Effect of Subcritical Solvent Extraction Conditions on Amount of γ-Oryzanol and γ-Tocopherol in Dawk Pa-Yom Rice Bran Oil

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Abstract

This research focused on the subcritical solvent extraction of phytochemicals γ-oryzanol and γ-tocopherol from upland rice bran (Dawk Pa-yom variety, DY). Subcritical solvent extraction was conducted using a batch reactor. The temperature was varied in range from 80 to 120°C for 20 to 60 min. The solvents in use were ethanol, methanol, and water. γ-Oryzanol and γ-tocopherol were simultaneously analyzed by HPLC. Antioxidant activity was determined by a DPPH radical assay. Methanol was able to extract a higher level of γ-tocopherol than ethanol and water. Extraction by methanol gave higher antioxidant activity than ethanol. Even though water could not extract γ-oryzanol, it was able to extract substances with higher yield of antioxidant activity than either methanol and ethanol. Increasing the extraction temperature increased the oil yield, γ-oryzanol and γ-tocopherol in parallel with an increased antioxidant activity. During the extraction process, some degradation of antioxidants was observed, and this showed the importance of kinetics parameters in this research.

Keywords: extraction; kinetics; oil recovery; solvent extraction; γ-oryzanol; γ-tocopherol; rice bran
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1. Introduction

Nowadays, many people have concerns about health, and therefore there is great interest in valuable food products and food supplements. Especially sources of vitamin and supplement are from nature or biological source. Other than the source of raw material, the extraction method used is also a crucial part of getting high quality of supplement. There are many interesting extraction methods; soxhlet extraction, maceration, subcritical solvent extraction, and supercritical carbon dioxide extraction. The last two methods are expensive to operate; however, they are efficiency ways of extracting high quality of product. Liquefied solvent extraction or subcritical solvent extraction involves a solvent heated under pressure that depends on the solvent type. Such condition has changed the solvent properties; they generally decrease permittivity (polarity), increase diffusion rate, and decrease viscosity and surface tension. In addition, extraction under subcritical condition differs quite significantly from conventional extraction methods; it is very fast and of a hydrolytic nature [1-3].

Rice bran is a by product of the milling process and is a well-known source of phytochemicals. Bran contains large numbers of vitamins, minerals, and other nutritious items including phenolic compounds, vitamin E, and its associated components: tocols, tocopherol, tocotrienol and γ-oryzanol, which are substanes that can reduce Alzheimer's disease, cholesterol, cancer and heart disease [4-8]. Oryzanol and tocopherol are famous antioxidants and have often been found in rice. A group of ferulic acid esters of phytosterols, called γ-oryzanol and α-tocopherol, are responsible for the antioxidant activities of flavonoids and are considered excellent antioxidants. Jasmine rice, or in common name, Dawk-Mali (MA), is a famous, conventional rice variety that grows in lowland areas where flooding is regular in Thailand. Because of the drought that occurred in 2015 in the middle part of Thailand, it was impossible to grow jasmine rice. Rice variety, Dawk Pa-yom variety (DY), was able to withstand drought were grown in its place. The color of DY rice bran is dark and purplish-red color. Previous research studies have shown that the color of a bran is correlated to its phytochemical content. Specifically, the genotypes of a purple bran are significantly correlated with higher total flavonoid content (TFC) and total phenolic content (TPC), and oxygen radical absorbance capacity value than a red rice bran (attributed mainly to its flavonoid content) [6-9]. Moreover, previous researchers found that DY had higher antioxidant activity than Iranian rice bran oil and wheat oil; however, it had less activity compared to rice oil from Pone-Sai district, Thailand [1, 10, 11].

