Optimization of Cholesterol Removal Conditions from Homogenized Milk by Treatment with Saponin

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ABSTRACT : This study was carried out to determine the optimum conditions for cholesterol removal from homogenized milk by treatment with saponin using a response surface methodology (RSM). The effects of temperature, reaction time, and amounts of celite or saponin added on cholesterol removal from milk were investigated. The level of cholesterol removal from milk increased with saponin concentration and varied from 57.4 to 73.3%. The optimum reaction time, amount of celite addition determined by a partial differentiation of the model equation, and amount of saponin addition were 30min, 0.95% and 1.5%, respectively. Under these conditions, the predicted cholesterol removal by RSM was estimated to be 73.4%. The experimental removal value was 73.7%. Thus, there was no appreciable difference between the experimental value and the predicted value based on RSM. (*Asian-Aust. J. Anim. Sci. 2001. Vol. 14, No. 6 : 844-849*)

Key Words : Cholesterol Removal, Saponin, Homogenized Milk, Response Surface Methodology

INTRODUCTION

A strong positive correlation exists between increased serum cholesterol concentration and risk of coronary heart disease. Animal and human experiments show that plasma cholesterol is raised by an increased intake of cholesterol and saturated fat (Sieber, 1993). Therefore, consumers are increasingly concerned about the cholesterol level in their diets and there have been dramatic increases in no-, low- and reduced-cholesterol products in the market place (Ahn and Kwak, 1999).

The reduction of cholesterol levels in milk fat can be accomplished by many different means, including supercritical fluid extraction (Bradley, 1989; Mohamed et al., 2000), solvent extraction (Borges et al., 1996), steam distillation (Arul et al., 1988; Sundfeld et al., 1993a), melt crystallization (Sperber, 1989; Sundfeld et al., 1993a), formation of complexes and/or adsorption with digitonin (Micich, 1990), treatment with cyclodextrins (Ahn and Kwak, 1999; Lee et al., 1999; Makoto et al., 1992; Yen and Tsui, 1995), and enzymatic conversion (Morris, 1990; Sperber, 1989). However, removal of complexed cholesterol by saponin has the advantage of being implemented with equipment already available in the dairy industry at lower operating costs compared to the other methods (Sundfeld et al., 1993a).

Several studies show that saponins effectively remove cholesterol from dairy products. Sundfeld et al. (1993a, b) removed cholesterol from butteroil by treatment with saponins from *Quillaja saponaria*. Digitonin is the best known saponin for its ability to interact with cholesterol, forming compounds (digitonides) insoluble in aqueous systems. Digitonin-impregnated diatomaceous earth has been successfully used in the selective separation of cholesterol from butterfat (Schwartz et al., 1967). Micich (1990) also studied removal cholesterol from butteroil using polymer-supported digitonin. However, very little information is available on the use of saponin to remove cholesterol from milk.

We determined the optimal conditions for removing cholesterol from homogenized milk in order to prepare low-cholesterol milk, using saponins from the bark of *Quillaja saponaria* with response surface methodology (RSM).

MATERIALS AND METHODS

Materials

Commercial milk (3.6% milk fat) was purchased from a retail store as needed. Saponin, obtained from the bark of *Quillaja saponaria,* was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). This saponin is permitted for food use and generally recognized as safe (GRAS) in USA and is used commercially as a foaming agent in beverages and confectioneries, bakery products, and dairy desserts. Diatomaceous earth (Celite 545) was purchased from Shinyo Pure Chem. Co. (Osaka, Japan) and was used as an absorbent for the cholesterol-saponin complex. Cholesterol was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). All solvents were HPLC grade.

Experimental design

RSM was used to investigate conditions that might affect cholesterol removal from milk by saponin treatment. A central composite fractional factorial

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design with 4 factors and 3 levels was used on the program Stat-graphics (STSC Inc., Rockville, MD, USA). As shown in table 1, the four independent variables were X_1, X_2, X_3, and X_4 and the coded values chosen for the independent variables were -1 (the lowest level), 0, and +1 (the highest level). These ranges were chosen either from literature data or after performing preliminary experiments. The complete design consisted of 30 experimental points, which included 6 center points (0, 0, 0, 0), 8 axial points, and 16 corner points (table 3).

