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Study of extraction of phytosterol from masson pine raw tall oil

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Abstract The conditions for the extraction of phytosterol from masson pine raw tall oil were studied by experimenting with the procedural steps that included saponification, extraction, crystallization, and recrystallization. The influences of alkali dosage, saponifying temperature, saponifying time, and variety and dosage of extracting agent on the yield of unsaponifiables were investigated. The effects of various solvents used for removing residual soap from unsaponifiables and the effects of crystallization temperature, time, and solvent on the yield and composition of crystal in the processes of crystallization and recrystallization were examined to determine suitable technical conditions. The phytosterol obtained using optimal conditions contained 92% β-sitosterol and 8% campesterol. Its melting point and yield were 135.7°–136.8°C and 0.54% (based on the weight of crude tall oil), respectively.

Key words Phytosterol · Extraction · Masson pine · Raw tall oil

Introduction

Phytosterol is an important compound and has wide application. In particular, β-sitosterol, one of the sterols, has curative effects that decrease blood cholesterol levels, inhibit the growth of tumors, and exert antiinflammatory and antibacterial actions.[1–6] β-Sitosterol is an important intermediate for synthesizing some medicines,[7,8] and it has good physiological activity for stimulating hair growth, moisturizing skin, and promoting skin renewal.[9–11] β-Sitosterol can also be consumed as a functional food, is an antioxidant, and is used for producing artificial butter containing low cholesterol, among other uses.[12,13]

Natural β-sitosterol widely exists in the shell of cereals, bark, and leaves of plants in the forms of the free state, glucoside, and fatty acid ester. The main resources used for extracting β-sitosterol are soap residues of vegetable oil (e.g., soybean oil, rapeseed oil, cottonseed oil) and the by-product of softwood kraft pulping tall oil. Among them, tall oil is a favored material for extracting β-sitosterol because of its high content of phytosterol. In addition, the main phytosterol in tall oil is β-sitosterol. There are two kinds of tall oil in China; one is from masson pine, and the other is from mixed materials from larch and Korean pine. Analyzing the compositions of the unsaponifiable matter showed that the β-sitosterol content is higher in the unsaponifiable matter of masson pine tall oil than in that of larch or Korean pine. In addition, the contents of cycloartenol and other impurities in the latter two are higher than in masson pine tall oil. Hence, masson pine tall oil is more suitable for extraction of β-sitosterol. Furthermore, there were no detailed reports about extracting β-sitosterol from masson pine tall oil up to now.

The main purpose of this study was to determine suitable conditions for extracting phytosterol from masson pine raw tall oil to obtain a product with high yield and high β-sitosterol content.

Materials and methods

Raw materials and agents

Masson pine raw tall oil was obtained from the pulp and papermaking factory in Qingzhou (China). Bis-(trimethylsilyl)trifluoroacetamide (BSTFA), a silylation agent, was chromatography grade (E. Merck, Darmstadt, Switzerland). The other agents and solvents were all of analytical grade.

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Extraction of unsaponifiables from raw tall oil

The experimental process for separating phytosterol from raw tall oil[14–16] is shown in Fig. 1. Saponification was carried out in a flask fitted with an agitator, a reflux condenser, and a thermometer. The kettle was charged with raw tall oil (20 g), water (10 g), alcohol (10 g), and KOH solution (3.6 g dissolved in 3 ml water) in that order. The saponification of raw tall oil was carried out at 80°–120°C for 3–4 h. The reacted mixture was transferred to a separating funnel and extracted with a suitable solvent several times (see Results and discussion for solvent types). The organic phase was washed with distilled water until neutrality was obtained. The organic layer was dried, and the solvent was evaporated to obtain the raw unsaponifiables. The raw unsaponifiables were treated with acetone and butanone to remove the residual soap and to obtain the refined unsaponifiables.

Crystallization and recrystallization of phytosterol from refined unsaponifiables

Raw phytosterol was obtained from the refined unsaponifiables by crystallization in ethyl acetate or other solvents. Unsaponifiable extract (6.0 g) was placed in a 50-ml triangular flask and dissolved in ethyl acetate or other solvents to give a 40% (w/w) solution. The solution was crystallized at 10°–30°C. The raw phytosterol crystals were obtained by centrifugation, filtration, and drying.