There is some previous research on plant extraction under subcritical water and subcritical organic solvent extraction [12, 13]; however, there are no reports concerning rice bran extraction. A subcritical solvent can be defined as hot solvent put under high pressure to maintain it in its liquid state. Under subcritical condition, the ion product increase and dielectric constant decrease in which non-polar bioactive compounds can be extracted. However, this could be the effect of the degradation of bioactive compound during the extraction under limited temperature. Previous reseach discussed the degradation kinetics of substances that occurred during extraction [14-16]; however, no information relevant to rice bran oil extraction was mentioned, and thus there exists the need for research into the kinetics of rice brand extraction. This research will examine subcritical extraction of rice bran under three solvent types; ethanol, methanol and water in order to increase the extracted yield of rice bran oil, γ-oryzanol and α-tocopherol. In addition, their antioxidant ability would be evaluated by DPPH assay. This research should provide new knowledge in the area of subcritical extraction.

2. Materials and Methods

2.1 Upland rice bran

Upland rice bran was used as material in this research. Upland rice bran from Dawk Pa-yom variety (DY) was obtained from the southern part of Thailand, Suratthani province. The rice bran was sieved through a standard mesh (ASTM-E11-09, Endecotts, Endecotts Logistic Center, Inc.) with a mesh size of 850 µm, then kept at -20˚C until use.

2.2 Chemicals and materials

AR grade hexane, ethyl acetate, acetone, isopropanol and ethanol were used for extraction. HPLC grade methanol, isopropanol, ethyl acetate were used for HPLC analysis. Solvents of all types were ordered from RCI Labscan Limited, Bangkok, Thailand. For antioxidant activity assay, 2,2-diphenyl-1-picrylhydrazyl (DPPH) from Sigma-Aldrich, St.Louis, Missouri was used. γ-Oryzanol was purchased from Wako, Japan. γ-Tocopherol standard grade was purchased from Wako Pure Chemical Industries Ltd, Japan.

2.3 Subcritical solvent extraction

The subcritical solvent extraction was conducted using a 500-ml SS-316 batch reactor (Parr Instrument Co., Moline, IL, USA), as shown in Figure 1. A pressure limit was set with and protected by a rupture disc. The temperature and pressure were measured and recorded over time with Parr software. A constant ratio of bran sample and solvent at 1:10 (w/v) with solvent volume of 100 ml was maintained. The mixture was placed in the pressure vessel. Solvents in use were water, methanol and ethanol. After the lid had been tightly closed, oxygen gas in the reactor was replaced with nitrogen gas, and the reactor was warmed up until the pressure reached about 1 MPa, and then the extraction began. The extraction was performed for 20-60 min under various temperatures ranging from 80 to 120°C. Although some extraction had occurred during heating, the extraction time was recorded when the temperature had already reached the desired level. After the desired extraction time was achieved, the vessel was immediately removed from the oven and cooled down for 5 min. The mixture was then filtered through a 45 µm filter paper. The solvent in the extract was evaporated with a rotary evaporator (Hei-Vap Precision, Heidolph, Germany), and then the extract was kept in a refrigerator at -20°C until further analysis. Oil yield was determined by determining the ratio between grams of crude oil and grams of dried rice bran, and expressed in dimension (g/g dried rice bran).

2.4 γ-Oryzanol and γ-tocopherol determination

The γ-oryzanol and γ-tocopherol contents were simultaneously determined with RP-HPLC system following the procedure reported in Ruen-ngam [17] and Chen and Bergman [18]. The determination system consisted of a Shimadzu 2690 Alliance separation module and a Waters 2487 dual wavelength UV/Vis absorbance detector. Chromatograms were recorded and processed with LC Solution Chromatography Software (Shimadzu, Japan). The extracted crude oil was prepared into a concentration of 30 µg/ml by using a mobile phase of 47.5% methanol, 40% isopropanol, and 12.5% ethyl acetate. Then, a volume of 20 µl was injected into ACE 5 C18 column (250×4.6 mm, ACE, Scotland), which was the stationary phase. The mobile phase flow

rate was 1.0 ml/min. The contents were detected at the wavelength of 330 nm. The γ-oryzanol concentration was calculated based on the area under the peak of a standard of known concentration, which had been prepared in the range of 0.05-50 µg/ml.

