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Extraction conditions for bioactive compounds from germinated Black Jasmine rice using aqueous two-phase system

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Abstract

Black Jasmine rice is rich in bioactive compounds such as anthocyanins, phenolics, and flavonoids. It can be germinated to gain more bioactive compounds especially gamma-aminobutyric acid (GABA). Generally, bioactive substances can be extracted using several methods, and the aqueous two-phase system (ATPS) is one of the most environmentally friendly extraction methods. Therefore, this work focused on studying the conditions for extracting bioactive compounds from germinated black jasmine rice using the ATPS by studying ethanol concentrations of 32%, 34%, 36%, 38%, 40%, 42%, and 44%, and the sample-to-solvent ratio of 1:50, 1:40, 1:30, 1:20, and 1:10 using ammonium sulfate at 16%. The results showed that an ethanol concentration of 34% and a sample-to-solvent ratio of 1:40 were optimal for extracting a high amount of GABA. Ammonium sulfate concentration of 14%, 16%, 18%, 20%, and 22% were subsequently studied, using an ethanol concentration of 34% and a sample-to-solvent ratio of 1:40., It was found that GABA, phenolics, flavonoids, and Fe^{2+} chelating activity decreased as the concentration of ammonium sulfate increased., However, the amount of anthocyanin increased with the concentration of ammonium sulfate. This indicated that ATPS can be an alternative environmentally friendly extraction method for bioactive compounds from germinated rice.

Keywords: Aqueous two-phase system; Gamma-aminobutyric acid; Phenolics compounds; Flavonoid; Antioxidant activity

1. Introduction

Rice (*Oryza sativa* L.) is an important food source for humans. Thailand is the 6th highest rice-producing country in the world, contributing 4.0% to global rice production. It is also the second largest rice exporter with a market share of 13.5%, behind India with a market share of 38.8% Rice contains biologically active substances such as phenolics, flavonoids, anthocyanin, and other antioxidants. Especially, brown rice that is purple-black has more bioactive substances than normal white rice (Peanparkdee *et al.*, 2019). Rice can also be processed in many ways to add value to it, such as producing various rice flours, instant food, and germinated rice. Germination can increase the accumulation of important substances in rice, especially GABA, a substance that helps prevent neurological disorders and reduces blood pressure in the human brain, such as seizures, Parkinson's disease, and schizophrenia, and can also reduce stress or anxiety symptoms (Eamarjharn *et al.*, 2016). In addition, the important substances in rice can greatly increase its value. These extracts can be used to make various dietary supplements and cosmetic products.

The extraction can be done in several ways, such as soaking (maceration) with solvents. This method is easy and low cost, but has disadvantages such as solvents being flammable and dangerous to workers and also destroying the environment. Moreover, it has been reported that using the traditional extraction method still has many disadvantages, such as low yields and high temperatures, high solvent consumption, long time extraction, and high energy use (Xi *et al.*, 2023). Furthermore, the residue of solvent may affect its use in further products. In addition, according to the Thai Food and Drug Administration regulations of 2005, only water and alcohol are allowed for extraction. Therefore, we are interested in extraction using the ATPS, a two-phase water system using inorganic salts and short-chain alcohols which have low viscosity. It has high extraction efficiency and relatively good phase separation. ATPS is an environmentally friendly alternative technique as alcohol and salt can be recycled (İşçimen and Hayta, 2021). ATPS is widely used in the extraction of low molecular weight active ingredients, such as phytochemicals, from plants (Xi *et al.*, 2023). However, many factors that affect the extract yields, such as ATPS composition, solid-liquid ratio, and extraction temperature or time.

However, there has been no study of the extraction using ATPS to extract GABA in Nong Khai Black Jasmine brown rice. Therefore, this research aims to study ethanol concentration, ammonium sulfate concentration, and the sample-to-solvent ratio for extracting GABA from germinated Black Jasmine rice using ATPS.

