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Original Article

ANTIOXIDANTINDIVIDUAL γ-ORYZANOLSCREENING INCOLDPRESSEDRICEBRANOILOFDIFFERENTTHAIRICEVARIETIESBY HPLC-DPPH METHOD

APIRAK SAKUNPAK*[,a], JIRAPORNCHAI SUKSAEREE[a], PATHAMAPORN PATHOMPAK[a], TOSSATON CHAROONRATANA[a], NAMFA SERMKAEW[b]

[a]Facultyof Pharmacy and Sino-Thai Traditional Medicine Research Center (Cooperation between Rangsit University, Harbin Institute of Technology, and Heilongjiang University of Chinese Medicine), Rangsit University, PathumThani12000, THAILAND, [B]Schoolof Pharmacy, Walailak University, Nakhon SiThammarat, 80161, THAILAND.
Email: apirak.s@rsu.ac.th

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ABTRACT

Objective: The aim of this study was to evaluate the antioxidant capacity of cold pressed rice bran oil of six different Thai rice varieties using online-HPLC-DPPH method.

Methods: The target-guidance of DPPH-HPLCexperimentwas used for screening of antioxidant γ-oryzanol in cold pressed rice bran oil. An HPLC method was developed and successfully used to simultaneously determine individual γ-oryzanol.

Results: Four peaks were detected in rice bran oil, and there were identified as cycloartenylferulate, 24-methylcycloartenylferulate, campesterylferulate and *β*-sitosterylferulate by LC-MS/MS.24-methylcycloartenylferulate possessed the strongest radical scavenging capacity.Base on HPLC-DPPH method, Sang-Yot rice bran oil demonstrated the highest antioxidant capacity with the IC_{50} value of 0.77mg/mL. Further more, HPLC method for quantitative analysis of γ-oryzanol was developed and validated. The method involvesthe Agilent 1260 series andseparation was achieved at 25 ºC on a Poroshell 120 EC-C18 column. Acetonitrile and methanol (60:40 v/v) were used as mobile phase and was pumped at a flow rate of 0.8 mL/min.The quantitation wavelength was set at 325 nm.

Conclusions: Online-HPLC-DPPH method was successfully developed in this study for determining of antioxidant γ-oryzanol in rice bran oil. A high degree ofspecificity as well as repeatability and reproducibility were also achieved by the developed HPLC method.

Keywords: Antioxidant, Rice bran oil, γ-oryzanol, HPLC-DPPH, Thai rice.

INTRODUCTION

Oxidative stress enhances pathological processes contributingto cancer, cardiovascular disease, and neurodegenerative diseases,and dietary antioxidants could counteract these deleterious processes[1, 2].Rice bran oil is also a good andreadily available source of natural antioxidants, which has attractedmuch attention from nutritional and chemical researchers[3].

The people of Thailand have been using the brown outer layer of the rice kernel, known as rice bran, for generations. Rice bran is rich in oil and frequently sold as a dietary supplement[4]. It is a plentiful source of many bioactive compounds, including γ-oryzanol, phytosterols, ferulic acid and phytic acid. γ-oryzanol (sterylferulates) has been shown to be a major bioactive compound in rice[5]. The four major γ-oryzanol constituents in rice were determined to be cycloartenylferulate, 24-methylenecycloartanyl ferulate, campesterylferulate, and *β*-sitosterylferulate. They have been previously shown to display various biological activities, anti-inflammatory, anti-tumor and antioxidant activities[6].Rice bran oil was demonstrated to be rich in antioxidants and otherpotential health promoting compounds, which could be extractedfor use in food. Therefore, investigation into antioxidant activity of rice bran oil and their γ-oryzanolis very important for its quality control[7]. The reported values of γ-oryzanol and antioxidant properties, depending on the method of extraction, rice variety, weather, and area of cultivation[8, 9].In order to discover natural antioxidants from various rice bran oil products,the development of qualitative and quantitative analytical techniquesare important. Previously, severalanalytical methods have been developed for determination of antioxidant capacityfrom plants and plant extracts, such as 2,2̀ -azinobis (3-ethylbenzthiazoline-6-sulfonic acid)($ABTS^{+}$) and 1,1-diphenyl-2-picrylhydrazyl ($DPPH^{\bullet}$)[10]. However, these methods could not determine a complex mixture in plant materials. To avoid these problems, a methodcombining separation and activity evaluation would present a remarkable benefit for such investigation[11]. Recently, rapid and sensitiveon-line HPLC methods for analyzingthe bioactivity of individual compounds have been developed.The hypothesiswas that upon reaction with DPPH, the peak areas of compounds with potential antioxidanteffects in the HPLC chromatogram would be significantly reducedor disappeared[12]. The aim of this study were to evaluate the antioxidantactivity of different Thai rice varieties, find out the γ-oryzanol which contributed to the antioxidant activity by the combination of online HPLC-DPPH method.

