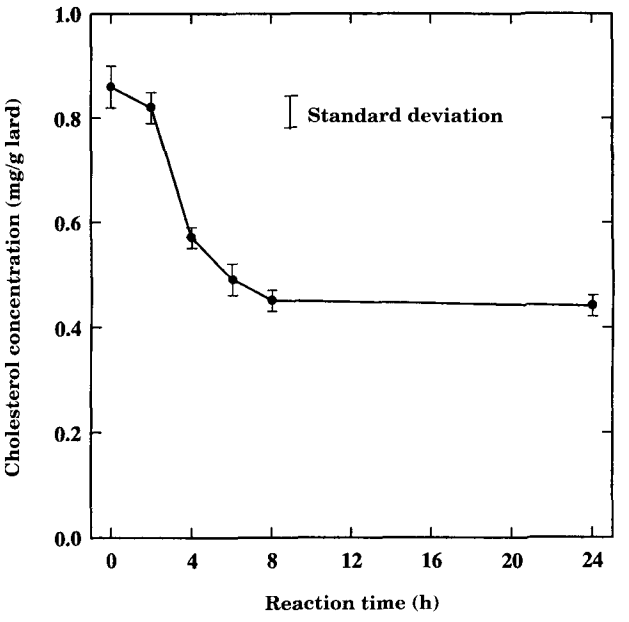

FIG. 1. The disappearance of cholesterol from lard when reacted with succinic anhydride.

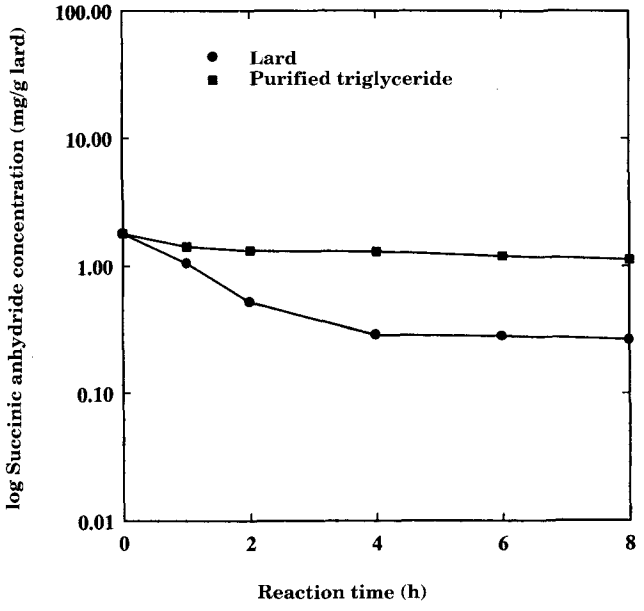

FIG. 2. The disappearance of succinic anhydride when heated with lard and purified lard triglycerides with an acetic acid catalyst.

The disappearance of succinic anhydride also was examined when purified lard triglycerides were used, and Figure 2 compares the results with purified and normal lard. The reduction of succinic anhydride in purified lard was much slower than that in normal lard during the first 4 h, but the rates of the two samples were similar during the later part of the reaction. The disappearance of succinic anhydride in even purified lard triglycerides indicated that a transacylation reaction between succinic anhydride and acetic acid may have resulted in the formation of acetic anhydride and succinic acid (21). Such formation of acetic anhydride would interfere with the determination of succinic anhydride from a standard curve as described by Goddu *et al.* (16).

To overcome the effect of various reactive compounds, other than cholesterol in the lard, the amount of succinic anhydride was increased to three times the molar amount of hydroxy compounds as determined by the hydroxy value. The hydroxy value was 0.59 meq KOH/50 g of fat, so 0.16 g of succinic anhydride was used per 50 g lard. The use of the greater amount of succinic anhydride slightly increased the amount of cholesterol removed to about 50%. But if the succinic anhydride was added to the reaction mixture in small amounts throughout the course of the reaction, so that the amount of succinic acid remained approximately constant, cholesterol removal was increased to 60–70%. Three different addition schedules were tried with similar results (Fig. 3). Schedule A gave the best result, and the cholesterol concentration was reduced by 70%.