2. Materials and Methods

2.1 Chemicals

Sodium hypochlorite solution, ammonium persulfate, boric acid, γ-aminobutyric acid analytical standard, di-sodium tetraborate decahydrate (borax), hydrochloric acid, 2,2-diphenyl-1-picrylhydrazyl, gallic acid, quercetin, ferrozine, ethanol, acetone, sodium carbonate anhydrous, sodium hydroxide, sodium nitrite, aluminium nitrate, iron (II) cholide, and Folin–Ciocalteu reagent were of analytical grade.

2.2 Germination of Black Jasmine rice

The de-husk Black Jasmine rice seeds were obtained from Nongkhai Rice Research Center. They were washed and soaked in 1% (v/v) sodium hypochlorite for 30 min to sterilize them. Then they were soaked in water for an initial period of 4 h, followed by soaking for 48 h, with water changes every 8 hours. Throughout the germination period, the rice seeds were incubated for 12 hours and washed with water every 4 h. The germinated rice grains were then steamed at 90°C for 10 min and dried at 50°C for 4 h. The germinated rice was dried and stored in a plastic bag for further analysis.

2.3 Extraction of bioactive compounds by aqueous two-phase system (ATPS)

2.3.1 Effect of ethanol concentration and sample-to-solvent ratio

This study used ATPS with 16% ammonium sulfate dissolved in ethanol (w/w) at 32%, 34%, 36%, 38%, 40%, 42% and 44% and sample-to-solvent ratios of 1:50, 1:40, 1:30, 1:20, and 1:10. The germinated Black Jasmine rice was coarsely ground and, extracted with ATPS at specified conditions in a water bath at 60 °C for 15 min. The suspension was then filtered with filter paper. It was left for about 10 min for the phase separation. The top phase extract was collected for GABA analysis.

2.3.2 Effect of ammonium sulfate concentration

To determine the optimal ammonium sulfate concentration for GABA extraction, concentrations of 14%, 16%, 18%, 20% and 22% was determined. This utilized the best ethanol concentration and sample-to-solvent ratio obtained from Section 2.3.1. The suspension was heated at 60°C for 15 min. After removal of the germinated rice samples, the solution was allowed to stand for approximately 10 min for phase separation. The top phase extract was then analyzed for bioactive compounds content.

2.4 Determination of bioactive compound content

2.4.1 Total γ-aminobutyric acid content

The γ-aminobutyric acid (GABA) content was determined following the method described by Songsamoe *et al.* (2021) with some modifications. Briefly, 0.5 mL of extract was mixed with 0.2 mL of 0.2 M borate buffer (pH 9), 1 mL of 6% phenol, and 0.4 mL of 9% sodium hypochlorite. The mixture was boiled for 10 min and then cooled for 20 min. After that, 2 mL of 60% ethanol was added, and the absorbance of the mixture was measured at a wavelength of 645 nm. Calibration curves of standard GABA were used to determine the concentration of GABA in the samples. The result was expressed as mg/g germinated rice.

2.4.2 Total phenolic content

The total phenolic content of the extracts was analyzed according to Işçimen and Hayta (2021) with modifications. A 0.2 mL of extract was mixed with 5 mL of distilled water, and 0.5 mL of Folin-Ciocalteu reagent and left for 5 min. After that, 1.5 mL of sodium carbonate. (75g/L) was added and the mixture was then left in a dark place at room temperature for 90 min. The absorbance of the mixture was measured at 725 nm. The value was compared with the gallic acid standard curve. The result was expressed as mgGAE/g germinated rice.

2.4.3 Total flavonoid content

The total flavonoid content of the extracts was analyzed according to Lasunon *et al.* (2022) with modifications. A 0.3 mL of extract was mixed with 2 mL of distilled water and 0.15 mL of sodium nitrite and then left for 5 min. Then, 0.15 mL of 10% aluminum nitrate was added to the mixture and left for 5 min. After that, 1 mL of 1 M sodium hydroxide was added. The absorbance of the mixture was measured at 420 nm. The value was compared with the quercetin standard curve. The result was expressed as mgQE/g germinated rice.

