

**Properties of Extracts Obtained from Rice Straw
by Its Subcritical Fluid Treatment**

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General Introduction

I.1 Production of Rice Straw

Rice is one of the main staple plants in the world and was cultivated for 8 million tons in the years 2011 in Japan [1]. Due to the growth of the world human population, rice production is set to increase in the future. The production process of rice generates 1 million dry tons of agricultural residues, mainly straw. Rice straw is typically collected after retrieving kernels during harvest [2]. The rice straw has many potential usages because its three major constituents are cellulose, hemicellulose, and lignin [3], which can become the sources for extraction of carbohydrates and phenolic compounds. However, it is subject to burning or incorporation into soil and has not been effectively utilized [4,5]. One of its reasons is that these constituents are poorly soluble in water due to strong hydrogen bonds between polysaccharide molecules and covalent bonds in the cell wall structure [6].

I.2 Subcritical Fluids

Subcritical fluid extraction, also known as pressurized liquid extraction or accelerated solvent extraction, is an efficient method to recover the functional compounds from rice straw. This method uses an extractant at elevated temperature under high pressure to maintain their liquid states (subcritical state). Under this state, the surface tension and viscosity of the extractants decrease with increasing temperature. The decrease in the surface tension would promote the penetration of the extractant into the stem matrix and enhance the solubility of the constituents in the extractant [7].

When water is employed as the extractant, the specific name of ‘subcritical water extraction’ or ‘hot compressed water extraction’ is used. Under subcritical conditions, ion

product of water increases to 1×10^3 -fold greater than that of water at room temperature [8]. Moreover, dielectric constant of subcritical water approximates to those of polar organic solvents with increasing temperature [9], and it further decreases by adding an organic solvent, such as ethanol and acetone [10]. The change in the dielectric constant of the extractant would change the solubility of the extracted substances, which results in the different compositions of the extract.

Additionally, lignocellulosic materials submitted to a subcritical organic solvent and/or water produces black liquor, whose blackness is due to the degradation products of hemicellulose and lignin that are solubilized during the extraction [11]. This black liquor has been reported to possess the radical scavenging ability [12,13].

I.3 Objectives of the Thesis

The objective of this thesis is to optimize the conditions for the subcritical fluid treatment of rice straw to efficiently obtain carbohydrates and phenolic compounds. In chapters 1-3, the effects of treatment conditions in subcritical water on the properties of the extracts were extensively studied. The properties of the extracts recovered from the different parts of rice straw, *i.e.* stem and leaf, were investigated in the treatment temperatures ranging from 140 to 260°C in Chapter 1. Chapter 2 focuses on the effects of the treatment time and re-treatment after the first subcritical water treatment on the properties of the extracts. The 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging-, iron (II) chelating-, and tyrosinase inhibition-abilities of the extracts were also extensively studied.

In order to improve the extraction efficiency, a mixture of an organic solvent and water under subcritical state was used for the extraction in Chapters 3-5. Chapters 3 and 4 deal with the effects of the content of an organic solvent (ethanol and acetone) in water, treatment temperature and time on the properties of the extracts. Relationship between the

color and DPPH radical scavenging ability of the extracts was also established in Chapter 3. In Chapter 5, subcritical treatment was repeated 3 times toward the same rice-straw sample using subcritical water, ethanol, and 75% (v/v) aqueous ethanol in different orders to investigate the roles of water and ethanol.

Chapter 1

Properties of Rice Straw Extract after Subcritical Water Treatment

1.1 Introduction

This chapter deals with the temperature effect of subcritical water treatment on the properties of rice straw extract from different parts of the straw. The node I and internode I of the stem were defined as upper parts of the stem, and the node II and internode II were as the middle part, and the node III and internode III were as the lower part (Fig. 1-1). Though the constitution of the stem parts is the same, their contents in the different parts vary [14]. Hence, the properties of the rice straw extract obtained from different parts were examined. Yield, total carbohydrate content, total protein content, total phenolic content, DPPH radical scavenging ability, and UV absorption spectra were chosen as the properties to be considered in characterizing the rice straw extract.

1.2 Materials and Methods

1.2.1 Materials

A rice straw sample was obtained from rice (*Oryza sativa*) cultivated in Hyogo Prefecture, Japan. It was dried under the sun and kept at 4°C in a storage room. They were cut into 1-cm long pieces. L-Ascorbic acid (purity > 99.5%) was purchased from Nacalai Tesque (Kyoto, Japan). Folin-Ciocalteu reagent was from ICN Biochemicals (Aurora, OH, USA). Gallic acid was from Sigma-Aldrich Japan (Tokyo). 1,1-Diphenyl-2-picrylhydrazyl (DPPH), crystallized bovine serum albumin (BSA, 99% purity), and all other chemicals

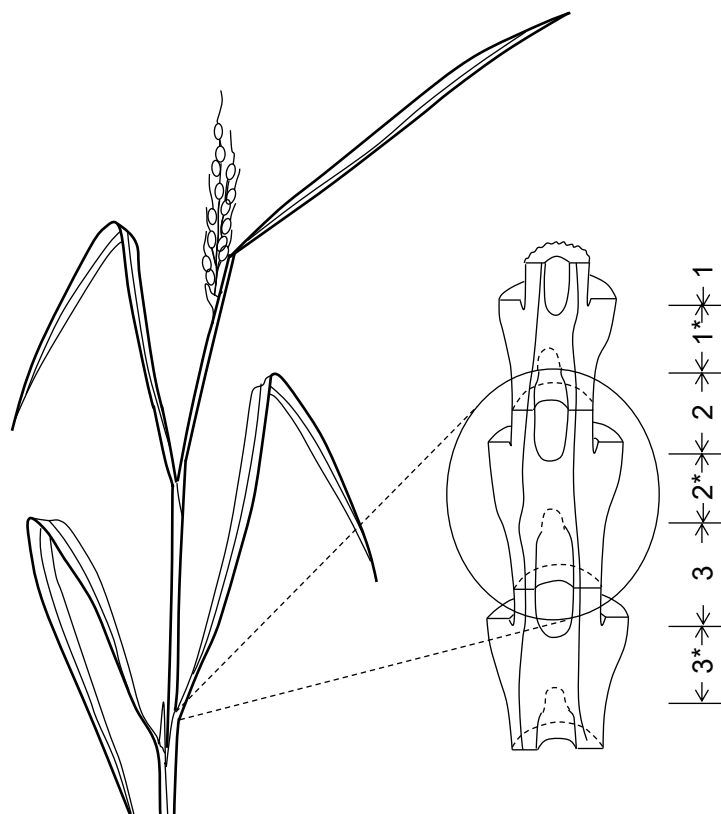


Fig. 1-1. Diagrammatic composition of rice stem. 1: internode I, 1*: node I, 2: internode II, 2*: node II, 3: internode III, and 3*: node III.

of reagent grade were from Wako Pure Chemical Industries (Osaka, Japan). Distilled water was used in all the experiments.

1.2.2 Preparation of extract

Subcritical water treatments were conducted in an apparatus as shown in Fig. 1-2. A 5-g sample of rice straw and 55 mL of water were inserted into a vessel (117-mL SUS-316 stainless steel vessel (i.d. 30 mm, height 165.5 mm)) assembled by Taiatsu Techno (Osaka, Japan). The tightly closed vessel was heated to temperatures ranging from 140 to 260°C using a mantle heater (200 W, Sogo Laboratory Glass Works, Kyoto, Japan) equipped with a TXN-700B temperature-controller (As One, Osaka). The internal vessel temperature was measured

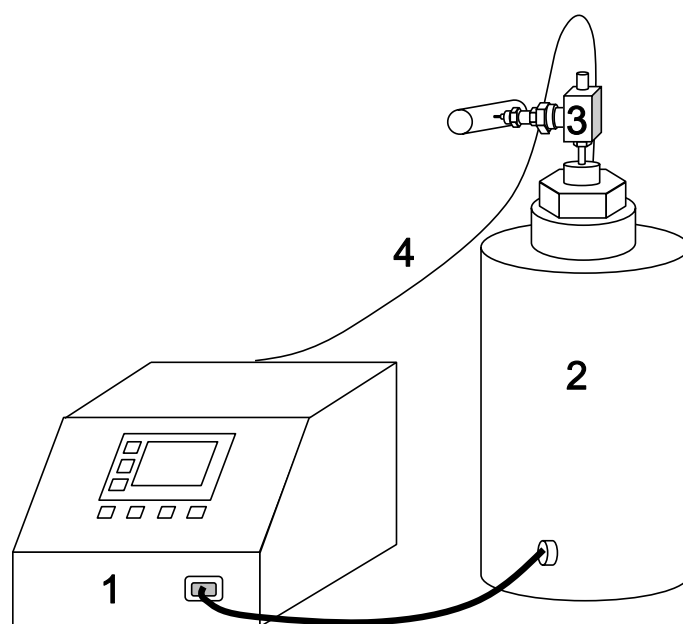


Fig. 1-2. Schematic diagram of the experimental apparatus. 1: temperature-controller, 2: mantle heater, 3: stainless steel vessel, 4: thermocouple.

during the treatment with a thermocouple inserted into a tube installed in the vessel. Exclusive of heat-up time (7.2 °C/min), the desired temperature was maintained for 5 min. Although the pressure inside the vessel was not measured, it was estimated to be 0.33 to 3.6 MPa at 120 to 260°C from the vapor pressure and the expansion of air in the head space. After the treatment, the vessel was cooled immediately to room temperature in an ice bath. The straw extracts were clarified by vacuum filtration through an Advantec filter paper (diameter 110 mm, Toyo Roshi, Tokyo, Japan). The straw extracts were kept in a refrigerator at 4°C until analysis.

1.2.3 Ash content

One gram of cut rice straw was placed in a porcelain crucible and burned with a

Bunsen burner until free of carbon. The white ash obtained was weighed to calculate the percentage of ash in the rice straw [15].

1.2.4 Yield

A 15-mL portion of the liquid straw extract was lyophilized using an Eyela FDU-1200 freeze-dryer (Tokyo Rikakikai, Tokyo, Japan). The lyophilized extracts were placed in a hot-air oven (DN 400, Yamato Scientific, Tokyo, Japan) at 105°C for 3 h to ensure dryness. The yield of the extract was calculated by dividing the weight of the dry solid extract by that of the dry rice straw.

1.2.5 Total carbohydrate content

The total carbohydrate content of the rice straw extract was measured by phenol-sulfuric acid method [16] with some modifications. A 25- μ L aliquot of an 80% (w/w) aqueous phenol solution and 2.5 mL of sulfuric acid were added to 1 mL of the diluted extract or glucose standard solutions and then the solution was mixed well. The mixture was placed at ambient temperature for 10 min and then placed in the 25°C water bath for further 10 min. The total carbohydrate content was measured using a UV-1200 spectrophotometer (Shimadzu, Kyoto, Japan) at 490 nm.

1.2.6 Total protein content

Total protein content was determined by Lowry-Folin method [17]. A standard curve was prepared using a BSA solution. Absorbance of all the straw extracts and the protein standard solution was measured using the spectrophotometer at 500 nm.

1.2.7 Total phenolic content

The diluted straw extract (100 μ L) was mixed with freshly prepared Folin-Ciocalteu reagent (400 μ L) and 75 g/L of sodium carbonate (1 mL) to measure the total phenolic content of the straw extract [18,19]. After filling the sample to 5 mL with distilled water, the mixture was kept in the dark at ambient temperature for 2 h to complete the reaction, and the absorbance at 765 nm was measured. Gallic acid was used as the standard, and the total phenolic content was expressed as gallic acid equivalent (mg-gallic acid/g-straw).

1.2.8 Radical scavenging ability

A 4-mL aliquot of the diluted rice straw extract or L-ascorbic acid (vitamin C) standard solution was mixed with a 1-mL aliquot of 0.5 mmol/L DPPH in ethanol. The mixture was agitated well and kept in the dark for 20 min at ambient temperature. The remaining radical quantity was then estimated by measuring absorbance at 516 nm. Radical scavenging ability as a percentage was calculated as follows [20]:

$$\text{Radical scavenging ability} = (A - B + C) / A \times 100 \quad (1-1)$$

where A is the initial absorbance of the blank (a 4-mL aliquot of 50% (v/v) aqueous ethanol), B is the absorbance at 20 min of the mixture of the diluted extract or vitamin C solution and DPPH solution, and C is the absorbance of the diluted extract without the DPPH solution. The radical scavenging ability of the straw extract was described as the amount of the extract necessary to decrease the initial DPPH concentration by 50%, and was expressed in mmol-vitamin C/g-straw.

1.2.9 UV absorption spectra

The straw extracts were diluted with distilled water to attain an absorbance of less than unity for both UV absorption spectra measurements. The absorbance of the extracts was

then measured at 200-400 nm.

1.2.10 Statistical analysis

All the experiments were conducted in triplicate. The results obtained were analyzed using Microsoft[®] Excel 2010 by two-way analysis of variance (ANOVA).

1.3 Results and Discussion

1.3.1 Effects of temperature on the properties of the extract

Due to decomposition of the cell-wall structure of rice straw, subcritical water could dissolve the substances embedded within the cell walls. The extraction yields from the stem parts increased with increasing temperature until it reached maximum at 200°C, and then sharply decreased at higher temperatures (Fig. 1-3). The yield from the leaf part also showed the same trend, but decrease was gradual over the maximum yield. The higher yield for the stem parts suggests that the rice straw stem contained more soluble substances than the leaf.

The total carbohydrate content of the extracts also increased up to a treatment temperature of 200°C, and decreased sharply at temperatures higher than 200°C. This decrease can be explained by degradation of carbohydrates at high temperatures [21]. The total protein content of the extracts was very low. This corresponds to the fact that rice straw itself contains proteins at low contents [22].

