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Novel Synthesis of Steryl Esters from Phytosterols and Amino Acid

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ABSTRACT: The feasibility of esterification of phytosterol with the amino acid L-glutamic acid was established. The influence of various organic solvents was investigated, and *n*-butanol was selected as an ideal solvent for phytosteryl esters synthesis with L-glutamic acid. The reaction conditions were further optimized by orthogonal experiments, and a 92.3% degree of esterification was obtained when optimum conditions were used. FT-IR spectral, GC-MS, and NMR analyses were adopted to determine the steryl esters of L-glutamic acid. The FT-IR spectrum indicated the presence of ester bonds in the phytosteryl esters with L-glutamic acid, and on the basis of the detailed mass spectrography analysis, GC-MS and NMR offered an efficient and reliable way to confirm the steryl esters. This novel synthesis approach of phytosteryl esters with amino acid supplied a promising alternative to the substrate on esterification of phytosterols and thus can be readily applied to further studies of functional food ingredients of phytosteryl esters.

KEYWORDS: phytosterols, amino acid, esterificaiton, steryl esters

INTRODUCTION

Phytosterols, which are derived from vegetable oils, have proved to be natural active substances. Phytosterols play major roles in pharmaceuticals and nutrition because they are known as cholesterol-lowering agents and to have anti-inflammatory, anti-oxidation, and anticancer functions.[1−5] In addition, phytosterols have a wide use in cosmetics.[6,7]

The promising application of phytosterols benefits from the esterification of phytosterols with fatty acids (FAs) because the phytosteryl esters possess much a greater solubility in oil and a much lower melting point, as compared to the corresponding free phytosterols, and can be easily incorporated into a variety of lipid matrices.[8,9] Phytosteryl esters of FAs are presently synthesized by chemical esterification, transesterification, and enzymatic procedures using lipases (triacylglycerol hydrolases EC 3.1.1.3), and both preparation methods achieved a certain degree of success.[10−12] The synthesis of sterol esters of FAs mentioned above is based on esterification between the 3-OH of phytosterols and −COOH of fatty acids, and due to this reaction principle, it is feasible that compounds with −COOH can theoretically undergo esterification with sterols.

Amino acids, which play an important role in the normal growth of humans, are typical compounds with −COOH. Acidic amino acids are logically considered to be more appropriate to esterify with sterols because two −COOH groups exist in the structure. Also, the water solubility of the steryl esters with amino acid seemed to increase, presumably with the introduction of $-NH_2$.

The purpose of the present study was to develop a novel synthesis of phytosterol esters with amino acid. Esterification of acidic amino acid L-glutamic acid with phytosterols wss investigated. The influences of several solvents were compared first and, thereafter, optimum of reaction conditions were established. FT-IR spectral and NMR analyses were adopted to evaluate the functional ester bond of the product, whereas the structure and degree of esterificaiton were determined by gas chromatography−mass spectrometry (GC-MS).

MATERIALS AND METHODS

Materials. Phytosterols (*β*-sitosterol, 46.41%; stigmasterol, 21.76%; campesterol, 26.12%; total phytosterol content, 94.3%) were purchased from Zhejiang Dawei Co., Ltd. (China). L-Glutamic acid, sodium bisulfate ($NaHSO_4 \cdot H_2O$), and the 4 Å $^1/_{16}$ molecular sieves were purchased from Shanghai Yuanju Chemical Co., Ltd. (China). All other reagents such as *n*-hexane, cyclohexane, *n*-butanol, chloroform, acetone, and *n*-heptane were of analytical grade.

Esterification Reactions. *Solvent Screening and Selection.* Different solvents were first compared using the same substrate concentration. Due to the polarity, *n*-hexane, cyclohexane, *n*-butanol, chloroform, acetone, *n*-heptane, and water were tested in the same substrate concentrations and reaction conditions. Each reaction flask included 0.2 g of total phytosterols, 1 g of L-glutamic acid, 0.2 g of sodium bisulfate, and 2 g of 4 Å $^1/_{16}$ molecular sieves, to which was added 5 mL of each of the solvents mentioned above. The flasks were placed in an orbital shaking water bath at 150 rpm and 60 °C for 24 h. After the reaction, the solvents were first filtered to remove the dissolved reactants and then passed through a sodium sulfate column to remove the water during reaction and then completely evaporated under a flow of nitrogen. Samples were then dissolved in *n*-hexane for further gas chromatographic analysis.