Attempts to speed the reaction by increasing concentrations of acetic acid led to the formation of significant amounts of cholesteryl acetate. Fumaric and succinic acids and monomethyl succinate were too insoluble in lard to be effective catalysts. Propionic acid and cholesteryl hemisuccinate were equivalent to acetic acid in catalytic activity. The effect of the pK of acid catalysts on the reaction rate was studied by comparing equimolar concentrations of monoethyl fumarate, chloroacetic acid and acetic acid by using a model system in xylene. The disappearance of cholesterol with the three catalysts is shown in Figure 4. This indicated that the greater the acidity of the catalyst, the faster the reaction. There was little side reaction

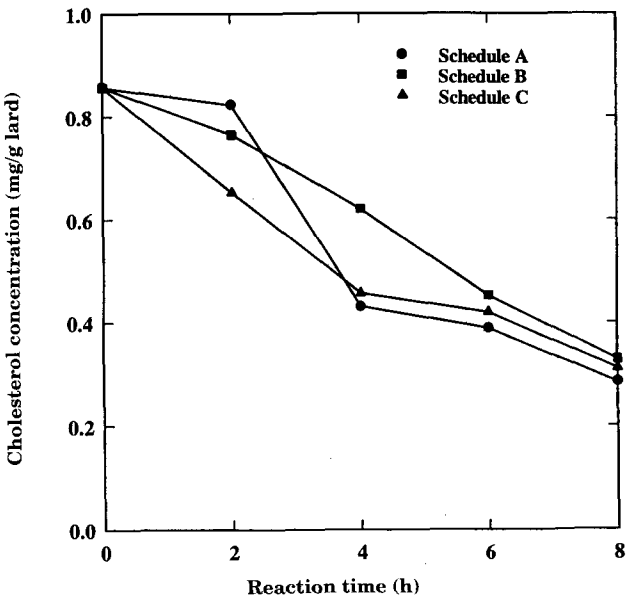

FIG. 3. The disappearance of cholesterol from lard when succinic anhydride was added throughout the reaction period according to three addition schedules.

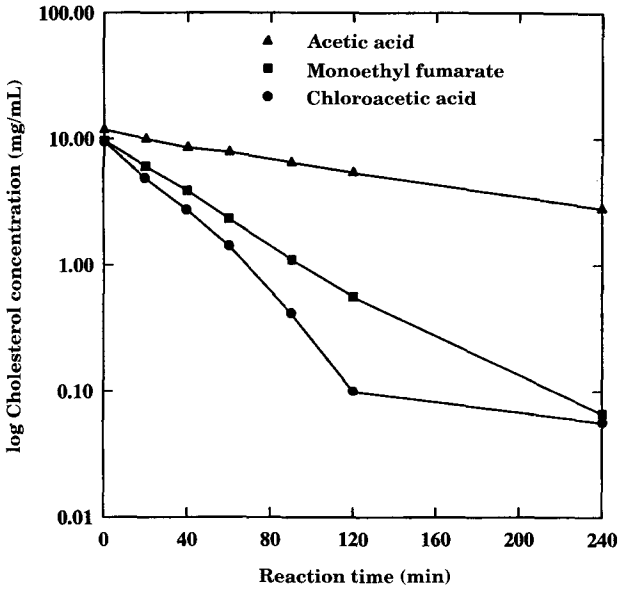

FIG. 4. A comparison of acetic acid, monoethyl fumarate, and chloroacetic acid as catalysts for the reaction of cholesterol with succinic anhydride.

with the concentrations of monoethyl fumarate needed to catalyze the reaction; however, monoethyl fumarate could not be used alone in fat systems without distillation of succinic anhydride from the reaction. When a mixture of monoethyl fumarate and acetic acid was used, over 65% reduction of cholesterol was achieved in only 4–5 h.