The yield and total carbohydrate and protein contents of the extracts from the stem did not depend on the kind of stem parts, and they were different from those of the extract from the leaf, which showed lower values for all the properties ($p < 0.05$). The leaf part showed approximately a 2-fold higher value for ash content (Table 1-1). The high

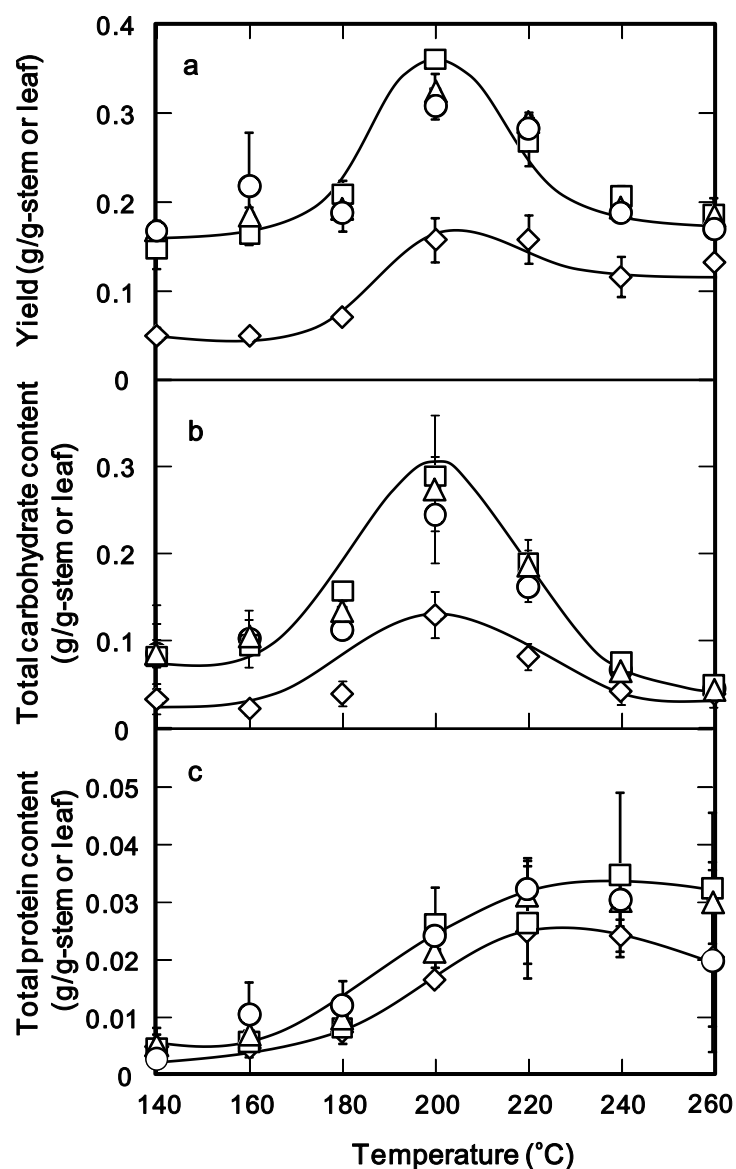


Fig. 1-3. (a) Yield and (b) total carbohydrate and (c) protein content of the rice straw extracts obtained at different treatment temperatures. (○) upper, (△) middle, and (□) lower parts of the stem and (◇) leaf.

Table 1-1 The ash content of rice straw

Part of straw	Ash (%w/w)
Upper stem	7.2 ± 0.15
Middle stem	6.4 ± 0.60
Lower stem	8.5 ± 0.17
Leaf	15.2 ± 0.41

ash content represents compensation in lower percentages for the carbohydrate and protein contents.

1.3.2 UV absorption spectra

UV spectra were measured for the extracts treated at various temperatures (Fig. 1-4). The spectra of the extracts were divided into three groups for expedience according to the treatment temperature: low (140-180°C), moderate (200°C), and high (220-260°C). The spectral profiles within a given group showed a little difference, and hence the spectral profiles of the extracts obtained at temperatures which gave the highest absorbance, 180, 200, and 240°C, were chosen to represent each group. The extracts prepared at low temperature and those at moderate temperature showed absorption maxima at about 280 nm and a small shoulder at about 220 nm, while the extracts obtained at high temperature showed maxima at about 280 nm and a shoulder at about 220 nm. The similarity in the UV spectra within each group suggests that their constituents are probably identical substances, although the concentrations were different.

Maximum absorption at about 220 nm might indicate the presence of carboxyl and carbonyl groups [23,24]. The absorption maximum around 280 nm probably indicates various phenolic substances originating from lignin and protein [25,26].

Figure 1-5 shows the ratio of the absorbance at 280 nm to that at 220 nm for the extracts obtained at different temperatures. At temperatures from 140 to 180°C, the ratios were found to show less variation and were below 1. This suggests that the extracts at low temperatures contained phenolic substances at lower amounts. The ratios rose at up to 240°C, and then dropped at 260°C. This drop in the absorbance ratio must be due to the following reasons: thermal degradation of phenolic substances at high temperature, which led to

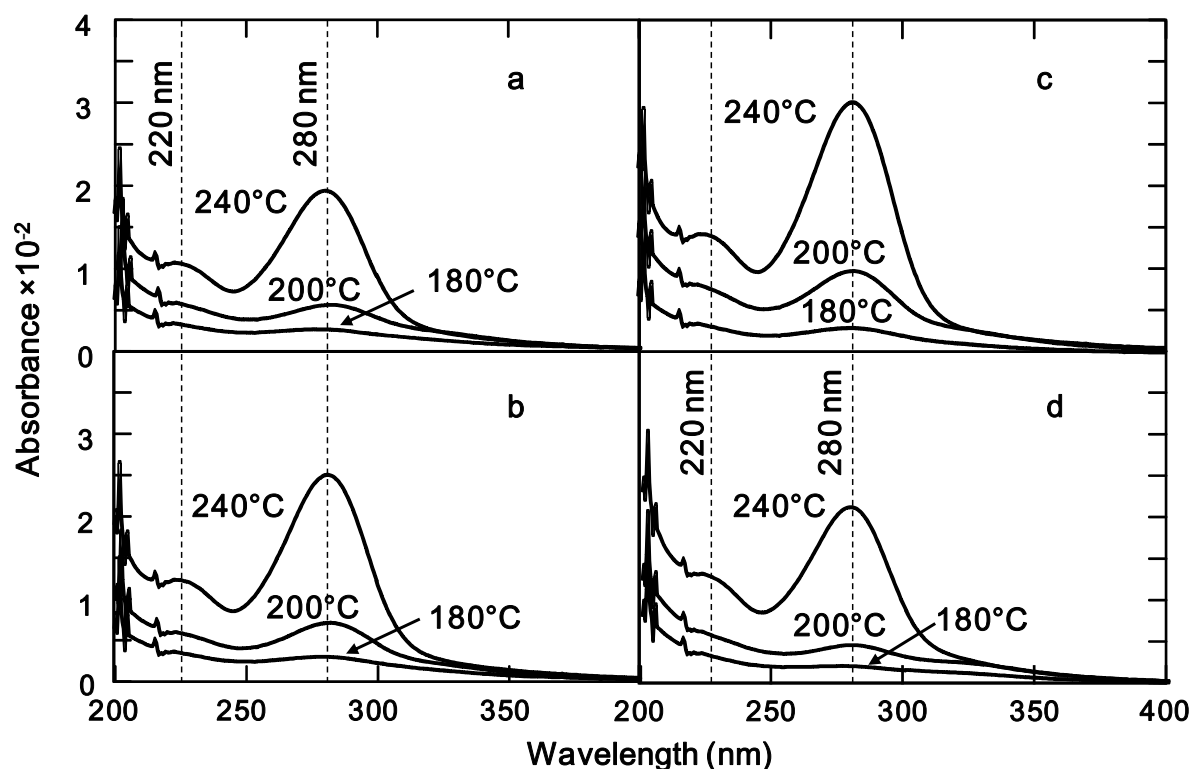


Fig. 1-4. UV spectra of rice straw extracts prepared from (a) upper, (b) middle and (c) lower parts of the stem and (d) leaf at 240, 200 and 180°C. The dotted lines indicate 220 nm and 280 nm.

a decrease in absorbance at 280 nm, and an increase in carbonyl products.

1.3.3 Phenolic content and radical-scavenging ability

The total phenolic contents of the extracts were measured because the UV spectra suggested the presence of phenolic substances. The phenolic content of the extracts increased with increasing treatment temperature, and a drastic increase was observed when the treatment temperature exceeded 180°C (Fig. 1-6a) because most of the phenolic substances in rice straw are bound each other as lignin, forming the structure of the cell walls of rice straw [5,27,28]. Deformation of the straw cell walls at high temperatures might have been

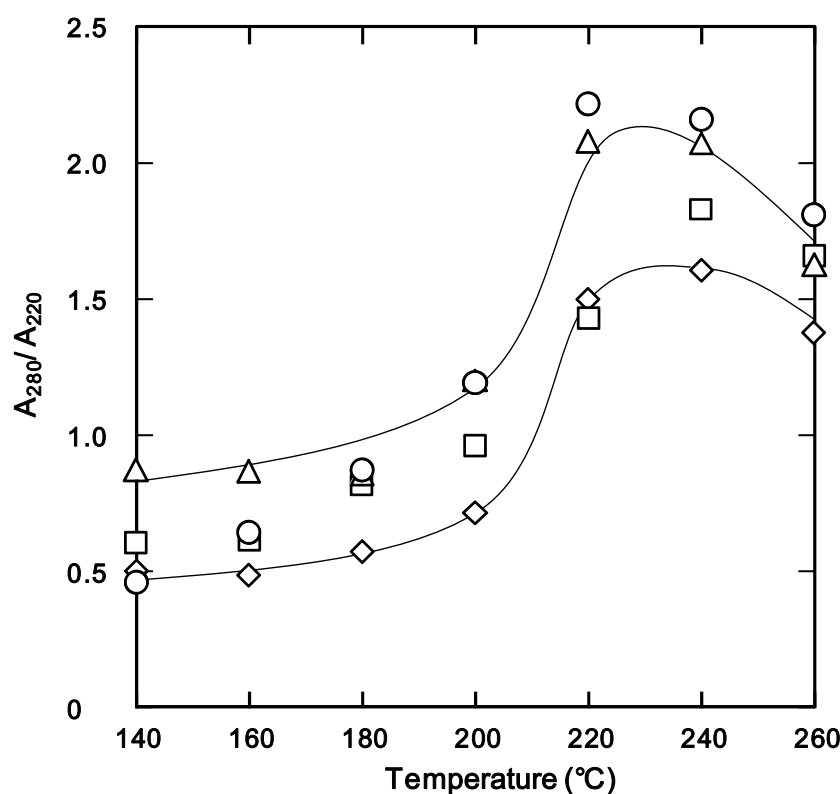


Fig. 1-5. Ratio of absorbance at 280 nm to that at 220 nm for the rice straw extracts prepared at different treatment temperatures. The symbols are the same as in Fig. 1-3.

the reason for the increase in total phenolic content. The sharp increase in phenolic content over 200°C suggests decomposition of the cell walls due to subcritical treatment. The phenolic contents of the extracts of all the stem parts had the same properties, but they were different from those of the leaf ($p < 0.05$).

Figure 1-6b shows the effects of treatment temperature on the DPPH radical scavenging ability. Statistical analysis revealed that the stem and leaf parts of the straw showed different DPPH radical scavenging ability, and that this ability of the extract depended on the treatment temperature ($p < 0.05$). The DPPH radical scavenging ability of the extract increased gradually with increasing treatment temperature. Several studies have shown that the DPPH radical scavenging ability is ascribable to phenolic substances in the extracts [27,29,30]. The plot between DPPH scavenging ability and total phenolic content indicates

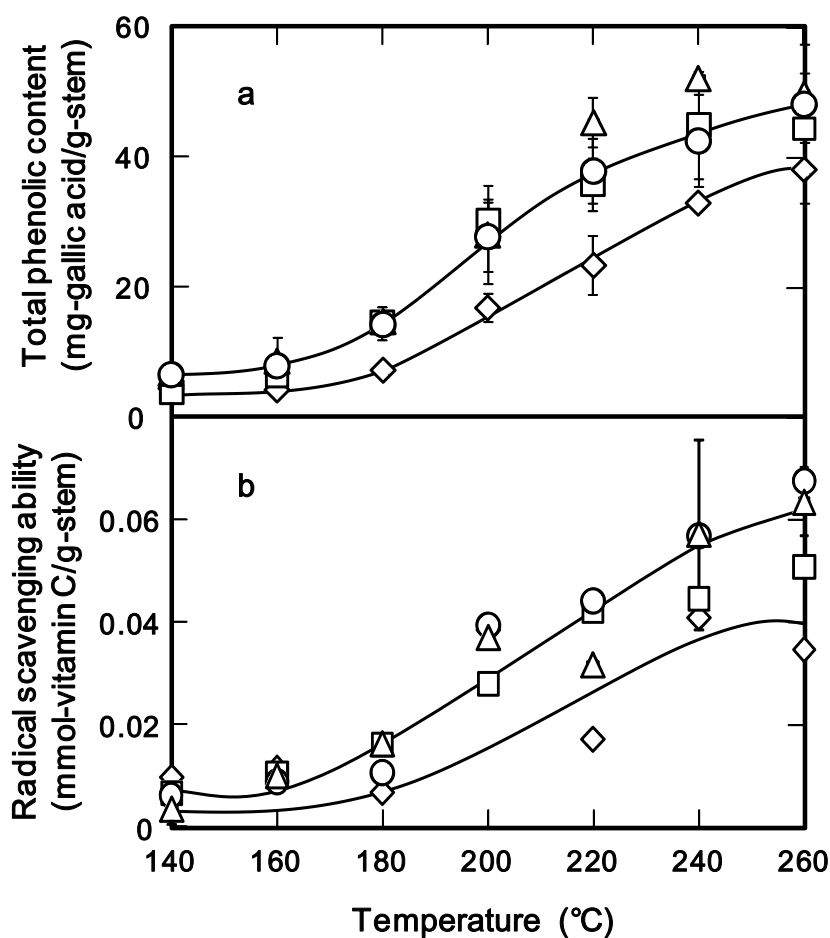


Fig. 1-6. (a) Total phenolic content and (b) radical scavenging ability of the rice straw extracts prepared at different treatment temperatures. The symbols are the same as in Fig. 1-3.

a strong positive correlation ($R = 0.92$) (Fig. 1-7). The plot also indicates that there is no difference between the stem and leaf parts in relation to DPPH scavenging ability and total phenolic content. Hence it is suggested that phenolic substances are also major compounds responsible for the antioxidative ability of the rice straw extract.

1.4 Conclusion

The major decomposition of the rice straw cell wall structure occurred at 200°C. This was evidenced by the maxima of the yield and total carbohydrate content at 200°C and

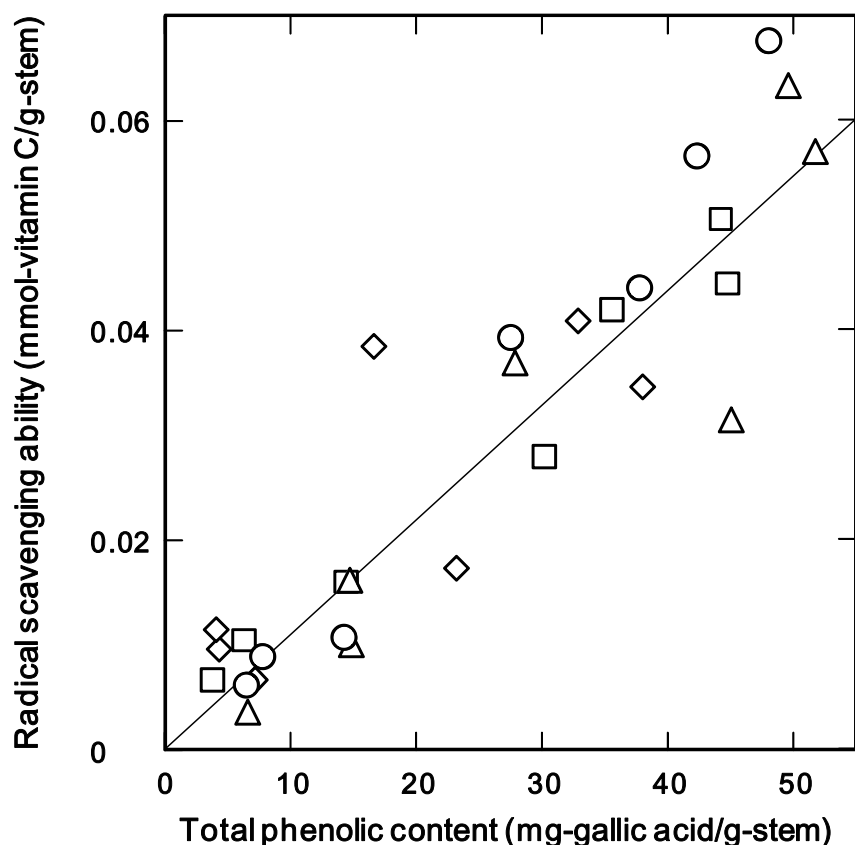


Fig. 1-7. Radical scavenging ability versus total phenolic content of the rice straw extract. The symbols are the same as in Fig. 1-3.

the drastic increase of the total phenolic content at 180°C. The decomposition of carbohydrates also occurred at high treatment temperature. Hence, the optimum point for extracting carbohydrates from the rice straw by the subcritical water would be at 200°C. The total phenolic content increased with the increasing treatment temperature. The highest total phenolic content and radical scavenging ability were achieved when the rice straw was treated with the subcritical water at 260°C. The extracts obtained from the stem parts of rice straw showed no significant difference in yield or in carbohydrate, protein, or phenolic contents ($p < 0.05$). However, the extracts obtained from the leaf part displayed lower values for all these features.