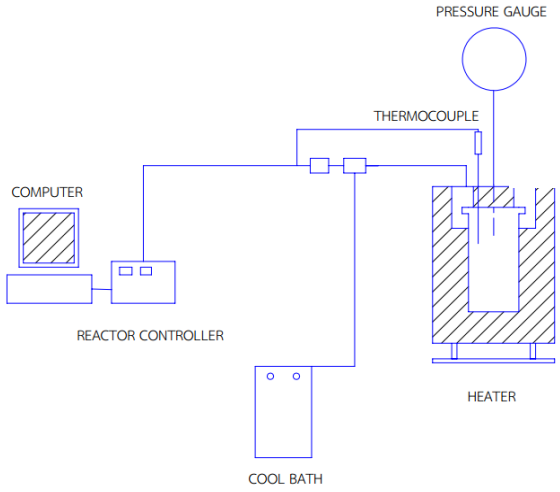

Figure 1. Experimental setup

2.5 Antioxidant activity assay

The free-radical scavenging activity of the rice bran extract was determined by DPPH radical assay according to previous research [6]. An aliquot of the sample solution was adjusted into a 0.2 mM ethanolic solution of DPPH at a ratio of 1:1 (v/v) in a 96-well plate. The well-mixed solution was then incubated for 30 min in the dark at room temperature. The absorbance was measured at 517 nm against a blank, using a UV-Vis microplate reader (EMS Reader MF, Labsystems). The results were expressed as half maximal inhibitory concentration (IC_{50}). A lower value of IC_{50} indicates a higher antioxidant activity. Percentage of inhibition of the DPPH radical was calculated by the following equation;

$$\%\ Inhibition\ = \frac{(A_{DPPH} - A_{Blank\ DPPH}) - (A_{Sample} - A_{Blank\ sample})}{(A_{DPPH} - A_{Blank\ DPPH})} \times 100 \qquad (1)$$

Where A_{Sample} is the absorbance of the extract in DPPH solution; $A_{Blank\ Sample}$ is the absorbance of the extract; A_{DPPH} is the absorbance of the DPPH solution; and $A_{Blank\ DPPH}$ is the absorbance of the solvent without DPPH.

The IC_{50} values obtained were compared to high-activity antioxidant standards: Ascorbic acid (Vit C), Trolox and BHT.

2.6 Statistical analysis

All experiments were performed in three replicates, and the mean and standard deviation were calculated. The significant differences between extraction conditions-solvent, temperature and time-were analyzed by one-way ANOVA and Duncan's new multiple range test at a significant level of 95% ($p < 0.05$).

3. Results and Discussion

3.1 Effect of solvent type

The effect of different solvent types on the amounts of extracted oil and the nutrients it contained at 100ºC subcritical extraction temperature and 40 min extraction time were tested. The preliminary results showed that methanol gave a higher (but not significantly higher at the 95% significance level) oil yield than ethanol did, which in turn, gave a higher yield than water did. In addition, the effect of solvent type on oil yield was also tested at various extraction temperatures (80, 100 and 120°C). It was found that methanol gave a higher oil yield than the other solvents did for every other temperature except at 100°C. At 100°C, methanol and ethanol gave higher ranges of yield and their yields were in the same range.

Both methanol and ethanol have similar dielectric constant values which are lower than that of water (as shown in Table 1). Therefore, they should be able to extract oil, a non-polar substance, better than water can. In general, dielectric constant, viscosity, and density of a solvent all strongly affect extraction efficiency. However, the results from this study indicate that the dielectric constant was a more predominant property than viscosity and density with regard to our extraction procedure, as methanol and ethanol (oil like non-polar solvents) with low dielectric constants produced higher oil yields than water did (polar solvent). Moreover, even though ethanol has a higher viscosity than water, it gave a higher oil yield than water did. Additionally, even though the density of water is the highest among the three solvents, it was not able to pull out the oil as well as the other two solvents.