2.4.4 Total anthocyanin content

The anthocyanin content of the extracts was analyzed according to Ranganna *et al.* (1977) with modifications. A 2 mL of extract was mixed with 2 mL of 1% hydrochloric acid (HCl) in ethanol, and the absorbance of the mixture was measured at 542 nm. The anthocyanin was calculated according to the following equation:

$$A = (B \times C \times D \times 100) / (E \times F) \quad (1)$$

$$\text{Total anthocyanin (mg/100 mL)} = 98.2 / A \quad (2)$$

A = Total optical density (OD) value per sample (100 mL)
B = The OD value of the sample is at 535 nm (Absorbance)
C = Volume of sample from volume-adjusted extraction (mL)
D = Total amount of extract obtained (mL)
E = Sample volume used to measure light absorption (mL)
F = The initial amount of sample used for extraction (100 g or mL)
98.2 = Average Extinction coefficient of anthocyanins

2.5 Antioxidant activity

2.5.1 DPPH radical scavenging activity

The DPPH radical scavenging activity of the extracts was analyzed according to Jalali-Jivan and Abbasi (2020) with modifications. A 1 mL of the extract was mixed with 1 mL of 0.1 mM DPPH, shaken well, and left in the dark for 30 min. The absorbance of the mixture was measured at 517 nm. A control sample was prepared by replacing the extract with ethanol. The radical scavenging activity of DPPH was calculated according to the following equation:

$$\%DPPH = (A_0 - A_1) \times 100 / A_0 \quad (3)$$

Where A_0 is the absorbance value at 517 nm of control and A_1 is the absorbance value at 517 nm of the sample.

2.5.2 Fe^{2+} chelating activity

The Fe^{2+} chelating activity of the extracts was analyzed according to Dinis *et al.* (1994) with modifications. A 400 µL of extract was mixed with 50 µL of 2 mM $FeCl_2$ and left it stand in the dark for 30 min. Then, 200 µL of ferrozine and 3350 µL of ethanol were added to the mixture. After 10 min of incubation in the dark, the absorbance of the mixture was measured at 542 nm. The result was expressed as % Fe^{2+} chelating activity compared to the control sample (ethanol).

2.6 Statistical analysis

The factorials design (2 factors and 6 levels) was used to study the effect of ethanol concentration and solid-to-solvent ratio. A completely randomized design (CRD) was employed to study the effect of ammonium sulfate concentration. The experiments were performed in triplicate and results were expressed as the mean and standard deviation (SD). The statistical analysis was conducted using analysis of variance (ANOVA) by the Statistical Analysis System (IBM SPSS Statistics version 28.0.1.0). The differences between the mean values were performed by Duncan's test with statistically significant values of $P<0.05$.

3. Results and Discussion

3.1 Effect of ethanol concentration and sample-to-solvent ratio on GABA concentration

Traditional extraction methods, such as soaking with solvents, have been reported to have several disadvantages, such as low yields, requiring high temperatures, long duration, and solvents, and high energy consumption. The ATPS, generally composed of inorganic salts and short-chain alcohols, has been widely used in the extraction of low molecular weight active ingredients, such as phytochemicals, from plants (Xi *et al.*, 2023). However, many factors, such as ATPS composition and the solid-to-solvent ratio, could influence the yield of bioactive compounds. Therefore, the effects of ethanol concentration and solid-to-solvent ratio were first studied. The GABA content of each extract was determined to find the optimal ethanol concentration and sample-to-solvent ratio, and the results are shown in Fig 1.

