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Effect of acid-catalyzed methanolysis on the bioactive components of rice bran oil

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ABSTRACT

The change in bioactive components in oil derived from rice bran oil after acid-catalyzed methanolysis was investigated in this study. The effects of catalyst amount, molar ratio of methanol to oil, reaction time, and nitrogen purging on acid-catalyzed methanolysis were investigated to find the optimum condition in converting all free fatty acids and acylglycerides into biodiesel with minimum loss of bioactive components.

Acid-catalyzed esterification at 60 °C using 5 wt% of sulphuric acid as the catalyst can convert all free fatty acids (initial content = 59.19%) and acylglycerides (initial content = 19.31%) into fatty acid methyl esters in 5 h with a molar ratio of methanol to oil = 40. After the reaction, the losses of squalene, α-tocopherol, γ-tocotrienol, campesterol, stigmasterol, β-sitosterol, and γ-oryzanol are 50.07%, 18.06%, 63.09%, 21.68%, 28.74%, 25.42%, and 35.43%, respectively. When nitrogen purging was applied during the reaction, the losses of the aforementioned bioactive components became 42.54%, 0.00%, 43.47%, 23.47%, 26.66%, 24.07%, and 29.76%, respectively. In addition, oxidation products were not detected by GC–MS during acid-catalyzed methanolysis. From the present investigation, loss of bioactive components can be mitigated by carried out the reaction under nitrogen atmosphere.

1. Introduction

Biodiesel is becoming an attractive alternative fuel for petro diesel. Its non-toxic, biodegradable, has low emission profiles, and therefore is environmentally benign [1,2]. However, biodiesel cost is still significantly higher than that of petro diesel. Even though biodiesel can be prepared from a variety of sources including vegetable oils, animal fats, and waste greases, most commercial biodiesel productions use refined edible oil. About 60–75% of the biodiesel production cost is from the raw material [3–5].

Rice bran oil (RBO) is one of the most nutritious oil because of its favorable fatty acid composition and a unique combination of naturally occurring biologically active and antioxidant components. However, crude RBO has been difficult to refine because of its high free fatty acids (FFAs), acetone-insoluble content and dark color [6]. Since rice bran is a low value byproduct of rice milling with lipid content of 15–20%, RBO is a potential candidate for biodiesel production.

Biodiesel obtained from rice bran oil yields large amount of byproduct solid (defatted rice bran) and liquid (biodiesel residue). The utilization of defatted rice bran for the production of proteins [7], carbohydrates [8], phytochemicals [9], and isolation and purification of value added nutraceutical from biodiesel residue, such as squalene, tocols, phytosterols, and γ-oryzanol [10] are attractive options to lower further the cost of biodiesel.

Bioactive components are gaining importance in food, pharmaceuticals, and cosmetics due to its antioxidant activity. The antioxidant activity of γ-oryzanol from rice bran was evaluated at mild condition under air flow. γ-Oryzanol from rice bran evidenced significant antioxidant activity when it mixed with linolenic acid in a certain molar ratio [11]. γ-Oryzanol also has a potential application for the stabilization of lipidic raw material [12]. γ-Oryzanol, which degrades at a lower rate than α-tocopherol, is a promising antioxidant for high temperature application [13]. Phytosterol fatty acyl esters have been granted as a rare health claim by the US Food and Drug Administration (FDA) for lowering both LDL-cholesterol levels and the risk of heart disease. Squalene and tocols, its application in food, pharmaceuticals, and cosmetics is well known. However, there is still very few information regarding how much those bioactive components are degraded/retained and oxidation products produced during the production of biodiesel *via* acid catalysis.