At extraction temperature higher than 100°C, ethanol gave nearly the same range of γ-oryzanol yield as methanol did, whereas at an extraction temperature of 80°C, the ranges were quite different. It was astonishing that no content of γ-oryzanol was extracted by water as solvent even at different extraction temperatures. It might be that γ-oryzanol has a low polar structure of an alkyl group, but water does not have an alkyl group (like dissolves like). In the case of γ-tocopherol yield, methanol gave the significantly highest yield at a significant level of 95% among the the three solvents tested. Previous studies have found that both γ-oryzanol and γ-tocopherol showed a DPPH radical scavenging capability, and one substance was more dominant than the other depending on the type of the biological system [19, 20].

3.2 Effect of temperature

The extraction yield results of bran oil, γ-oryzanol and γ-tocopherol in previous sections demonstrated that methanol was able to achieve the highest yield of these compounds. Therefore, in the investigation of the effect of temperature, the focus was on methanol. Increasing the extraction temperature increased the oil yield because doing so decreased the density and viscosity of methanol (results not show here). Moreover, its dielectric constant seems to be decreased, as shown in the properties of water in Table 1. The lower density and viscosity of methanol enabled it to easily penetrate through the porous material, pull the oil out, and make the oil dissolved in the solvent on the outside. At the same time, the substances that were dissolved in the oil-γ-oryzanol and γ-tocopherol also came out. The highest amounts of γ-oryzanol and γ-tocopherol achieved by methanol extraction were around 12.12±0.48 and 397.70±2.82 mg/g dried rice bran, respectively. When the extraction temperature was increased up to 120°C, the extract still had good quality as shown in Figure 2. The IC_{50} values at 100 and 120°C were in almost the same range which were due to nearly the same amounts of γ-oryzanol extracted out at these temperatures (1.74±0.14 mg/ml). It was expected that with further increase of extraction temperature over 120°C,

Table 1. Solvent properties

Solvent types	T (°C)	p_v[1]	ρ_l[2]	ρ_v[3]	μ_l[4]	μ_v[5]	σ_l[6]	ε_r[7]
Ethanol	80	1.086	0.757	1.43	0.432	1.03	17.3	25.3 (20°C)
	100	2.26	0.73	3.41	0.318	1.092	15.5	
	120	4.29	0.71	6.01	0.243	1.157	13.4	
Methanol	80	1.819	0.7355	0.00208	0.271	115	17.5	33.0 (20°C)
	100	3.731	0.714	0.00398	0.214	123	15.7	
	120	6.551	0.69	0.00714	0.17	130	13.6	
Water	80	0.47359	0.97182	0.2932	0.351	113	62.69	80.1 (20°C)
	100	1.01325	0.95877	0.5974	0.279	121	58.91	10 (360°C)
	120	1.9854	0.94339	1.121	0.23	128	54.96	

Remarks: Ethanol: Boiling point = 78.1°C, Critical point = 241°C, 6.3 MPa
Methanol: Boiling point = 64.7°C, Critical point = 240°C, 7.7 MPa
Water: Boiling point = 100°C, Critical point = 374°C, 21.3 MPa
Data in Table were available from https://www.engineeringtoolbox.com/liquid-dielectric-constants-d_1263.html
[1]Saturation pressure (10^5 Pa)
[2]Liquid density (10^3kg/m³)
[3]Vapor density (kg/m³)
[4]Liquid viscosity (10^{-3}N-s/m²)
[5]Vapor viscosity (10^{-5}N-s/m²)
[6]Liquid surface tension (10^{-3}N/m)
[7]Dielectric constant

the antioxidant activity from γ-oryzanol might be degraded. At 120°C, γ-tocopherol might be the dominant substance that provided the antioxidant activity which was the reason that the IC_{50} value at this temperature did not decrease.

