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Novel Fractionation Method for Squalene and Phytosterols Contained in the Deodorization Distillate of Rice Bran Oil

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1. Introduction

To obtain the valuable constituents from natural products, "fractionation" is very important step in industrial processing. In addition, how it is efficient, how it is green for the environment, animals, nature and human, has been required for its standard. Especially in food industry, there is limitation of usage of organic solvent, which is well adopted for extraction of hydrophobic constituents from natural products. Although, hexane or ethanol has used for extraction in food industry at least in Japan, it is often less efficient than chloroform or methanol for extraction of low polar constituents such as lipids. In addition, it is difficult to remove residual solvent. Safe and simple extraction or fractionation methods have been required. In this point, supercritical fluid extraction method may be useful. In this chapter, after introducing about the key words of our study, we will present about - novel fractionation method for squalene and phytosterols contained in the deodorization distillate of rice bran oil.

2. Rice bran and rice bran oil

Rice bran, ingredient of rice bran oil (RBO), is co-product of milled rice containing pericarp, seed coat, perisperm and germ. In Japan, 1,000,000 t/year of rice bran have produced. One third is used for production of RBO and the residue is for feeding stuff, fertilizer, Japanese pickle and etc. Rice bran contains ~20% of RBO, and in RBO, unsaponifiables such as squalene and phytosterol are specifically highly contained compare to other seed oils (Table 1). Fatty acid in RBO is mainly composed by oleic acid (C18:1) and linoleic acid (C18:0). Palmitic acid (C16:0) is contained higher compare to other edible oils. In addition lower content of linolenic acid (C18:3) makes RBO stable for oxidation.

It is known that RBO exerts cholesterol lowering effect, for example, Chou et al. reported that RBO diet improves lipid abnormalities and suppress hyperinsulinemic responses in rats with streptozotocin/nicotinamide-induced type 2 diabetes (Chou et al., 2009). Physiological function of RBO is well documented in several reviews (Cicero and Gaddi, 2001; Jariwalla, 2001; Sugano et al., 1999).

In the process of produce RBO, crude RBO is steam-distillated for its flavor. The volatile component is called "deodorization distillate of RBO". The content of deodorization distillate of RBO is 0.15-0.45% varied with its condition of distillation. The deodorization

distillate of RBO is viscous and typical smelled liquid. Besides diacylglyceride and free fatty acid, deodorization distillate of RBO has nearly 40% of unsaponifiable substances such as squalene, phytosterols and tocopherol. Particularly it contains ca 10% of squalene in deodorization distillate of RBO.

	RBO	Soy been	Canola	Corn
Unsaponifiables/Oil (%)	2.31	0.46	0.87	0.96
Fatty acid composition (%)				
C16:0	16.4	10.5	4.2	10.4
C18:0	1.6	3.9	2.0	1.9
C18:1	42.0	23.3	60.8	27.5
C18:2	35.8	53.0	20.6	57.2
C18:3	1.3	7.6	9.2	1.2
Others	2.9	1.7	3.2	1.8

Table 1. Chemical properties and fatty acid compositions of major edible oils.

3. Squalene

Squalene is widely found in marine animal oils as a trace component, and has been extensively studied their preventive effect in many diseases such as cardiovascular diseases and cancer (Esrich et al., 2011; Smith, 2000). Recently it is also attracted attention as food factor (Bhilwade et al., 2010). Since it has been well known that the liver oil of some varieties of shark (*Squalidae* family), especially those inhabiting the deep sea, is rich in squalene, this substance has been fractionated from shark liver oil. On the other hand, squalene obtained from shark liver oil has not been fully utilized recently on humane grounds, due to the unstable supply of shark liver oil as an industrial material, the characteristic smell of fish oil, and the large variation of the constituents. Then, attention has shifted to squalene of plant origin, and the application of this type of squalene to cosmetics, medicine and functional foods has been attempted. For example, squalene originating from olive oil is being produced in Europe. However, the production is poor to make up for the sagging production of squalene from shark liver oil. Therefore, the possibility of extracting squalene from RBO is being investigated. Furthermore, because squalene is easily oxidized owing to its structural character having a lot of carbon double bond (Fig. 1.), novel fractionation method conducting in mild condition is required.

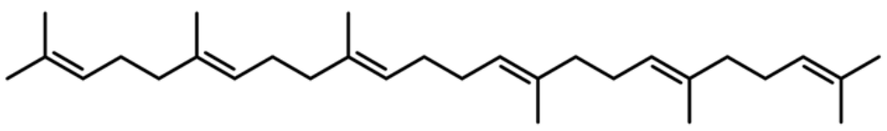

Fig. 1. Chemical structure of squalene.