MATERIALS AND METHODS

Materials

Solvents used for chromatography were acetonitrile (HPLC grade), methanol (HPLC grade) and water (HPLC grade). All obtained from B&J (Korea). A standard of γ-oryzanol (analytical grade) was purchased from TCI, Tokyo, Japan. Rice bran samples were provided by a local milling company in Thailand. The rice bran samples were passed though sieve number 20 and immediately extracted under cold press conditions (Table1).

Isolation of individual γ-oryzanol

The four major of sterylferulates were isolated form standard γ-oryzanol mixture by HPLC. A 50 mg of γ-oryzanol mixture was dissolved in isopropanol 10 mL. An aliquot 30 µL was separated on HPLC (Agilent Technologies, USA) with a Poroshell120 EC-C18 column (3.0×150 mm, 2.7µm). Elution was achieved by using a solvent mixture of acetonitrile and methanol (60:40 v/v), with a flow rate of 0.8 mL/min. Four peaks were collected (peak 1 t_R10.6 min; peak 2 t_R12.2 min; peak 3 t_R13.8; peak 4 t_R15.9 min). The isolated compounds were used as external standard for HPLC quantitative analysis.

Table 1: Thai rice varieties were used in this study

Rice varieties	Type of rice as ecosystem	Location
Hom-Pathum	Lowland rice	PathumThani Province
Hom-Mali	Lowland rice	Lopburi Province
Hom-Mali Gorkor	Lowland rice	Suphanburi Province
Chainat 1	Lowland rice	Chainat Province
Sang-Yot	Lowland rice	Phatthalung Province
White rice	Lowland rice	AyutthayaProvince

Identification of individual γ-oryzanol

LC-MS was carried out using Dionex UltimateTM3000equipped. The sample was separated at 25 ºC on a Poroshell 120 SB-C18 (2.1×150 mm, 2.7 µm)using a mobile phase consisting of acetonitrile and methanol (60:40 v/v). Injection volume was 10 µL and the UV detector was at 325 nm (variable wavelength detector). Individual γ-oryzanol was identified with High capacity 3D quadrupole ion trap(Bruker Amazon SL).The mass spectrometer was equipped with an ESI ion source. The ESI-MS spectra were acquired in negative ionization mode recorded on a mass range of m/z 100-800. Capillary voltage was 4500 V. Drying gas temperature was set at 200 ºC with a flow rate of 7.0 L/min and nebulizing pressure was of 2 bar. Data were processed by HPLC/MSD Trap software 4.2 and Data Analysis 2.2.

HPLC conditions

HPLC analysis was carried out using the Agilent 1200 series equipped with an Agilent 1200 series Photodiode-array detector (PDA) and autosampler. Data analysis was performed using Open LABCDS EZChrom software. Separation was achieved at 25 ºC on aPoroshell120 EC-C18, 3.0×150 mm, 2.7µm (Agilent Technologies, USA). The mobile phase consisted of acetonitrile-methanol (60:40v/v) and was pumped at a flow rate of 0.8 mL/min. The injection volume was 10 µL.The quantitation wavelength was set at 325 nm.