Raw phytosterol was recrystallized in a mixed solvent of methanol and *n*-hexane (1:2–4:1, v/v) to obtain the refined phytosterol. Raw phytosterol was first dissolved in the mixed methanol/*n*-hexane solvent, and the solution was then crystallized at 5°–25°C for 10–30 h. The crystals of refined phytosterol were obtained by filtration, washed with the mixed solvent, and dried at room temperature under vacuum.

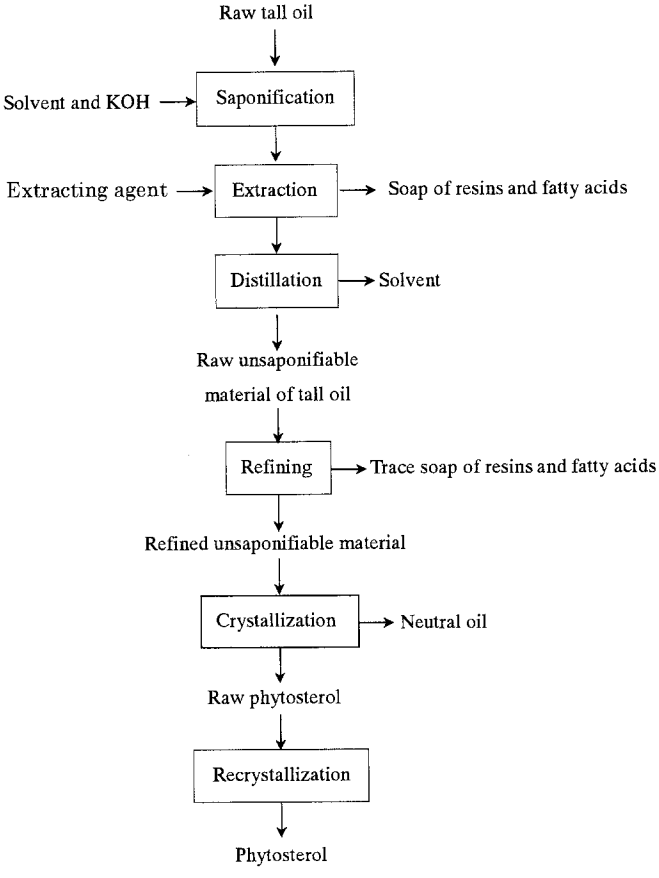

Fig. 1. Flow diagram for extracting phytosterol from raw tall oil

Composition analysis and melting point measurement of crystals

Before undergoing gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS) analyses, the components of the isolated phytosterol were derivatized by silylation.[17] The silylation operation was as follows: phytosterol (6 mg) was dissolved in 0.5 ml of trichloromethane, and the solution was injected into a capillary tube. The capillary tube was sealed after 0.05 ml of BSTFA (0.97 g/ml) was injected into it. The mixture was then left at 120°C for 4 h. The resultant solution was used for GC and GC-MS analyses.

A Shimadzu GC-9A GC equipped with hydrogen flame ionization detector (FID) was used for analyzing the composition of the obtained phytosterol. Separation was conducted on a DB-1 glass capillary column (30 m × 0.25 mm i.d.). The operating conditions of the column were as follows: helium was used as the carrier gas at a flow rate of 1 ml/min, and the split ratio was 1:50; the temperature of the gasifying chamber and the detector were both 305°C; the inlet temperature was 280°C; and the column temperature was elevated from 150°C to 320°C at a rate of 5°C/min, after which the temperature was held at 320°C for 30 min.

Component identification of crystals was conducted on a JEOL JMS-D300 GC-MS system. The glass capillary column and GC operating conditions were the same as stated above. The operating conditions of the mass spectrometer were as follows: electron ionization as an ion source, electron energy 70 eV, accelerating voltage 3 kV, ionization current 300 μA, scanning frequency 2 s, temperature of ionization source 210°C, scanning scope from m/z 40–400 to m/z 40–600, temperature of the joint between GC and MS 300°C.

The GC peaks were identified primarily on the basis of MS data obtained from online GC-MS by comparison with published MS spectra data.[18–20] In addition, some peaks were identified by comparing their relative retention times and MS data with those of known compounds.

The melting point of phytosterol was measured with a B-shaped melting point apparatus.