4. Phytosterol

The main phytosterols in RBO are β-sitosterol, campesterol, stigmasterol and isofucosterol (Fig. 2.), and the content in phytosterols were ca 50%, 20%, 15%, 5%, respectively. It is well known that phytosterols inhibit cholesterol absorption on small intestine which results

cholesterol-reducing activity of phytosterols (Gupta et al., 2011; Niijar et al., 2010; Malinowski & Gehret, 2010). In addition, the sterol content of RBO is specifically high compare to other major oils from plant- ca 1% in RBO, and 0.2-0.5% in soy bean, canola, and corn oil. These phytosterols are effective substances for utilization for functional foods called foods for specified health use.

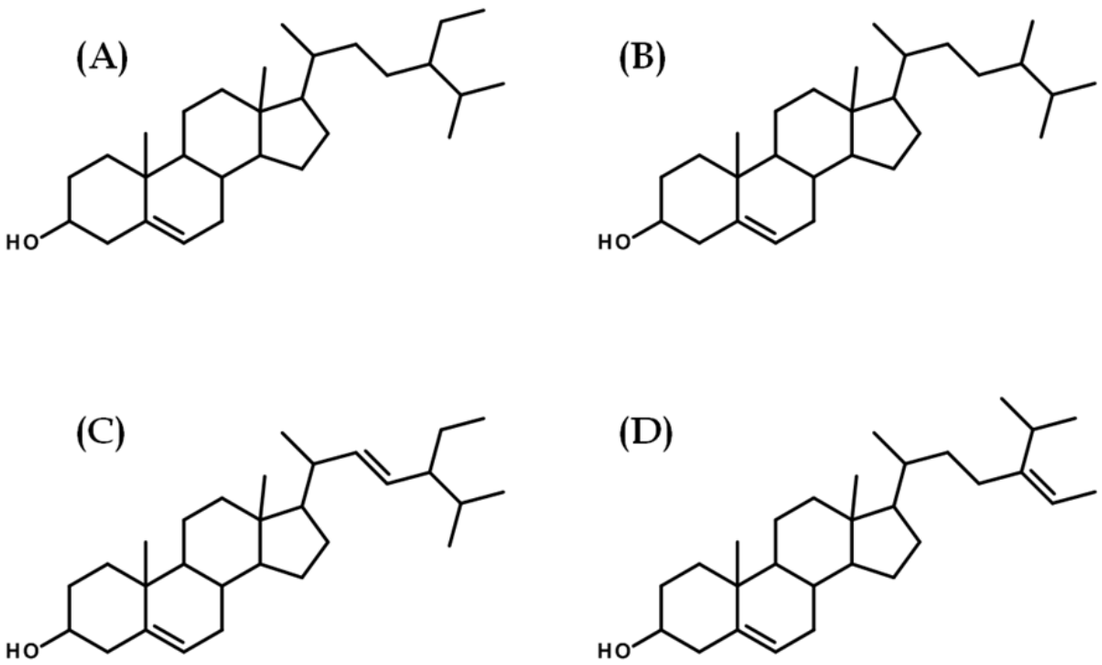

Fig. 2. Chemical structures of main phytosterols in RBO; (A) β-sitosterol, (B) campesterol, (C) stigmasterol, (D) isofucosterol.

5. Supercritical fluid extraction and supercritical fluid chromatography

Supercritical fluid is any substance at a temperature and pressure above its critical point, where distinct liquid and gas phases do not exist. It can effuse through solids like a gas, and dissolve materials like a liquid. There are mainly two techniques, supercritical fluid extraction (SFE) and supercritical fluid chromatography (SFC). For high fractionation selectivity, combination of SFE and SFC is recently adopted. Following advantages of SFE or SFC are known.

- Carried at low temperature-thermal denaturation of substances is poorly occurred.
- Inactive gas (CO_2)-denaturation and oxidation are almost ignored.
- Supercritical fluid is whole diminished after extraction.
- Bland, innocuous and harmless- applicable for food industry.

Carbon dioxide and water are the most commonly used supercritical fluids, being used for decaffeination of coffee been (Khosravi-Darani, 2010), extraction of eicosapentaenoic acid and docosapentaenoic acid from fish oil (Higashidate et al., 1990) and fractionation of terpenes from lemon peel oil (Yamauchi & Saito, 1990), respectively. For the detail of the application of supercritical fluid for variety of field including food industry, several reviews are available (Herrero et al., 2010; Khosravi-Darani, 2010; Zhao & Jiang, 2010).