Optimization of Esterification Conditions. Once the suitable solvent was selected, the orthogonal experiments were carried out to optimize the esterification conditions. Four key parameters including the reaction temperature (A), molar ratio of phytosterol to L-glutamic acid (B), reaction time (C), and catalyst load (D) were optimized according to the

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Table 1. Factors and Levels Selected for Orthogonal Experiments

	factor			
level	A (temperature, °C)	B (phytosterol/L-glutamic acid, M/M)	C (reaction time, h)	D (catalyst load, wt %)
1	70	1:2	6	3
2	80	1:3	8	5
3	90	1:4	10	7

Table 2. Organic Solvent Screening for the Esterification of Phytosterols with L-Glutamic Acid

solvent	log *P*	DE (%)
acetone	−0.23	−8.95
n-butanol	0.9	89.2
chloroform	2.0	2.1
cyclohexane	3.2	—[a]
n-hexane	3.5	—
n-heptane	4.0	—

[a] —, not detected.

Table 3. Detailed Schemes and Variance Analysis of Orthogonal Test on Degree of Esterification[a]

	factor				
	A	B	C	D	DE (%)
1	70	1:2	6	3	88.035
2	70	1:3	8	5	89.26
3	70	1:4	10	7	90.795
4	80	1:2	8	7	90.625
5	80	1:3	10	3	91.915
6	80	1:4	6	5	88.52
7	90	1:2	10	5	91.58
8	90	1:3	6	7	88.58
9	90	1:4	8	3	89.245
k_1	89.3633	90.08	88.3783	90.17	
k_2	90.3533	89.9183	89.71	89.7867	
k_3	89.8017	89.52	91.43	89.75	
R	0.99	0.56	3.0517	0.2683	
order of influence: C > A > B > D					
optimal scheme: $A_2B_1C_3D_1$					

[a] The value of DE represents the mean of triplicate experiments. k_i represents the average of the experiment values of DE corresponding to level *i*. *R* represents the range, which indicates how far it is from the lowest k_i to the highest k_i for a certain factor.

orthogonal test schedule in Table 1. Every factor had three levels to be optimized.

The esterification process comprised the following steps: first, phytosterol L-glutamic acid, and sodium bisulfate were weighed according to Table 1 in a round-bottom flask, and then the organic solvent was selected (as detailed under Solvent Screening and Selection) and the 4 Å $^{1}/_{16}$ molecular sieves were added into the flask to react at tested reaction temperatures and times with a reflux device while the reaction mixtures were magnetically agitated at 220 rpm.

Isolation and Purification of Steryl Esters. Once the esterification reaction was complete, the reactants were first filtered to remove the molecular sieves and undissolved substances in a Buchner flask. Filtrate was then evaporated completely by rotary evaporator under vacuum to remove the solvent *n*-butanol, and subsequently, *n*-hexane was added to redissolve the crude product of steryl esters. Another filter step was then carried out to remove the undissolved amino acid, and the liquor was applied to a silica gel 60 column (30 × 250 mm, Merck, Darmstadt, Germany); steryl esters were eluted with a mixture of *n*-hexane/diethyl ether (90:10, v/v). Steryl esters were then obtained after the eluate was evaporated.

Analysis Methods. FT-IR spectra were recorded on Nicolet 6700 spectrometer (Nicolet Instrument Corp., USA) with a DTGS detector. Phytosterol and the phytosterol ester samples were diluted in KBr and measured using attenuated total reflectance method with the spectral scanning scope for 400−4500 cm^{-1}.

GC-MS was employed to analyze the phytosteryl esters. A Shimada GC-MS-QP2010 (Shimadzu Co., Kyoto, Japan) equipped with a capillary column (HP-5, 30.0 m × 0.32 mm, 0.25 μm film thickness, Agilent) was used. The GC parameters were as follows: carrier gas, high-purity helium; injector temperature, 200 °C; detector temperature, 320 °C; split ratio, 20:1; column flow rate, 1 mL/min; inject volume, 1 μL. The column temperature was programmed to increase to 285 °C from the initial 200 °C at the rate of 7 °C/min and then maintained for 12 min. The mass spectrometer was operated in electron impact ionization (70 eV), full scan (m/z 40−800) mode. The MS parameters were as follows: scan speed, 1666; interscan, 0.5 s; source temperature, 250 °C; interface temperature, 285 °C. Compounds were identified by comparison of their retention indices and mass spectra with the mass spectral library.