Our previous work reported that in the isolation of γ-oryzanol from RBO, a byproduct liquid phase (LP2) was generated [14]. This liquid phase comprises FFAs, acylglycerides (AGs), and bioactive components such as γ-oryzanol, phytosterols, squalene, tocopherols and tocotrienols. Compositions of LP2 similar as soybean oil deodorizer distillate (SODD), a byproduct of the deodorization step during the refining of soybean oil which is most bioactive components are concentrated. Therefore, they can be a good raw material for the production of tocopherols, phytosterols and

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fatty acids [15]. Crude RBO usually comprises saponifiable lipids (90–96%) and unsaponifiable lipids (4–5%) [16–18]. Since bioactive components such as phytosterols, tocols, squalene and γ-oryzanol are unsaponifiable lipids, their change after acid-catalyzed methanolysis were difficult to identify using HTGC due to their contents are low. Isolation of γ-oryzanol from residue obtained during the production of biodiesel from RBO needed a series of steps only to increase γ-oryzanol content to 16%. After applying solvent extraction, modified soxhlet extraction, and finally applying silica gel column chromatography, they can increase γ-oryzanol to 83% [10]. Probably, the possibility of re-isolation of bioactive components after acid-catalyzed methanolysis was higher if LP2 used as starting material, because after distillation the composition of residue was only bioactive components. Therefore, in this study, LP2 used as a model to investigate the effect of acid-catalyzed methanolysis on the loss of bioactive components on oil (LP2) derived from crude RBO.

2. Materials and methods

2.1. Materials

Rice bran was donated by a rice mill located in Kaoshiung County, Taiwan. Analytical thin layer chromatography (TLC) aluminum plate (20 cm × 20 cm × 250 μm), silica gel $60F_{254}$, was purchased from Merck (Darmstadt, Germany). Advantec filter paper was obtained from Toyo Roshi Kaisha Ltd. (Tokyo, Japan). Standards of methyl linoleate, squalene, oleic acid, α-tocopherol, δ-tocopherol, γ-tocopherol, γ-tocotrienol, monooleylglycerol, dioleylglycerol, and trioleylglycerol were obtained from Sigma Chemicals Company (St. Louis, MO). Stigmasterol and sulphuric acid (H_2SO_4) were supplied by Across Organics (USA). γ-Oryzanol and practical grade β-sitosterol was obtained from Wako (Japan) and MP Biomedicals, LLC (Aurora, OH), respectively. All solvents and reagents were either of HPLC grade or of analytical reagent grade and were obtained from commercial sources.

2.2. Acid-catalyzed methanolysis of LP2

Acid-catalyzed methanolysis was carried out in a 50 ml screw-capped vessel with 1 g of LP2 at 60 °C and atmospheric pressure with magnetic stirrer at 600 rpm. Nitrogen purging was carried out in the screw-capped vessel before the reaction was started. The molar ratios of methanol to LP2 used were 10 and 40. Fatty acid composition of LP2 was found to be similar to that of RBO with palmitic acid (C16:0, 17.70%), stearic acid (C18:0, 1.99%), oleic acid (C18:1, 47.30%), and linoleic acid (C18:2, 31.26%) as the major fatty acids. The average molecular weights of fatty acid and fatty acid methyl ester of LP2 were estimated to be 276.40 and 290.40, respectively. Sulphuric acid (0.01 and 0.05 g or 1 and 5 wt% of oil) was dissolved in methanol and stirred at 600 rpm for 30 min before added into the reaction vessel. At the end of the reaction, the solution was cooled to ambient temperature and 10 ml of *n*-hexane was added. The mixture was stirred at 600 rpm for 10 min, then transferred to a separator funnel and let alone for another 10 min to separate water phase and *n*-hexane phase. The extraction steps were repeated until no lipids were found in the water phase. All *n*-hexane phases were pooled and washed with 10 ml 50 °C distilled water under stirring at 100 rpm for 10 min. Afterwards, the mixture was transferred to a separator funnel and let alone for another 10 min to separate water phase and *n*-hexane phase. The extraction steps were repeated until no catalysts were found in the *n*-hexane phase. After washing, *n*-hexane phase was dried over magnesium sulfate and subjected to a rotary evaporator. The recovered products were purged with nitrogen and analyzed by TLC, HTGC and UV-VIS spectrophotometer.

