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Separation of Campesterol and β-Sitosterol from a Sterol Mixture

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Abstract: By solvent crystallization using diethyl ether as the solvent on sterol mixture, brassicasterol and stigmasterol that contains a side chain with double bond were separated from campesterol and β-sitosterol with a saturated side chain. The total campesterol and β-sitosterol content in the liquid phase was more than 97% with a recovery of 12%. Multistage crystallization using acetone as the solvent could increase the recovery of campesterol and β-sitosterol to 30%. By employing zeolite selective adsorption on the campesterol and β-sitosterol fraction, β-sitosterol can be recovered in the liquid phase with a purity of 95.2% and a recovery of 3% (overall recovery 1%). After desorbing in ethanol, campesterol adsorbed on the zeolite can be recovered with a purity of 95.4% and a recovery of 3.7% (overall recovery 1.6%).

Keywords: Adsorption, phytosterol, solvent crystallization, zeolite

INTRODUCTION

Phytosterols (plant sterols) are important structural components of plant membranes. Free phytosterols serve to stabilize phospholipid bilayers in

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plant cell membranes just as cholesterol does in animal cell membranes. Their function appears to be to control membrane fluidity, permeability, and signal transduction (1). Phytosterols and their fatty acid esters have water-holding property and are widely used as ingredients of cosmetics and bath additives (2). Recently, phytosteryl ester was found to be effective in lowering plasma cholesterol concentration by inhibiting the absorption of cholesterol from the small intestine. This physiological activity has led to the development of functional foods, such as salad oil dressing with added sterol and margarine blended with fatty acid sterol ester (FASE) (3).

Instead of a sterol mixture, either purified β-sitosterol or campesterol, has applications in biological studies. A chemical synthesis of pure sterol usually involves the use of carcinogenic chemicals and the reactions are unselective, resulting in unwanted side products which results in extra purification steps. Alternatively, pure sterol can be obtained from a natural sterol mixture by physical and/or chemical separation. However, the isolation of pure sterol from a sterol mixture is difficult. This is due to the similarity of the structures of the sterols involved. Few reports on the purification and isolation of individual sterols from a sterol mixture are available in the literature.

Koskenniska (4) isolated β-sitosterol from the unsaponifiable matter of crude sterol mixture by treating the sterol with a solvent mixture containing an aromatic hydrocarbon, a polar organic solvent, and water. The recovery of β-sitosterol was over 70% with a purity of 93% (original β-sitosterol content 65%). Hamunen (5) isolated β-sitosterol from the unsaponifiable matter in crude soap obtained from the sulfate cellulose process by dissolving the sterol in a mixture of organic solvent and water. From a sterol mixture, Xu et al. (6) were able to obtain β-sitosterol (88% purity and 39% recovery) and stigmasterol (90% purity and 7% recovery) by using a 3-stage and a 5-stage solvent crystallization, respectively.

In the separation of sterols involving silica gel, the strong affinity between the hydroxyl group of sterol and silica gel can be overcome by the acetylation of the hydroxyl group using silver nitrate. The silver ion in the silica gel can form a complex with the carbon double bond and sterols can be separated based on the number of double bonds in the sterol structure. Campesterol with 98% purity and 1.8% recovery was obtained by Kirche and Rosenstein by using silica gel column chromatography (7). Preparative silver impregnated thin layer chromatography was employed to obtain the desired high purity sterols (8). However, the use of silver hindered the use of this method, especially in large scale operation.

Zhou et al. (9) obtained β-sitosterol with 97% purity and 50% recovery from a mixture of sterols by high speed countercurrent chromatography (HSCC). There are no other reports on sterol separation using HSCC

despite their claim that HSCC is capable of separating compounds with similar physical properties.

Since the MW of campesterol is about 3% less than that of β-sitosterol and the melting point of β-sitosterol is about five degrees centigrade higher than that of campesterol, molecular distillation can be employed for the separation of these two compounds from a crude phytosterol mixture (10).