Results and discussion

It was found from the experimental processes that the yield and purity of phytosterol obtained from raw tall oil were

Table 1. Effect of extraction solvents on the yield and color of unsaponifiables

Extraction effects	Benzene	Toluene	Petroleum ether	*n*-Hexane	Cyclohexane	Diethyl ether
Yield of unsaponifiables (%)	5.0	5.1	3.5	4.8	4.7	4.7
Color of obtained unsaponifiables	Light brown	Hazel	Light yellow	Light yellow	Light yellow	Light yellow

Table 2. Effect of saponification temperature on the yield of unsaponifiables

Saponification temperature (°C)	Yield of unsaponifiables (%)
60	4.3
70	4.6
80	4.8
90	7.1
100	7.9
110	10.3
115	10.4
120	10.4

influenced by many factors, such as saponification temperature, saponification time, alkali dosage for saponification, extracting agent, solvent for crystallization, crystallization temperature, and crystallization time.

Influence of saponification conditions on the yield of unsaponifiables

During the process of extracting phytosterol, the yield of unsaponifiables directly influences the total yield of phytosterol. It was found that the yield of unsaponifiables was mainly influenced by such factors as the sort of solvent used for extraction, the saponification temperature and time, and the alkali dosage for complete saponification.

Alkalis adopted for saponification may be any one of LiOH, NaOH, KOH, or RbOH. From the point of view of cost, sodium hydroxide and potassium hydroxide are preferable to others. Although calcium hydroxide is much less expensive, it is unsuitable for tall oil saponification because the calcium salt produced is insoluble in an organic solvent such as alcohol. Moreover, it easily contaminates the final product. In this study, KOH was chosen to saponify the raw tall oil.

Influence of various solvents on the yield of unsaponifiables

The extraction effects of various solvents are shown in Table 1. The reaction temperature was 80°C, and the reaction time was 4h. It was found that the solvents had different extraction effects. The yields of unsaponifiables obtained using aromatic hydrocarbons such as benzene and toluene were slightly higher than those using aliphatic hydrocarbons such as *n*-hexane, cyclohexane, or petroleum ether. However, the color of the former extracts were much deeper than those of the latter. Moreover, if an aromatic solvent such as benzene and toluene was used as an extracting agent, the reacted liquid easily caused emulsification; but if diethyl ether was used as the extracting agent, the solvent was difficult to recover because of its low boiling point. The extracting effect of *n*-hexane was close to that of cyclohexane, but cyclohexane was much more expensive than *n*-hexane. Therefore, using *n*-hexane as the extracting agent was more suitable.

Influence of solvent on the saponification reaction

A mixture of water and alcohol was used as solvent in the saponification reaction. Alcohol plays a role in defoaming and accelerating the dispersal of tall oil in the basic liquid, especially at the beginning of saponification. The test results indicated that the alcohol/water ratio greatly affected the reaction. When the water/alcohol ratio was 2:1 by weight, and when the tall oil/mixed solvent ratio was 1:1 by weight, the reactant liquor was difficult to agitate because it stuck to the stirring bar at the initial saponification stage. When the tall oil/mixed solvent ratio increased to 1:2 to 1:4, an accompanying disadvantage was an increase in foaming when the reaction temperature was over 90°C, although agitation was greatly improved. The reaction temperature was difficult to raise over 90°C when the water/alcohol ratio was reduced to 1:2 and when the tall oil/mixed solvent ratio was 1:1. However, the reacting material was also difficult to stir if the tall oil/mixed solvent ratio increased to 2:1. Only when both ratios (water/alcohol and tall oil/mixed solvent) were within 1:1–1:2 the foregoing difficulty could be overcome; the reaction temperature could reach 115°–130°C and the reactant material could be stirred easily.

Influence of saponification temperature on the yield of unsaponifiables

The effect of the saponification temperature on the yield of unsaponifiables is shown in Table 2. The water/alcohol ratio was 1:1 (w/w), the concentration of tall oil was 50% by weight, and *n*-hexane was used as the extracting agent. Test results showed that the saponification temperature greatly influenced the yield of unsaponifiables. The yield increased along with the increase in reaction temperature during the same period of time. When the temperature reached 110°C, the yield reached 10.3% and did not increase any more as the temperature rose further. The suitable temperature was thus 110°–115°C.

Influence of alkali dosage on saponification time

The influence of the alkali dosage on the reaction time is described in Table 3. The saponification temperature was

110°–115°C. Test results showed that the reaction time decreased with the increase in alkali dosage. When the alkali dosage was threefold the saponification value for raw tall oil, the time needed for complete saponification was only 30 min. However, the alkali dosage directly influenced the washing of the unsaponifiables: the higher the alkali dosage, the more difficult it was to wash up to neutrality. Hence, judging from both the washing time and the reaction time, the suitable alkali dosage was 1.5-fold the saponification value for raw tall oil.