2.3. Analysis by UV-spectrophotometer

γ-Oryzanol content in the sample was determined spectrophotometrically (V-550 UV-VIS Spectrophotometer, JASCO, Japan). The sample was dissolved in hot *n*-hexane (60 °C) in a 1 cm quartz cell and absorptions at multi wavelength (200–400 nm) were measured [19,20]. The analysis was performed under 100 nm/min, bandwidth = 1 nm, and data pitch = 1 nm. The calibration curve was obtained with pure γ-oryzanol in a concentration range of 4–56 mg/l. In this concentration range the absorption obeys Beer's law and the calibration curve at λ_{316} is a straight line passing through the origin ($R^2 = 0.9954$) and the slope represents the specific extinction coefficient [$E_{(316\ nm)} = (36.84 \pm 0.28)$ g/l/cm].

2.4. Analysis by TLC and HTGC

Compounds in each fraction were identified by TLC and HTGC using authentic standards. TLC plates were developed in a mixture of *n*-hexane/ethyl acetate/acetic acid (90/10/1, v/v/v). After air-drying, spots on each plate were visualized by exposing the chromatogram in iodine vapor. FASEs, phytosterols, and γ-oryzanol spots were detected by spraying with a fresh solution of 50 mg ferric chloride in a mixture of 90 ml water, 5 ml acetic acid, and 5 ml sulphuric acid. After heating at 100 °C for 3–5 min, it was indicated by a red-violet color. The contents of squalene, tocopherols, tocotrienols, phytosterols, FFAs, and acylglycerols in each fraction were determined by HTGC. External standard calibration curves were obtained by using 0.02–20 mg pure standard. In this concentration range, each external calibration curve is a straight line passing through the origin ($R^2 > 0.99$). Chromatographic analyses were performed on a TLC plate and a Shimadzu GC-17A (Kyoto, Japan) gas chromatograph equipped with a flame ionization detector. Separations were carried out on a DB-5HT (5(-phenyl) – methylpolysiloxane non-polar column (15 m × 0.32 mm i.d.; Agilent Tech. Palo Alto, CA). The temperatures of injector and detector were both set at 370 °C. Temperature of the column was started at 80 °C, increased to 365 °C at 15 °C/min, and maintained at 365 °C for 8 min. The split ratio was 1:50 and the carrier gas was nitrogen. Twenty milligrams sample was dissolved in 1 ml ethyl acetate, heated until clear solution was observed, and 1 μl sample was injected into the HTGC.

2.5. Analysis by low temperature gas chromatography (LTGC)

The content of FAMEs, which is the same as fatty acid composition, in each fraction was determined by LTGC. External standard calibration curves were obtained by using 0.02–20 mg pure standard. Chromatographic analysis of fatty acid composition was performed on a *China Chromatography* 8700F (Taiwan) gas chromatograph equipped with a flame ionization detector. Separations were carried out on an Rtx-2330 10% cyanopropylphenyl-90% biscyanopropyl polysiloxane column (30 m × 0.25 mm i.d.; *Restek*, Bellefonte, PA). The temperatures of injector and detector were both set at 250 °C. Temperature of the column was started at 150 °C and held for 2 min, increased to 245 °C at 5 °C/min, and maintained at 245 °C for 14 min. Capillary head pressure, purge velocity, and vent velocity were 150 kg/cm^2, 2–3 ml/min, and 100 ml/min, respectively. Twenty milligrams sample was dissolved in 1 ml *n*-hexane, heated until clear solution was observed, and 1 μl sample was injected into the GC.

2.6. Analysis by GC–MS

The fragmentations of compounds were determined by a *Shimadzu* GC-17A (Kyoto, Japan) GC equipped with a 5970 mass

selective detector, and a 5990A MS Chemstation (HP-UX) (Hewlett-Packard, North Hollywood, CA, USA). Separations were carried out on a DB-5HT (5(-phenyl)-methylpolysiloxane non-polar column using the same temperature program as described in HTGC analysis. All mass spectra were acquired using the electron impact (EI) mode at 70 eV, with an ion current of 50 μA, and an ion source temperature of 200 °C. The MS scanned in the range of m/z 10–490 at 2.25 scan/s.