Berezin et al. (11) found that Y-type zeolite preferentially adsorbs campesterol over β-sitosterol in hexane. Zeolite separation is a promising method for the separation of β-sitosterol and campesterol from a sterol mixture. Berezin's work focused mostly on showing the possibility of using zeolite for sterol separation as well as clarifying the adsorption mechanism. Due to the highly importance of obtaining high purity of β-sitosterol and campesterol especially for biological studies (12), a systematic investigation of this method is necessary for the efficient separation of β-sitosterol and campesterol from a sterol mixture. In this study, solvent crystallization and zeolite adsorption were employed for the isolation of campesterol and β-sitosterol from a commercially available sterol mixture. Figure 1 shows chemical structures of the phytosterols involved in this work.

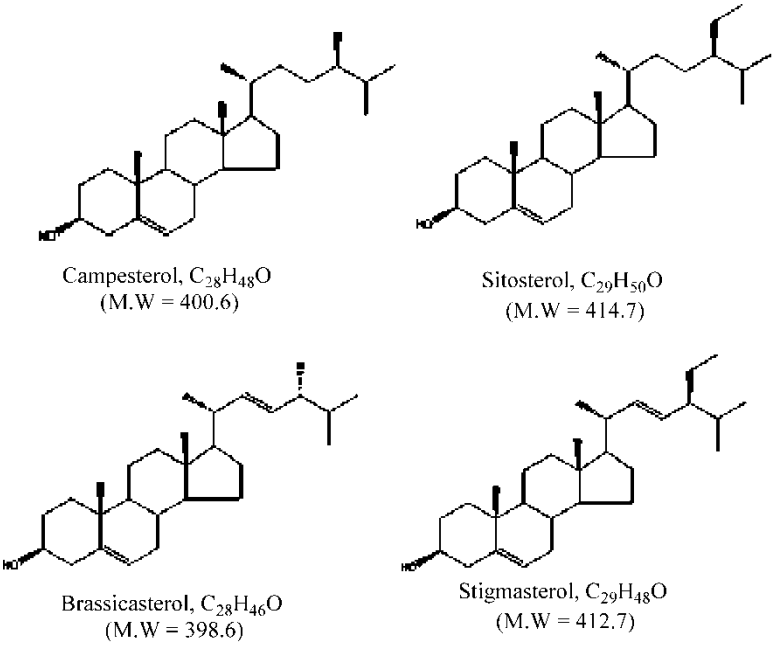

Figure 1. Chemical structure of sterols in the sterol mixture used in this study.

MATERIALS AND METHODS

Materials

A sterol mixture which consists of 5% brassicasterol, 15% stigmasterol, 30% campesterol, and 50% β-sitosterol is a product of MP Biomedicals (ICN), Germany. Y type zeolite (sodium ion) was purchased from Sigma-Aldrich (MO, USA). All solvents and reagents were either of HPLC grade or analytical reagent grade. All other chemicals used were obtained from commercial sources.

Crystallization using Diethyl Ether as the Solvent

A sterol mixture (10 g) was dissolved in diethyl ether (500 mL) at 25°C. The solution was then stored in an ultra-low temperature freezer (Sanyo MDF-192; Gunma, Japan) for 24 h. Immediately after removing from the freezer, solid and liquid phases were separated by vacuum filtration using an Ace Buchner funnel (25–50 μm). The sterols in the liquid phase were recovered by removing the solvent in a vacuum rotary evaporator at 35°C. The sterol composition in the liquid phase was analyzed by gas chromatography (GC-17A, Shimadzu, Japan) using DB5-HT (15 m × 0.32 mm I.D) column with a flame ionization detector. The solid phase still contained more than 75% saturated sterols (campesterol and β-sitosterol) and was subjected to repeated crystallization as described above using acetone as the solvent. The liquid phases obtained from all solvent crystallization steps were pooled together to form the saturate-sterol fraction.

Adsorption by Zeolite

The saturate-sterol fraction obtained from solvent crystallization was the starting material used in zeolite adsorption. Selective adsorption of campesterol over β-sitosterol on Y type zeolite in organic solvent resulted in the separation of these two sterols. The composition and physical properties of the Y type zeolite used in this study are: pore diameter = 7.4 Å, surface area = 920 m^2/g, porosity = 0.3, particle size = 2 microns. Its chemical composition is: 63.8% SiO_2, 22.9% Al_2O_3, 13.0% Na_2O, 0.13% Fe_2O_3, and 0.38% CaO. Fresh zeolite was activated at 500°C for 18 h before use. The sterol mixture (0.5 g) was dissolved in hexane (250 mL) in a stoppered glass vessel in a water bath at 30°C. To this solution, zeolite was added and the solution was stirred with a magnetic stirrer. At regular intervals, 100 μL of solution was withdrawn into a micro centrifuge tube. To this sample, 50 μL standard (10 mg cholesterol in 10 mL ethyl acetate) was added and zeolite was separated by centrifugation at 800 g for 1 min. The product composition and recovery was determined by GC. At the end of the

adsorption, zeolite was separated by filtration. The composition of sterols in the liquid phase was analyzed and the zeolite containing adsorbed sterols was subjected to sterol desorption experiments.