The compositions of unsaponifiables obtained under the aforesaid suitable saponification conditions were analyzed by GC and GC-MS. Analysis results showed that the unsaponifiables consisted of 3.6% sesquiterpenes, 4.4% diterpenes, 31.2% resin aldehydes, 6.5% fatty alcohols, 1.5% phenoloid, 32.7% steroid, and 6.0% potassium soap of resin and fatty acids. In the steroid, the contents of β-sitosterol, β-sitostanol, campesterol, campestanol, and cycloartenol were 70.6%, 10.1%, 4.9%, 0.3%, and 3.6%, respectively.

Separation of residual soap from raw unsaponifiables

It was difficult to remove the residual soap completely from the unsaponifiables by simply washing with water because of the lower solubility of some soap of resins and fatty acids. Separating soap from unsaponifiables was carried out by choosing solvents in which phytosterol had good solubility but in which the soap was insoluble. The test conditions were as follows: raw unsaponifiables (10 g) were dissolved in 30 ml of solvent and refluxed for 1 h; the insoluble soap was then removed by centrifugation filtration at 30°C. The obtained filtrate was distilled to recover the solvent. The separation effects of the solvents are shown in Table 4. It is known that most of the residual soap can be removed by this method. The solvents had different separation effects. Among them, the most preferable was butanone, which thoroughly removed the soap.

Separation of phytosterol from unsaponifiables

Phytosterol was separated from unsaponifiables by means of crystallization. Because the phytosterol obtained was used mainly in the fields of cosmetics and medicine, the solvent selected usually should be suitable under the following conditions. First, the solubility of the phytosterol, especially β-sitosterol, must be much higher at high temperatures than at low temperatures, and there must be a great solubility difference between the impurity and the phytosterol in the solvent. Second, the solvent cannot react with phytosterol, and it must be inexpensive, nontoxic to humans, and easy to remove from phytosterol. In consideration of the foregoing conditions, the crystallization effects of some common solvents were explored.

Crystallization behavior of phytosterol in ethyl acetate

The crystallization behavior of phytosterol in ethyl acetate is shown in Table 5. The solution of unsaponifiables was prepared at a concentration of 40% by weight. It was found that the crystallization temperature had a great influence on the crystal yield. For the first crystallization, the crystal obtained was only about 0.1% (based on the weight of unsaponifiables) when the crystallization temperature was 28°–30°C. The crystal yield increased as the temperature decreased, but the melting point of the crystal decreased slightly, indicating that some impurities contaminated the isolated phytosterol at lower temperatures. The melting point of the crystals increased to 135°–137°C when the crystals obtained in Exp. 3 were used for the second crystallization. The composition of crystals obtained in Exp. 3 and Exp. 4 are shown in Table 6. It was found that the main

Table 3. Relation of alkali dosage and saponification time

Alkali dosage (multiple of saponification value)	Saponification time (min)
1.0	240
1.5	180
2.0	90
2.5	60
3.0	30

Table 4. Effect of various polar solvents on the separation of unsaponifiables from soap

Solvent	Content of soap in unsaponifiables (%)
1,2-Dichloroethane	0.8
Dichloromethane	0.4
Acetone	0.3
Butanone	0

Table 5. Effect of ethyl acetate solvent on crystallization of phytosterol

Exp. no.	Crystn temperature (°C)	Crystn time (h)	Crystn yield (%)	Melting point (°C)
1	28–30	50	0.1	136–138
2	20–22	50	1.2	134–137
3	10–12	50	12.7	132–134
4	10–12	40	8.2	135–137

Crystn, crystallization; Exp., experiment
The crystal obtained in Exp. 4 was the product of recrystallization of Exp. 3

phytosterols were β-sitosterol, β-sitostanol, and campesterol, and the β-sitosterol content reached 78.8%. If it was recrystallized in Exp. 4, the β-sitosterol content could be increased to 84.4%. This showed that using ethyl acetate as solvent for crystallization had good selectivity for β-sitosterol, but the crystal yield is low.