2.7. Statistical analysis

Reliability of the results was checked by statistical analysis. The excel spreadsheet program was used to calculate mean, standard deviation, and variance. The significance level was $p < 0.05$. The experimental data represent mean ± standard deviation of three independent experiments.

3. Results and discussion

3.1. Composition of LP2

LP2 was collected during isolation of γ-oryzanol from RBO. The composition of LP2 used in this study (Table 1) shows that FFAs is the major component (*ca.* 59.19%). Acylglycerides (*ca.* 19.31%) contain mainly diacylglycerides (*ca.* 16.43%). Bioactive components (*ca.* 21.5%) comprised squalene (*ca.* 1.06%), α-tocopherol (*ca.* 0.43%), γ-tocotrienol (*ca.* 0.60%), phytosterols (campesterol, stigmasterol and β-sitosterol) (*ca.* 8.24%), and γ-oryzanol (*ca.* 9.11%).

The composition of LP2 is similar to that of soybean oil deodorizer distillate (SODD) which contains about 45.38% FFAs, 23.30% AGs, and 17.50% bioactive components [15] and can be used as feedstock for biodiesel production.

3.2. Effect of catalyst amount

The acid-catalyzed methanolysis does not enjoy the same popularity in commercial biodiesel production as the base-catalyzed methanolysis because acid-catalyzed methanolysis is slower. However, it holds an important advantage over the base-catalyzed one in that its performance is not affected by the presence of FFAs in the reactant. In fact, acid catalyst can simultaneously catalyze both esterification and transesterification. Thus, a great-advantage with acid-catalyzed methanolysis is that it can directly produce biodiesel from low-cost feedstocks, generally associated with high FFAs content. Typically, H_2SO_4 is used as the catalyst in the amount of 1–5 wt% [3]. Therefore, 1 and 5 wt% of H_2SO_4 were used in this work to study the effect of catalyst amount on the loss of bioactive components after acid-catalyzed methanolysis and the results are shown in Fig. 1.

Table 1
Composition of LP2.

Compound	wt%
FFAs	59.19 ± 0.19
MGs	1.88 ± 0.31
DGs	16.43 ± 0.61
TGs	1.00 ± 0.08
Squalene	1.06 ± 0.03
α-Tocopherol	0.43 ± 0.06
γ-Tocotrienol	0.60 ± 0.05
Campesterol	1.75 ± 0.05
Stigmasterol	1.87 ± 0.08
β-Sitosterol	4.62 ± 0.09
Oryzanol	9.11 ± 0.14
Others[a]	2.06 ± 1.01

[a] Tocols, phytosterols and fatty acid steryl esters (FASEs).

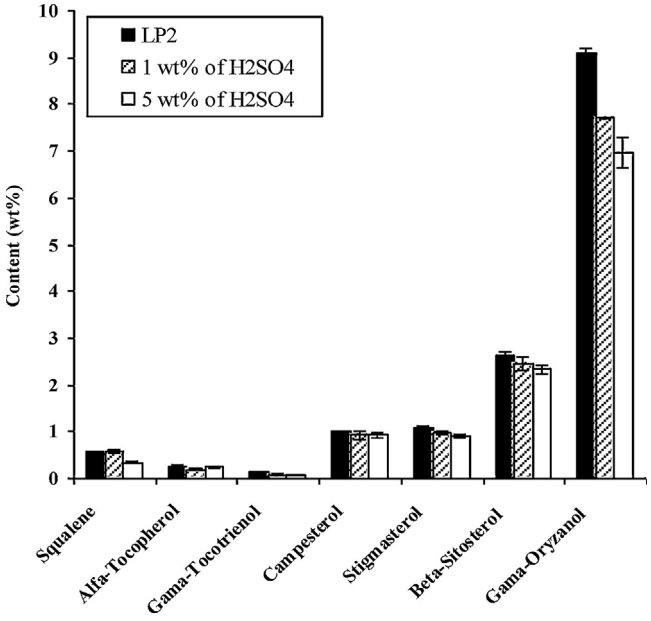

Fig. 1. Effect of catalyst amount on the content of bioactive components. Reaction conditions: methanol/LP2 = 40/1 (mol/mol), T = 60 °C, stirrer speed = 600 rpm, reaction time = 5 h and under air atmosphere.