Desorption of Sterols from Zeolite

The sterol-adsorbed zeolite was dried in oven at 60°C for 2 h. The zeolite was then put into ethanol (zeolite: ethanol = 1:10 g/mL) at 80°C and stirred at 200 rpm for 2 h. After the zeolite was separated by filtration, campesterol-enriched sterol was obtained by the evaporation of ethanol. This process was repeated until all sterols adsorbed on the zeolite were desorbed. The desorbed zeolite can be reused after being calcined at 500°C for 24 h.

Figure 2 shows the flow chart of the purification steps involved in this study for separating campesterol and β-sitosterol from commercial sterol mixture.

RESULTS AND DISCUSSION

Crystallization using Diethyl Ether as the Solvent

Figure 3 shows the effects of solvent (diethyl ether) to sterol ratio and storage temperature on the contents and yields of campesterol and β-sitosterol in the liquid phase. The results show that at a solvent/sterol ratio of 50:1 (mL/g) and a storage temperature of −80°C, most brassicasterol and stigmasterol were separated as solid phase. The sterol compositions before and after the

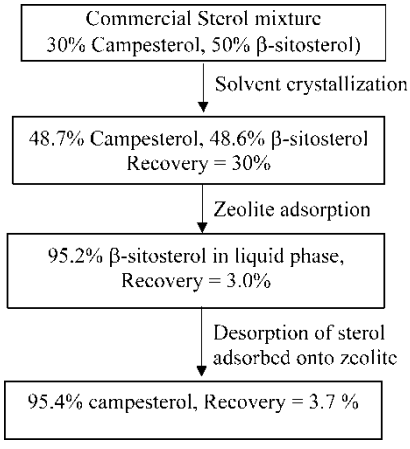

Figure 2. Flow chart of the purification steps involved in this study for separating campesterol and β-sitosterol from commercial sterol mixture.

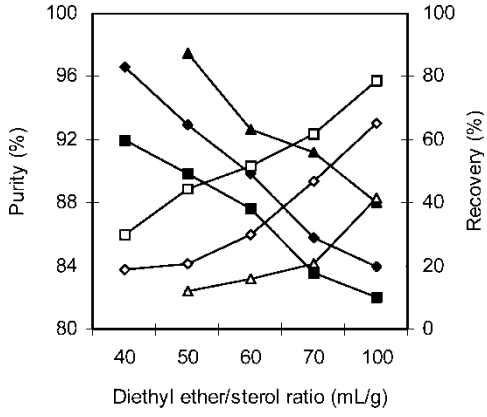

Figure 3. The effects of solvent (diethyl ether) to sterol ratio and storage temperature on the separation of campesterol and β-sitosterol. Symbols: (■, □) = −40°C (◆, ◇) = −60°C (▲, △) = −80°C. Solid symbols: sterols content in liquid phase. Open symbols: sterol recovery in liquid phase. Purity was calculated as w/w ratio of the sum of campesterol and β-sitosterol to all sterols in liquid phase after crystallization. Recovery was calculated as w/w ratio of the sum of campesterol and β-sitosterol in liquid phase after crystallization to the initial amount of both campesterol and β-sitosterol before crystallization.

crystallization are presented in Table 1. Crystallization studies carried out at solvent/sterol ratio <50:1 (mL/g) did not result in distinct separation of solid and liquid phases and the solution became hazy and turbid. Though the total campesterol and β-sitosterol content in the liquid phase was more than 97%, the recovery of campesterol and β-sitosterol in the liquid phase was low (12%). Most campesterol and β-sitosterol remained in the solid phase.