Nuclear magnetic resonance (NMR) experiments were conducted on a Bruker AVANCE III 400 spectrometer operating at 400.23 and 100.63 MHz for proton and carbon, respectively, at 27 ± 1 °C. NMR signals were measured in $CDCl_3$ using tetramethylsilane as the internal standard.

Calculation of Degree of Esterification. The degree of esterification (DE, mol %) of phytosterols with amino acid to form phytosteryl esters was calculated with reference to the synthesis of phytosteryl esters with fatty acid,[13] that is, from the amount of phytosterols consumed during the reaction. The phytosterol concentrations in the calibration solution were 0.2, 0.4, 0.6, and 0.8 mg/mL, and each calibration point was done in triplicate. 5α-Cholesterol was used as internal standard. The ratio of integrated peak areas of phytosterols to the peak area of internal standard was used to draw the calibration curve. The calibration curve was then established, and a correlation coefficient of 0.9912 was obtained from the linear regression equation.

RESULTS AND DISCUSSION

Solvent Screening and Selection. The esterification reaction between phytosterols and amino acid is feasible in theory due to the −OH of the phytosterol and the −COOH of the amino acid. Therefore, in our experiment, first, L-glutamic acid was dissolved in water, and then phytosterol and the catalyst sodium bisulfate were added. The solution with reactants turned yellow to light red during the reaction because of the formation of the acidamide of the −COOH and $-NH_2$ within L-glutamic acid. Thus, the esterification reaction is difficult to carry out with water as solvent.

Six organic solvents with log *P* ranging from −0.23 to 4.0 were then investigated to explore the influence on the esterification of phytosterol and L-glutamic acid. The degree of esterification is

shown in Table 2. From the results, it seemed that the chemical bond rather than the log *P* of the organic solvent was the essential element. The hydroxide radical in *n*-butanol promoted the positive reaction effectively, and the ketone group in acetone also had a positive effect. *n*-Butanol was selected as an ideal solvent for phytosteryl ester synthesis with L-glutamic acid; furthermore, the reaction conditions with *n*-butanol as solvent were optimized by the following orthogonal test.

Optimization of Esterification Conditions. The effects of four parameters in Table 1 were investigated by means of orthogonal tests and variance analysis. An orthogonal table $L_9(3^4)$ was used to array the parameters, and the detailed results are listed in Table 3. The DE was taken as the index point to evaluate the esterification efficiency under different factors and levels.

It can be seen from Table 3 that the influence on the DE was in the order C > A > B > D. The esterification reaction time (C) and temperature (A) showed an obvious effect on DE. The molar ratio of phytosterol to L-glutamic acid (B) and catalyst load (D) had no significant effect on DE. The optimal esterification reactions to obtain highest DE were $A_2B_1C_3D_1$ when the reaction temperature was 80 °C, the molar ratio of phytosterol to L-glutamic acid was 1: 2, the reaction time was 10 h, and the catalyst load was 3%. Following the above optimized esterification conditions, a 92.3% DE was obtained.

Table 4. FT-IR Spectrum Analysis of Phytosterols

wavenumber[a]	adscription[b]	potential functional groups
3426.88 (s)	ν_{OH}	−OH
2937.06 (s)	ν_{CH}	$-CH_3$, $-CH_2-$
1465.64 (m)	δ_{CH}	$-CH_3$, $-CH_2-$
1382.71 (m)	δ_{CH}	$-CH_3$
1062.59 (m)	ring vibration	polycyclic compounds
958.45 (w)	ν_{C-C}	−C−C−
740.53 (w)	ν_{CH}	$-(CH_2)_n$, $n > 4$

[a] s, strong intensity; m, middle intensity; w, weak intensity. [b] ν, stretching vibration; δ, deformation vibration.

Table 5. FT-IR Spectrum Analysis of Phytosteryl L-Glutamic Esters

wavenumber[a]	adscription[b]	potential functional groups
2937.06 (s)	ν_{CH}	$-CH_3$, $-CH_2-$
1737.55 (s)	$\nu_{C=O}$	R−CO−OR′
1465.64 (m)	δ_{CH}	$-CH_3$, $-CH_2-$
1382.71 (m)	δ_{CH}	$-CH_3$
1191.80 (m)	ν_{C-O-C}	ROOR′
1062.59 (m)	ring vibration	polycyclic compounds
958.45 (w)	ν_{C-C}	−C−C−
619.04 (w)	δ_{NH}	−NH−

[a] s, strong intensity; m, middle intensity; w, weak intensity. [b] ν, stretching vibration; δ, deformation vibration.