Chapter 2

Effects of Treatment Time during Subcritical Water Treatment and Its Re-Treatment on the Properties of Rice Stem Extract

2.1 Introduction

Rice straw consists of cellulose, hemicellulose, lignin, and other minerals. Phenolic compounds in rice straw, which cross-link with lignin in the cell wall structure [5], may have potential usages for their antioxidative abilities. This chapter aims to investigate the effects of treatment time during subcritical water treatment on the properties of the rice stem extract. The rice stem without leaves was selected as the specimen to undergo the subcritical water treatment, because the rice stem has a higher total phenolic content than the rice leaf. The re-treatment of the extract was also employed to understand the effect of subcritical water treatment on the properties of the extract without extraction. The total carbohydrate and phenolic contents, radical scavenging ability, metal chelating ability, tyrosinase inhibition ability, and antioxidative ability of the extract against autoxidation of linoleic acid were evaluated.

2.2 Materials and Methods

2.2.1 Materials

Tyrosinase from mushroom were from Sigma-Aldrich Japan (Tokyo, Japan). Iron (II) chloride tetrahydrate ($\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$), 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4',4''-disulfonic acid monosodium salt (ferrozine), and L-tyrosine were from Wako Pure Chemical Industries (Osaka, Japan). All other materials were the same as described in 1.2.1.

2.2.2 Preparation of extract

Extraction procedure was done in the same manner described in 1.2.2. In this chapter, 5 g of rice stem and 55 mL of water were used. The treatment temperature of 260°C, in which the highest total phenolic content was achieved, was selected according to the previous study. Excluded from a heat-up period (7.2 °C/min), the designated temperature was maintained for 0-20 min. The resulting extracts treated for 5 min were subjected to re-treatment at 260°C. Similar to the previous treatment, the desired temperature was maintained for 0-60 min exclusive of the heat-up period.

2.2.3 Total carbohydrate and phenolic content and radical scavenging ability

Determinations of total carbohydrate and phenolic contents and radical scavenging ability of the extracts were the same as described in 1.2.5, 1.2.6, and 1.2.8, respectively.

2.2.4 Iron (II) chelating ability

Iron (II) chelating ability of the extract was measured by the modified method of Carter [31]. A 500-μL sample of the rice stem extract was mixed with 25 μL of 1 mmol/L $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ and 425 μL of distilled water. The mixture was left for 1 min at room temperature, and then was mixed with 50 μL of 5 mmol/L ferrozine. After being left at room temperature for another 10 min, the mixture was centrifuged at 3000 rpm for 5 min. The absorbance of the supernatant was measured at 562 nm. The percentage of chelating ability was calculated using an equation similar to Eq. (1-1), where A is the absorbance of the $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$, B is the absorbance of the mixture of sample and $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$, and C is the absorbance of the sample without $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$.

2.2.5 Tyrosinase inhibition ability

The tyrosinase inhibition ability was measured according to the method by Mason and Peterson with some modifications [32]. A 0.1-mL sample of the stem extract was mixed with 1 mL of 1 mmol/L L-tyrosine and 0.8 mL of 1/15 mol/L potassium phosphate buffer (pH 6.8) in a test tube. The test tube was put into the water bath for 10 min at 37°C, and then 0.1 mL of a 400 U/mL mushroom tyrosinase solution was added. The mixture was left in the water bath for 30 min at 37°C, and then its absorbance at 475 nm was measured. The percentage inhibition of tyrosinase was calculated using the equation similar to Eq. (1-1) in 1.2.8, where *A* is the absorbance of the enzyme, *B* is the absorbance of the mixture of sample and enzyme, and *C* is the absorbance of the sample without enzyme. The ability of the extract for inhibiting the tyrosinase activity by 50% was compared with that of kojic acid, and expressed in mol-kojic acid/g-stem.

2.2.6 HPLC analysis

The extract was analyzed by HPLC using an LC-10AD HPLC pump (Shimadzu, Kyoto, Japan) equipped with a Hydrosphere C18 separation column (3 mm i.d. × 150 mm, YMC, Kyoto, Japan), a guard column (10 mm i.d. × 30 mm, YMC), and an SPD-10A UV detector (Shimadzu). A 20-μL sample of the 100-fold diluted extract was applied to the column and eluted at 0.3 mL/min under gradient mode. From 0 to 10 min, the methanol concentration in the mobile phase linearly increased from 0 to 61%. The methanol concentration was kept at 61% from 10 to 25 min, and was raised to 70% from 25 to 35 min. The rice stem extracts showed the absorption maximum around 280 nm, which probably indicates various phenolic substances originating from lignin. Hence, the absorbance at 280 nm of the effluent was recorded.

2.2.7 Statistical analysis

All the experiments were done in triplicate. The results obtained were analyzed by using Microsoft[®] Excel 2010 with a two-way analysis of variance (ANOVA). The standard derivations for total phenolic content, radical scavenging ability, tyrosinase inhibition ability, total carbohydrate content, and metal chelating ability were about 11.58%, 16.91%, 11.45%, 15.34%, and 13.64%, respectively.

2.3 Results and Discussion

2.3.1 Effects of treatment time on the properties of the extracts

Figure 2-1 shows the properties of the extracts obtained at the different treatment times. The total phenolic content of the extracts scarcely depended on the treatment time. The phenolic compounds in the extracts would be produced by hydrolyzing lignin in rice stem cell wall. It was reported that the phenolic compounds extracted from lignin are degradable under the sub- and supercritical conditions [33,34]. However, no significant degradation was recognized in this study due to the relatively low temperature compared with the temperatures in the previous studies. The radical scavenging ability of the extract did not change with increasing treatment time. It also showed a linear correlation with the total phenolic content ($R^2 = 0.76$), suggesting that the phenolic compounds in the rice stem extract were the main sources of its radical scavenging ability. Tyrosinase inhibition ability of the extract gradually increased with increasing treatment time up to 10 min, and then it became steady. The steadiness of tyrosinase inhibition ability of the extracts obtained after 10 min might be because the compounds responsible for the tyrosinase inhibition ability have high stabilities in subcritical water. Rice straw was also reported to contain *p*-coumaric acid and salicylic acid, which possess the mushroom tyrosinase inhibition ability [35-39]. However, those phenolic

compounds are reported to decompose in subcritical water with the temperature above 150°C [40,41]. Therefore, one of other reasons for the steady tyrosinase inhibition could be that the decomposition products of the phenolic compounds also possess the tyrosinase inhibition ability.

Total carbohydrate content in the extract decreased almost linearly with increasing treatment time. The carbohydrate content originated from hydrolyzed products of cellulose and hemicellulose in subcritical water. Cellulose and hemicellulose were hydrolyzed to shorter poly-, di-, or monosaccharides, and the resulting saccharides would be further decomposed [42,43]. Decomposition of these saccharides is, therefore, the reason for decrease in the total carbohydrate content in the extract after elongated treatment time.

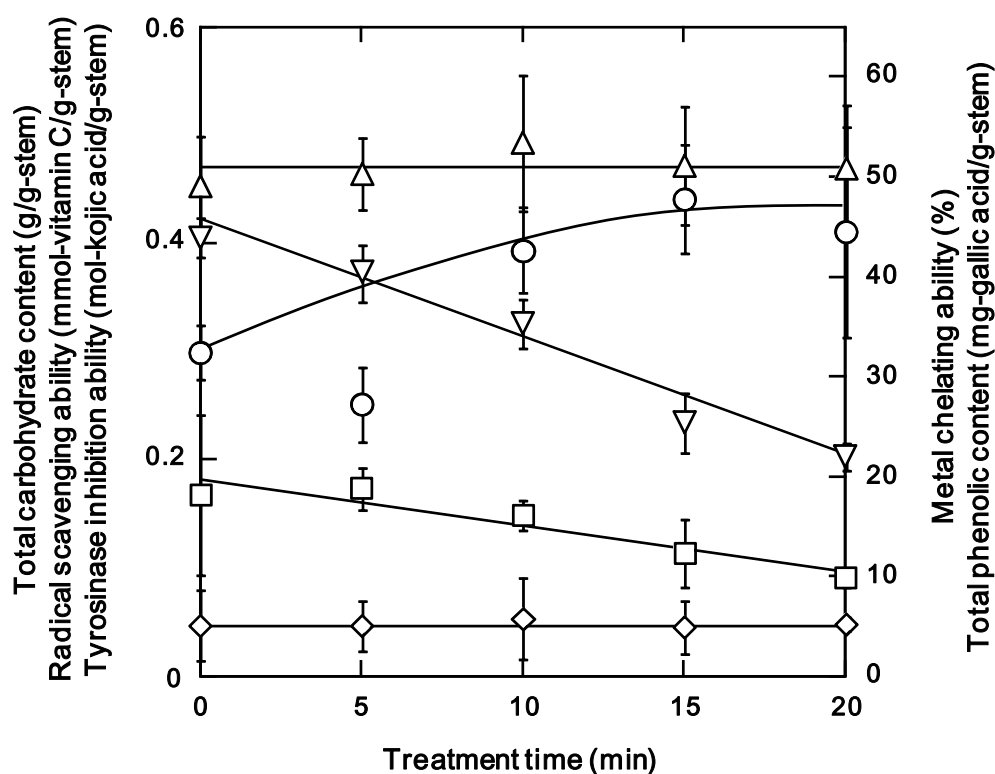


Fig. 2-1. (Δ) Total phenolic and (▽) carbohydrate content, (◇) radical scavenging ability, (□) metal chelating ability, and (○) tyrosinase inhibition ability of the extracts obtained at different treatment time. The treatment time indicates the time elapsed after reaching 260°C.

Metal chelating ability also decreased gradually with increasing treatment time. This was in similar tendency to that of the total carbohydrate content, and there was a linear correlation between them ($R^2 = 0.94$). Carbohydrates are potential ligands to metals, and their chelating ability would come from the interactions with iron to form complexes through deprotonated hydroxyl groups [44,45].

2.3.2 Properties of the extracts after re-treatment

The extracts obtained at a treatment time of 5 min were subject to further treatment in subcritical water. During the treatment, the time required to reach the desired treatment temperature is defined as heat-up time. Generally, the heat-up time of *ca.* 36 min was needed to reach 260°C. The extract without re-treatment obtained just after reaching the desired temperature had the highest total carbohydrate content, and its total phenol content was not statistically different from those obtained with increasing treatment time (Fig. 2-1). This would suggest that the extraction would already occur during the heat-up period. The total carbohydrate and phenolic contents of the extracts obtained without re-treatment at 5 min (Fig. 2-1) were compared with those of the extracts with re-treatment obtained just after reaching the desired temperature (Fig. 2-2), and they were not statistically different ($p < 0.05$). Hence, it suggests that the heat-up time for the re-treatment would have negligible effect on the properties of the rice stem extract.

In Fig. 2-2, the total phenolic content in the extract decreased with increasing re-treatment time. In subcritical water, it has been reported that lignin in rice stem is hydrolyzed into phenolic compounds, and the resulting phenolic compounds are further decomposed to degraded products [33,34]. It has also been reported that the major phenolic compounds, which are *p*-coumaric acid, *p*-hydroxybenzoic acid, ferulic acid, and vanillic acid [36,37], are rapidly decomposed in subcritical water [40,41], and they would become smaller phenols,

which are more stable under subcritical condition. The radical scavenging ability also decreased progressively with extended treatment time. The total phenolic content and the DPPH radical scavenging ability showed strong linear correlation ($R^2 = 0.95$). This would suggest that the decomposed products from phenolic compounds would not possess the DPPH radical scavenging ability.

The extracts submitted to further treatment for 0, 20, 40 and 60 min were chosen for HPLC analysis at 280 nm. The HPLC chromatograms for the extracts exhibited two major peaks at retention time of *ca.* 16 min (Fig. 2-3). These two major peaks decreased with increasing treatment time. However, the minor peaks materialized between *ca.* 18-22 min were stable. The decrease of the major peaks can be explained by the total phenolic content,

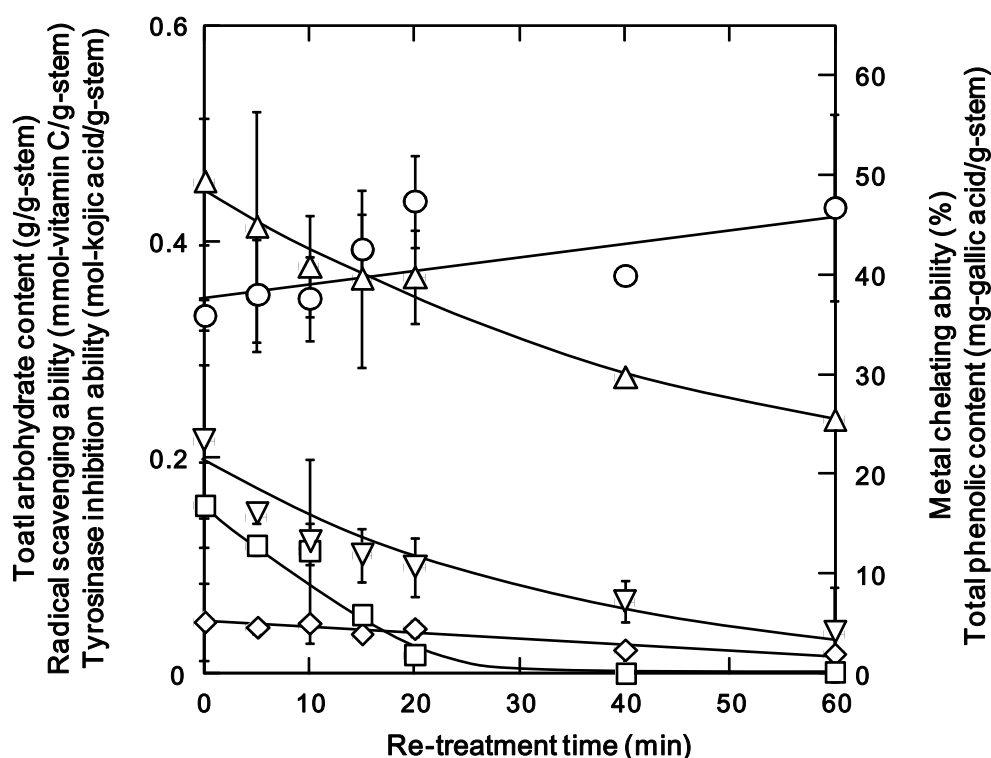


Fig. 2-2. Total phenolic and carbohydrate content, radical scavenging ability, metal chelating ability, and tyrosinase inhibition ability of the extracts obtained after its re-treatment. The symbols are the same as in Fig. 2-1.

which decreased rapidly at the beginning and became steady at longer treatment time. Most of the phenolic compounds in the rice stem extracts showed their peaks, when the concentration of methanol in the mobile phase was at 51%. This suggests that the phenolic compounds in rice stem have moderate polarity.