Almost all bioactive components contents decrease with increasing catalyst amount. However, the difference of phytosterol content between using 1 and 5 wt% of H_2SO_4 is not significant ($p > 0.05$). Acid-catalyzed methanolysis of LP2 using 5 wt% of H_2SO_4 can converted all FFAs and AGs into FAMEs in 5 h while unreacted AGs (*ca.* 3%) was detected when 1 wt% H_2SO_4 was used. This is because the reaction rate of AGs was much slower than that of FFAs in acid catalyzed reaction. Increasing the amount of catalyst did increase the reaction rate of AGs; however more bioactive components will be destroyed.

Fig. 2 shows the effect of H_2SO_4 amount on bioactive components recovery. By increasing H_2SO_4 amount from 1 to

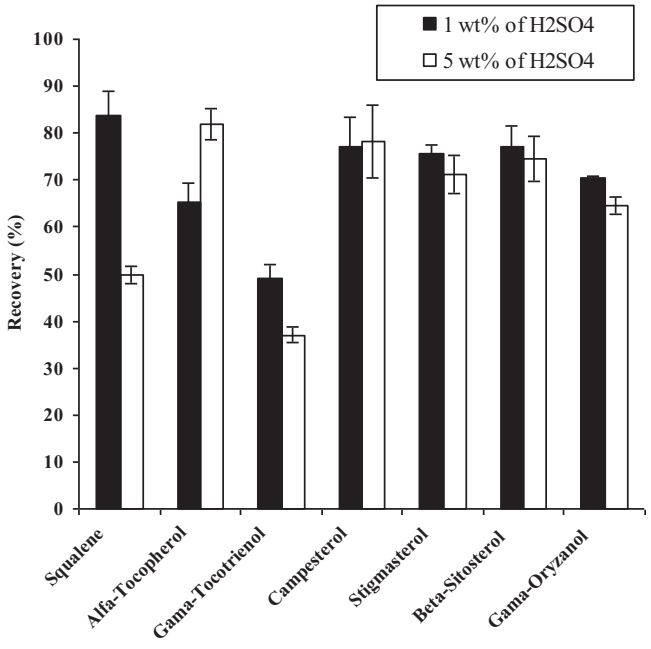

Fig. 2. Effect of catalyst amount on the recovery of bioactive components. Reaction conditions: Mthanol/LP2 = 40/1 (mol/mol), T = 60 °C, stirrer speed = 600 rpm, reaction time = 5 h and under air atmosphere.

Table 2
Effect of molar ratio of methanol to LP2 on loss of bioactive components.[a]

Compound	Methanol/LP2 = 10/1		Methanol/LP2 = 40/1	
	Content (%)	Recovery (%)	Content (%)	Recovery (%)
Squalene	0.63 ± 0.02	90.90 ± 3.49	0.34 ± 0.02	49.93 ± 1.78
α-Tocopherol	0.29 ± 0.03	100.00 ± 10.00	0.24 ± 0.00	81.94 ± 3.28
γ-Tocotrienol	0.11 ± 0.01	59.31 ± 2.92	0.07 ± 0.00	36.91 ± 1.66
Campesterol	0.96 ± 0.05	80.59 ± 6.32	0.92 ± 0.07	78.32 ± 7.72
Stigmasterol	1.07 ± 0.02	84.18 ± 3.32	0.90 ± 0.04	71.26 ± 3.95
β-Sitosterol	2.62 ± 0.15	82.80 ± 6.84	2.33 ± 0.09	74.68 ± 4.81
γ-Oryzanol	7.64 ± 0.12	70.07 ± 2.79	6.97 ± 0.31	64.57 ± 1.94

[a] Reaction conditions: H_2SO_4 = 5 wt%, T = 60 °C, stirrer speed = 600 rpm, reaction time = 5 h.

5 wt%, squalene recovery decreased from 84 to 50% and γ-tocotrienol from 49 to 37%, while the recovery of α-tocopherol increased from 66% to 81%. The recoveries of phytosterol and γ-oryzanol decreased slightly.