Analysis of Phytosterols and Phytosteryl Esters. Phytosterols and phytosteryl esters synthesized under optimum conditions were analyzed by FT-IR, GC, and GC-MS, respectively.

Analysis of Phytosterols and Phytosteryl Esters by FT-IR. The FT-IR spectra of phytosterols and the potential functional groups are shown in Table 4. The ring vibration at 1062.59 cm^{-1} corresponds to the character of steroid. The stretching vibration at 3426.88 cm^{-1} indicates the presence of a −OH group. The bands at 2937.06 and 1465.64 cm^{-1} are the stretching vibration and the deformation vibration of CH_2 or CH_3 groups. The band 1382.71 cm^{-1} shows the symmetrical bending model of a CH_3 group. The band at 740.53 cm^{-1} reveals that there are four or more CH_2 groups in the side chain.

The FT-IR spectra of phytosteryl esters and the potential functional groups are shown in Table 5. The absorbance $\nu_{C=O}$ and ν_{C-O-C} stretching bands at 1737.55 and 1191.80 cm^{-1} of phytosteryl esters and without the −OH group characteristic absorption band above 3000 cm^{-1}, which are different from the characteristic absorption of phytosterols, indicate the presence of steryl esters of the sample.[14]

Analysis of Phytosteryl Esters by GC and GC-MS. GC analysis was employed to determine the phytosterol and phytosterol esters of L-glutamic acid. Gas chromatography of free phytosterols as substrate showed that the campesterols, stigmasterols, and

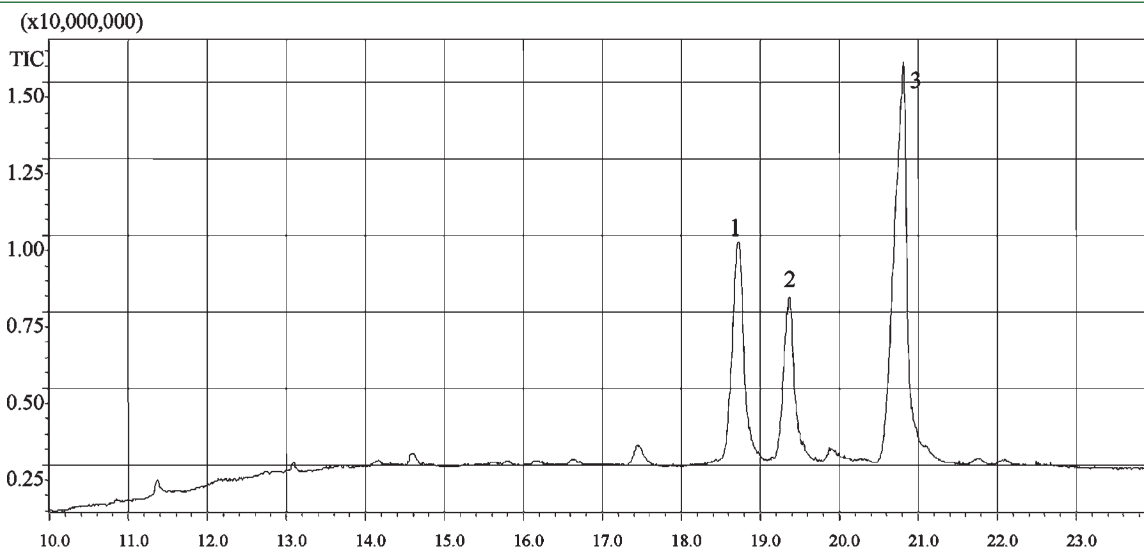

Figure 1. Gas chromatographic separation of the substrate phytosterols; peaks and RRT: (1) campesterol, 18.729 min; (2) stigmasterol, 19.367 min; (3) β-sitosterol, 20.808 min.