After removing the solvent from the solid phase, the sterol mixture was used as substrate in repeated solvent crystallizations. This is a modification of the work by Poulos et al. (13). By employing repeated crystallization

Table 1. Sterol compositions before and after low temperature solvent crystallization

	Brassicasterol (%)	Stigmasterol (%)	Campesterol (%)	β-Sitosterol (%)
A[a]	5	15	30	50
B	1.2	1.5	48.7	48.6
C	5.7	16.8	27.9	50

[a]Operation conditions: Solvent = diethyl ether, solvent to sterol mixture ratio = 50:1 (mL/g), storage temperature = −80°C, storage time = 24 h. A: Initial sterol composition (1 g), B: Liquid phase composition (0.104 g), C: Solid phase composition (0.896 g).

using acetone as the solvent, the recovery of campesterol and β-sitosterol in the liquid phase can be increased from 12% to 30% with negligible effect on the total content of campesterol and β-sitosterol.

Effect of Stirring on Adsorption

The stirring correlates directly to the power consumption in which P is proportional to N^3, where P and N represent power consumption and stirring speed, respectively (14). Figure 4 shows the effect of stirrer speed on the zeolite-adsorption of sterol at 30°C. It is apparent that adsorption rate is slower without stirring. A stirrer speed of 100 rpm is enough to overcome the external mass transfer resistance. In this study a stirrer speed of 200 rpm was employed.

Effect of Temperature on Adsorption

The effect of temperature on adsorption was investigated and it was found that temperature has negligible effect on the selectivity for zeolite adsorption of campesterol and β-sitosterol (data not shown).

Effect of Solvent on Adsorption

The Y-type zeolite (sodium ion) has an affinity for molecules with a permanent dipole. Polar solvent molecules have a tendency to occupy the active site of

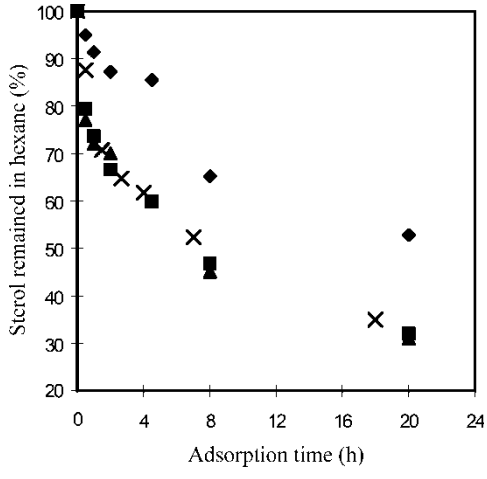

Figure 4. The effect of stirrer speed on the zeolite adsorption of sterols at 30°C. Sterols: 20 mg, zeolite: 200 mg, hexane: 10 mL. Stirrer speed: (◆) 0 rpm (■) 100 rpm (▲) 200 rpm and (×) 400 rpm. Sterol remained was calculated as w/w ratio of sterols that remained in hexane to the initial amount of sterols before zeolite adsorption.

zeolite. Adsorptions carried out with a sterol mixture dissolved in polar solvent, such as ethyl acetate, acetone, ethanol, and methanol, were not successful. Significant adsorption of sterol did occur when carried out in a non-polar solvent of pentane, hexane, heptane, n-octane, and iso-octane. Figure 5 shows the time courses of campesterol adsorption on zeolite using alkane as the solvent. The highest adsorption rate was observed with pentane as the solvent.

Separation factor for campesterol relative to β-sitosterol α, is defined as (15) $\alpha = (\chi_{z,1}/\chi_{z,2})(\chi_{s,2}/\chi_{s,1})$, where $\chi_{z,1}$ and $\chi_{z,2}$ represent weight fractions of campesterol and β-sitosterol adsorbed on zeolite, respectively; while $\chi_{s,1}$ and $\chi_{s,2}$ represent weight fractions of campesterol and β-sitosterol in solution, respectively. Separation factors for pentane and hexane are 2.58 and 1.76, respectively. Hexane was employed as the solvent in this study since it has higher boiling point (65°C). Campesterol can be preferentially adsorbed onto zeolite leaving β-sitosterol.