Cholesterol removal

To examine the effect of each combination of 4 factors, 50 ml of milk and saponin were placed in a 300 ml Erlenmeyer flask and 5 mm glass marbles were added to the flasks to enhance mixing. The flasks were placed in a shaking water bath at 100 rpm. After milk and saponin were contacted, Celite 545 was added directly to the flask, which was shaken in a water bath for 60 min at 100 rpm. Four factors were applied to the cholesterol removal process. After processing, each mixture was centrifuged at 5000×g, 4℃, for 20 min in an HMR-220 centrifuge (Hanil Industrial Co., Seoul, Korea). The supernatant containing cholesterol-reduced milk fat was decanted and used for cholesterol determination (figure 1).

Extraction and determination of cholesterol

To extract cholesterol (Bachman et al., 1976), 0.2 g of saponin-treated sample was placed in a screw-capped glass tube (15×180 mm) and saponified at 60℃ for 30 min with 3 ml of 95% ethanol and 1 ml of 80% KOH solution (in H_2O, w/v). After cooling to room temperature, cholesterol was extracted with 5 ml hexane and 2 ml distilled water. The process was repeated three times. The hexane layers were transferred to a round-bottom flask and dried under vacuum. The extract was re-dissolved in 1 ml of HPLC grade methanol and filtered using a 0.45 μm filter. The operation conditions for cholesterol analysis by high performance liquid chromatography are shown

Table 1. Variables and levels for a rotatable central composite design used in process optimization for cholesterol removal from milk treated with saponin

Variable		Coded-variable levels		
	Symbol	-1	0	+1
Reaction time with saponin (min)	X_1	10	30	50
Amount of celite addition (%)	X_2	0.25	0.75	1.25
Reaction temperature (℃)	X_3	25	35	45
Amount of saponin addition (%)	X_4	0.5	1.0	1.5

Table 2. Instrument and operation conditions for cholesterol analysis by high performance liquid chromatography

Instrument	:	Waters HPLC
Column	:	Nova-Pak C18 (3.9×300 nm)
Detector	:	Waters 486 absorbance detector (205nm)
Solvent	:	Methanol:Acetonitrile:Isopropanol =7:2:1 (v/v/v)
Flow rate	:	1.6 mL/min
Temperature	:	35℃
Injection vol.	:	20 μL

Table 3. The fractional factorial block design used for cholesterol removal(%) from milk treated with saponin

Treatment No.	Coded var.				Removal of cholesterol (%)
	X_1	X_2	X_3	X_4	
1	-1	-1	-1	+1	64.7
2	-1	-1	+1	-1	61.1
3	-1	+1	-1	-1	62.5
4	-1	+1	+1	+1	68.2
5	+1	-1	-1	-1	61.3
6	+1	-1	+1	+1	66.3
7	+1	+1	-1	+1	67.3
8	+1	+1	+1	-1	64.9
9	0	0	0	0	72.1
10	0	0	0	0	72.1
11	-1	-1	-1	-1	57.4
12	-1	-1	+1	+1	64.2
13	-1	+1	-1	+1	66.9
14	-1	+1	+1	-1	65.2
15	+1	-1	-1	+1	64.4
16	+1	-1	+1	-1	62.3
17	+1	+1	-1	-1	58.3
18	+1	+1	+1	+1	65.2
19	0	0	0	0	72.1
20	0	0	0	0	72.1
21	+1	0	0	0	61.6
22	-1	0	0	0	66.6
23	0	+1	0	0	71.1
24	0	-1	0	0	63.0
25	0	0	+1	0	67.0
26	0	0	-1	0	66.1
27	0	0	0	+1	73.3
28	0	0	0	-1	70.5
29	0	0	0	0	72.1
30	0	0	0	0	72.1

X_1: reaction time with saponin (min), X_2: amount of celite addtion (%), X_3: reaction temperature (℃), X_4: amount of saponin addition (%)

in table 2 (Fillion et al., 1991). All treatments were performed in triplicate.