Preparation of standard solutions

γ-Oryzanol was accurately weighed to 25 mg in a volumetric flask (size 25 mL) and the volume was adjusted to 25 mL with isopropanol. The stock solution was serial two-fold diluted to six concentrations and filtered through a membrane filter (0.45 µm) before HPLC analysis.

Preparation of samples

Rice bran oils were accurately weighed to 25 mg in a volumetric flask (size 25 mL) and the volume was adjusted to 25 mL with isopropanol. The samples were filtered through a membrane filter (0.45 µm) before HPLC analysis. The experiments were carried out in triplicate.

Method validation

For validation of the analytical method, the guidelines of the International Conference on Harmonization of Technical Requirement for the Registration of Pharmaceuticals for Human Use were followed (ICH, 2005). Calculated data were checked for their linearity, accuracy, intra-day and inter-day precision specificity, LOD and LOQ to validate the HPLC method.

Calibration curve and linearity

The calibration curves were analysis of a mixture containing each of the standard γ-oryzanol at six concentrations and plotting peak areas against the concentration of each reference standard. The linearity of the detector response for the standards was determined by means of linear regression analysis. The calibration curve should show a coefficient of correlation (R^2) ≥ 0.999.

Accuracy

Rice bran oil sample solutions were fortified with three concentrations of known quantities of the standard γ-oryzanolin order to check the accuracy of the data. Prior to fortification with standard γ-oryzanol the background levels of standard γ-oryzanolin the rice bran oil were determined so as to calculate actual recoveries. The amounts of γ-oryzanolwere determined in triplicate, and the percentage recoveries were then calculated.

Precision

Precision experiments were conducted to ascertain any intra-day and inter-day variability. The solution of one rice bran oil sample was used to check the intra-day precision. Six separate injections of this sample were carried out on the same day. The data were used to calculate the % R.S.D. (not more than 2%) for intra-day precision. The inter-day precisions were validated by repeating the extraction procedure on the same sample. An aliquot of each extract was then injected and quantified. This parameter was evaluated by repeating the extraction in triplicate on three different days with a freshly prepare mobile phase and sample. The data was used to calculate % R.S.D. (not more than 5%) for inter-day precision.

Specificity

Peak identification was carried out using authentic standards and scanning the UV spectrum of each peak using the photodiode-array detector. The UV spectra were taken at various points of the peaks to check the peak homogeneity.

Limits of detection (LOD) and quantification (LOQ)

Serial dilutions of a reference standard were prepared in isopropanol and were then analyzed by the HPLC method. LOD and LOQ were the concentrations that give a signal to noise ratio equal to 3 and 10, respectively.

Predominant antioxidant γ-oryzanol screening with DPPH-HPLC experiment

Five gram of rice bran oil samples were dissolved in DMSO and adjusted to 10 mLwith DMSO in volumetric flask. Five milliliter of rice bran oil solutions were reacted with 5 mL of DPPH(0.1 mMin DMSO), then the mixture was incubated at25 °C in a constant temperature for 30 min, and thenthe mixture was passed through a 0.45 µm filter and subjected toHPLC analysis. The rice bran oil sample in DMSO 10 mLwas used as a control. The analyticalHPLC systems for determination of γ-oryzanol are described as above.