6. Novel fractionation method for squalene and phytosterols contained in the deodorization distillate of rice bran oil

Although there are already some reports on methods of concentrating squalene originating from plants, the thermal and oxidative deterioration of squalene poses significant problems for its use in foods and medicines. In addition, solvent fractionation bears some difficulties related to proper solvent selectivity during processing, the elimination of the residual solvent from the squalene fraction, and the disposal of the waste fluid. In this chapter, we presented a novel method of concentrating the squalene and phytosterols contained in the deodorization distillate of rice bran oil by combining SFE, SFC and solvent fractionation. Under the supercritical condition, gas acquires unique characteristics: upon a slight pressure change, it becomes more dense, less viscous and more soluble (Xiao-Wen, 2005). Therefore, supercritical fluid has the excellent ability to separate and extract specific components from a mixture. Since the critical temperature and pressure of carbon dioxide are 31.1^{o}C and 72.8 kg/cm^{2}, respectively, there are many advantages to using supercritical carbon dioxide for extraction at room temperature: the extract undergoes no thermal and oxidative deterioration, no remnant gas is generated in the extract at atmospheric pressure and etc as described above. SFC is determined here that the method extracting from the solution mixed with silica gel as a stationary phase, not capillary column SFC or packed column SFC.

6.1 Experimental

6.1.1 Samples and reagents

The deodorization distillate of RBO used to concentrate squalene and phytosterols was supplied from Boso Oil & Fat Co., Ltd., (Funabashi, Japan). The composition of the deodorization distillate was as follows: squalene, 8.5%; phytosterols, 3.5% (sitosterol: 2.05%, stigmasterol: 0.69%, campesterol: 0.76%); tocopherol (Toc), 1.6%; triacylglycerol (TG), 58.0%; diacylglycerol (DG) + fatty acids (FA), 26.5%. Squalene standard of special grade and 2-propanol of HPLC grade were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), and all other reagents used were of special grade or first grade, as commercially available, and used without further purification, unless otherwise specified.

6.1.2 Condensation of squalene from the deodorization distillate

6.1.2.1 Condensation of squalene by SFE

To fractionate squalene from the deodorization distillate, SFE with supercritical carbon dioxide was employed. SFE makes it possible to extract the target compound by changing the density of supercritical carbon dioxide under certain conditions of temperature and/or pressure. In the present experiment, SFE was carried out with an SFC apparatus, model 880-81 (JASCO, Tokyo, Japan), and a 10- or 50-mL stainless vessel. The extractor was kept in a thermostatic oven, model CH-201 (Scinics Corporation, Tokyo, Japan) and temperatures ranging from 30 to 80^{o}C and pressures ranging from 80 to 220 kg/cm^{2} were examined at an extraction time of 2 h and a supercritical carbon dioxide flow rate of 7 mL/min. The squalene concentration in the extract was determined by TLC-FID analysis with hexane as the primary developing solvent and benzene/ethyl acetate (9:1, v/v) as the secondary developing solvent.

6.1.2.2 Condensation of squalene by SFC

The optimal conditions for squalene concentration by SFE were determined to be as follows: extraction temperature, 30°C; supercritical carbon dioxide pressure, 100 kg/cm^2; flow rate, 7 mL/min. However, squalene was only concentrated to 25%, and the other components in the extract had higher polarity, such as TG, DG and FA. Therefore, 1 to 3-fold higher quantities of activated silica gel as compared with the deodorization distillate were placed in the extraction vessel to adsorb the components with higher polarity, and then squalene was extracted and/or concentrated under the optimal conditions listed above by means of SFC as shown in Fig. 3.