Therefore, for energy saving, the extraction temperature at 100°C was deemed to be the optimum temperature for extraction of this bran oil extraction. However, the γ-oryzanol concentration achieved was low for the material pretreated, implying some degradation, while other studies report that the amount of γ-oryzanol remained quite constant as the heating temperature was increased from 60°C to 110°C. Thus, it was expected that with the extraction temperature of more than 120°C, degradation of γ-oryzanol would be pronounced [21, 22]. This research also demonstrated the γ-oryzanol degradation kinetics in Section 3.4.

3.3 Effect of time

The amounts of rice bran oil, γ-oryzanol, γ-tocopherol yield and IC_{50} values for different periods of extraction time and at each temperature in methanol are shown in the extraction curves in Figures 2 (a)-(d). The oil yield increased sharply with time for the first 20 min and the rate of increase gradually slowed until the final yield. This behavior was the same for all temperatures. However, the extraction time of 60 min gave the significantly highest oil yield at the level of 95%.

The amounts of γ-oryzanol and γ-tocopherol followed a similar trajectory to the oil yield and reached the maximum at 60 min at the significant level of 95% as shown in Figures 2 (b)-(c). This behavior might be because initially the oil was extracted out easily from the immediate mass with which the solvent was in contact, but as time passed, it was difficult for the subcritical solvent to reach the inside mass of the material, and this corresponded to the period of gradual increase after the first 20 min.

In addition, the overall extraction curve in Figure 2 was used to calculate the solubility of the oil by dynamic method from the slope of the initial part of the overall extraction curve. The solubilities of all of the extracted substances—oil, γ-oryzanol and γ-tocopherol—increased when a higher extraction temperature was used. Initially, the γ-oryzanol yield increased sharply with time and then changed to more gradual increase in the second section, and this curve will be further used in the activation energy calculation in the following section. This increase in the solubility increased the driving force in the fluid phase. The maximum solubility of all substances were found at 120°C and the solubilities of oil, γ-oryzanol and γ-tocopherol were 0.0185, 0.4275 and 9.080 mg/g dried rice bran, respectively. It is surprising that the amount of γ-tocopherol continuously increased at extraction time longer than 40 min at the temperature of 120°C. It might be that the increased extraction temperature softened the hard mass of the rice bran and decreased the viscosity of the solvent so that it was able to penetrate deeply and easily into the material inside [22-25].

The amount of antioxidants increased significantly with time at120°C; howver, they contrasted with IC_{50} value which gradually increased. The IC_{50} at 120°C was in the same range as the IC_{50} at 100°C. This may indicate some degradation of substance. The IC_{50} antioxidant activity of extracted oil (1.74 mg/ml) was lower compared to other well-known antioxidants; Vit C (5.99 $\times 10^{-3}$ mg/ml), Trolox (14.47 $\times 10^{-3}$ mg/ml) and BHT (0.18 mg/ml).

3.4 Overall degradation of substances

The overall extraction curve in Figure 2 shows that for extraction times longer time than 20 min, the amounts of oil, γ-oryzanol and γ-tocopherol gradually decrease because of substance degradation. Therefore, this section offers information about the kinetic of degradation of such compounds. The graph plotted according to equation (2) shows that the fraction of γ-oryzanol is linearly related to time at constant temperature ranging from 80 to 120°C as shown in Figure 3. The trend of the curve was similar to that found by Debnath *et al*. [26]. The previous section in this work demonstrated the high extraction rate of γ-oryzanol in the first 20 min of extraction, which was followed by a decreased extraction rate. There was a similar trend occurred in the cases of the oil and γ-tocopherol. This indicates that the degradation process can be expressed by pseudo-first order kinetics. Activation energy and pre-exponential factor were calculated according to a pseudo-first order reaction kinetics by plots based on the natural logarithm of a Arrhenius equation as follows:

A pseudo-first order reaction:
$$\ln\left(\frac{C}{C_0}\right) = -kt \quad (2)$$

Arrhenius equation:
$$k = \mathrm{A}e^{\frac{-E_a}{RT}} \quad (3)$$

Taking the natural logarithm of (3):
$$\ln(k) = \frac{-E_a}{R}\left(\frac{1}{T}\right) + \ln(A) \quad (4)$$