Total antioxidant capacity assay by HPLC

DPPH stock solution (1 mM) was made in DMSO. Gallic acid solution was prepared in the rage of 6.25-0.78 µg/mLin DMSO. The cold pressed rice bran oil solutions were diluted to different concentration (8.0-1.0 mg/mL) by DMSO. The total antioxidant capacity assay was determined by usingHPLC. Briefly, an aliquot of 50 µL cold pressed rice bran oil solutions (at the final concentration of 4.0-0.5 mg/mL)with different diluted ratios was added to 50 µLof DPPH solution (at the final concentration of 0.5 mM). The mixturewas mixed and left to stand in the darkfor 30 min at room temperature. Then the sample was filteredthrough 0.45 µm filter and 10 µLof the sample was analyzed by HPLC. The blank control was prepared by adding DMSO 50 µL in 50 µLof DPPH stock solution.Chromatographic analysis was carried out by using an Agilent 1200 series equipped with an Agilent 1200 series Photodiode-array detector (PDA). Separation was achieved at 25 ºC on a ZORBAX Eclipse Plus C18 column (3.5 µm, 100 mm × 4.6 mm i.d.) and elution with 100% acetonitrile at a flow rate of 1.0 mL/min. TheDPPH peaks were measured at 517 nm. The difference in thereduction of peak areas (A) for DPPH between the blank andthe sample was used to determine radical scavenging activity ofthe sample according to the following equation:

Radical scavenging (%) =[($A_{control}$ - A_{sample})/$A_{control}$] × 100

IC_{50} values were calculated by nonlinear regression analysis andexpressed by the mean of thrice determination results. Gallic acid was used as a positive control for the evaluation of freeradical scavenging activity.

RESULTS AND DISCUSSION

Peak identification and assignment

Peak identification and assignment in HPLC fingerprints of rice bran oil were based on the comparison mass spectral data with published data. The four characteristic peaks were identified as cycloartenylferulate (peak 1 t_R 10.6 min, $[M-H]^-$ (m/z) 601.5; MS/MS (m/z) 586.5), 24-methylcycloartenylferulate (peak 2,t_R 12.2 min, $[M-H]^-$ (m/z) 615.6; MS/MS (m/z) 600.5), campesterylferulate (peak 3,t_R 13.8 min, $[M-H]^-$ (m/z) 575.6; MS/MS (m/z) 560.5), β-sitosterylferulate(peak 4,t_R 15.9 min, $[M-H]^-$ (m/z) 589.6; MS/MS (m/z) 574.5). The chemical structures and MS spectra of individual γ-oryzanol are shown in Fig. 1 and 2, respectively.

Validation method

We examined the optimal conditions for the simultaneous quantitative determination of cycloartenylferulate, 24-methylenecycloartanyl ferulate, campesterylferulate, and β-sitosterylferulate in cold pressed rice bran oil using an isocratic RP-HPLC system. As all four compounds have good absorption at 325 nm, this wavelength was used for quantitation. Mixtures of acetonitrile and methanol (60:40 v/v) were examined as the mobile phase. This mobile phase and ratio provided a good resolution. All four compounds were eluted within 17 min with satisfactory resolution (Fig. 3). The linearity, accuracy, intra-day and inter-day precision, specificity and limits of detection and quantitation were determined to validate the RP-HPLC method. The calibration curve was linear over the concentration rang 500-15.125 µg/mL. Cycloartenylferulate, 24-methylenecycloartanyl ferulate, campesterylferulate, and β-sitosterylferulate exhibited linearity over the evaluated ranges with correlation coefficients 0.9996.Both intra-day and inter-day precision were estimated by the relative standard deviation were less than 2% and 5%, respectively. Recoveries in the range of 100.1-101.9% were observed for all compounds. Utilising the photodiode array (PDA) makes it possible to obtain the UV spectra. Specificity of the method was evaluated using the UV-absorption spectra produced by the diode-array detector. The spectra were taken at different three points of the peak for cycloartenylferulate, 24-methylenecycloartanyl ferulate, campesterylferulate, and β-sitosterylferulate. When it was compared with the standard, the spectra of the peak were observed to be homogenous. Finally, it was found that the RP-HPLC method was very sensitive for detecting cycloartenylferulate, 24-methylenecycloartanyl ferulate, campesterylferulate with LOD and LOQ values of 0.156 and 0.312 µg/mL, and The LOD and LOQ of β-sitosterylferulate were 0.781 and 1.562 µg/mL, respectively (Table 2).