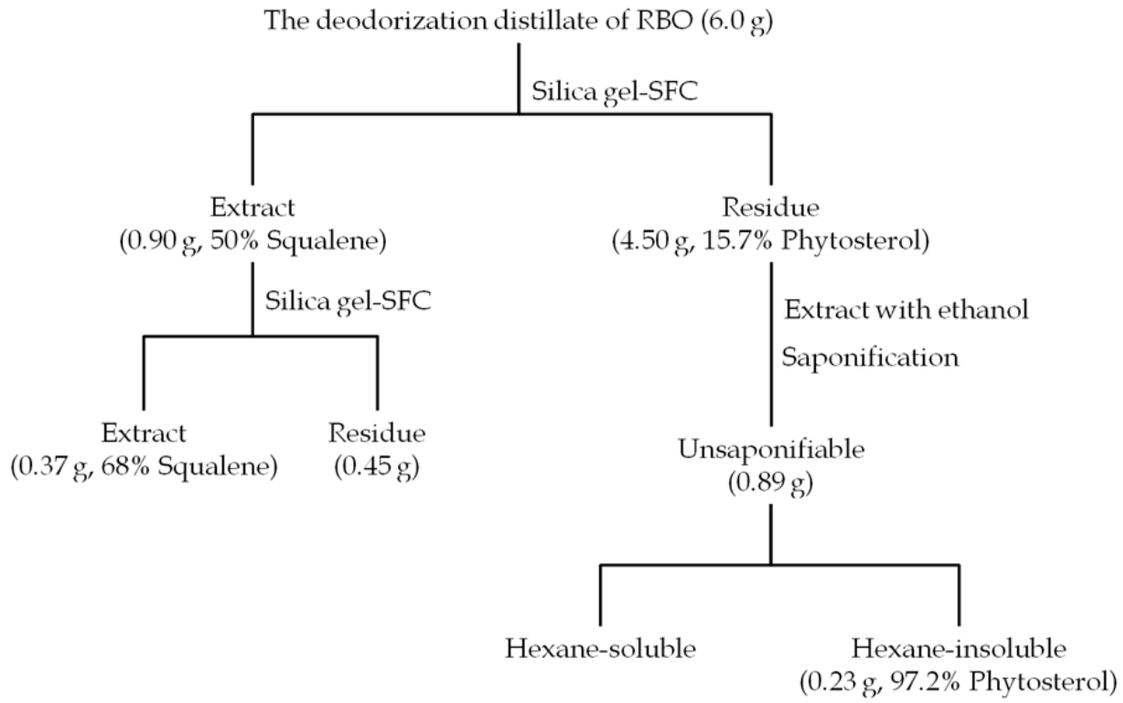

Fig. 3. Fractionation of squalene and phytosterols from deodorization distillate of RBO.

6.1.3 Condensation of phytosterols from the deodorization distillate

6.1.3.1 Condensation of phytosterols by SFC

Since phytosterols were concentrated in the residue after the extraction of squalene by SFC, further concentration of the phytosterols contained in 6 g of residue was attempted. In addition, the residue recovered as a result of SFE was also subjected to SFC to concentrate the phytosterols adsorbed onto the silica gel. In this case, the concentration ratio of phytosterols was compared with the amounts of residual phytosterols before and after the further extraction of squalene for 2 h, because phytosterols remained in the residue.

6.1.3.2 Condensation of phytosterols by solvent fractionation

The residue remaining in the vessel packed with silica gel were extracted with ethanol and then saponified by refluxing for 4 h with 3 mL of 25% potassium hydroxide aqueous

solution and 40 mL each of ethanol and hexane. After the saponification, the reactant was separated into a hexane layer and a hydrated ethanol layer.

6.1.3.3 Condensation of phytosterols from the unsaponifiable components of the deodorization distillate

Another means of concentrating phytosterols was also examined, as shown in Figs. 4: the phytosterols were concentrated after the saponification of the deodorization distillate.