3.3. Effect of molar ratio of methanol to LP2

LP2 contains about 20% AGs. It is known that ester formation increased with increasing molar ratio of methanol to oil [3]. Therefore, in this study high methanol to oil molar ratios of 10 and 40 and its effect on the content and recovery of bioactive components is shown in Table 2. It can be seen that the content and recovery of bioactive components decreases with increasing molar ratio of methanol to oil. Table 2 also shows that degradation of bioactive components increased as more methanol was used in the reaction. However, at a lower molar ratio of methanol to oil of 10, all FFAs and AGs cannot be converted into biodiesel (data not shown). As this ratio is raised to 40, all FFAs and AGs can be completely reacted within 5 h.

3.4. Effect of reaction time

A shorter reaction time is preferable due to less loss of bioactive components. However, with too short reaction time, AGs cannot be completely removed even using higher amount of catalyst (5 wt%) and higher molar ratio of methanol to oil (40). In this work, the complete conversion of FFAs and AGs into BD and high content and recovery of bioactive components in the residue were chosen as criteria for determining the optimal reaction time.

The effects of reaction time on the contents and recoveries of bioactive components are given in Table 3. The contents of squalene, γ-tocotrienol, and γ-oryzanol decreased while those of α-tocopherol and phytosterols (campestreol, stigmasterol, and β-sitosterol) increased slightly as the reaction proceeded. Table 3 also shows that the contents of most bioactive components are not significantly different ($p > 0.05$) when reaction time increased from 1 to 3 h and from 3 to 5 h. However, the differences are significant from 1 to 5 h ($p < 0.05$).

As reaction proceeded from 1 to 5 h, recoveries of squalene, γ-tocotrienol, and γ-oryzanol decreased while those of α-tocopherol and phytosterols (campestreol, stigmasterol, and β-sitosterol) increased slightly. Phytosterols increased slightly due to degradation of γ-oryzanol. γ-Oryzanol is a mixture containing ferulate (4-hydroxy-3-methoxycinnamic acid) esters of triterpene alcohols and plant sterols. There are 5 major components of γ-oryzanol in RBO which were identified as ferulate esters of cycloartenol, 24-methylenecycloartanol, campesterol, β-sitosterol, and cycloartanol. Degradation of γ-oryzanol in the acid-catalyzed methanolysis resulted in phytosterols. Therefore, the content and recovery of phytosterols increased slightly with reaction time. The recovery of α-tocopherol was higher than that of γ-tocotrienol in the reaction due to their structure difference. γ-Tocotrienol contains more double bonds in their side chain than their counterpart α-tocopherol. The degradation rate of γ-tocotrienol is higher than that of squalene due to the fact that γ-tocotrienol is a potent antioxidant. Hence, the recovery of γ-tocotrienol is the lowest one. In addition, the recovery of γ-oryzanol is higher than that of squalene.

3.5. Effect of nitrogen

Most acid-catalyzed methanolysis of oil reported in literatures were conducted under air atmosphere at high temperature (≥60 °C) and with medium to high amount of H_2SO_4 (≥2 wt%) [3,4]. Depending on the composition of oil, reaction can take up to 8 h to reach 98% conversion [5]. Under such conditions, loss of bioactive components is inevitable. In this study, acid-catalyzed methanolysis was carried out under nitrogen atmosphere and its effects on the loss of bioactive components are given in Table 4. As can be seen, both content and recovery of bioactive components increased when the reaction was carried under nitrogen atmosphere. The effect is more pronounced especially for tocopherols. After acid-catalyzed methanolysis, the reaction product (crude biodiesel) usually is brownish [5,10]. The same reaction carried out under nitrogen atmosphere yield yellowish product.

Compositions of crude biodiesel obtained were biodiesel and bioactive components. Isolation of bioactive components from crude biodiesel can be done simply by vacuum distillation. More than 95% low-boiling point components, such as FFAs and biodiesel, were obtained as the distillate [10]. Therefore, the residue obtained was mainly bioactive components. They can be a good raw material for the production of squalene, tocols, phytosterols, and γ-oryzanol.