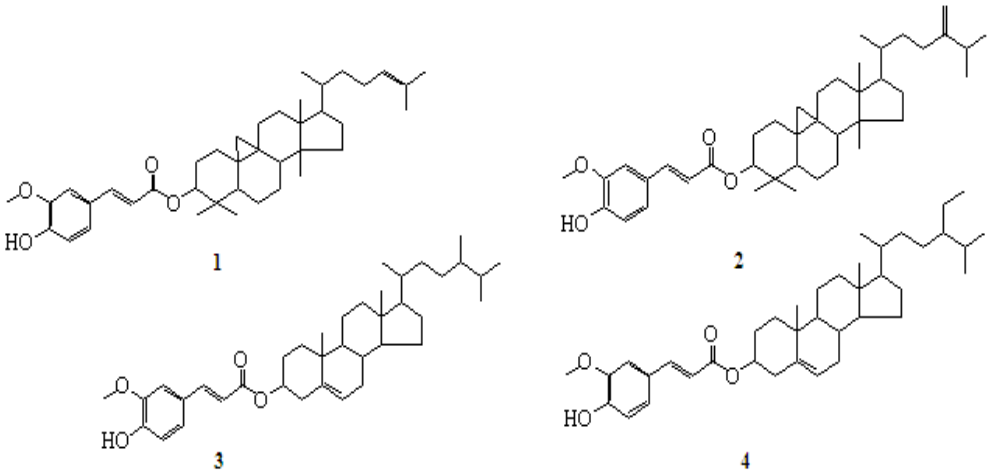

Fig. 1: Chemical structures of the sterylferulates identified from the commercial standard γ-oryzanol mixture (1) cycloartenylferulate; (2) 24-methylenecycloartanyl ferulate; (3) campesterylferulate; and (4)β-sitosterylferulate.

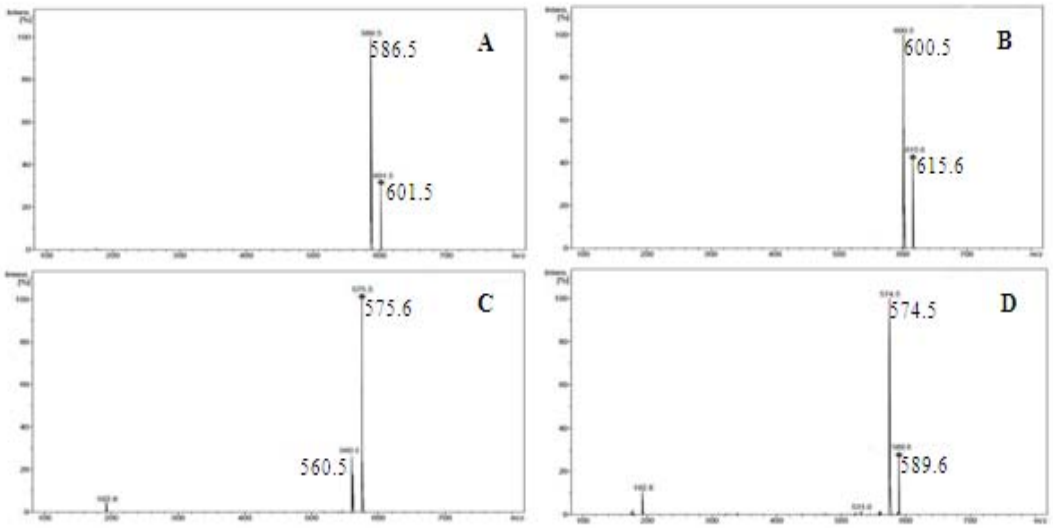

Fig. 2: MS/MS spectra of cycloartenylferulate (A), 24-methylenecycloartanyl ferulate (B), campesterylferulate (C), and β-sitosterylferulate (D)