Statistical analysis

The Stat-graphics (STSC Inc., Rockville, MD, USA) statistical analysis software was used to analyze variance (ANOVA) and to fit the second order model to cholesterol removal (Y) of the dependent variable using the following equation:

$$Y=B_o+\sum B_iX_i+\sum B_{ii}X_i^2+\sum B_{ij}X_iX_j$$

where B_o, B_i, B_{ii} and B_{ij} are constant and regression coefficients of the model, and X_i and X_j are independent variables in coded values.

The elimination of variables from a full regression equation was based on R^2 values and the significance of the *t*-test. A fitted equation was obtained for the predicted cholesterol removal score. The contour plots were generated using PS plotting software (Polysoft, Salt Lake City, UT) to illustrate the main effects of the independent variables in the process.

RESULTS AND DISCUSSION

Saponins are amphiphilic compounds in which sugars are linked to a hydrophobic aglycone steroid or triterpenoid. They occur in a variety of plants and are known as surface-active agents, forming stable foams in water and acting as oil-in-water emulsifying agents (Price et al., 1987). There is considerable evidence that some saponins have the ability to bind bile salts and cholesterol. This ability has been proposed to explain the plasma cholesterol-lowering effect of the ingestion of saponin containing foods (Oakenfull, 1986; Oakenfull and Sidhu, 1983). Food-grade saponins have also been proposed as cholesterol removing agents.

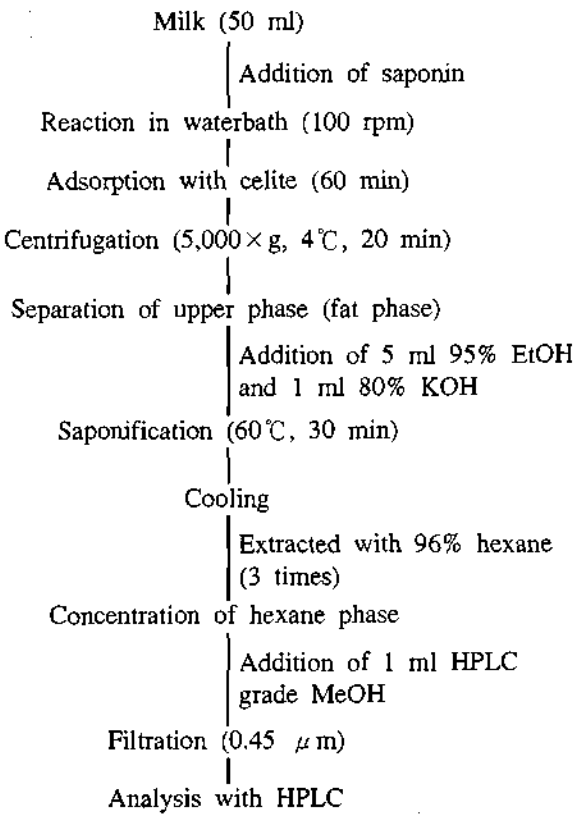

Figure 1. Schematic diagram for the process of saponin treatment and cholesterol extraction

It was reported that the effectiveness of cholesterol adsorption by saponin is dependent on several factors, including reaction time and temperature, the amounts of celite and saponin added (Oh et al., 1998a; Oh et al., 1998b; Sundfeld et al., 1993a; Sundfeld et al., 1993b). This study investigated the optimal conditions for cholesterol removal from milk by treatment with saponin using RSM. Using RSM, one can evaluate the effects of multiple parameters, alone or in combination, on response variables. It has been successfully applied to the optimization of conditions in food, chemical, and biological processes. Average mean values for cholesterol removal were determined (table 3). In treatment condition 27 (30 min reaction time, 0.75% celite, 35℃ and 1.5% saponin), cholesterol removal was the highest (73.3%). Analysis of the significance using the F-test and R^2 values suggested that only three out of four independent variables (reaction time with saponin, amount of celite added, and amount of saponin added) appeared to influence the response (table 4). According to significance tests on estimated coefficients, reaction time with saponin and amount of saponin added were the most important factors influencing cholesterol removal from homogenized milk. When reaction temperature was excluded, the R^2 value was 0.7641, and the F-test still revealed significance ($p<0.001$) (table 5). The model equation included linear and quadratic terms for X_1 and X_2, and a quadratic term for X_4. The best-fit equation describing cholesterol