The treatment time negligibly affected the tyrosinase inhibition ability of the extracts. It was reported that *p*-coumaric and ferulic acid derived amides, which were obtained by coupling reactions, also possess the ability to inhibit tyrosinase activity [46,47]. The black melanin was caused by transformation of L-tyrosine to *ortho*-dopaquinone. This transformation occurs in two steps: hydroxylation of L-tyrosine to L-dopa, and then oxidation of L-dopa to *ortho*-dopaquinone [48]. In the re-treatment, the free radical scavenging ability of rice stem decreased, while its tyrosinase inhibition ability was being steady. This may suggest that the tyrosinase inhibition ability of rice stem extract did not involve the inhibition of catalytic reactions of L-dopa to *ortho*-dopaquinone caused by tyrosinase.

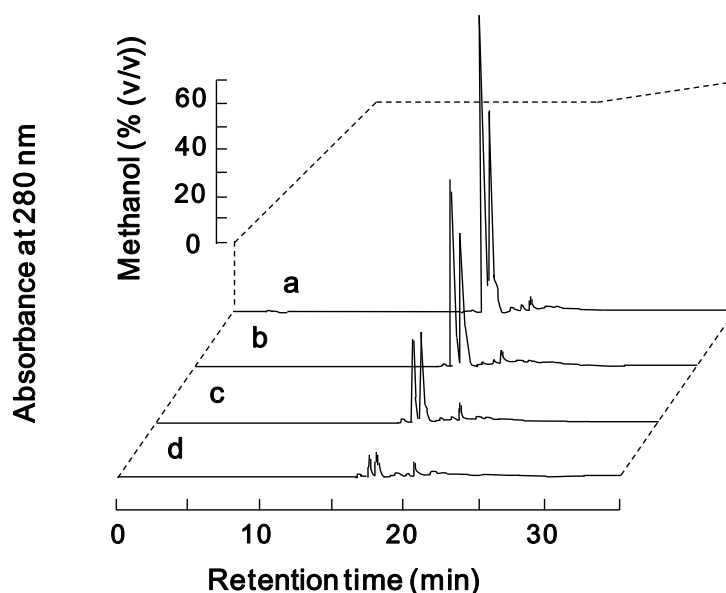


Fig. 2-3. HPLC analysis of the extracts obtained after its re-treatment for (a) 0 min, (b) 20 min, (c) 40 min, and (d) 60 min.

The total carbohydrate content and the metal chelating ability almost linearly decreased with increasing re-treatment time and showed a strong correlation between them ($R^2 = 0.86$). However, the metal chelating ability disappeared after 20 min of re-treatment time. In subcritical water, carbohydrates are decomposed [42,43], and the longer reaction time would result in the formation of more degraded products. The disappearance of metal chelating ability after 20 min of re-treatment would mean that the degraded products from carbohydrate do not possess the metal chelating ability.

2.4 Conclusion

The long-term treatment and re-treatment resulted in decrease in the total phenolic content, total carbohydrate content, radical scavenging ability, and metal chelating ability of the rice stem extracts. However, the tyrosinase inhibition ability of the extracts did not decrease with increasing treatment time. This would indicate that the degradation products of the re-treatment possess the ability to inhibit tyrosinase activity. Therefore, the short-time treatment would be more suitable for obtaining carbohydrates and phenolic compounds with higher contents from rice stem using subcritical water at 260°C.

Chapter 3

Properties of Rice Stem Extracts Obtained Using Subcritical Fluids

3.1 Introduction

Chiou *et al.* have reported that subcritical aqueous ethanol and acetone could effectively extract carbohydrates and phenolic compounds from defatted rice bran [49]. In this chapter, we investigated the effects of the ethanol or acetone content in a subcritical fluid on the carbohydrate and phenolic contents of the rice stem extract. The relationship between the color and radical scavenging ability of the extracts was also established.

3.2 Materials and Methods

3.2.1 Materials

All materials were the same as described in 1.2.1.

3.2.2 Preparation of extract

Extraction procedure was done in the same manner described in 1.2.2. Rice stem was subjected to treatment using aqueous ethanol or acetone with concentration of 0, 20, 40, 60, 80, and 100% (v/v) in subcritical conditions. A 5-g sample of rice stem and 55 mL of an extractant were used in this study. The vessel was heated to 230°C at a heating rate of 7.2 °C/min. This treatment temperature was selected because ethanol and acetone could still be in their subcritical conditions at 230°C by applying pressure.

3.2.3 Yield, total carbohydrate and phenolic content, and radical scavenging ability

The yield, total carbohydrate and phenolic content, and radical scavenging ability of the extracts were determined using the same methods described in 1.2.4-6 and 1.2.8, respectively.

3.2.4 Color measurement

The 0.5-mL stem extract was placed in a quartz cuvette (10 × 10 × 43 mm). A white-color screen was immersed into the extract to attain the height of 5 mm above the cuvette bottom to reflect light back to its source. The loaded cuvette was then placed in an opaque measurement chamber. Color of the rice stem extracts was measured using colorimeter, NF 333 (Nippon Denshoku Industries, Tokyo, Japan). The measured color was reported in CIE color system of L^* (lightness), a^* (redness), and b^* (yellowness).

3.2.5 Statistical analysis

All the experiments were done in triplicate. The results obtained were analyzed using Microsoft[®] Excel 2010 with a two-way analysis of variance (ANOVA).

3.3 Results and Discussion

3.3.1 Yield and total carbohydrate content of the extracts

Figure 3-1 shows the yield and total carbohydrate content of the extracts obtained at different ethanol or acetone concentrations. The extracts obtained using subcritical ethanol/water and acetone/water mixtures demonstrated the exponential correlations between the yield and total carbohydrate content ($R^2 = 0.93$ and 0.95 for the extracts with water/ethanol and water/acetone mixtures, respectively) as shown in the inset of Fig. 3-1.

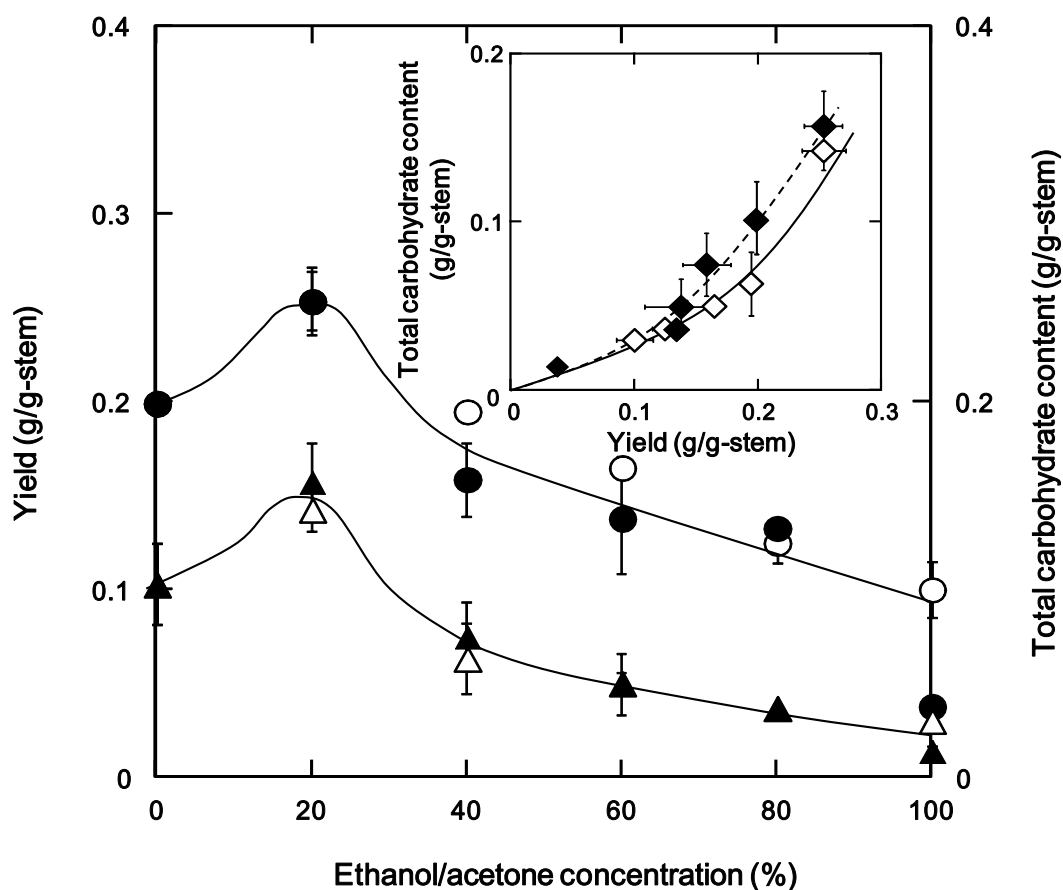


Figure 3-1. (○,●) Yield and (△,▲) total carbohydrate content of the rice stem extracts obtained using subcritical aqueous ethanol (open symbols) and aqueous acetone (closed symbols). The inset shows the relationship between the yield and total carbohydrate content of the rice stem extracts obtained using (◇) subcritical aqueous ethanol and (◆) aqueous acetone.

This strong correlation suggests that the majority of the extracted substances were carbohydrates. The yield and total carbohydrate content of the extracts obtained using ethanol or acetone were not statistically different ($p < 0.05$). This statistical indifference would indicate that the extraction of carbohydrates from rice stem may be governed by the water fraction in the extractant. It was reported that the subcritical water treatment is an effective means to hydrolyze hemicellulose, which is one of the major rice stem components [21,42,50].

The yield and total carbohydrate content of the extracts using 20% ethanol or acetone were higher than those of the extracts obtained using water, and then decreased with the increasing concentration above 20%. Under the subcritical conditions, the decomposition of the carbohydrates also occurs [21]. Therefore, the extraction and decomposition of carbohydrate could be balanced when the 20% (v/v) ethanol or acetone content in the extractant were used under the subcritical conditions.

The higher ethanol content in the extractant would require a longer treatment time in order to obtain a higher carbohydrate content. This was evidenced in Chapter 4, in which the highest carbohydrate content was obtained by a subcritical 50% (v/v) ethanol treatment at 230°C for 5 min.

3.3.2 Total phenolic content and DPPH radical scavenging ability

In Fig. 3-2, the total phenolic contents of the extracts obtained using subcritical ethanol/water and acetone/water show a similar tendency. The total phenolic content was linear up to 40% (v/v) ethanol or acetone in water. It then increased with the increasing ethanol or acetone concentration up to 80%, but drastically decreased thereafter. At a lower concentration of ethanol or acetone (0-40% (v/v)), the extracts obtained using the subcritical ethanol and acetone showed no difference in the total phenolic content. The differences in the total phenolic content between the extracts obtained using the subcritical ethanol and acetone were observed at the higher concentration of ethanol or acetone (60-100% (v/v)). The extracts obtained using the subcritical acetone showed a higher total phenolic content than those obtained using the subcritical ethanol. The majority of the phenolic compounds was reported to be less polar and more readily dissolved in a less polar extractant [51]. Therefore, a higher total phenolic content could be obtained in acetone, which is less polar than ethanol [52,53].

The DPPH radical scavenging ability of the extracts is shown in Fig. 3-2. It slightly decreased with the addition of ethanol or acetone up to 20% (v/v). This decrease was followed by the increase at a 40-60% (v/v) ethanol or acetone concentration and the decrease at 80-100% (v/v) ethanol or acetone concentration.

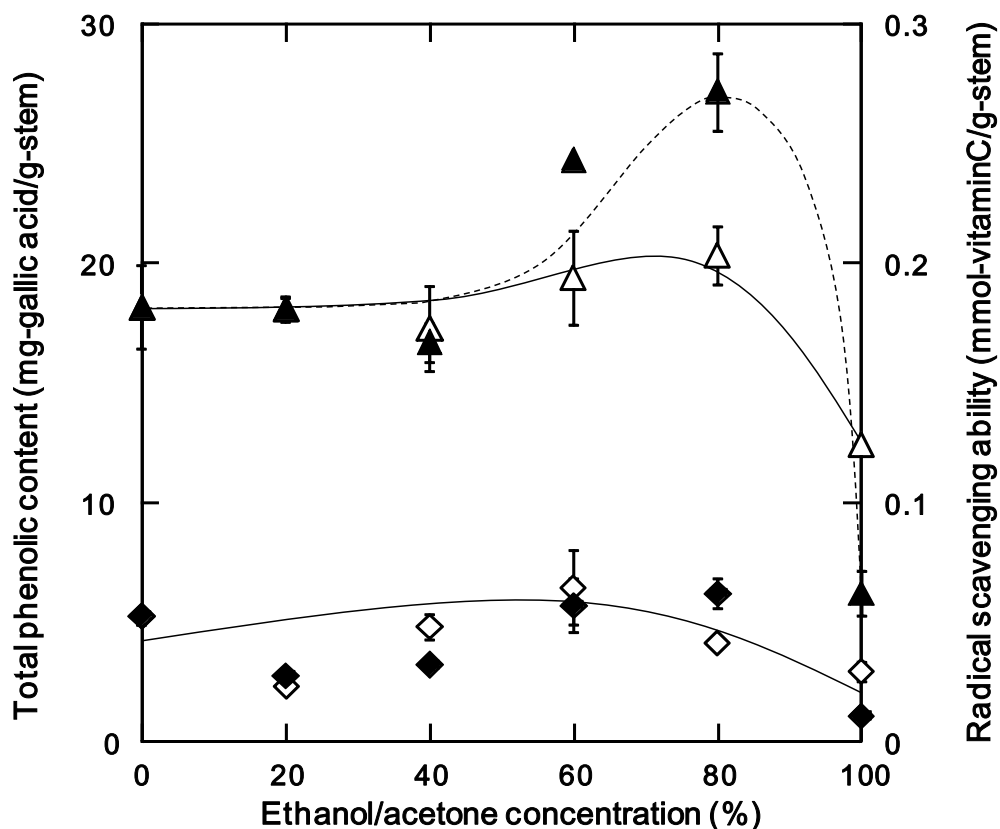


Figure 3-2. (△,▲) Total phenolic content and (◇,◆) radical scavenging ability of the rice stem extracts obtained using subcritical aqueous ethanol (open symbols) and aqueous acetone (closed symbols).