A search for possible side products. Comparison of the cholesterol removed with the amounts of cholesteryl hemisuccinate formed when the reaction was applied to lard indicated that as much as 10–20% of the cholesterol might be disappearing into side reactions. The formation of a cholesterol-containing product not extractable by sodium carbonate solution would account for our inability to remove more than about 70% of the cholesterol in animal fats. But the evidence for the occurrence of such a compound depends on the cumulative accuracy of a complicated analytical scheme. Because of the small amounts of cholesterol present in lard, the reliability of this analysis was questionable. Radioactive tracers provided a more direct and sensitive test of the existence of such compound(s). 4-^{14}C-Cholesterol was added to the lard, and the usual reaction was carried out. The product was analyzed by an HPLC equipped with a radioisotope detector. The results showed that there were only minor unknown compounds (<3%) that contained ^{14}C other than cholesterol and cholesteryl hemisuccinate. Thus, the evidence obtained by mass balance that suggested that as much as 20% of an unknown cholesterol-containing product was formed seems to be in error, and cholesteryl hemisuccinate is the only product of consequence. Probably the reason that cholesterol cannot be reduced to lower concentrations is that as the cholesterol concentration in the fat becomes low, competing reactions use up the succinic anhydride.

The effect of the process on fat stability. The reaction between cyclic anhydride and the hydroxyl group might have additional uses aside from its application in removing cholesterol from fats. α-Tocopherol, the major neutral antioxidant present in animal fats, also has a free hydroxyl group and may be subject to removal by the proposed procedure. If so, the α-tocopherol removed should be added back to processed fats to maintain the proper oxidative stability.

Lard for this experiment was prepared by wet-rendering adipose tissue to obtain a product without added synthetic antioxidants. Both fresh lard and the product after 8-h reaction were saponified and analyzed for α-tocopherol. The results showed that the 8.0 $\pm$ 0.1 mg/kg of α-tocopherol in fresh lard was reduced to 3.7 $\pm$ 0.1 mg/kg lard by the reaction. Thus, more than 50% of the tocopherol was removed by the procedure.

To determine the effect of the procedure to oil oxidative stability, PVs were determined during storage, and the results are plotted in Figure 5. In the first 8 d of storage, there were no significant differences in PVs of the treated and control lard, but, after 8 d, PVs of lard reacted with succinic acid were significantly higher than those of the control. If α-tocopherol was added back to the reacted lard to make up for the amount removed by the succinic anhydride treatment, the oxidative stability of the reacted lard was restored, so that its PVs were not significantly greater than those of the untreated control.

Use of succinic anhydride to recover hydroxy compounds from milk fat. The possibility of using the reaction with cyclic anhydrides to concentrate hydroxy compounds other than cholesterol and tocopherol from milk fats was studied. One of the unique features of milk fat is the presence of γ- and δ-hydroxy fatty acids in its triglycerides. γ- and δ-Lactones, which contribute fruity and coconut notes to the flavors of dairy products, are formed when these hydroxy fatty acids are released (22).