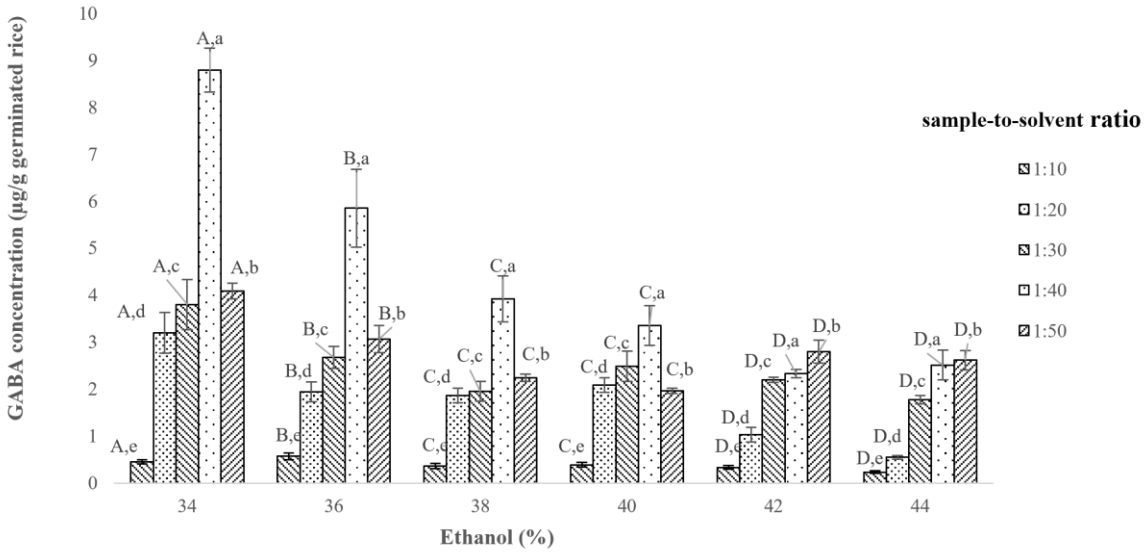

Fig 1 Effect of sample-to-solvent ratio and ethanol concentration on the amount of GABA extracted (ammonium sulfate concentration held constant at 16%). Values with different letters (a-d) were significantly different ($P<0.05$). Uppercase letters indicate ethanol concentration. Lowercase letters are sample-to-solvent ratio.

The ethanol concentration in ATPS varied across 6 concentration levels from 34-44 % with an ammonium sulfate concentration of 16%. The results (Fig 1) revealed that the GABA content gradually decreased with increasing ethanol concentration. The ethanol concentration at 34 % gave the highest GABA content in the extract. This result did not agree with the research conducted by Fu *et al.*, (2018) which reported that the extraction of polyphenols and lutein from marigold flower increased with the concentration of ethanol due to the enlargement of the upper phase. It could be explained by the variations in the solubility and polarity of the target compounds. GABA is a relatively polar molecule, and higher ethanol concentrations (less polar solvent) may have reduced its availability for extraction into the ATPS upper phase.

When considering the effect of sample-to-solvent ratio, it was found that the amount of GABA extracted increased as the sample-to-solvent ratio increased from 1:10 to 1:40. However, the GABA content slightly decreased at the sample-to-solvent ratio of 1:50. This can be explained by a reduction in the viscosity of the extraction medium and an increase in the diffusion rate due to the increased sample-to-solvent ratio ratios (Odabaş and Koca, 2021). The increasing of sample-to-solvent ratios enhanced the penetration of the solvent into the material and facilitated the dispersion of the target compounds into solvent (Liu *et al.*, 2013). However, excessively high solvent volumes can dilute the extracted components, leading to a decreased in the concentration of the substance. This phenomenon was also found in the anthocyanin extraction from *Rosa pimpinellifolia* L. fruits using ATPS (Odabaş and Koca, 2021).

Therefore, an optimal sample-to-solvent ratio of 1:40 (w/v) and an ethanol concentration of 34% yield the maximum GABA extraction, which could be extracted up to 8.80 µg/g of germinate rice. This optimized condition was then used in the next experiment.

3.2 Effect of ammonium sulfate concentration on bioactive compounds and antioxidant activities

Generally, the ATPS is composed of inorganic salts and short-chain alcohols, which are major factors affecting the target compound concentration. Ammonium sulfate has been studied in many research and was used in this study. The optimal ethanol concentration of 34% and the sample-to-solvent ratio of 1:40 (w/v), as reported in section 3.1, yield the highest GABA content. Consequently, the effect of ammonium sulfate concentration on bioactive compounds was studied. The results are shown in Fig 2.