Effect of Ethanol on Adsorption

It was reported that the addition of a small amount of ethanol to hexane enhances the adsorption rate of sterols by zeolite (11). The effect of ethanol on the adsorption rate of β-sitosterol on zeolite was investigated and the results in Fig. 6 show that a small amount of ethanol did enhance the adsorption. However, too much ethanol ($>0.4\%$) had a negative effect on the adsorption of sterols. When more than 0.5% ethanol was used, the adsorption of

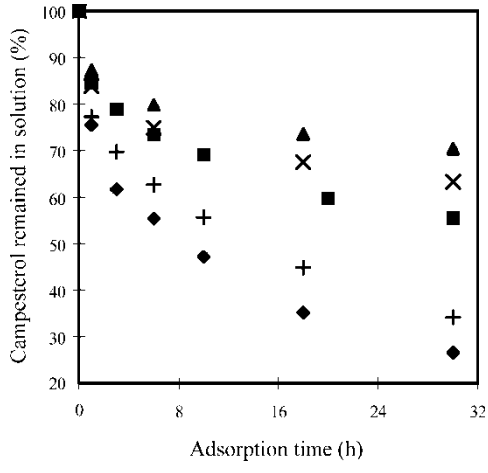

Figure 5. Time-course of zeolite-adsorption of campesterol in different solvents. Sterol mixture: 20 mg, zeolite: 200 mg, solvent: 10 mL, stirrer speed: 200 rpm, temperature: 30°C. Solvent: (◆) Pentane (■) Hexane (▲) Heptane (×) n-octane (+) Iso-octane.

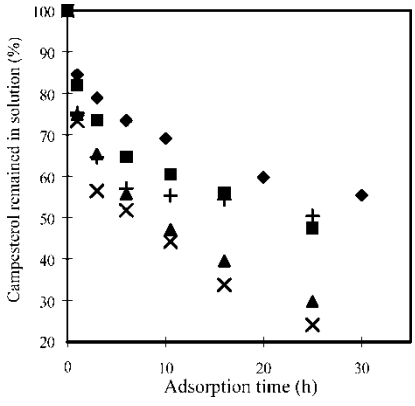

Figure 6. The effect of ethanol on the time courses of zeolite-adsorption of campesterol. Sterol mixture: 20 mg, zeolite: 200 mg, hexane: 10 mL, stirrer speed: 200 rpm, temperature: 30°C. Percentage ethanol added based on hexane volume: (◆) 0 (■) 0.1 (▲) 0.2 (×) 0.3 (+) 0.4. Campesterol remained in solution was calculated as w/w ratio of campesterol that remained in hexane to the initial amount of campesterol in hexane before adsorption started.

sterols by zeolite was completely inhibited. This could result from the competitive occupation of active site by ethanol molecules.

Multistage Adsorption of Sterols

A seven-stage adsorption was employed for the purification of β-sitosterol. In the first stage, 400 mg sterol mixture (1% brassicasterol, 49% campesterol, 1% stigmasterol and 49% β-sitosterol) was added into 200 mL hexane containing 4 g zeolite and 0.3 wt% ethanol. The sterol mixture was obtained from the solvent crystallization of commercial sterol mixture. The adsorption was conducted at 30°C for 12 h with 200 rpm stirring. At the end of the adsorption, zeolite was separated by filtration. Enriched β-sitosterol was recovered from the liquid phase by evaporating the solvent. This β-sitosterol-enriched sterol fraction was subjected to another six adsorptions under the same operation conditions as the first one. Purity and recovery of sterols obtained during this multi-stage adsorption are shown in Fig. 7. After seven stages of adsorption, β-sitosterol was obtained in the liquid phase with a purity of 95.2% and a corresponding recovery of 3%.

Desorption of Sterols from Zeolite

The recovery of adsorbed sterols from zeolite (desorption) is another important step in the separation and purification of sterols. The effect of

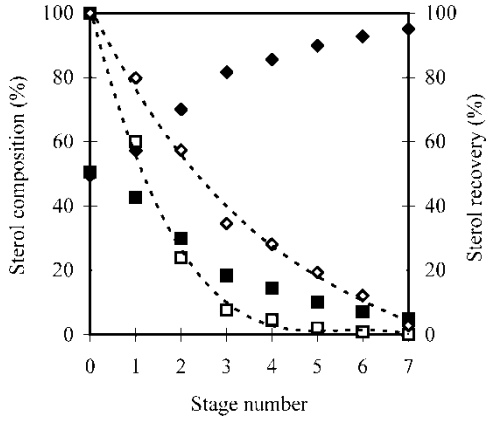

Figure 7. The enrichment of β-sitosterol by multi-stage zeolite adsorption. In the first stage, 400 mg sterol mixture (1% brassicasterol, 49% campesterol, 1% stigmasterol, 49% β-sitosterol), 4 g zeolite and 0.3 wt% ethanol was added into 200 mL hexane, and the adsorption was conducted at 30°C for 12 h under 200 rpm stirring. Solid symbols: sterol purity in liquid phase, hollow symbols: sterol recovery in liquid phase. (◆, ◇) β-sitosterol, (■, □) Campesterol.