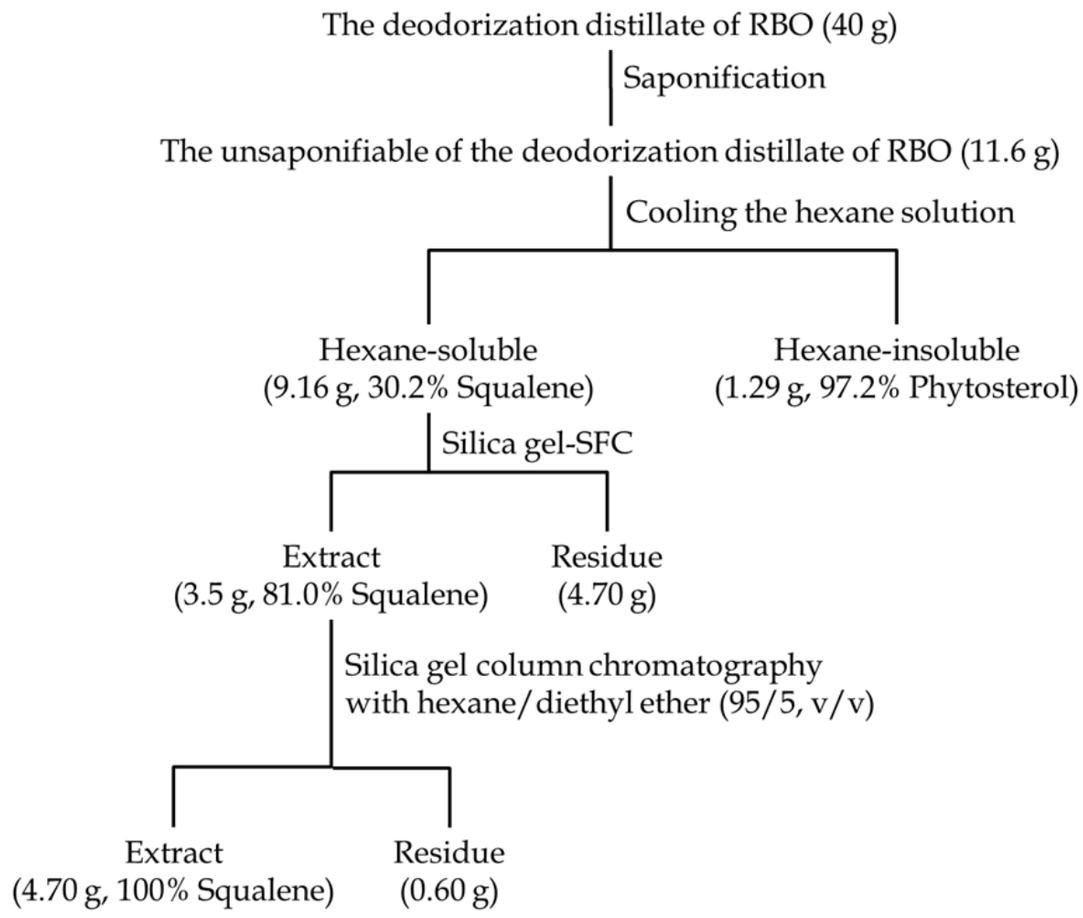

Fig. 4. Fractionation of squalene and phytosterols from unsaponifiable components of the deodorization distillate of RBO.

6.1.3.4 Preparation of highly purified squalene

To obtain more highly purified squalene, 6 g of the extract, which contained squalene having 81% purity and recovered by SFC of the unsaponifiable components, were column-chromatographed with hexane/diethyl ether (95:5, v/v) as an eluent by the procedure shown in Fig. 4.

6.1.4 Analysis

To confirm the composition of the deodorization distillate of rice bran oil, TLC-FID analysis was carried out on a Iatroscan, model MK-5 (Mitsubishi Kagaku Iatron Inc., Tokyo, Japan)

equipped with a Chromatopac C-R6A (Shimadzu Corp., Kyoto, Japan). The deodorization distillate, squalene, canola oil, oleic acid, phytosterols and Toc as standard samples were loaded on silica gel sintering quartz rods (Chromarod S-III, Mitsubishi Kagaku Iatron Inc., Tokyo, Japan). Developing solvents consisting of hexane as a primary solvent to detect squalene and benzene/ethyl acetate at a ratio of 9:1 (v/v) as a secondary solvent to detect the other components were used. The fatty acid composition of the lipids was analyzed by GLC. The constituent fatty acids were methyl-esterified (Jham et al., 1982), and a fused silica capillary column, CBP1-S25-050 (0.32 mm x 25 m; Sinwa-kagaku Co., Ltd.), was connected to a GLC (model GC-18A; Shimadzu Corp., Kyoto, Japan) for constant-temperature GLC analysis. The column temperature was set at 200°C, helium was used as the carrier gas, and FID was used as the detector. A normal-phase column, Finepak SIL-5 (4.6 × 250 mm; JASCO Corp.), was connected to an HPLC (model BIP-1; JASCO), and a mobile-phase solvent that consisted of hexane and 2-propanol at a ratio of 124:1 was allowed to flow through the column at a rate of 0.7 mL/min. A fluorescence detector (model FP-2020 Plus, JASCO) with excitation at 295 nm and emission at 325 nm was used. For the quantitative analysis of Toc, 2,2,5,7,8-pentamethyl-6-chromanol (PMC) was used as the internal standard, and the calibration curves prepared beforehand for the Toc isomers [for a-Toc, y=0.4508x-0.0927 (R2=0.9682); for b-Toc, y=0.7795x-0.3017 (R2=0.9488); for g-Toc, y=1.2532x-0.7692 (R2=0.9887); and for δ-Toc, y=1.2454x-0.9934 (R2=0.9752)] were used.