The radical scavenging ability of plant extracts was related to their phenolic contents [27,30]. In Fig. 3-3, two curves were obtained from the relationship between the total phenolic content and DPPH radical scavenging ability; one for the extracts obtained by the subcritical aqueous ethanol treatment and the other for the extracts obtained by the subcritical

aqueous acetone treatment. Excluding the extracts obtained using the subcritical 20% and 80% (v/v) ethanol, there was a strong exponential correlation ($R^2 = 0.98$) between the total phenolic content and DPPH radical scavenging ability of the extract obtained using subcritical ethanol/water. The positions below the curve of the subcritical 20% and 80% ethanol extracts may be due to its small portion of ethanol or water, which caused the precipitation of hydrophobic substances to occur. The precipitation also occurred in the extracts obtained

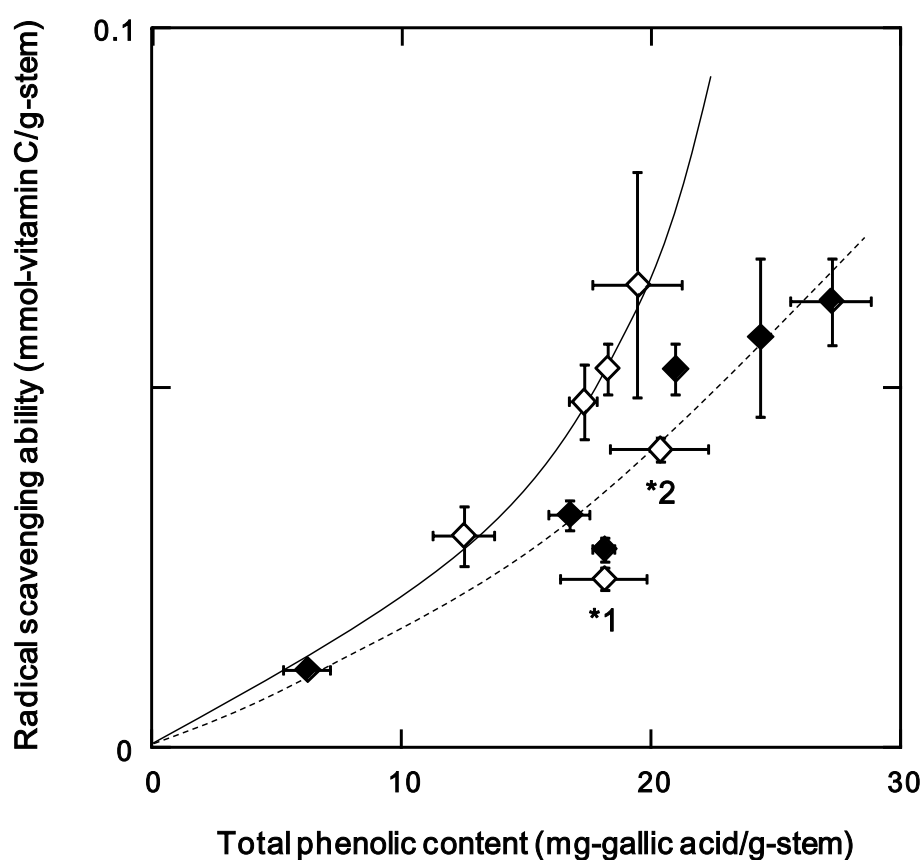


Figure 3-3. Relationship between the total phenolic content and radical scavenging ability of the rice stem extracts obtained using (◇) subcritical aqueous ethanol and (◆) aqueous acetone. *1 and *2 indicate the extracts obtained using subcritical 20% (v/v) and 80% (v/v) ethanol, which were excluded for the correlation between the total phenolic content and the radical scavenging ability.

using subcritical acetone. However, the total phenolic content of the extracts obtained using subcritical aqueous acetone still showed a strong exponential correlation to its DPPH radical scavenging ability ($R^2 = 0.92$).

3.3.3 Color of extracts

All the color parameters (L^* , a^* , and b^*) are in the positive region, indicating that the extracts are yellow-orange in color (Fig. 3-4). A single logarithmic curve ($R^2 = 0.93$) was obtained from the relationship between the lightness and yellowness of the extracts obtained using subcritical ethanol/water and acetone/water. This would mean that the extracts with

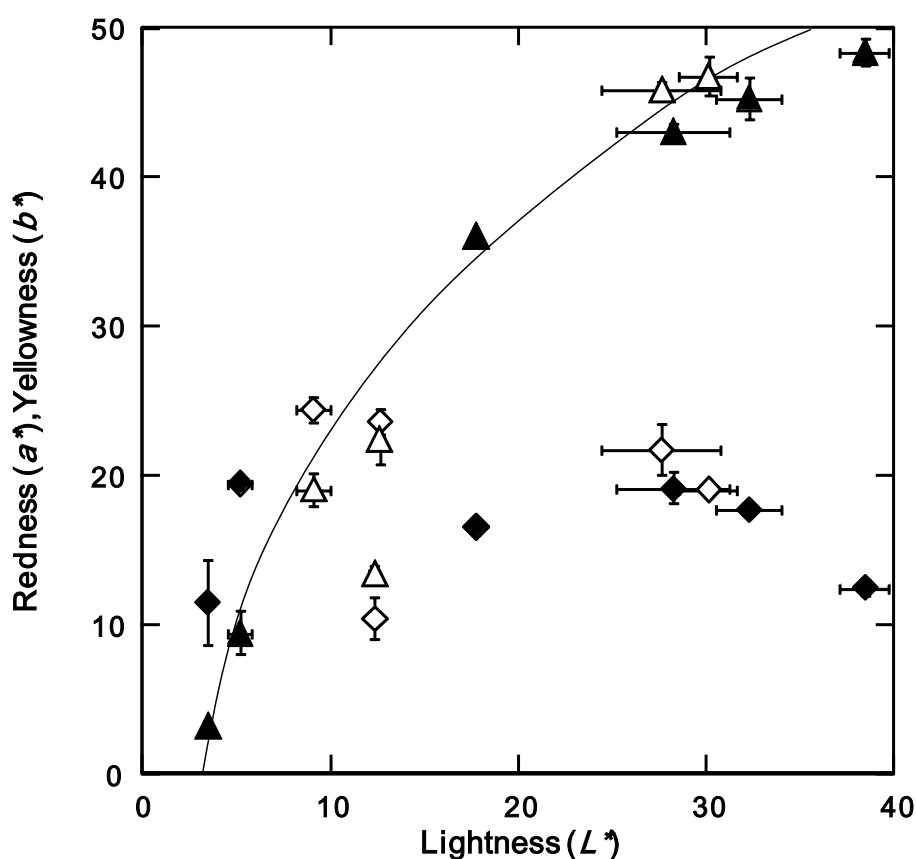


Figure 3-4. Relationship between ($\diamond, \blacklozenge$) redness or ($\triangle, \blacktriangle$) yellowness and lightness of the rice stem extracts obtained using subcritical aqueous ethanol (open symbols) and aqueous acetone (closed symbols).

a similar color could be obtained from the rice stem by the treatment either with subcritical aqueous ethanol or acetone. The redness of the extracts, however, did not show any relation to the yellowness or lightness of the extracts.

The relationship between the lightness of the extracts and DPPH radical scavenging ability is shown in Fig. 3-5. The lightness of the extracts shows a negative linear correlation to the DPPH radical scavenging ability ($R = -0.85$). The negative correlation would mean that the DPPH radical scavenging ability increased with darkening the rice stem extract. The blackness of the extract was due to the degradation products of hemicellulose and lignin that were solubilized into the extractant during the extracting process [11,54,55]. The lightness

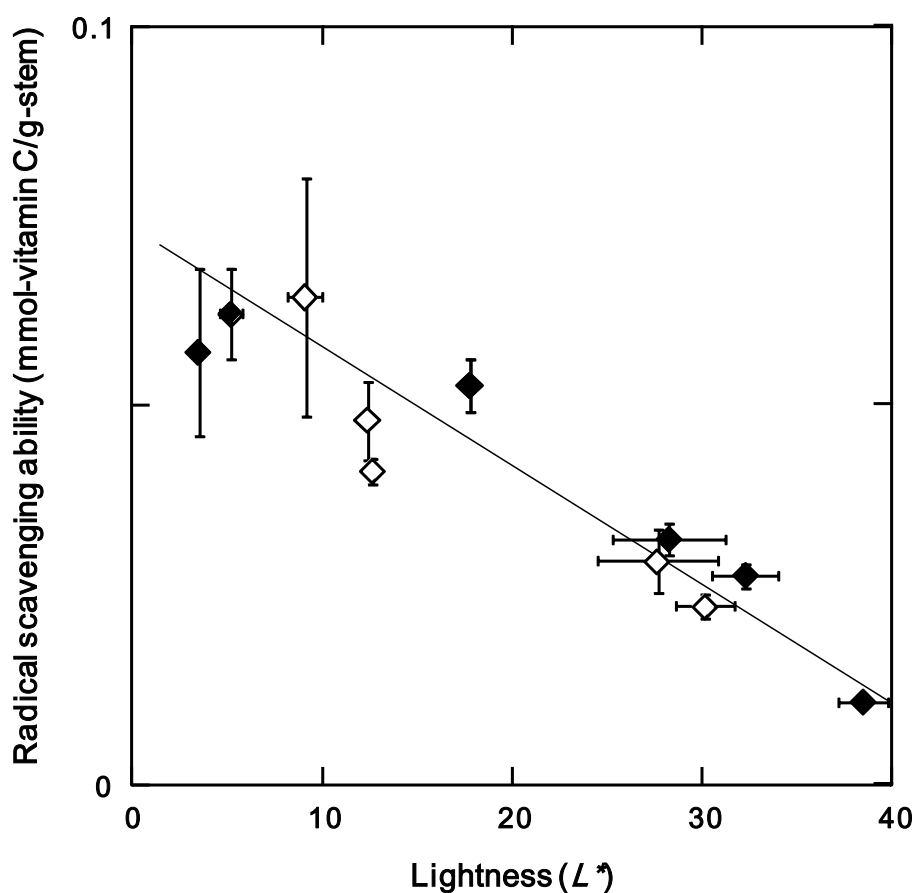


Figure 3-5. Relationship between radical scavenging ability and lightness of the rice stem extracts obtained using (◇) subcritical aqueous ethanol and (◆) aqueous acetone.

did not show any correlation to the total phenolic content (data not shown), suggesting that its DPPH radical scavenging ability may originate from other sources. Hence, the products obtained from the browning reactions, such as Maillard reactions or caramelization, which also relate to the color of the extract, would be other potential sources for the radical scavenging ability of the extract [56-58].

3.4 Conclusion

By employing ethanol and acetone, a non-statistical difference occurred in the total carbohydrate content of the extract from rice stem by its subcritical fluid treatment. This would suggest that extraction efficiency of carbohydrates under subcritical conditions might not be related to the type of an organic solvent. The highest total phenolic content was achieved with the extracts obtained by the treatment using subcritical 80% (v/v) aqueous acetone. In addition, the highest DPPH radical scavenging ability was found in the extracts obtained using subcritical 60% (v/v) ethanol and 80% (v/v) acetone. The DPPH radical scavenging ability could be related to the blackness of the extracts. However, the relationship could not be well established between the total phenolic content and the blackness of the extracts. This may indicate that, apart from the phenolic compounds, the sources of the color could be the degradation products and browning reaction products of hemicellulose and lignin.

Chapter 4

Properties of Rice Stem Extracts Obtained by Subcritical Water/Ethanol Treatment

4.1 Introduction

This chapter deals with the effects of the treatment temperature and ethanol content in the extractant on the properties of the rice stem extract. The addition of ethanol would enhance the extraction efficiency, because the constituents with different polarities would be obtained as the extract by a mixture of ethanol and water [7,59]. The other advantages of ethanol are that it is inexpensive, easy to remove, and has low toxicity. Based on these reasons, the mixture of water and ethanol was used as an extractant in this chapter. This chapter aims optimization of the extraction conditions in terms of yield, carbohydrate content as well as the content of phenolic compounds from rice stem.

4.2 Materials and Methods

4.2.1 Materials

All materials were the same as described in 1.2.1.

4.2.2 Preparation of extract

Extraction procedure was done in the same manner described in 1.2.2. A 5-g sample of rice stem was treated using aqueous ethanol with its concentration of 0, 25, 50, 75, and 100% (v/v) in subcritical conditions. Rice stem was treated at 170, 200, and 230°C for 5 min.

4.2.3 Yield, total carbohydrate and phenolic content, radical scavenging ability, and UV absorption spectra

The yield, total carbohydrate and phenolic contents, radical scavenging ability, and UV absorption spectra of the extracts were determined by the same methods described in 1.2.4-6, 1.2.8, and 1.2.9, respectively.

4.2.4 HPLC analysis

HPLC analysis was carried out in the same manner described in 2.2.6. The 100-fold diluted extract (20 μ L) was applied to a column (Hydrosphere C18, 3 mm i.d. $\times$ 150 mm, YMC, Kyoto, Japan) and eluted at 0.3 mL/min in gradient mode. Distilled water was employed as the eluent from 0 to 10 min. From 10 to 60 min, the concentration of methanol in the eluent linearly increased from 0 to 100% and was maintained at 100% from 60 to 80 min. The elution profile was monitored with absorbance at 280 nm.

4.2.5 Statistical analysis

All the experiments were conducted in triplicate. The obtained results were analyzed for means, standard deviations, and correlations using Microsoft[®] Excel 2010.

4.3 Results and Discussion

4.3.1 Effects of treatment temperature and concentration of ethanol on the properties of extracts

Figures 4-1a and 4-1b show the yields and total carbohydrate contents of the extracts obtained using extractants of different ethanol concentrations. The yield and the total

carbohydrate content of the extract demonstrated a similar tendency. One of the reasons for this similarity would be that the major sources of carbohydrates in the extracts is hemicelluloses, and that the rice straw consists of approximately 24% hemicellulose [4]. At 170°C, the yield and total carbohydrate content were constant until the 50% (v/v) ethanol and then they slightly decreased. The yield and total carbohydrate content linearly decreased with the increasing ethanol concentration at 200°C. According to Chapter 1, the major decomposition of the rice stem cell wall occurred at the treatment temperature. This decrease suggests that the extraction efficiency of subcritical water may be disrupted by the increasing ethanol concentration. When treated at 230°C, the yield and total carbohydrate content increased until 50% (v/v) ethanol and then drastically decreased. Under the subcritical water conditions, it was reported that the degradation reactions more rapidly occur at the higher temperature [21,30]. The subcritical 50% (v/v) ethanol at 230°C might be the point, where the extraction efficiency and carbohydrate degradation are balanced. This indicate that the increase of the yield and total carbohydrate content would be due to the presence of water, while ethanol reduces the degradation of the extracted carbohydrates. When the solvent contained more ethanol, the higher treatment temperature would be needed in order to achieve the maximum yield. This may be related to the presence of water in the system, in which the higher ion product of water would be required to disrupt the rice stem cell wall structure through hydrolysis. The decrease of the water fraction would result in a decreased ion product. Thus, the higher treatment temperature was necessary to increase the ionization constant of water. This could also be the reason for the lower yields and carbohydrate contents of the extracts obtained using subcritical ethanol than those of the extracts obtained using subcritical water and the ethanol/water mixtures at any treatment temperature.