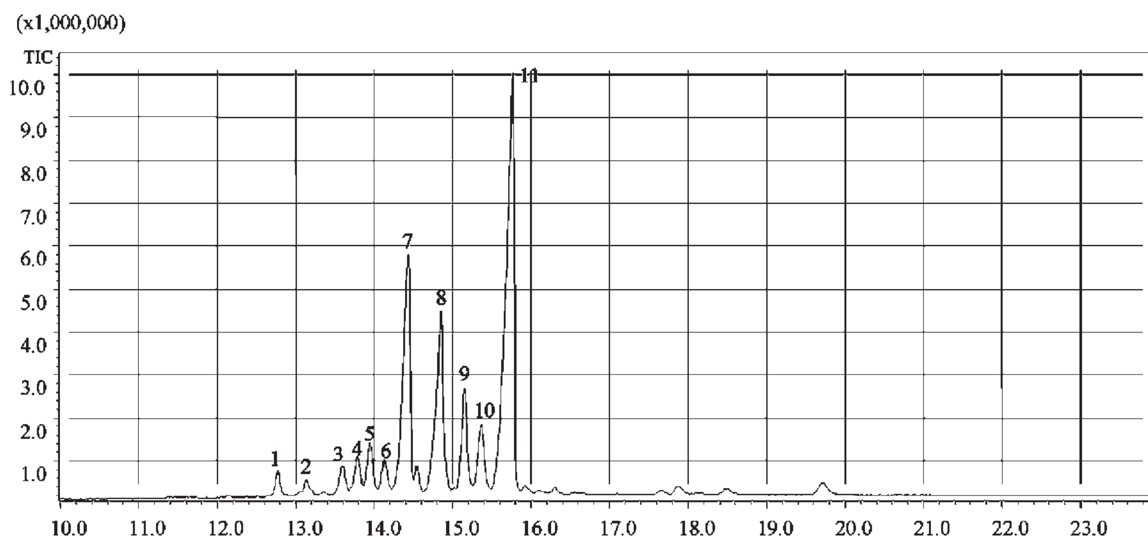

Figure 2. Gas chromatographic separation of the synthesized phytosteryl L-glumatic esters; peak numbering according to Table 6.

Table 6. Characteristic Fragment Ions of Phytosteryl L-Glutamic Esters (GC-EI MS; 70 eV)

phytosteryl ester[a]	RRT[b]	characteristic fragment ions, m/z (relative abundance)
1	12.773	43 (100), 57 (89), 71 (58), 145 (38), 81 (37), 105 (25), 159 (18), 261 (16), 383 (10), 450 (1)
2	13.135	55 (100), 83 (88), 69 (48), 159 (39), 255 (38), 41 (36), 121 (35), 213 (10), 395 (16), 380 (6), 339 (4)
3	13.604	55 (100), 81 (84), 43 (77), 105 (77), 145 (39), 159 (27), 213 (15), 255 (37), 281 (5), 337 (3), 381 (18)
4	13.791	43 (100), 145 (73), 81 (67), 57 (61), 145 (73), 159 (32), 213 (17), 275 (26), 339 (2), 382 (9), 397 (20)
5	13.944	43 (100), 105 (73), 91 (61), 145 (32), 213 (20), 255 (26), 329 (3), 341 (3), 368 (13), 383 (11), 450 (0.2)
6	14.132	43 (100), 135 (68), 143 (56), 119 (39), 211 (6), 253 (7), 261 (19), 284 (4), 340 (1), 366 (3), 381 (14), 397 (3), 604 (1)
7	14.438	43 (100), 55 (61), 81 (84), 105 (82), 145 (68), 147 (90), 159 (28), 199 (10), 213 (27), 274 (27), 341 (5), 368 (23), 383 (17), 395 (2)
8	14.854	55 (100), 81 (90), 83 (57), 105 (52), 147 (38), 159 (27), 213 (15), 255 (37), 282 (7), 352 (3), 395 (40), 450 (1)
9	15.152	43 (100), 57 (66), 91 (64), 145 (40), 201 (14), 213 (23), 229 (5), 255 (33), 275 (7), 343 (15), 382 (5), 397 (52)
10	15.368	57 (63), 81 (53), 95 (41), 119 (42), 135 (75), 147 (29), 163 (14), 211 (7), 253 (10), 275 (19), 281 (3), 339 (4), 380 (4), 395 (42)
11	15.771	147 (100), 43 (83), 57 (54), 105 (84), 121 (37), 159 (30), 213 (34), 255 (32), 274 (6), 288 (29), 355 (6), 382 (32), 397 (78)

[a] Peak numbers. [b] Relative retention time correspond to Figure 2.

β-sitosterols were eluted with relative retention times of 18.729, 19.367, and 20.808 min (Figure1).