solvent on desorption of sterols was investigated in this study and the result suggested that desorption rate depends on the polarity of solvent and the solubility of sterols. It was found that the rates of desorption of sterol in ethanol and acetone are comparable. Ethanol was employed as the solvent in this

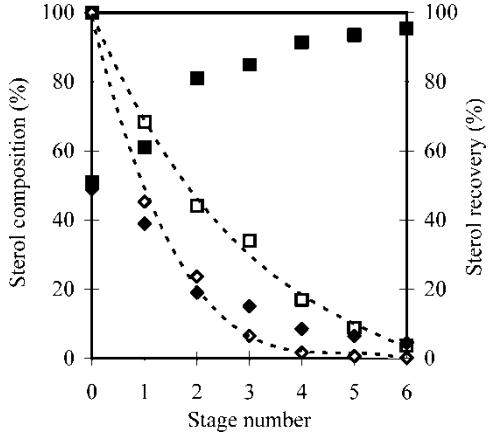

Figure 8. The enrichment of campesterol by zeolite desorption. Enriched campesterol was obtained by the desorption of zeolite in the multi-stage adoption of sterol studies. Solid symbol: sterols purity, hollow symbol: sterols recovery. (◆, ◇) β-sitosterol, (■, □) campesterol.

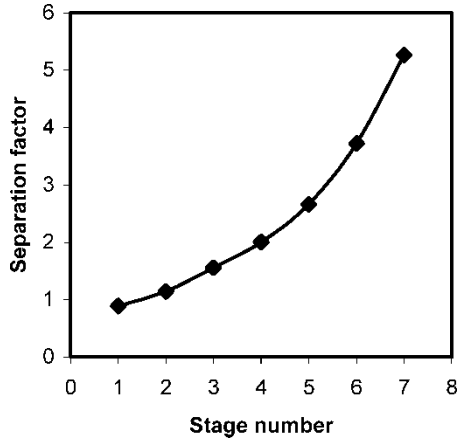

Figure 9. Separation factor as function of number of adsorption stage. Separation factor is defined as $\alpha = (\chi_{z,1}/\chi_{z,2})(\chi_{s,2}/\chi_{s,1})$, where $\chi_{z,1}$ and $\chi_{z,2}$ represent weight fractions of campesterol and β-sitosterol adsorbed on zeolite, respectively; while $\chi_{s,1}$ and $\chi_{s,2}$ represent weight fractions of campesterol and β-sitosterol in solution, respectively.

work for desorption studies. Campesterol can be enriched from the desorption of zeolite in each adsorption operation during the repeated adsorption. Purity and recovery of sterols during the desorption process were shown in Fig. 8. After six desorption steps, campesterol with a purity of 95.4% and a corresponding recovery of 3.7% was obtained.

Figure 9 shows the separation factor for each step in the multistage zeolite adsorption. Separation factor increases with increasing number of adsorption stage which indicates that a lower campesterol concentration or higer β-sitosterol concentration in the solution is favorable to improving the separation factor for campesterol relative to β-sitosterol.

CONCLUSION

High purity of β-sitosterol and campesterol could be successfully separated and purified from a commercial sterol mixture. Low recoveries for both campesterol and β-sitosterol were obtained in the present study. This is the result of high similarity in the chemical and physical properties of the four sterols involved, as well as the result of multi-step separation employed in this study. Considering the fact that the price of 97% pure β-sitosterol or even lower purity of campesterol (65%) is at least several thousand times more expensive than that of commercial sterol mixture, a recovery of 1.6% for campesterol with purity of 95% by the process developed in this study is attractive. Further investigation to achieve higher yield with simpler procedure remains a challenging research.

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