Table 4. Values of regression coefficients calculated by RSM program for cholesterol removal from milk treated with saponin

Independent variables	Coefficient	Standard error	Sig. level
Constant	49.928791	2.177342	0.0000
X_1	0.770465	0.193596	0.0006
X_1^2	-0.013082	0.003193	0.0004
X_2	16.463051	7.743846	0.0440
X_2^2	-9.130923	5.108651	0.0865
X_4^2	2.112201	0.555377	0.0009

X_1: reaction time with saponin (min), X_2: amount of celite addtion (%), X_3 : reaction temperature (℃), X_4: amount of saponin addition (%)

removal in coded values was as follows:

$$\text{Cholesterol removal (\%)} = 49.928791 + 0.770465X_1 - 0.013082X_1^2 + 16.463051X_2 - 9.130923X_2^2 + 2.112201X_4^2$$

The relationship between reaction factor and response can best be understood by examining the series of contour plots generated by holding constant either reaction time (figure 2) or amount of celite added (figure 3).

Figure 2 shows the interrelationship between the amounts of celite and saponin added on cholesterol removal when reaction time with saponin was held at 10, 30, and 50 min. When reaction time and amounts of celite and saponin added were 30 min, 0.9%, and 1.5%, respectively, cholesterol removal (73.0%) was greatest. Removal of cholesterol increased up to 30 min and decreased thereafter. This result is in agreement with that of Oh et al. (1998a) who reported that higher levels of cholesterol removal from milk were found at a 30 min (70.50%) reaction time versus 10 (62.55%) and 50 min (66.73%). These results may be due in part to the instability of the adsorptive complex formed between saponin and cholesterol during longer reaction times. In contrast, Sundfeld et

Table 5. Analysis of variance for full regression of cholesterol removal from milk treated with saponin

Source	Sum of squares	DF	Mean squares	F-value
Model	435.879	5	87.1758	15.5485***
Error	134.561	24	5.60671	
Total	570.440	29		

R^2=0.76411, *** p<0.001.