Table 2: Validation data of HPLC method

Substances	Linear equations	%Recovery	%RSD		LOQ (µg/mL)	LOD (µg/mL)
			Intra-day	Inter-day		
R1	y = 44978x - 38615	101.15 + 1.02	0.06	0.52	0.312	0.156
R2	y = 44989x - 54136	101.97 + 1.25	0.42	0.60	0.312	0.156
R3	y = 45040x - 19571	101.35 + 1.15	1.12	4.00	0.312	0.156
R4	y = 44983x - 9316	100.10 + 1.43	0.02	0.58	1.562	0.781

R1, cycloartenylferulate; R2, 24-methylenecycloartanyl ferulate; R3, campesterylferulate; R4, *β*-sitosterylferulate

Table 3: Individual γ-oryzanol content in cold pressed rice bran oilof different Thai rice varieties

Rice varieties	% Content; Mean ± SD (%w/w)				
	R1	R2	R3	R4	Total
Hom-Pathum	0.53 ± 0.08	0.87± 0.13	0.45± 0.06	0.31± 0.11	2.17± 0.28
Sang-Yot	0.36 ± 0.02	1.13 ± 0.09	0.37 ± 0.07	0.29 ± 0.03	2.14± 0.07
Hom-Mali	0.14 ± 0.02	0.43 ± 0.01	0.24 ± 0.02	0.08± 0.11	0.89 ± 0.04
Hom-Mali Gorkor	0.15 ± 0.01	0.61 ± 0.06	0.33± 0.03	0.16 ± 0.02	1.24± 0.06
Chainat	0.14± 0.01	0.44 ± 0.02	0.22 ± 0.01	0.12 ± 0.01	0.91± 0.09
White rice	0.19± 0.01	0.43± 0.03	0.27± 0.02	0.12 ± 0.01	1.02 ± 0.02

n=3, R1, cycloartenyl ferulate; R2, 24-methylenecycloartanyl ferulate; R3, campesteryl ferulate; R4, *β*-sitosteryl ferulate

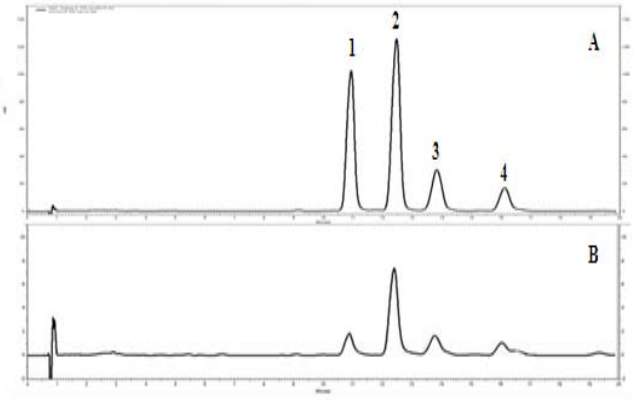

Fig. 3: HPLC chromatograms of standard γ-oryzanol (cycloartenylferulate (1), 24-methylenecycloartanyl ferulate (2), campesterylferulate (3), and *β*-sitosterylferulate (4)) (A) and cold pressed rice bran oil (B)