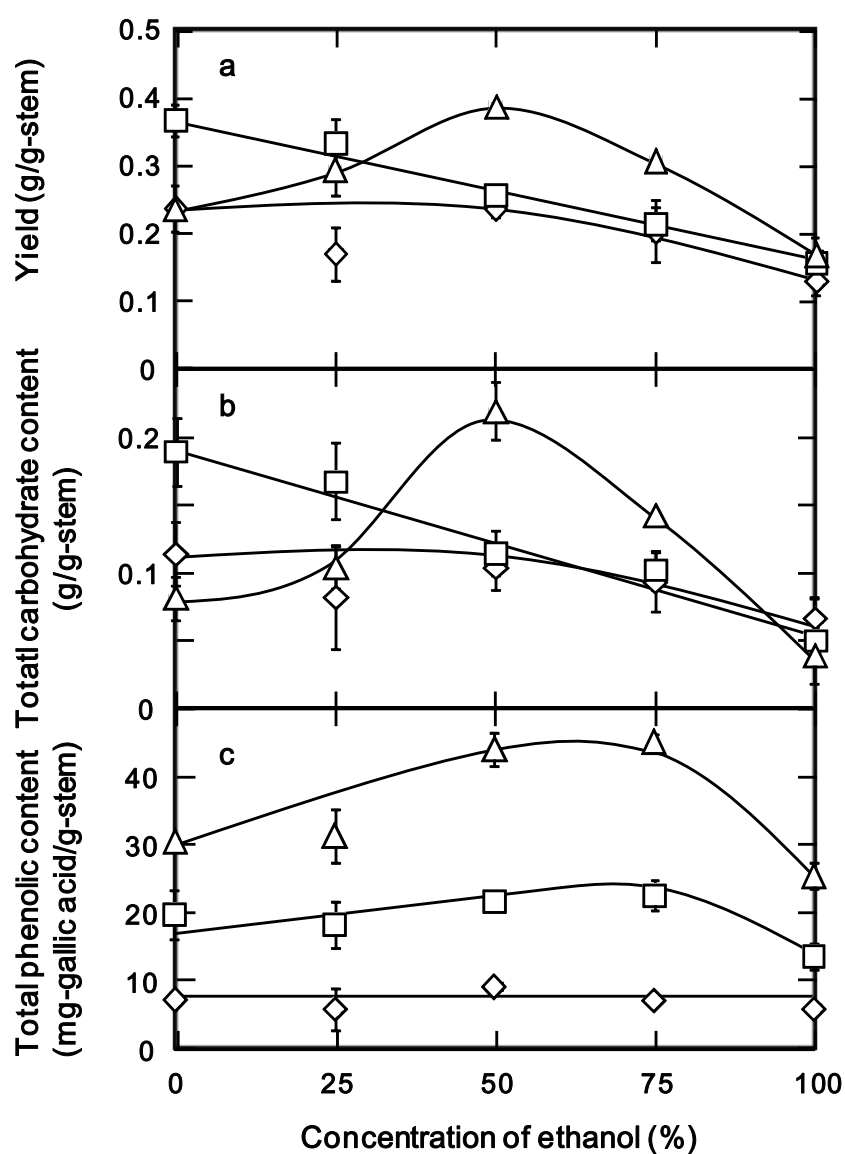


Fig. 4-1. (a) Yield and (b) total carbohydrate and (c) phenolic content of extracts obtained using different subcritical fluids obtained at ($\diamond$) 170°C, ($\square$) 200°C, and ($\triangle$) 230°C.

At the lower treatment temperature of 170°C, the ethanol concentration did not significantly affect the phenolic content of the extracts (Fig. 4-1c). This may be because disruption of the cell wall did not significantly occur at this temperature. However, at the higher temperatures, the total phenolic content increased with the increasing ethanol concentration up to 75%. This increase might be because the main source of the phenolic compounds in vascular plants is lignin, which cross-links to cellulose and hemicellulose to

form the cell wall structure [60]. At elevated temperatures, the disruption of the cell wall occurred and subsequently released the phenolic substances from the cell wall into the extract [30]. The total phenolic content in the extract treated with a subcritical ethanol/water mixture was higher than that with the subcritical water or ethanol. The highest total phenolic content was achieved when the rice stem was treated with a subcritical 75% (v/v) ethanol/water mixture at 230°C. The polarity of solvent plays an important role in the extraction process. Ethanol is less polar than water [52,53], and the phenolic compounds more readily dissolve in a less polar solvent [51]. Additionally, the decomposition of the cellular structure also occurs during the subcritical water treatment [30,61], and this would permit an extractant to easily

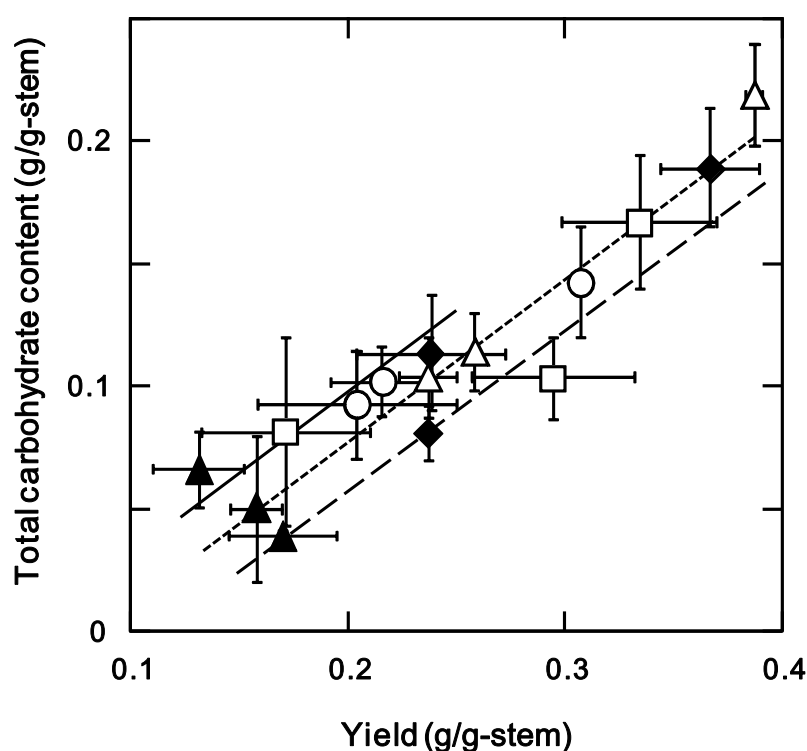


Fig. 4-2. Relationship between the yield and the total carbohydrate content of the rice stem extracts obtained at (—) 170, (-----) 200, and (— —) 230°C using (◆) subcritical water, (□) 25% (v/v) ethanol/water, (△) 50% (v/v) ethanol/water, (○) 75% (v/v) ethanol/water, and (▲) ethanol.

access to the inner phenolic compounds. Solubility of phenolic compounds is higher in the mixed subcritical solvent than in the subcritical water, therefore, it was advantageous to improve extraction efficiency.

The plots of the total carbohydrate content and the yield gave three straight lines (Fig. 4-2); one for the extracts obtained at 170°C ($R^2 = 0.96$), and the others for those obtained at 200 and 230°C ($R^2 = 0.98$ and 0.95, respectively). The slope of those lines would indicate the degradation rate of carbohydrates. With increasing treatment temperature, the lines became steeper, suggesting that the degradation of carbohydrate compounds occurred more rapidly.

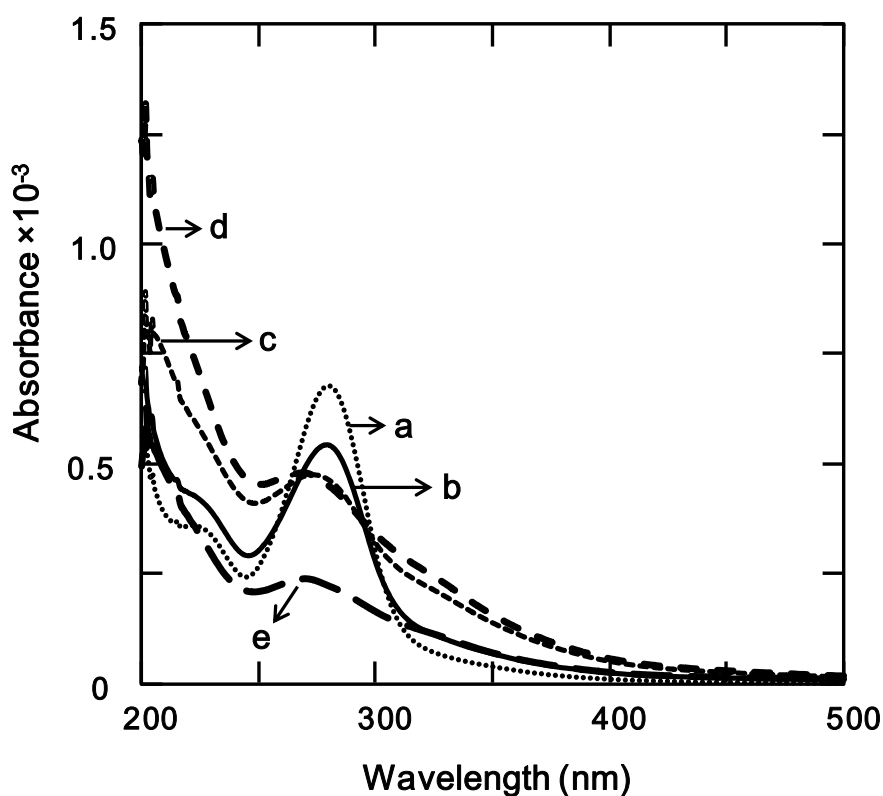


Fig. 4-3. UV-Vis spectra of the rice stem extracts obtained at 230°C using subcritical (a) water, (b) 25% (v/v) ethanol/water, (c) 50% (v/v) ethanol/water, (d) 75% (v/v) ethanol/water, and (e) ethanol.

4.3.2 UV absorption spectra

The UV spectra of the extracts showed that the extracts obtained using different concentrations of ethanol gave different spectra (Fig. 4-3), indicating the different chemical compositions of the extracts. The UV spectra of the extracts obtained using subcritical water and a subcritical 25% (v/v) ethanol/water mixture showed absorption maxima at about 280 nm and a small shoulder at about 220 nm. The spectra of the extracts obtained using the subcritical 50 and 75% (v/v) ethanol/water mixtures and subcritical ethanol showed almost the same tendency, *i.e.*, a plateau around 240–260 nm. Absorbances from 200 to 500 nm of the extracts obtained using the subcritical 50 and 75% (v/v) ethanol/water mixtures were higher than that obtained using the subcritical ethanol. The absorbance at 280 nm suggests the presence of phenolic compounds, which may be derived from lignin [7,62]. The lower absorbance around 250 nm indicates non-lignin materials, such as the decomposition products of the carbohydrates and other extractives [6].

4.3.3 Effects of treatment temperature and ethanol concentration on the radical scavenging ability of the extracts

Many reports have related the radical scavenging ability of plant extracts to their phenolic components [27,29,30]. The radical scavenging ability of the rice stem extracts also demonstrated a high correlation ($R^2 = 0.97$) to their total phenolic content (Fig. 4-4). Similar to the total phenolic content, the radical scavenging ability of the extract also increased with the increasing treatment temperature, and the highest radical scavenging ability of 0.3 mmol-vitamin C/g-stem was obtained when the rice stem was treated with a subcritical 75% (v/v) water/ethanol mixture at 230°C. The plots of the radical scavenging ability versus the total phenolic content gave two curves; one for the extract obtained using the subcritical water and ethanol/water mixtures, and the other for the extracts obtained using the subcritical ethanol.

These relationships suggest that the phenolic compounds are some of the sources for the radical scavenging ability in the rice stem extract. The radical scavenging ability of the extracts obtained using subcritical ethanol was comparatively lower than that of the extracts obtained using subcritical water and ethanol/water mixtures.

4.3.4 HPLC analysis of the extracts

The extracts obtained using different subcritical fluids at 230°C were analyzed by HPLC at 280 nm (Fig. 4-5). The major peak around 38 min was seen in all the extracts. These major peaks decreased with the increasing ethanol concentration in the solvent. Meanwhile, the area of minor peak that appeared around 40-70 min increased with the increasing ethanol

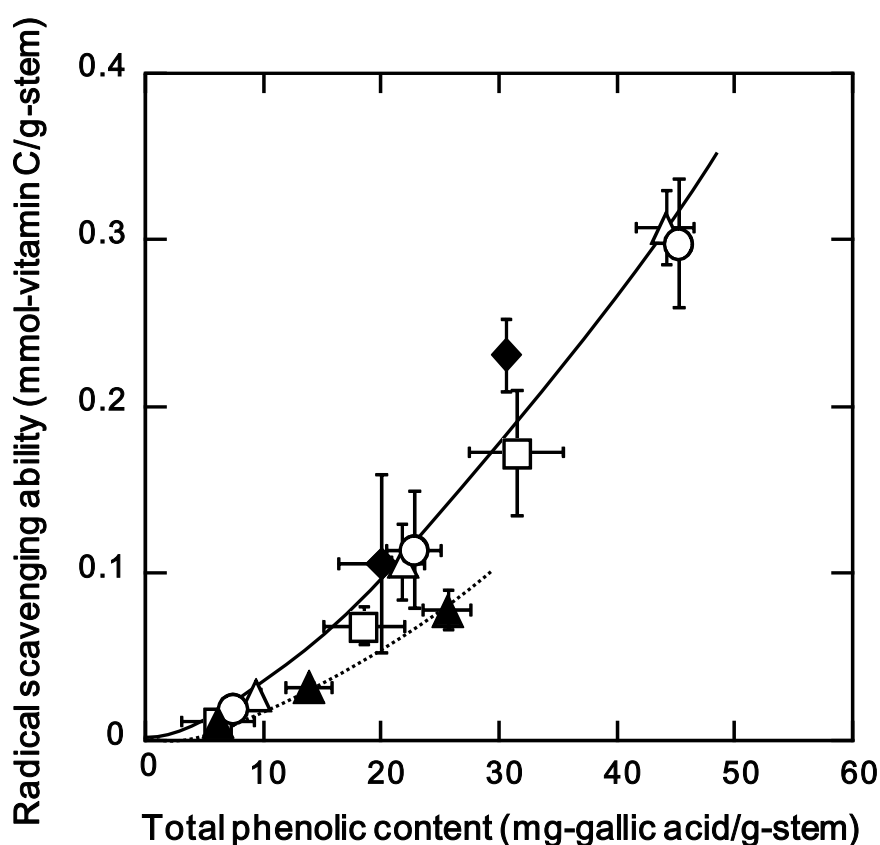


Fig. 4-4. Relationship between the radical scavenging ability and the total phenolic content of the rice stem extracts. The symbols are the same as those in Fig. 4-2.