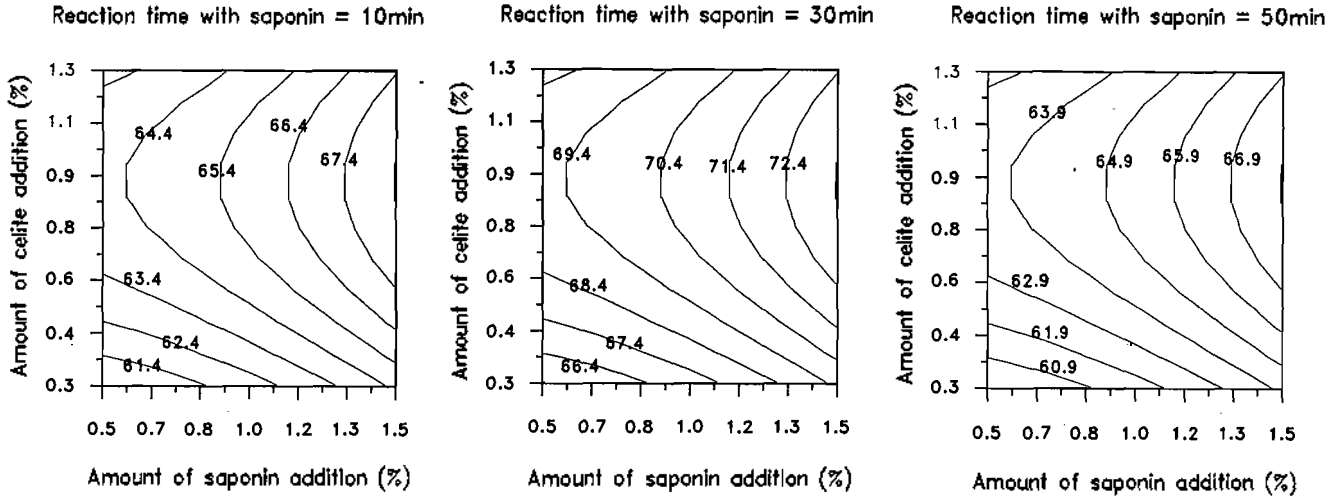

Figure 2. Contour plots showing response behavior of the removal of cholesterol from milk at a constant reaction time

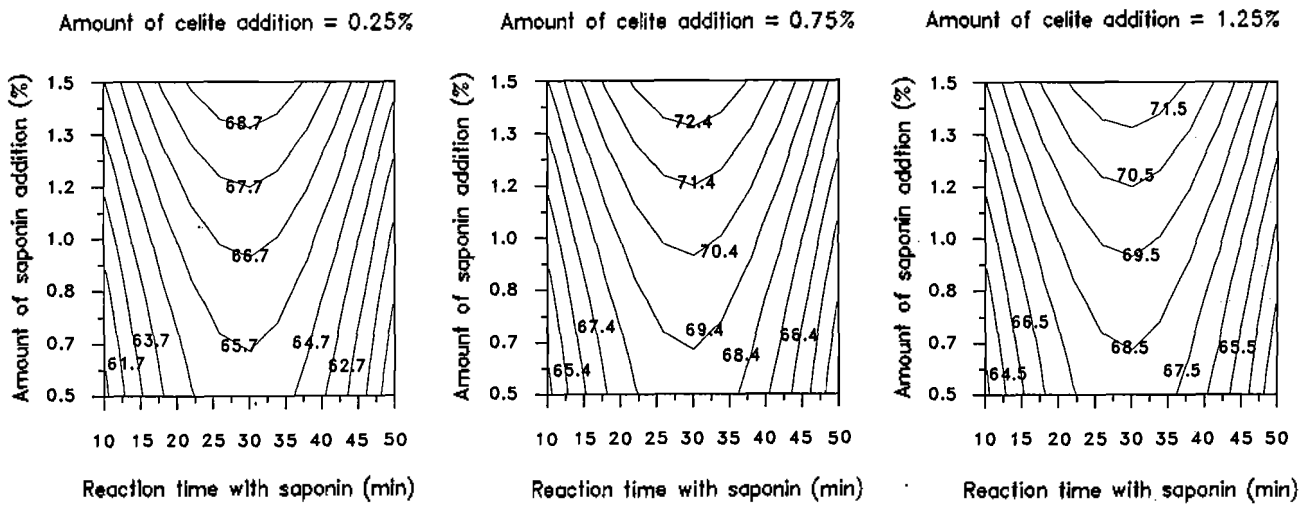

Figure 3. Contour plots showing response behavior of the removal of cholesterol from milk at a constant amount of celite addition

al. (1993a) reported that removal of cholesterol from butteroil with saponin was not influenced by reaction time with saponin. Therefore, the optimum reaction time might vary with different saponin and the type of samples treated. Similar results (Ahn and Kwak, 1999; Lee et al., 1999; Makoto et al., 1992; Yen and Tsui, 1995) were reported in cholesterol removal studies using β-cyclodextrin (CD) for cholesterol adsorption in milk, cream, lard, and cheese.

In figure 2, cholesterol removal continuously increased with increased saponin concentration up to 1.5%. Sundfeld et al. (1993b) showed that treatment of butteroil with 0.40 g/ml saponin significantly lowered mean cholesterol content compared with 0.04 g/ml saponin. Oh et al. (1998a) also reported that cholesterol removal from milk by treatment with saponin was best at 1.5% saponin (69.94%) and decreased thereafter. In a similar study using digitonin for cholesterol adsorption, Micich (1990) indicated that above a certain concentration, digitonin decreases cholesterol removal from butteroil. Our previous work (Oh et al., 1998a) suggests that excess amounts of adsorption materials may cause the compounds to compete with each other for cholesterol binding, which may lower the efficiency of cholesterol removal. And, cholesterol removal further increased with addition of up to 0.9% celite. Higher celite concentrations decreased cholesterol removal values.