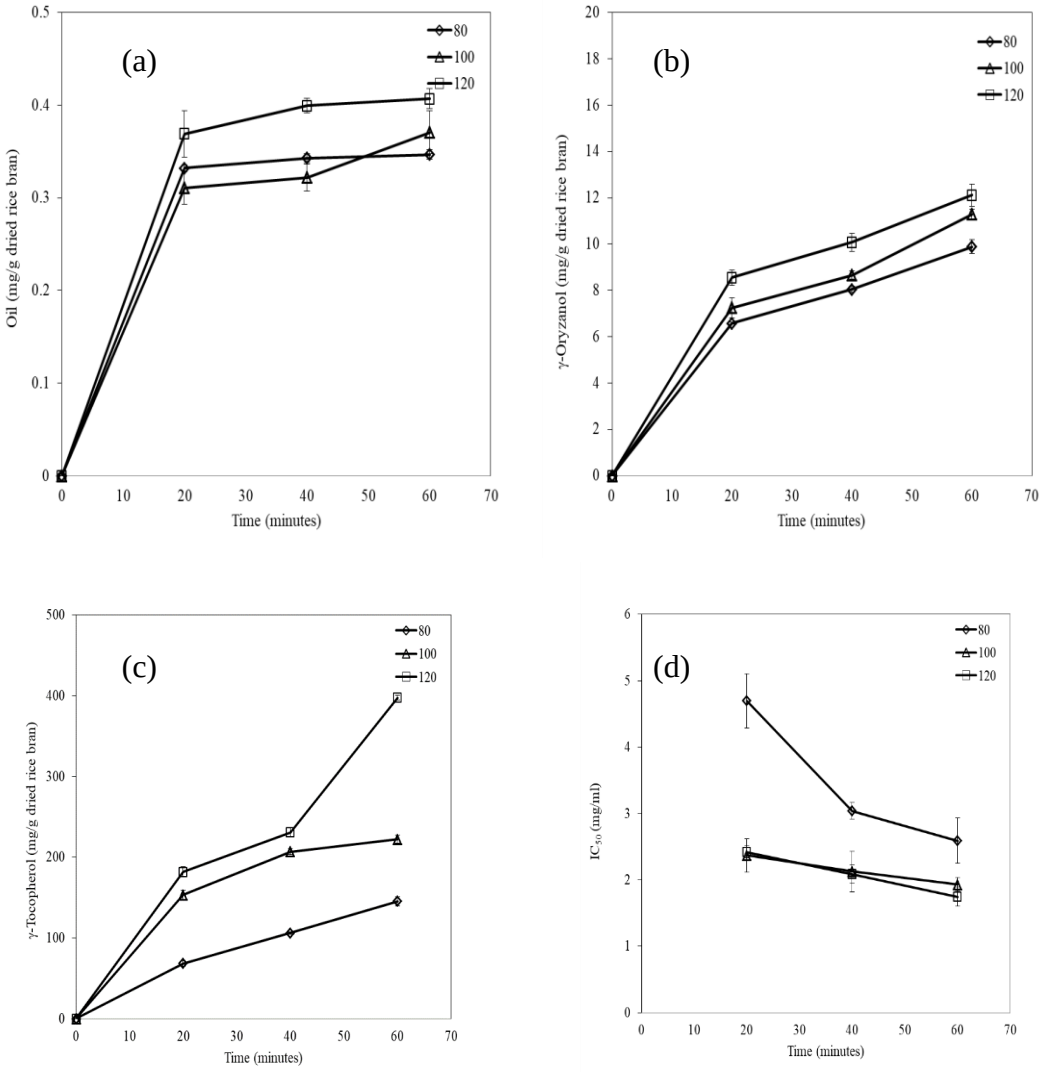

Figure 2. Effect of time and temperature on the yields in extracted DY rice bran oil under subcritical methanol; (a) oil, (b) γ-oryzanol, (c) γ-tocopherol, and (d) IC_{50}