concentration up to 75%, suggesting that more moderate or less polar phenolic compounds were obtained using the solvent with a higher ethanol content. During the extraction, the substances are dissolved in the solvent possessing a similar polarity. The addition of ethanol and the increase in temperature decreased the polarity of the solvent [63], hence, resulting in less polar phenolic compounds being dissolved into the system. The minor peaks pattern appeared around 40-70 min of extract obtained using subcritical ethanol were different from those of the extract obtained using subcritical 50 and 75% (v/v) water/ethanol mixtures. This would indicate another reason for the extracts obtained using subcritical ethanol to have

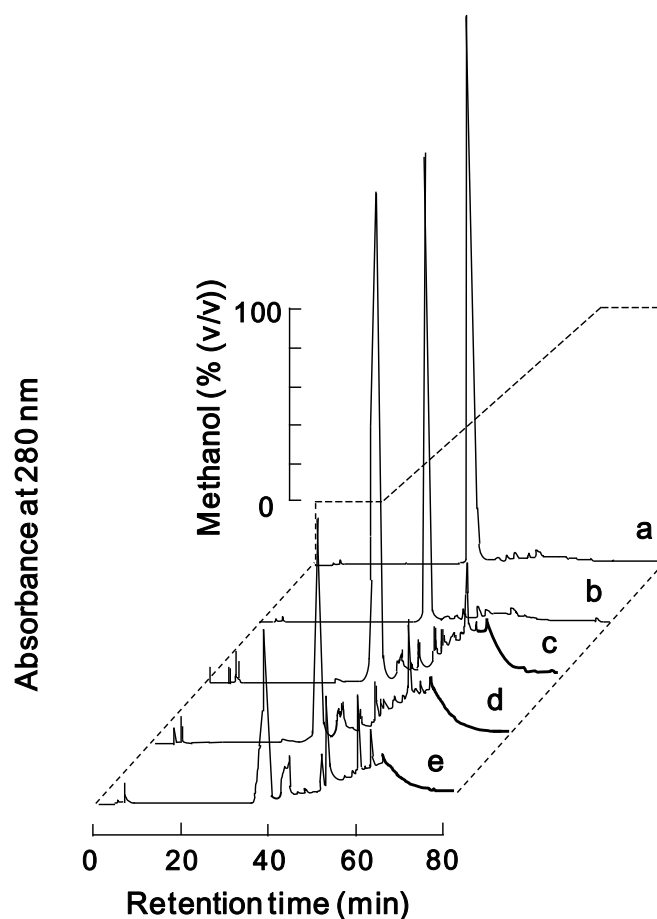


Fig. 4-5. HPLC chromatograms of rice stem extracts obtained using different subcritical fluids at 230°C. Labels are the same as those in Fig. 4-3.

a lower radical scavenging ability.

4.4 Conclusion

The treatment temperature and ethanol concentration affected the properties of the rice stem extract. The yield and total carbohydrate content increased with increasing treatment temperature and ethanol concentration up to 75% (v/v). The higher treatment temperature also provided the extracts with higher total phenolic content and radical scavenging ability. The ethanol concentration had a slight effect on the content of the extracted phenolic compounds, but did affect the type of phenolic compounds.

Chapter 5

Effects of Repeated Treatment on the Properties of Rice Stem Extract Using Subcritical Water, Ethanol, and their Mixture

5.1 Introduction

The aim of this chapter is to investigate the roles of water and ethanol in extracting process by repeating subcritical treatments using subcritical water, ethanol, and 75% (v/v) ethanol in different orders. Additionally, the properties of the rice stem extract were measured, and the relationship between the color and radical scavenging ability of the extract was investigated.

5.2 Methods and Materials

5.2.1 Materials

All materials were the same as described in 1.2.1.

5.2.2 Preparation of the extract

Rice stem was repeatedly treated 3 times using subcritical water, ethanol, and 75% (v/v) ethanol in different orders. The experimental runs are expressed in Table 5-1. Extraction procedure was done in the same manner described in 1.2.2. A 5-g sample of rice stem and 55 mL of an extractant were added to an extraction vessel. The vessel was heated to 230°C, and the temperature was maintained for further 5 min. The vessel was then put in an ice bath to immediately cool down to room temperature after the treatment to stop the extraction. The crude extracts were filtered through an Advantec filter paper to obtain the clarified extracts. The treated stem was dried in a hot-air oven at 105°C for 3 h before the next treatment. Until

Table 5-1 Treatment run

Run	1st treatment	2nd treatment	3rd treatment
1	Ethanol	75% Ethanol	Water
2	Ethanol	Water	75% Ethanol
3	Water	75% Ethanol	Ethanol
4	Water	Ethanol	75% Ethanol
5	75% Ethanol	Ethanol	Water
6	75% Ethanol	Water	Ethanol

used for the analysis, the extracts were stored in a refrigerator at 4°C.

5.2.3 Yield, total carbohydrate and phenolic contents, and radical scavenging ability

The yield, total carbohydrate and phenolic contents, and radical scavenging ability of the extracts were measured by the same methods described in 1.2.4-6 and 1.2.8, respectively.

5.2.4 Color measurement

The determination of color was the same as described in 3.2.4.

5.2.5 UV absorption spectra

The stem extracts were diluted with distilled water to acquire the solution with an absorbance of less than unity for the UV measurement. The absorbance was then measured from 200 to 350 nm using a UV-1200 spectrophotometer.

5.2.6 Statistical analysis

All the experiments were done in triplicate. The obtained results were analyzed using Microsoft® Excel 2010 with a one-way analysis of variance (ANOVA).

5.3 Results and Discussion

5.3.1 Effects of the repeated treatment on the yield and total carbohydrate content

Figure 5-1 shows the relationship between the yield and total carbohydrate content of the rice stem. The yield and total carbohydrate content of the extracts obtained from the 1st treatment showed a strong linear correlation ($R = 0.91$). Their correlations decreased with more repeated treatments: $R = 0.74$ and 0.69 for the 2nd and 3rd treatments, respectively.

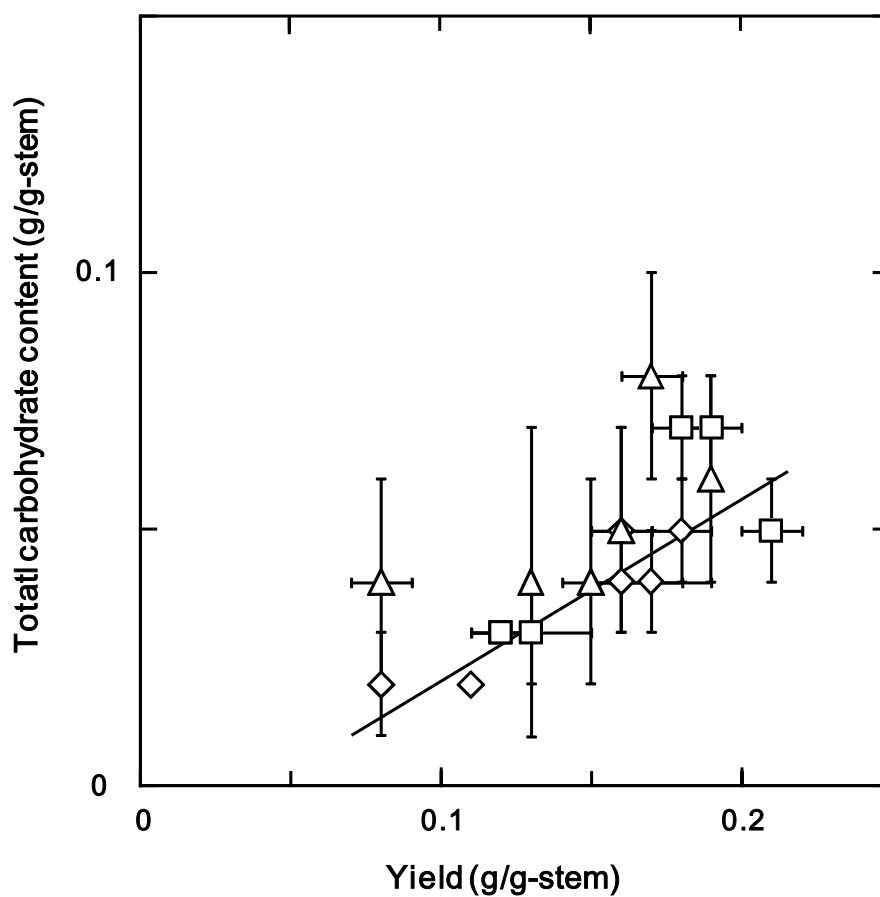


Fig. 5-1 Relationship between the yield and total carbohydrate content of the rice stem extracts obtained from (◇) 1st, (□) 2nd, and (△) 3rd treatments. The line indicates the relationship obtained from the 1st extraction.

The decrease in the correlation might be due to the decrease in the carbohydrate content in the stem sample resulting from the repeated treatment.

The yield and total carbohydrate content of the stem extracts obtained using subcritical water and 75% (v/v) ethanol for the different treatment steps were not statistically different ($p < 0.05$). For the extracts obtained using the subcritical ethanol, the yield and total carbohydrate content were affected by the sequence of the extraction, *i.e.*, the yield and total carbohydrate content increased when subcritical ethanol was employed after the subcritical water treatment. The previous treatment with subcritical water would loosen the cell wall structure of the rice stem due to hydrolysis of the hemicellulose and amorphous part of the cellulose. The hydrolysis of the amorphous part of the cellulose opened the cellulose surface cracks [50]. This would facilitate the extraction by subcritical ethanol in the following step. The yield and total carbohydrate content of the extracts obtained using the subcritical ethanol were usually inferior to those obtained using subcritical water and 75% (v/v) ethanol. This might suggest that the water in an extractant would be required to extract the carbohydrate from the rice stem.

5.3.2 UV absorption spectra

The different UV absorption spectral patterns of the extracts obtained using the different extractants suggest different chemical compounds (Fig. 5-2). The extracts obtained using subcritical water had an absorption maximum at *ca.* 280 nm and a shoulder at *ca.* 220 nm, while those of the extracts obtained using subcritical ethanol and 75% (v/v) ethanol showed a plateau around 240-260 nm. The absorbance at 280 nm would suggest the presence of various phenolic compounds, *i.e.*, phenol acids and flavonoids [26,62,64]. The non-lignin compounds, *e.g.*, the decomposition products of the carbohydrates and other extractives, would be suggested by the absorbance from 200 to 250 nm [6].

The absorption spectra of the extracts obtained using the same extractants in the different repeated treatments showed different absorbances. The highest absorbance of the extracts obtained using the subcritical water and ethanol were those of the extracts from the 2nd treatment of runs 2 (Fig. 5-2a, 4) and 4 (Fig. 5-2b, 2), respectively. Both the subcritical water and ethanol have been reported to be able to partially hydrolyze/alcohololyze or decompose hemicellulose, lignin, and cellulose [4,50,65]. The hydrolysis and decomposition

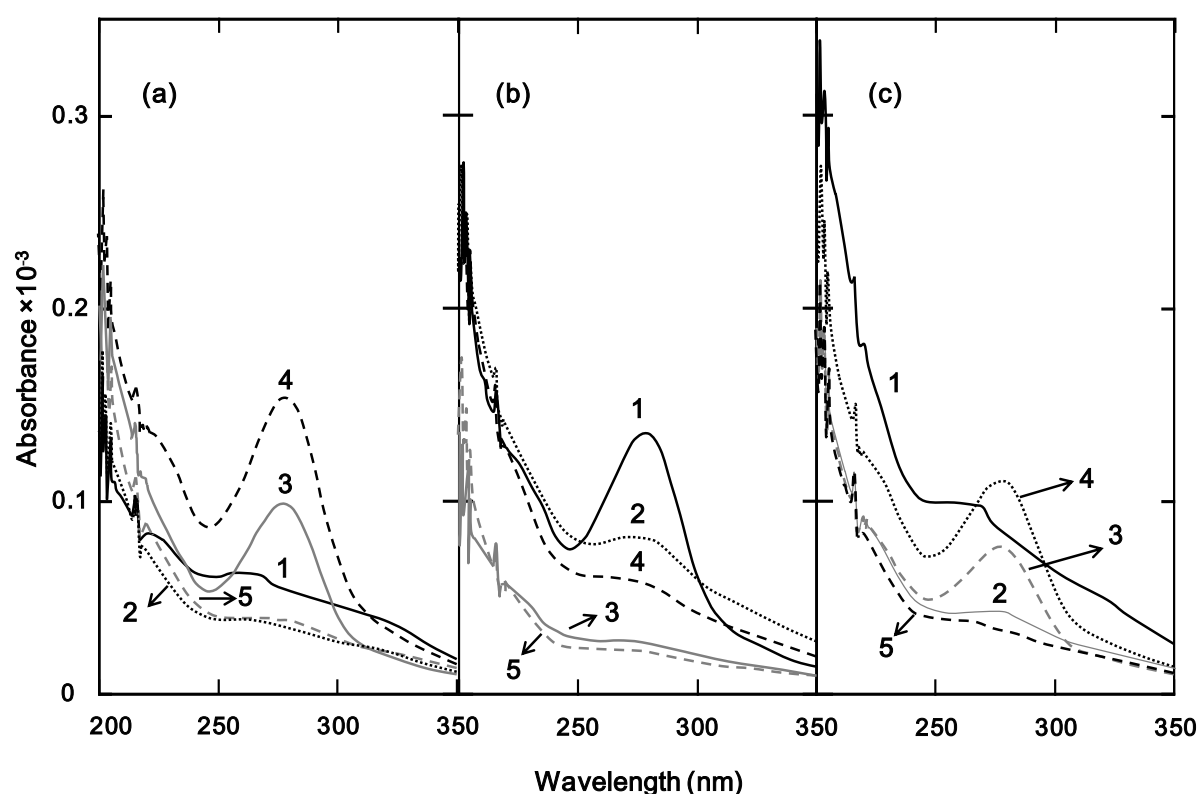


Fig. 5-2 UV-Vis spectra of the rice stem extracts obtained after treated with (a, 1) 1st treatment of runs 1 and 2, (a, 2) 2nd treatment of run 1, (a, 3) 3rd treatment of run 1, (a, 4) 2nd treatment of run 2, (a, 5) 3rd treatment of run 2, (b, 1) 1st treatment of run 3 and 4, (b, 2) 2nd treatment of run 3, (b, 3) 3rd treatment of run 3, (b, 4) 2nd treatment of run 4, (b, 5) 3rd treatment of run 4, (c, 1) 1st treatment of run 5 and 6, (c, 2) 2nd treatment of run 5, (c, 3) 3rd treatment of run 5, (c, 4) 2nd treatment of run 6, and (c, 5) 3rd treatment of run 6.

of the primitive cell wall structure would allow the extractant in the following treatment to extract more substances, and consequently, increase the absorbance. The highest absorbance of the extract obtained using the subcritical 75% (v/v) ethanol was that of the extract obtained from the 1st treatment (runs 5, 6 in Fig. 5-2c, 1). This implies that the subcritical 75% (v/v) ethanol does not require a pre-treatment to enhance its extraction efficiency.