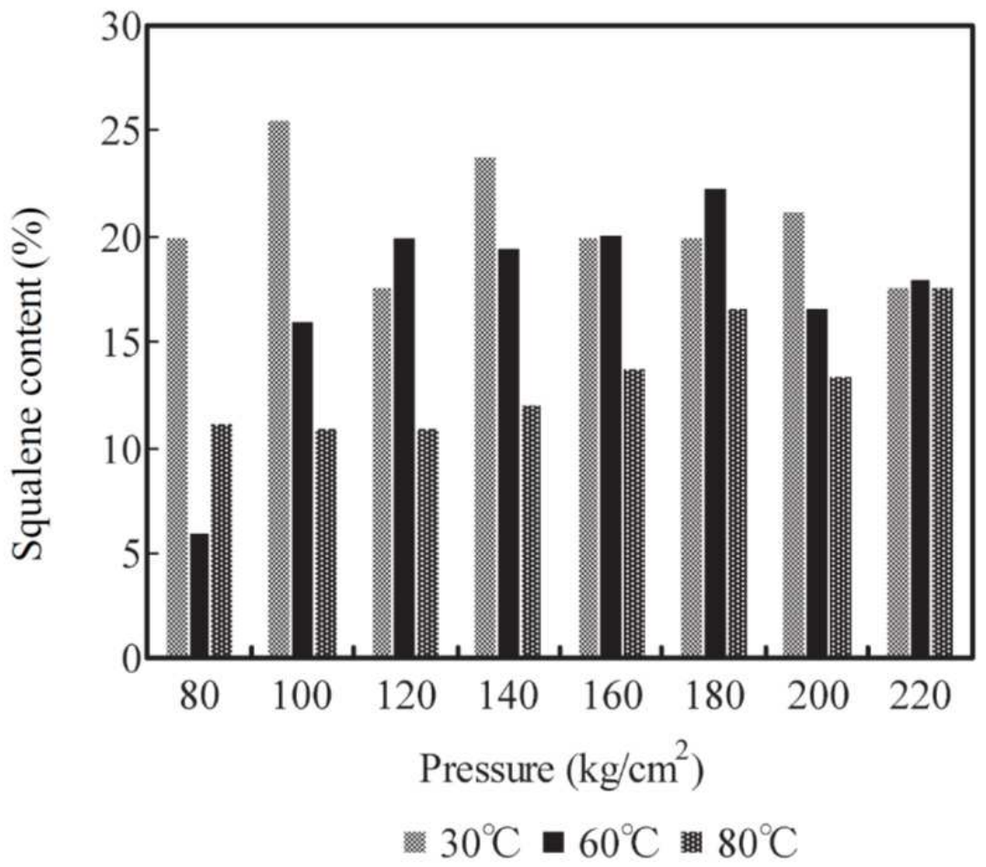

Fig. 5. Changes in squalene content under various conditions of SFE.

6.2 Results and discussion

6.2.1 Condensation of squalene from the deodorization distillate

6.2.1.1 Condensation of squalene by SFE

As shown in Fig. 5, squalene was concentrated to an average concentration of 25% with quantitative recovery under the following conditions: extraction temperature, 30°C; pressure, 100 kg/cm²; extraction time, 2 h. It was confirmed that squalene could be concentrated to 3 times the original content (8.5%) in the deodorization distillate with peroxide value (PV) of 3.0 meq/kg.

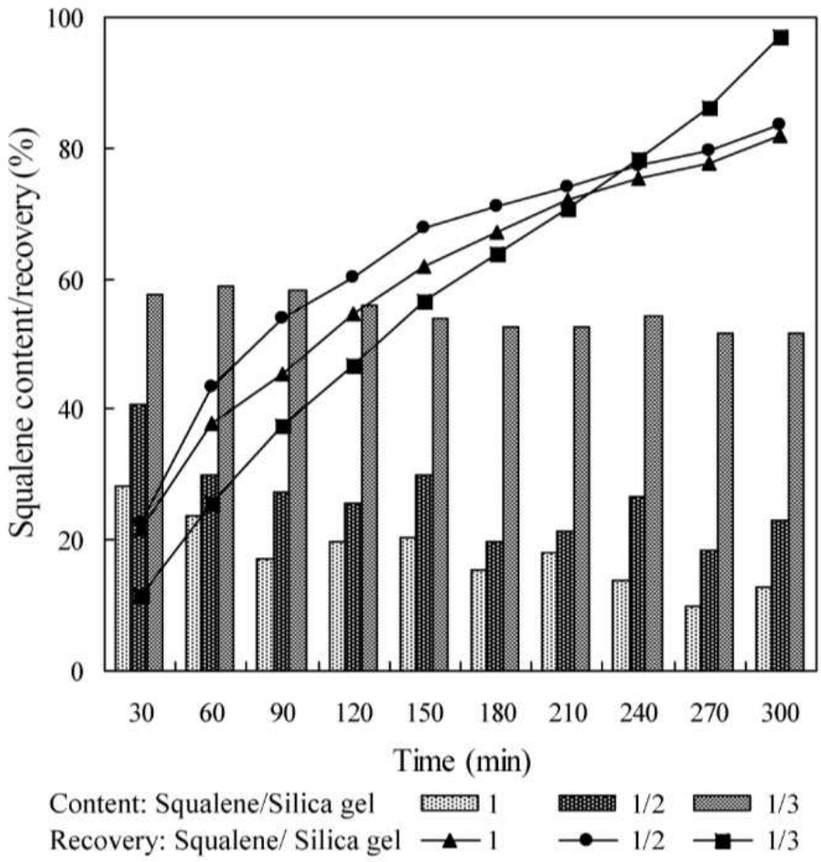

Fig. 6. Changes in the squalene content and recovery at various amounts of activated silica gel in SFC.