Crystallization behavior of phytosterol in methanol

The crystallization behavior of phytosterol in methanol is shown in Table 7. The test results demonstrated that the crystallization effect of methanol was much better than that of ethyl acetate. The yields were all more than 14.2%. The composition of crystals obtained at different crystallization temperatures were also different. At 60°–62°C the crystal yield reached 14.2%, and the recovery ratio of β-sitosterol reached 51.1% (based on the total amount of β-sitosterol contained in unsaponifiables). In addition, the purity of β-sitosterol was a little higher than that obtained when using ethyl acetate.

Crystallization behavior of phytosterol in ethanol, isopropanol, and nitromethane

The experimental conditions when using ethanol, isopropanol, or nitromethane were the same as those when using methanol as the solvent for crystallization. The test results appear in Table 8, which shows that the crystallization behavior of phytosterol in nitromethane was better than that in ethanol or isopropanol at the same temperature and over the same time frame. For nitromethane, the crystal yields at different temperatures were different. Table 9 reveals that the lower the crystallization temperature, the lower was the content of phytosterol in crystals obtained.

Table 6. Compounds and contents of crystals obtained during Exp. 3 and Exp. 4 in Table 5

Compounds	Crystals obtained (%)	
	Exp. 3	Exp. 4
Campesterol	5.5	7.4
Campestanol	0.3	0.2
β-Sitosterol	78.8	84.4
β-Sitostanol	11.1	6.7
Cycloartenol	2.1	0.7
Other compounds	2.3	0.8

Comparing the crystallization effect of ethyl acetate, methanol, and nitromethane from the point of view of β-sitosterol purity, methanol was better than either ethyl acetate or nitromethane. The β-sitosterol content in crystals obtained by crystallization in methanol reached 83.1% (Table 7). However, from the point of view of crystal yield, the nitromethane was preferable to methanol. Generally, methanol has an effect similar to that of nitromethane and much better than that of ethyl acetate. However, nitromethane has the fatal drawbacks of high price and strong toxicity, and it easily explodes. Accordingly, methanol was considered the ideal solvent for phytosterol crystallization.

The β-sitosterol content in the crystals obtained by the foregoing method was only 83.1% by analysis. Recrystallization is necessary to improve the content of β-sitosterol.

Recrystallization of raw phytosterol

The mixed solvents of *n*-hexane and methanol were adopted for recrystallization of raw phytosterol. The polarity of the mixed solvents was adjusted by controlling the methanol/*n*-hexane ratio to improve the crystallization selectivity of β-sitosterol. The influence of the methanol/*n*-hexane ratio as well as the temperature and time on recrystallization were explored.

Table 8. Effect of ethanol, isopropanol, and nitromethane crystallization solvents on the yield of crystals

Crystn temperature (°C)	Crystn time (h)	Crystal yield[a] (%)
Ethanol		
60–62	1	–
50–52	1	1.1
40–42	1	1.7
30–32	2	2.3
20–22	5	15.4
Isopropanol		
50–52	1	–
30–32	2	1.8
20–22	5	11.3
Nitromethane		
60–62	1	14.5
50–52	1	18.7
40–42	1	39.6
30–32	1	70.3

[a]The yield of crystal was based on the total unsaponifiables

Table 7. Effect of methanol solvent on crystallization of phytosterol

Crystn temp (°C)	Crystn time (h)	Crystal yield[a] (%)	Composition of crystal (%)							
			CE	CA	SE	SA	CY	DA	TA	OT
60–62	1	14.2	5.6	0.1	83.1	10.7	0.5	–	–	–
50–52	1	17.6	5.4	0.2	80.2	9.8	2.3	–	0.3	1.8
40–42	1	37.6	3.5	0.3	51.3	7.4	2.7	3.6	8.4	22.8
30–32	1	68.3	2.5	0.2	36.2	5.3	1.9	2.5	5.9	54.5

[a]The yield of crystal was based on the total unsaponifiables
CE, campesterol; CA, campestanol; SE, β-sitosterol; SA, β-sitostanol; CY, cycloartenol; DA, docosanol; TA, tetracosanol; OT, other compounds

Table 9. Effect of crystallization temperature on the composition of crystals obtained with nitromethane solvent

Compound	Content (%)			
	60°–62°C	50°–52°C	40°–42°C	30°–32°C
Campesterol	6.1	5.7	3.4	1.8
Campestanol	0.2	0.3	0.3	0.1
β-Sitosterol	80.3	78.8	51.7	34.1
β-Sitostanol	12.7	11.3	7.6	4.9
Cycloartenol	0.7	1.3	1.8	1.6
Docosanol	–	–	3.7	2.7
Tetracosanol	–	0.9	8.8	5.6
Other compounds	–	1.7	22.7	49.2