GC-MS was utilized to investigate phytosterol esters with L-glutamic acid. The result of gas chromatography is shown in Figure 2, and characteristic fragment ions of steryl L-glutamic esters with mass spectrometry according to the peak number in Figure 2 are summarized in Table 6. Steryl acetates can be separated sufficiently by using the HP-5 as the stationary phase, and the relative times were earlier than those of the corresponding phytosterols. Mass spectrometry of the corresponding campesteryl, stigmasteryl, and β-sitosteryl acetates as examples are shown in Figure 3. It can be seen that all steryl acetates had primary fragment peaks of m/z 255, 213, 145, indicating that the steroidal nuclei were possessed. The maximum detectable masses of the campesteryl and stigmasteryl acetates were less than that of the molecular ion by 60 mass units, indicating that they lost one molecule of acetate. Other fragment ions of the steryl acetates remain consistent with the description in the literature concerning the electron impact mass spectra of steryl esters.[15−19]

NMR Analysis. Phytosterols and synthesized and isolated steryl esters of L-glutamic acid were identified by ^{1}H and ^{13}C NMR: ^{1}H NMR, δ 7.706 (2H, d, $N\underline{H}_2$), 5.377 (1H, m), 5.353 (1H, m), 4.583−4.754 (1H, m, >C$\underline{H}$−O−CO−), 3.652 (2H, m, >C$\underline{H}_2$−), 2.782−2.910 (10H, m), 2.271−2.286 (6H, m), 1.995−2.117 (4H, m), 1.378−1.396 (3H, m, CH_3), 1.007 (3H, s, CH_3), 0.919 (3H, d, J = 6.6 Hz, CH_3), 0.844 (3H, d, J = 6.6 Hz, CH_3); ^{13}C NMR δ 11.60, 13.85, 18.89, 19.40, 20.23, 23.09, 24.31, 26.13, 28.26, 29.18, 31.90, 34.77, 36.16, 37.28, 39.8, 42.33, 50.14, 56.77, 62.62, 64.89, 66.57, 68.41, 73.74, 79.52, 121.66, 122.35, 140.02, 171.41, 172.09, 172.84, 173.23. Signals from the carbonyl carbon of the steryl ester carbon at 173.23, 172.84, 172.09, and 171.41 ppm and the methine adjacent to oxygen at δ 62.62, 64.89, 66.57, and 68.41 were observed, and these signals were not detected in the NMR spectrum of phytosterols. The secondary alcohol carbon signal at C-3 of phytosterol was observed at 71.73 ppm, but this signal shifted to δ 73.74 for the steryl ester. Also, methine peaks from the steroid skeleton were observed at δ 56.77 and 50.14. It was therefore

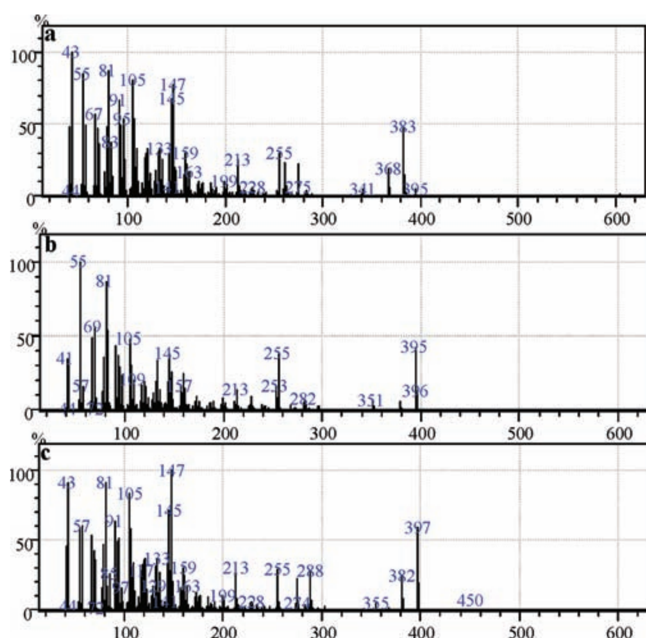

Figure 3. Electron-impact ionization mass spectra of campesteryl L-glumatic acid ester (a), stigmasteryl L-glumatic acid ester (b), and sitosteryl L-glumatic acid ester (c).

shown that L-glutamic acid was bound at the hydroxy group at C-3 of the phytosterol.[13,20] In combination with the gas chromatographic separation of the steryl esters in Figure 2, it can be inferred that there formed different kinds of carbonyls between phytosterols and different carboxyls of L-glutamic acid.

This work has shown that phytosteryl esters with L-glutamic acid were synthesized, supplying a promising alternative to the substrate on esterification of phytosterols. The steryl esters of amino acids are expected to have lower melting points and greater solubility in oils compared to the corresponding phytosterols with free hydroxyl groups. Furthermore, this synthesis approach of phytosteryl esters with amino acid can be readily applied to further steryl ester studies.

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