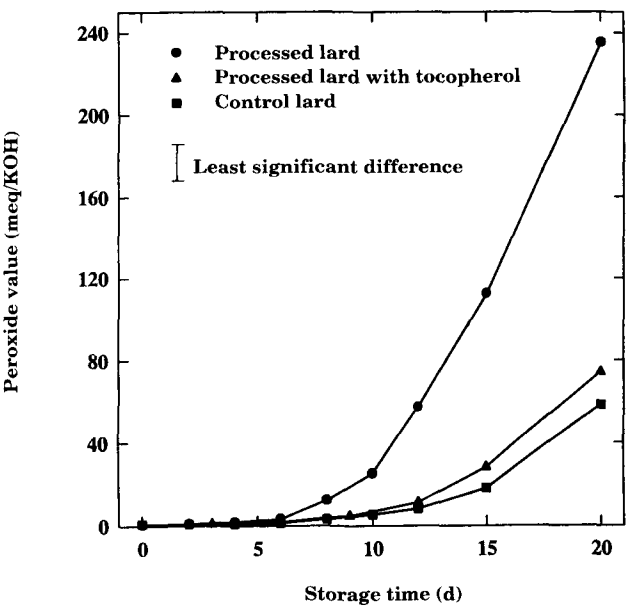

FIG. 5. The stability of lard before and after treatment with succinic anhydride to remove hydroxy compounds compared with the stability of treated lard whose level of α-tocopherol had been restored to its original concentration.

If these hydroxy fatty acids are removed from milk fat along with cholesterol by the succinic anhydride treatment, they may need to be added back to processed milk fat to maintain the proper flavor. Alternatively, this reaction might provide a way to prepare a concentrate of these flavors from milk fat.

When reacted fats are extracted with aqueous sodium carbonate and centrifuged, the soaps of the succinylated products accumulate in an emulsion layer between clear oil and water layers. The total ion chromatograms obtained by GC/MS were used to estimate the quantity of lactones from reacted and control samples (Table 1). An average three- to fourfold greater concentration of lactones was found in the reacted milk fat emulsion layer compared with the control on a weight basis, and the emulsion layer in the succinic anhydride-treated sample weighed about four times more than the control emulsion layer. Thus, about 8.8 mg of lactones can be concentrated from one gram of reacted milk fat, about 16-fold more than was isolated from the control. Thus, there may be a significant loss of certain flavor compounds in milk fat during the reaction with succinic anhydride, but this reaction provides a way to concentrate lactone flavor precursors from milk fat.

Economic analysis. This process could be carried out with simple equipment by using a batch reactor with a carbon dioxide atmosphere to prevent oxidation. The process uses 0.050 lb of acetic acid and 0.0032 lb of succinic anhydride to react with 1 lb of fat. The costs of acetic acid and succinic anhydride are $0.33/lb and $1.71/lb (23), respectively. Thus, the cost of reagents for the proposed reaction is $0.022/lb of fat. The cost can be reduced further by recovering reagents and reaction product. The cholesteryl hemisuccinate extracted can be hydrolyzed to cholesterol and succinic acid with sodium hydroxide. Although the cholesterol is likely to be contaminated with small amounts of fatty acids, it should be possible to recover the cholesterol removed from animal fats for use in feeds, pharmaceuticals and cosmetics. Current prices for cholesterol suggest that the approximately 0.3 g recovered from 1 lb of lard may equal or exceed the cost of the process reagents and equipment, so that the process may cost nothing or produce a small net profit. The acetic acid and the succinic acid can be recovered from the hydrolyzate, and the latter can be converted to succinic anhydride and recycled. α-Tocopherol recovered from the hydrolyzate can be added back to the fat to restore its stability.

TABLE 1

Lactone Concentrations in Reacted and Control Milk Fats

Sample	Lactones in emulsion (mg/g)	Emulsion layer formed (g/10 g fat)	Total lactones (mg/g fat)
Reacted milk fat	43	2.01	8.83
Control milk fat	10	0.56	0.56

ACKNOWLEDGMENTS

Since submission of this paper, we have become aware that P.W. Wrezel, R.G. Krishnmurthy and G.L. Hasenhuettl were issued a patent "Method for Removing Cholesterol from Edible Oils," U.S. Patent 5,128,162, dated July 7, 1992 and assigned to Kraft General Foods. This patent discloses technology very similar to that of this paper for which we also have been issued a patent (E.G. Hammond, and Y. Chen, "Process for Reducing Cholesterol in Animal Fats", U.S. Patent 5,264,599, dated November 23, 1993).

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