Contour plots of cholesterol removal from milk at constant celite concentrations of 0.25, 0.75, and 1.25% are shown in figure 3. Diatomaceous earth, in various forms and grades, is extensively used in the food industry in filtration operations. It is also used as an adsorbent in the removal of a variety of organic and inorganic compounds. Our proposed process uses celite, which has been successfully used in combinations with digitonin (Katz and Keeney, 1967; Schwartz et al., 1967) and saponin (Oh et al., 1998a; Oh et al., 1998b; Sundfeld et al., 1993a) in the separation of cholesterol, to adsorb the cholesterol-saponin complex from milk. At a constant amount of celite addition, cholesterol removal increased with increasing reaction time up to 30 min, and then decreased thereafter. Removal of cholesterol also increased with an increase in the amount of saponin addition at different reaction times. The highest cholesterol removal (72.8%) was achieved at 30 min reaction time, 0.75% celite addition, and 1.5% saponin addition. The addition of 0.75% celite tended to increase cholesterol removal compared with 0.25% and 1.25% addition. However, cholesterol removal was affected only slightly by celite addition (table 4).

Sundfeld et al. (1993a) reported that higher levels of celite significantly increased cholesterol removal. Oh et al. (1998a) also reported that addition of 0.25% celite was maximally efficient in removing cholesterol from milk with saponin, which is in disagreement with our results. We evaluated multiple variables and their interactions, whereas in the aforementioned study (Oh et al., 1998a), the authors evaluated the effect of one single variable on cholesterol removal. This may explain why our results differ.

We found that reaction time with saponin (X_1) and the amount of saponin added (X_4) produced the greatest cholesterol removal at the designed 3-level (-1, 0, +1), at values of 30 min and 1.5%, respectively, whereas the amount of celite added (X_2) did not. Therefore, the amount of celite added was determined by partial differentiation of the model equation. Consequently, the most favorable conditions for cholesterol removal from homogenized milk by treatment with saponin were 30 min reaction time with 1.5% saponin and 0.95% celite addition. Under these conditions, the predicted cholesterol removal was 73.4%.

To confirm the predicted level of cholesterol removal, a verification experiment was performed independently using the optimal conditions determined by RSM; the resulting experimental value was 73.7%. There was no appreciable difference between the predicted value from RSM and the experimental results. This indicates that the optimal conditions for cholesterol removal from homogenized milk with saponin developed by RSM are adequate and useful.

Dietary fat and cholesterol intakes has been shown to have a positive effect on serum cholesterol levels of individuals and to increase the risk of coronary heart disease and atherosclerosis. So, many of today's consumers are concerned about reducing cholesterol and fat in the daily diet (Sperber, 1989). Strategies to lower coronary heart disease routinely concentrate on reducing cholesterol concentrations through lowering the intake of dietary cholesterol. Reducing cholesterol intake in our foods appears to be the simplest approach to lowering bodily cholesterol levels and the apparent associated disorders (Sieber, 1993). As a result, there have been dramatic increases in no-, low- and reduced-cholesterol and fat products available in the market place. Therefore, daily consumption of cholesterol reduced milk which is processed according to the optimum conditions will lower serum cholesterol level as compared to untreated milk and possibly be useful in reducing the risk of coronary heart disease for hypercholesterolemic humans.

CONCLUSIONS

These data show that the optimal conditions for cholesterol removal from homogenized milk using RSM consist of a 30 min reaction time with 1.5% saponin and 0.95% celite. Under these conditions, cholesterol removal was predicted to be 73.4%, and

the experimental value was 73.7%. There was no appreciable difference between the predicted value of RSM and the experimental result. The data further suggest that the optimal conditions for cholesterol removal from homogenized milk with saponin as developed by RSM are adequate and useful.

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