Table 10. Effect of the methanol/*n*-hexane ratio on the yield and composition of crystals

Methanol/*n*-hexane (v/v)	Yield of crystals[a] (%)	Composition of crystals (%)				
		CE	CA	SE	SA	CY
1:2	27.1	5.2	–	94.8	–	–
1:1	36.3	8.0	–	92.0	–	–
2:1	44.5	6.3	–	92.3	1.4	–
3:1	63.2	6.1	–	89.1	4.8	–
4:1	70.1	5.9	0.1	86.3	7.4	0.3

[a] The yield of crystals was based on the raw crystal

Effect of the methanol/n-hexane ratio on the yield and composition of crystals

The influence of the methanol/*n*-hexane ratio on the yield and composition of crystals is shown in Table 10. The crystallization temperature and time were 10°–15°C and 20h, respectively. The test results showed that the methanol/*n*-hexane ratio had a great influence on not only crystal yield but also crystal composition. When the methanol/*n*-hexane ratio was 1:1 (v/v), the crystal obtained consisted of β-sitosterol and campesterol, and the β-sitosterol content reached 92%. Furthermore, the crystal yield was 36.3% (based on the weight of raw phytosterol).

Effect of recrystallization time and temperature on yield and composition of crystals

The influence of recrystallization time and temperature on the yield and composition of crystals is shown in Table 11. A methanol/*n*-hexane ratio of 1:1 (v/v) and a recrystallization temperature of 10°–15°C had been chosen when exploring the influence of the recrystallization time; and a recrystallization time of 20h had been chosen when exploring the influence of the recrystallization temperature. The test results indicated that the lower the recrystallization temperature, the higher was the crystal yield. However, the β-sitosterol content was higher at 10°–15°C. Consequently, a suitable temperature was thought to be 10°–15°C. As for the crystallization time, 20h was considered more suitable than other times from the points of view of the yield and the purity of the phytosterol obtained.

Table 11. Effect of recrystallization time and temperature on the yield and composition of crystals

Recrystallization condition	Yield of crystals[a] (%)	Composition of crystals (%)				
		CE	CA	SE	SA	CY
Time (h)						
10	21.4	8.6	–	91.4	–	–
20	36.3	8.0	–	92.0	–	–
30	39.7	7.6	–	91.7	0.7	–
Temperature (°C)						
4–5	40.4	6.3	0.1	89.4	3.8	0.4
10–15	36.3	8.0	–	92.0	–	–
20–25	17.8	9.7	–	90.3	–	–

[a] Yield of crystals was based on the raw crystal

Conclusions

The extraction conditions for phytosterol from masson pine raw tall oil obtained from the pulp and papermaking factory in Qingzhou (China) were investigated in detail. Suitable conditions for saponification of raw tall oil and crystallization and recrystallization of phytosterol were determined.

Suitable saponification conditions were as follows: a mixture of water and ethanol was used as the solvent for saponification; other conditions were as follows: ethanol/water ratio 1:1 (w/w); raw tall oil/solvent ratio 1:1–1.2 (w/w); saponification temperature 110°–115°C; and saponification time 3h. KOH was used for saponification, and its dosage was 1.5-fold the value for saponification of raw tall oil.

n-Hexane was used as an extracting agent for isolating unsaponifiables. The yield of unsaponifiables was 10.4% (based on the tall oil weight). Butanone could be used to remove residual soap from unsaponifiables completely.

Raw phytosterol was obtained under the following crystallization conditions: methanol was used as solvent, and the crystallization temperature was 60°–62°C. The content of β-sitosterol in raw phytosterol was 83.1%, and the yield of raw phytosterol reached 14.2% (based on the weight of the unsaponifiables). Phytosterol containing 92% β-sitosterol was obtained when the raw phytosterol was recrystallized under the following conditions: recrystallization temperature 10°–15°C, recrystallization time 20h, and a methanol/*n*-hexane ratio 1:1 (v/v). The yield of refined phytosterol was 36.3% (based on the weight of raw phytosterol); and the final yield of phytosterol was 0.53% (based on the weight of raw tall oil). This extraction technology has a low operating cost, a simple operating process, high purity of the product, and other advantages. It is now possible to undertake industrial-scale extraction of β-sitosterol from masson pine raw tall oil.

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