3.6. Oxidation and hydrolysis products

γ-Oryzanol and phytosterols content in LP2 are higher than the other bioactive components as can be seen in Table 1. Therefore, both compenents were investigated for their degradation products after they were subjected to acid-catalyzed methanolysis. In the model experiment, γ-oryzanol (0.1 g) was subjected to acid-catalyzed

Table 3
Effect of reaction time on the content and recovery of bioactive components.[a]

Compound	Content (%)			Recovery (%)		
	1 h	3 h	5 h	1 h	3 h	5 h
Squalene	0.46 ± 0.04	0.40 ± 0.02	0.34 ± 0.02	69.66 ± 5.87	57.78 ± 2.23	49.93 ± 1.78
α-Tocopherol	0.13 ± 0.01	0.24 ± 0.02	0.24 ± 0.00	46.69 ± 3.65	81.82 ± 7.39	81.94 ± 3.28
γ-Tocotrienol	0.09 ± 0.00	0.08 ± 0.01	0.07 ± 0.00	50.28 ± 2.06	43.49 ± 4.53	36.91 ± 1.66
Campesterol	0.74 ± 0.02	0.86 ± 0.07	0.92 ± 0.07	64.07 ± 1.47	72.73 ± 6.38	78.32 ± 7.72
Stigmasterol	0.72 ± 0.04	0.82 ± 0.05	0.90 ± 0.04	58.84 ± 3.28	65.13 ± 4.79	71.26 ± 3.95
β-Sitosterol	1.89 ± 0.12	2.21 ± 0.15	2.33 ± 0.09	61.85 ± 3.78	70.45 ± 5.22	74.68 ± 4.81
γ-Oryzanol	7.19 ± 0.20	6.79 ± 0.06	6.97 ± 0.31	68.33 ± 2.17	62.90 ± 0.09	64.57 ± 1.94

[a] Reaction conditions: methanol/LP2 = 40/1 (mol/mol), H_2SO_4 = 5 wt%, T = 60 °C, stirrer speed = 600 rpm, reaction time = 5 h and under air atmosphere.

Table 4
Effect of nitrogen on loss of bioactive components.[a]

Compound	Under air	Under N_2	Under air	Under N_2
	Content (%)		Recovery (%)	
Squalene	0.34[a]	0.39[a]	49.93[c]	57.46[d]
α-Tocopherol	0.24[a]	0.31[a]	81.94[c]	106.29[d]
γ-Tocotrienol	0.07[a]	0.10[b]	36.91	56.60[d]
Campesterol	0.92[a]	0.96[a]	78.32[c]	76.53[c]
Stigmasterol	0.90[a]	0.92[a]	71.26[c]	73.34[c]
β-Sitosterol	2.33[a]	2.36[a]	74.58[c]	75.93[c]
γ-Oryzanol	6.97[a]	7.51[a]	64.57[c]	70.24[c]

[a] Reaction conditions: methanol/LP2 = 40/1 (mol/mol), H_2SO_4 = 5 wt%, T = 60 °C, stirrer speed = 600 rpm, reaction time = 5 h. Different superscript letters within a row indicate a statistically significance difference ($p < 0.05$).

methanolysis (reaction conditions were the same as LP2) to determine its degradation products by GC–MS. Oxidation products were not detected, whereas campesterol and β-sitosterol were found as hydrolysis products. Meanwhile, oxidation and hydrolysis products were not detected by GC–MS when phytosterols (0.1 g) was subjected to the same acid-catalyzed methanolysis. When LP2 was subjected to the same acid-catalyzed methanolysis, no oxidation products were detected. Therefore, degradation products of minor components during acid-catalyzed methanolysis were the products of hydrolysis.

4. Conclusion

Loss of bioactive components in acid-catalyzed methanolysis of vegetable oil is inevitable. Increasing the amount of catalyst and molar ratio of methanol to oil can convert all FFAs and AGs into FAMEs; however, loss of bioactive components also increases. Loss of bioactive components can be mitigated by carried out the reaction under nitrogen atmosphere.

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