HPLC-DPPH quantitative analysis of individual γ-oryzanol antioxidant activity

HPLC-DPPH analysis was utilized on Agilent 1200 series. Separation was achieved at 25 ºC on a Poroshell120 EC-C18, 3.0×150 mm, 2.7 µm. A mixture of acetonitrile and methanol (60:40 v/v) were used as mobile phaseand was pumped at a flow rate of 0.8 mL/min. The detection wavelength was set at 325 nm. Reaction between an antioxidant and a DPPHcould result in the oxidation of the antioxidant, which thus changedthe molecular structure of the antioxidant. Basedon this, after reaction with DPPH, the peak area of the radical scavengingcompounds would obviously decrease in the HPLC chromatogram.While for those without antioxidant effects, nochange in their peak area was observed. As a result, four chromatographic peaks were detected in rice bran oil samples(Fig. 4),which indicated that it had free radical scavengingactivity. Four detected chromatographic peaks were identified as cycloartenylferulate, 24-methylenecycloartanyl ferulate, campesterylferulate, and *β*-sitosterylferulate by comparing their retention times and DAD spectra with reference compounds, respectively.In order to determine theradical scavenging capacity of the antioxidants of individual γ-oryzanol in cold pressed rice bran oil,it was reacted with DPPH of differentconcentrations and then followed by HPLC.

The peak area changes of cycloartenylferulate, 24-methylenecycloartanyl ferulate, campesterylferulate, and *β*-sitosterylferulate were shown in Fig. 5. With peak area of cycloartenylferulate, 24-methylenecycloartanyl ferulate, campesterylferulate, and *β*-sitosterylferulate of untreated sample setting as 100%. It was found that campesterylferulate peak decreased more sharply than another peak. It was indicated that antioxidants activity of campesterylferulate possessed strongest antioxidant capacity followed by *β*-sitosterylferulate, 24-methylenecycloartanyl ferulate, and cycloartenylferulate.

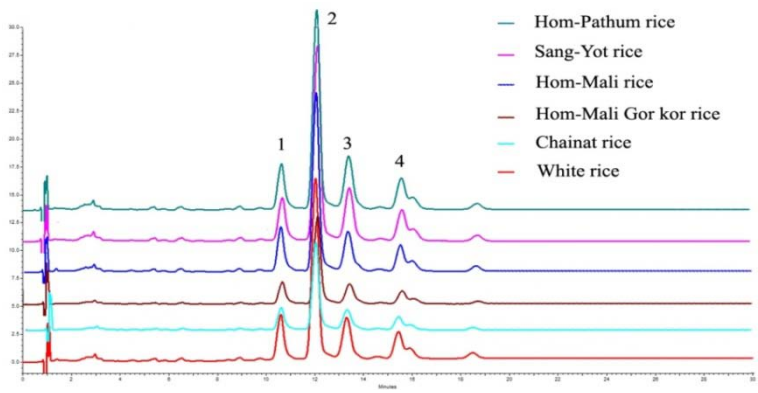

Fig. 4: HPLC chromatograms of cold pressed rice bran oil of various Thai rice varieties (cycloartenylferulate (1), 24-methylenecycloartanyl ferulate (2), campesterylferulate (3), and*β*-sitosterylferulate (4))

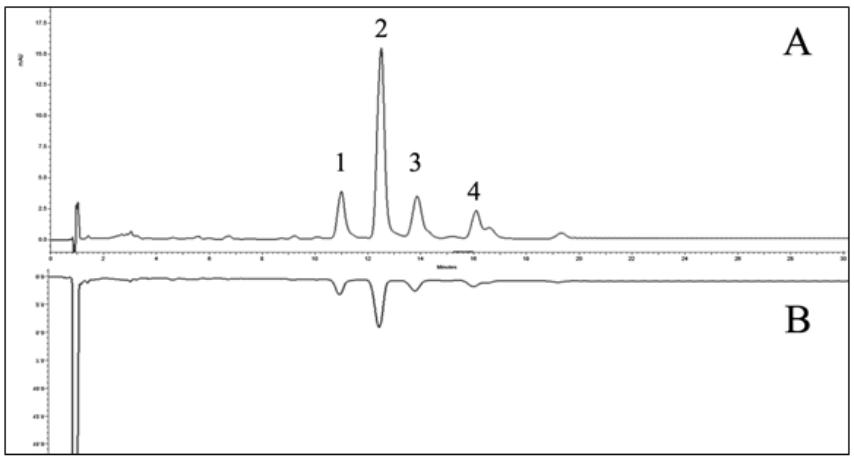

Fig. 5: HPLC chromatograms of cold pressed rice bran oil monitored at 325 nm. (A) Before reaction with DPPH free radicals, (B) after reaction with DPPH free radicals (cycloartenylferulate (1), 24-methylenecycloartanyl ferulate (2), campesterylferulate (3), and*β*-sitosterylferulate(4))