6.2.1.2 Condensation of squalene by SFC

As shown in Fig. 6, the higher squalene content was obtained with more than 95% recovery when 3 times more silica gel was added to the deodorization distillate and the squalene was extracted with supercritical carbon dioxide for 5 h. The extract obtained by silica-gel SFC contained, on average, 50% squalene and 38% components with higher polarity. Then, SFC of the extract obtained by the first SFC was repeated after mixing fresh silica gel. As a result, it was proved that squalene could be concentrated to 68% in the extract with PV of 3.1 meq/kg by repeating SFC with 3-fold more silica gel under the conditions stated earlier. The PV of intact squalene was 3.0 meq/kg as mentioned above.

From these results, it is suggested that the squalene was not oxidized under these conditions. Therefore, it was found that the present silica gel-SFC, with the addition of silica gel as a stationary phase into the supercritical vessel to create a chromatographic system, had a higher selectivity than mere SFE. The present silica gel-SFC is expected to become a very useful technique for concentrating squalene from the deodorization distillate of RBO as shown in Fig. 3.

6.2.2 Condensation of phytosterols from the deodorization distillate

6.2.2.1 Condensation of phytosterols by SFC

The composition of the residue with PV of 3.0 meq/kg recovered by SFC with silica gel packed as a stationary phase under the conditions of 30°C, 100 kg/cm², 7 mL/min and 5 h was 10.4% phytosterols, 3.9% Toc, 48.6% TG, and 37.1% DG + FA. In addition, the residue recovered from the procedure described in 6.1.2.1 contained 17.3% phytosterols under the following conditions: 30°C, 220 kg/cm², 7 mL/min and 7 h. From these results, it was considered that SFC did not suit the separation of phytosterols from a mixture of phytosterols, TG, DG and FA, which have nearly the same polarities, although SFC was suitable for the extraction of compounds with lower polarity, such as a squalene. Then, we examined solvent fractionation to concentrate the phytosterols from the deodorization distillate.

6.2.2.2 Condensation of phytosterols by solvent fractionation

The residual fraction shown in Fig. 3 contained 15.7% phytosterols in 4.5 g of recovered residue. The 4.5 g of residue remaining in the vessel packed with silica gel were extracted with ethanol and then saponified by refluxing for 4 h with 3 mL of 25% potassium hydroxide aqueous solution and 40 mL each of ethanol and hexane. After the saponification, the reactant was separated into a hexane layer and a hydrated ethanol layer. The unsaponifiable components thus obtained in the hexane layer were then cooled to obtain 0.23 g of crystalline phytosterols with 97.3% purity. As described above, squalene was fractionated by SFC with silica gel packed into the vessel, and phytosterols were highly concentrated from the residue by solvent fractionation. Therefore, it is considered that the combination of silica gel-SFC and solvent fractionation was a very effective means of obtaining both components with higher purity. This method, however, is rather time-consuming and costly, because SFC has to be repeated in order to concentrate the squalene, and the residue has to be extracted from the silica gel in the SFC column to concentrate the phytosterols.

6.2.2.3 Condensation of phytosterols from the unsaponifiable components of the deodorization distillate

After the saponification of the deodorization distillate (40 g) by refluxing for 4 h with 3 mL of 25% potassium hydroxide aqueous solution, 11.6 g of unsaponifiable components were recovered. Then, hexane was added to the components, and the crystalline phytosterols were recovered from the hexane-insoluble fraction under cooling. By a series of processes, 9.16 g of hexane-soluble fraction and 1.29 g of hexane-insoluble fraction were obtained and analyzed by TLC-FID. As a result, it was found that the phytosterols were concentrated to 97.2% in the hexane-insoluble fraction as shown in Table 2.

Fraction	Hexane soluble (9.16 g)	Hexane insoluble (1.29 g)
Less polar components	11.9	0
Squalene	30.2	0
Phytosterol	14.1	97.2
TG	17.5	2.8
DG	26.3	0
FA		
PV (meq/kg)	3.5	3.8

Table 2. Composition of the hexane-soluble and hexane- insoluble fractions by solvent fractionation (%).