Where C is concentration of γ-oryzanol in the solvent, C_o is initial concentration of γ-oryzanol in rice bran (mg/g rice bran), t is time, T is temperature, E_a is activation energy (kJ/mole) and A is pre-exponential factor (min^{-1})

The kinetic parameters are demonstrated in Table 2. The values of the kinetic parameters in Table 2 are in the same range as those for the degradation of folic acid (Ea = 69.9 kJ/mole) [24].

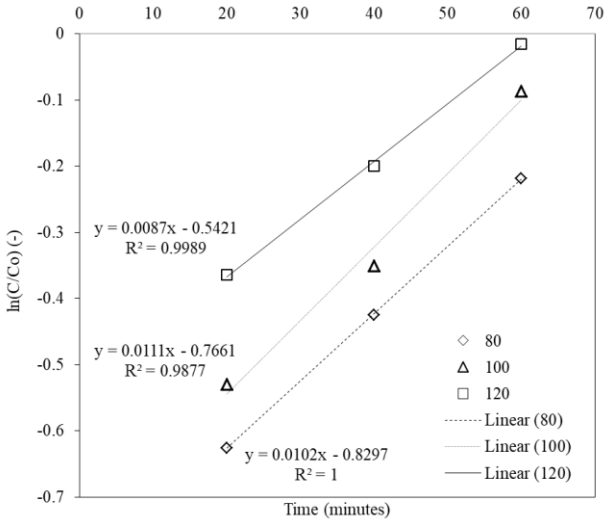

Figure 3. Plots of $\ln(C/C_o)$ vs. time for determining Arrhenius parameters of γ-oryzanol

Table 2. Activation energy (E_a) and pre-exponential factor (A) for pseudo-first order degradation kinetics of components in rice bran oil

Compositions	A (min^{-1})	E_a (kJ/mole)
Oil	0.0023	23.500
γ-Oryzanol	0.0024	4.413
γ-Tocopherol	0.0170	0.299

3.5 Comparison of conventional extraction

The results of extracted yield under methanolic subcritical extraction were then compared to the extraction results when using a soxhlet apparatus and done by ethanol extraction for 8 h at boiling point (78°C). The oil and γ-oryzanol were lower than those obtained from soxhlet extraction. The oil yield from methanolic subcritical extraction ranged from 81.03 to 99.25% of that produced by soxhlet extraction, and γ-oryzanol came in at 58.88 to 91.75% of Soxhlet extraction yield, a result with a wide range that might have been due to extraction time. The amount of γ-tocopherol at 60 min was higher (106.66%) than that obtained from soxhlet extraction. This experimental method used less time than did the conventional extraction method. Solvent type and solvent properties effected the amount of substance extracted. Solvent properties, especially viscosity and surface tension, seemed to be more effective at elevated temperatures as shown in the properties in Table 1. Moreover the solvent under high pressure changed phase to be ionic in nature, and therefore it could more easily penetrate and flow through the material than fluid phase [25, 26].

4. Conclusions

Subcritical solvent extraction was investigated as an alternative extraction method. Methanol produced the highest yield of oil, γ-oryzanol, and γ-tocopherol. The temperature and the time of 100°C and 60 min were optimal conditions for obtaining the highest yield of such compounds that also had the highest antioxidant activity. The highest oil yield obtained around 0.40±0.01 mg/g dried rice bran. γ-Oryzanol and γ-tocopherol had the highest value at around 12.12±0.48 and 397.70±2.82 mg/g dried rice bran, respectively. When extraction temperature was increased in range 80 to 120°C, there was some degradation of substance observed and the kinetics of degradation was also noted. Although methanol is not a green solvent, this research demonstrated its potential its potential use as a subcritical solvent that may be applied in further specific use.

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