The extracts obtained using the subcritical ethanol in runs 3, 5, and 6, (Fig. 5-2b, 3; Fig. 5-2c, 3; Fig. 5-2c, 5) and subcritical 75% (v/v) ethanol in runs 1, 2, and 4 (Fig. 5-2a, 3; Fig. 5-2a, 5; Fig. 5-2b, 5) showed almost the same UV spectral patterns. They were extracts obtained from the rice stem previously treated with subcritical 75% (v/v) ethanol or ethanol in either the 1st or 2nd treatment. This means that subcritical ethanol and 75% (v/v) ethanol extracted similar compounds, which reduced the amount of extractable compounds in the following repeated treatment. Therefore, similar UV absorption spectra were obtained.

5.3.3 Effects of the repeated treatment on the phenolic content and DPPH radical scavenging ability

The total phenolic content of the extracts is shown in Fig. 5-3 together with the DPPH radical scavenging ability. Similar to the UV spectra results, the phenolic contents of the extracts obtained using subcritical water and ethanol increased when they were subsequently used (runs 2 and 4). The highest phenolic content was achieved in the 2nd treatment of run 4. It was reported that the phenolic compounds more readily dissolve in a less polar solvent [51]. Therefore, the subcritical ethanol could retrieve more phenolic compounds from the rice stem pre-treated with subcritical water.

In previous chapter, the DPPH radical scavenging ability of the rice stem extracts were related to the phenolic content of the extract. In Fig. 5-3, the extracts obtained from the 3rd treatment showed a strong linear relation between the total phenolic content and

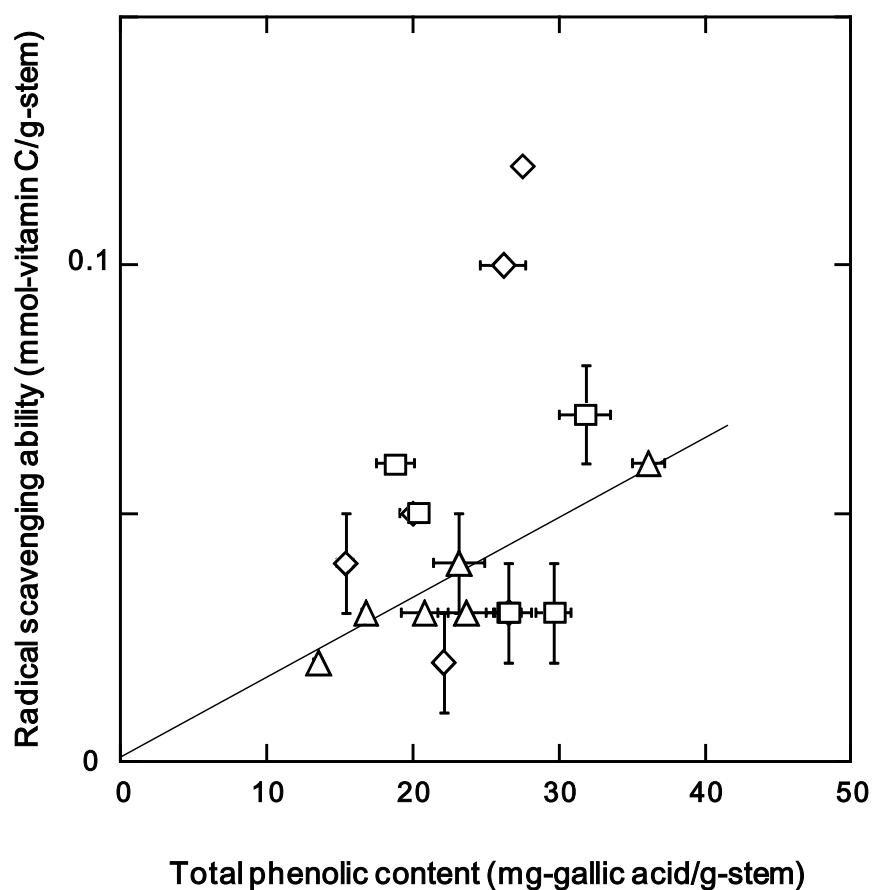


Fig. 5-3 Relationship between the total phenolic content and radical scavenging ability of the rice stem extracts from (◇) 1st, (□) 2nd, and (△) 3rd treatments. The line indicates the relationship obtained from the 3rd extraction.

DPPH radical scavenging ability ($R^2 = 0.97$). The reason behind this strong correlation could be that the compounds responsible for the DPPH radical scavenging ability in the extract obtained by treating with subcritical ethanol and 75% (v/v) ethanol in the 3rd treatment may be of the same origin, which was explained by their similar UV absorption spectral patterns as shown in Fig. 5-2.

5.3.4 Effect of the repeated treatment on the color of the extracts

The rice stem extracts obtained using the subcritical water were yellow, while the

color of the extracts obtained using the subcritical ethanol and 75% (v/v) ethanol were red and black, respectively. Figure 5-4 shows the relationships of the redness (a^*) and yellowness (b^*) to the lightness (L^*) of the extracts. The yellowness (b^*) and lightness (L^*) of the extracts obtained using the subcritical ethanol and 75% (v/v) ethanol showed a strong linear correlation ($R = 0.99$). However, the redness (a^*) of the extracts showed no relevance to any other parameters.

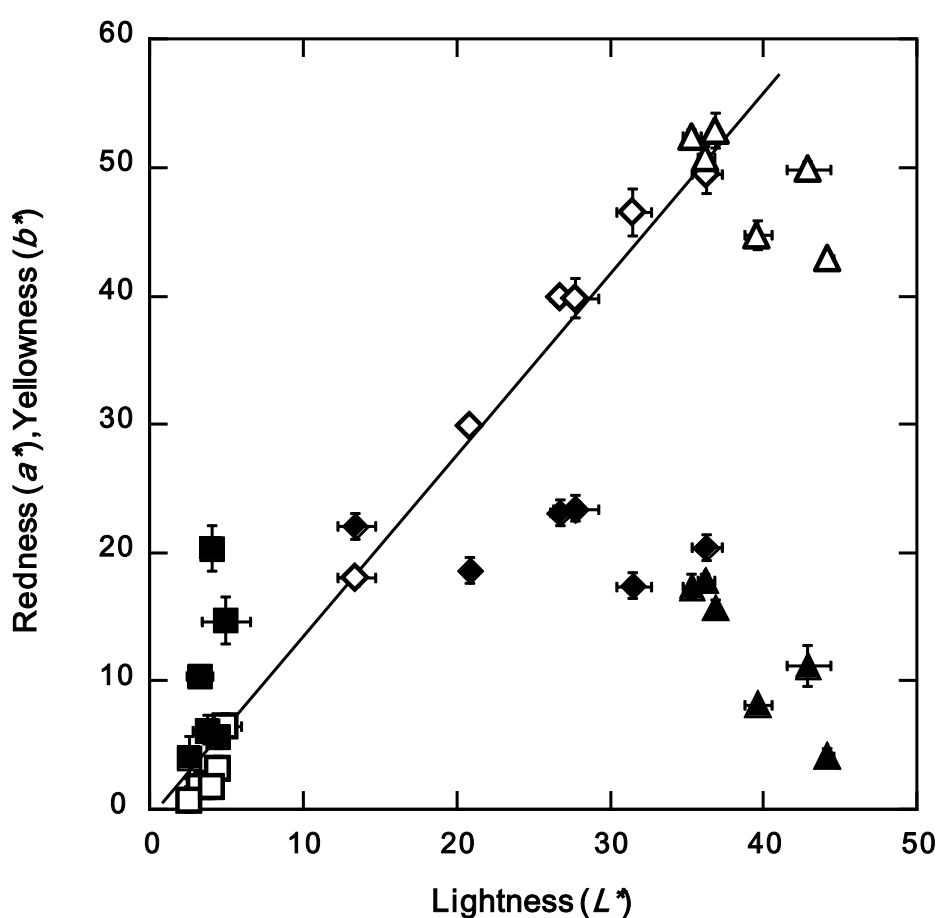


Fig. 5-4 Relationship between the yellowness (open symbols), redness (closed symbols), and lightness of the rice stem extracts obtained using ($\triangle$) subcritical water, ($\diamond$) ethanol, and ($\square$) 75% (v/v) ethanol. The line indicates the relationship between the yellowness and lightness of the extracts.

In Fig. 5-5, the correlation between the lightness and DPPH radical scavenging ability of the extracts linearly decreased with further treatment steps ($R = -0.96$, -0.81 , and -0.64 for the 1st, 2nd, and 3rd treatments, respectively). The negative correlation between the DPPH radical scavenging ability and lightness of the extracts would mean that the source of the radical scavenging ability were from black substances. It has been reported that the black liquor is obtained from the lignocellulosic materials treated in the organosolv process, which uses an organic solvent at elevated temperature and pressure [11-13]. During this process,

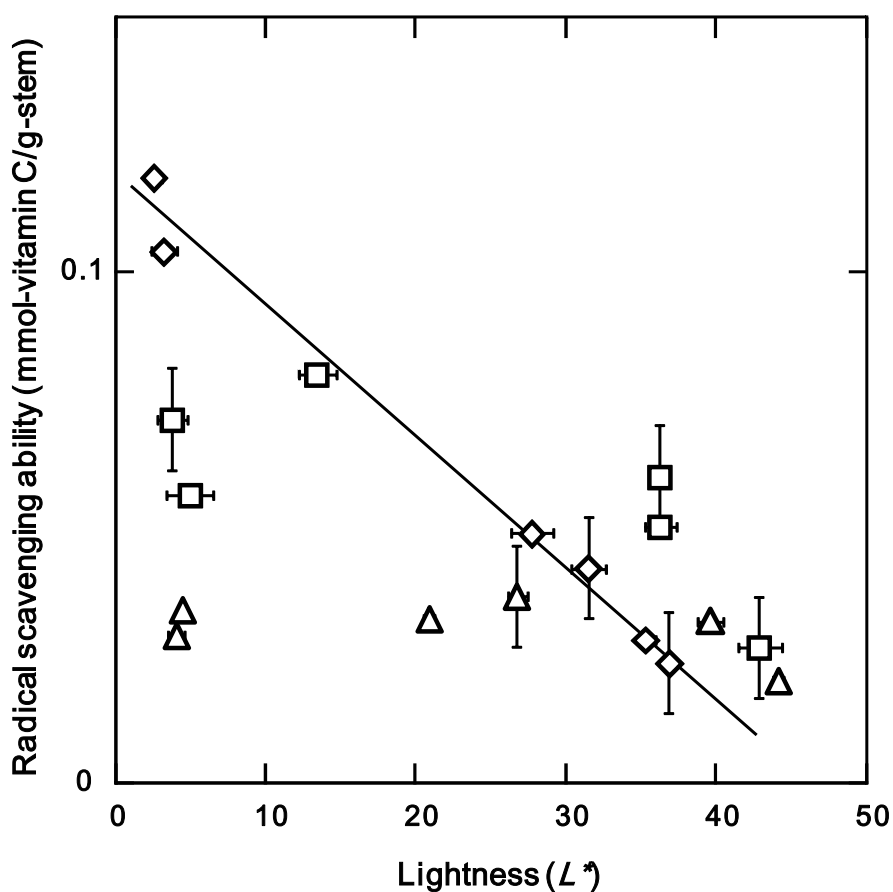


Fig. 5-5 Relationship between the radical scavenging ability and lightness of the rice stem extracts obtained from (◇) 1st, (□) 2nd, and (△) 3rd treatments. The line indicates the relation obtained from the 1st extraction.

the hemicellulose degradation products and lignin are solubilized into the black liquor. The hydrophobic black lignin can be separated from the black liquor by precipitation [54,55,66]. Therefore, one of the sources of the DPPH radical scavenging ability may be related to the hydrophobic lignin, which promotes the blackness in rice stem extract. The color pigments, carotenoids and chlorophyll, in the rice stem may also be the sources of the extract radical scavenging ability. Carotenoids and chlorophyll, their derived and degraded substances, have been reported to possess a radical scavenging ability [67-72]. Furthermore, the products resulting from browning reactions, such as Maillard reactions and caramelization during subcritical water treatment, were reported to demonstrate a radical scavenging ability [56-58].

5.4 Conclusion

Different compounds were extracted by subcritical water, ethanol and 75% (v/v) aqueous ethanol. However, similar UV spectral patterns were obtained from the extracts obtained using subcritical ethanol and 75% (v/v) ethanol, suggesting that the same compounds could be extracted. Subcritical water would be the most effective subcritical fluid to loosen a lignocellulosic structure and to obtain carbohydrates from rice stem. The highest phenolic content was achieved using subcritical ethanol after the cell wall structure was loosened by pre-treatment with subcritical water. However, the highest DPPH radical scavenging ability was obtained for the extracts obtained using subcritical 75% (v/v) ethanol in the 1st treatment. The DPPH radical scavenging ability could be related to the blackness and yellowness of the extracts. The sources of the color could be the hydrophobic lignin, stem color pigments, and browning reaction products.

Concluding Remarks

Chapter 1

Rice straw was separated into four parts: the upper, middle, and lower parts of the stem, and the leaf. They were treated with subcritical water. The yield and total carbohydrate, protein, and phenolic contents were obtained as well as the UV absorption spectra and the radical scavenging ability of the extracts. The extracts obtained from the stem parts had almost the same properties and were different from those of the leaf part. The extracts, prepared at higher temperature, exhibited higher radical scavenging ability. The radical scavenging ability and the phenolic content showed a linear correlation, suggesting that the phenolic substances in the extract cause its antioxidative ability.

Chapter 2

Effects of the treatment time and re-treatment on the properties of the rice stem extract were investigated. In the further treatment, total phenolic content, carbohydrate content, radical scavenging ability, and metal chelating ability decreased with extended treatment time. Tyrosinase inhibition ability, however, did not depend on the treatment time. There were linear correlations between the total phenolic content and radical scavenging ability and between the total carbohydrate content and metal chelating ability under any treatment conditions.

Chapter 3

Rice stems were subjected to a subcritical fluid treatment using subcritical aqueous ethanol or acetone. The yield and total carbohydrate content of the extracts obtained using subcritical ethanol and acetone did not show any statistical difference. However, the highest

yield, total carbohydrate content, total phenolic content, and DPPH radical scavenging ability were achieved using subcritical aqueous ethanol or acetone. The lightness of the extracts obtained using the subcritical ethanol and acetone showed a negative linear correlation to their DPPH radical scavenging ability. The relationship between the lightness and phenolic content of the extracts was not significant, suggesting that other substances in the extract could also possess a radical scavenging ability.

Chapter 4

The stem separated was treated with subcritical water, ethanol, and their mixture, and the properties of the extract were measured. The higher yield and total carbohydrate content of the stem extract were achieved when stem was treated with a subcritical ethanol/water mixture with a higher ethanol content at higher temperature. The extract obtained using 75% (v/v) aqueous ethanol at 230°C had the highest total phenolic content and radical scavenging ability.

Chapter 5

Rice stem was repeatedly treated 3 times with subcritical water, ethanol, and 75% (v/v) aqueous ethanol in different orders. The highest total carbohydrate and phenolic contents were achieved by treatments with subcritical water and with subcritical ethanol after pre-treatment with subcritical water, respectively. However, the extract with the highest radical scavenging ability was obtained by treatment with subcritical 75% (v/v) ethanol as the 1st treatment.

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List of Publications

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Tangkhavanich, B.; Kobayashi, T.; Adachi, S. Properties of rice stem extracts obtained using subcritical fluids. In submission.