In hexane soluble faction, saponifiables such as TG, DG and FA were contained. In this study, the condition of saponification was not finely examined. By controlling the reflux time and temperature or the concentration of potassium hydrate, TG, DG and FA could be well saponified.

It was confirmed that a combination of saponification and solvent fractionation of the deodorization distillate is an effective means of concentrating phytosterols. Since squalene was concentrated to 30.2% in the hexane soluble fraction, this fraction were subjected to SFC with silica gel under the following conditions to obtain higher purity squalene: flow rate of supercritical carbon dioxide, 3 or 7 mL/min; extraction pressure, 80-140 kg/cm^2. As results, it was found that higher squalene recovery tended to be obtained at faster flow rates and higher pressures. Furthermore, the squalene content in the extract reached 81.0%. From these results, it is considered that the deodorization distillate which is usually discarded as waste can be utilized for sources of functional components. In addition, the comparison of Fig. 3 with Fig. 4 indicates that the solvent fractionation of unsaponifiable components of the deodorization distillate is a practicable and convenient method of concentrating phytosterols and squalene. The combination of solvent fractionation and SFC developed in the present work is deemed to be an effective and safe means of fractionating squalene and phytosterols, which can then be used as additives in cosmetics and functional foods.

6.2.2.4 Preparation of highly purified squalene

The 3.50 g of extract containing 81.0% squalene obtained from the SFC were further purified by column chromatography with hexane/diethyl ether (95:5, v/v). As a result, 2.55 g of squalene with 100% purity and PV of 4.0 meq/kg could be obtained with 500 mL of eluate.

7. Conclusion

In this chapter, a novel method of fractionating squalene and phytosterols contained in the deodorization distillate of RBO without any oxidative rancidity was established by the combination of solvent fractionation and SFC after saponification of the deodorization distillate. Although there are some industrial production methods which are patented (Hirota & Ohta, 1997; Tsujiwaki et al., 1995; Ando et al., 1994) of squalene or squalane from the deodorization distillate of RBO, those methods have to perform many processes such as saponification, solvent fractionation, distillation, hydrogenation, and final molecular distillation to avoid the oxidative rancidity of squalene, or another is a cultivation method with yeast extracts for 6 days at 30^{o}C. A Japan patent (Kohno, 2002) for the production for phytosterols are released from Kao Corporation, in which phytosterols are concentrated to

90-94% purity from crude phytosterols (purity: ca. 80%) with hydrocarbon solvents. Commercial squalenes obtained from shark liver oil, olive oil, and rice bran oil are now on sale as 1,000-1,500 yen/kg, 2,500 yen/kg, and 15,000 yen/kg, respectively. The market prices of phytosterols are 3,500-15,000 yen/kg based on their purities. Therefore, the present method has some merits such as a fewer operation process, time-saving, no oxidative rancidity and continuous production of the two functional components. In addition, there is a strong possibility of lower prices production than existent methods, since carbon dioxide used as a supercritical gas is costly but recyclable. It was found that the present method very safely and effectively fractionates the functional components contained in deodorization distillate, which is usually regarded as waste.

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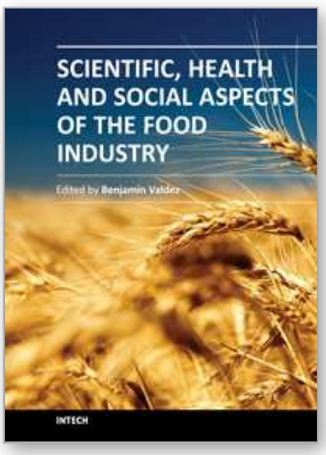

Scientific, Health and Social Aspects of the Food Industry
Edited by Dr. Benjamin Valdez

ISBN 978-953-307-916-5
Hard cover, 488 pages
Publisher InTech
Published online 01, February, 2012
Published in print edition February, 2012

This book presents the wisdom, knowledge and expertise of the food industry that ensures the supply of food to maintain the health, comfort, and wellbeing of humankind. The global food industry has the largest market: the world population of seven billion people. The book pioneers life-saving innovations and assists in the fight against world hunger and food shortages that threaten human essentials such as water and energy supply. Floods, droughts, fires, storms, climate change, global warming and greenhouse gas emissions can be devastating, altering the environment and, ultimately, the production of foods. Experts from industry and academia, as well as food producers, designers of food processing equipment, and corrosion practitioners have written special chapters for this rich compendium based on their encyclopedic knowledge and practical experience. This is a multi-authored book. The writers, who come from diverse areas of food science and technology, enrich this volume by presenting different approaches and orientations.

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