Table 4:Antioxidant activity of different Thai rice bran oil varieties by HPLC-DPPH method

Rice varieties	IC_{50}± SD (mg/mL)
Hom-Pathum	1.43 ± 0.12
Sang-Yot	0.77± 0.04
Hom-Mali	2.28 ± 0.14
Hom-Mali Gorkor	1.63 ± 0.09
Chainat	1.84 ± 0.03
White rice	1.84 ± 0.11
γ-Oryzanol	1.39 ± 0.01 µg/mL
Gallic acid	37.41 ± 0.47 µg/mL

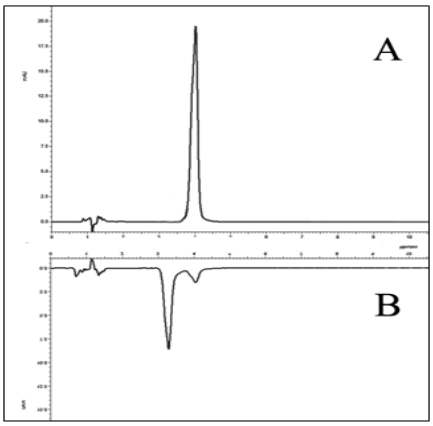

Fig. 6: HPLC chromatogram of pure DPPH detected at 517 nm. (A) Before reaction with cold pressed rice bran oil, (B) after reaction with cold pressed rice bran oil

Total antioxidant capacity assayed by HPLC analysis

The antioxidant activity of rice bran oil of different Thai rice varieties were determined by HPLC-DPPH method. Chromatographic analysis was carried out by using an Agilent 1200 series equipped with an Agilent 1200 series PDA detector. Separation was achieved at 25 ºC on a ZORBAX Eclipse Plus C18 column (3.5 µm, 100 mm × 4.6 mm i.d.) and elution with 100% acetonitrile at a flow rate of 1.0 mL/min. The detection wavelength was set at 517 nm. Gallic acid was used as positive control. The antioxidant activity is expressed in term of IC_{50}. The chromatograms of pure DPPH and scavenged DPPH in presences of rice bran oil sample have been present in Fig. 6. Sang-Yot rice bran oil has shown the highest peak area reduction of DPPH, indicating the highest scavenging activity, follow by Hom-Pathumrice bran oil with the IC_{50}value of 0.77 ± 0.44 and 1.43 mg/mL, respectively (Table 4).Sang-Yotexhibited stronger antioxidant activity than Hom-Pathum rice significantly, although, they contained the equivalent amount of γ-oryzanol. This was due to the fact that rice barn oil contains other antioxidant compound such asα-tocopherol, γ-tocopherol, δ-tocopherol and α-tocotrienol.

CONCLUSIONS

HPLC-DPPH method was used for screening the antioxidant activity of γ-oryzanol in rice bran oil. Four antioxidant peaks were detected and identified as cycloartenylferulate, 24-methylcycloartenylferulate, campesterylferulate and *β*-sitosterylferulate. In addition, 24-methylcycloartenylferulate possessed the strongest radical scavenging capacity.Total antioxidant capacity of six varieties of Thai rice branoil, Sang-Yot rice bran oil demonstrated the highest antioxidant capacity follow by Hom-Phathum rice. Moreover, an HPLC method was developed andsuccessfully used to simultaneously determine individual γ-oryzanol, which provided a good linearity, specificity, repeatability and reproducibility.

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