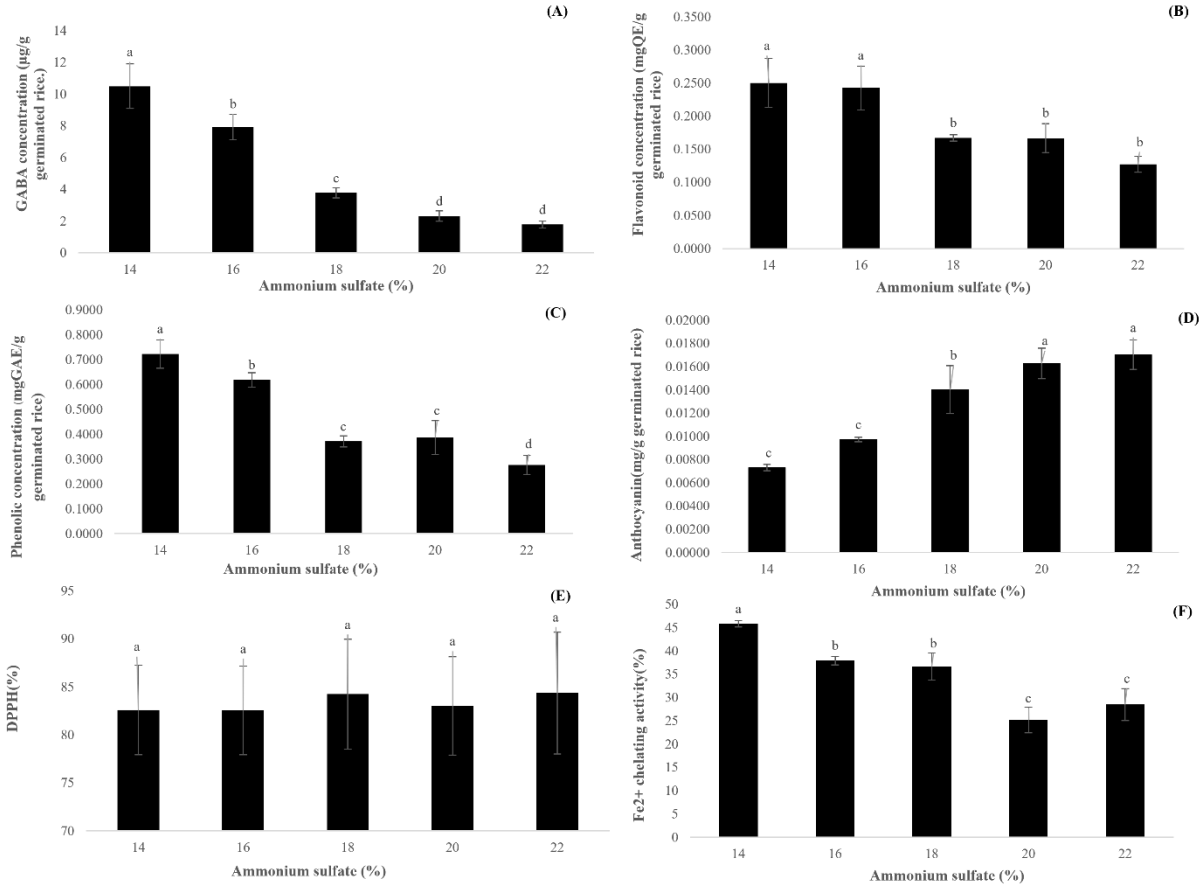

Fig 2 Effect of ammonium sulfate concentration. When using an ethanol concentration of 34 % and a sample-to-solvent ratio of 1:40 (w/v): GABA concentration (A), flavonoid concentration (B), phenolics concentration (C), anthocyanin (D) DPPH% (E), Fe^{2+} chelating activity (F)., Values with different letters (a-d) are significantly different ($P<0.05$).

The results found that the amounts of GABA, phenolics, and flavonoids significantly decreased as the concentration of ammonium sulfate increased (Fig 2A-C). This was probably due to the decrease of the upper phase volume as the concentration of ammonium sulfate increased leading to a decrease in concentration of the target compounds in the upper phase (Ma *et al.*, 2013). This result was similar to the phenolics extraction from dry beans (İşçimen and Hayta, 2021). However, the anthocyanin content increased when the ammonium sulfate concentration increases from 14% to 20% (Fig 2D). The increase in ammonium sulfate concentration caused the decrease in the volume and water content in the upper phase. As a result, the anthocyanin content increased as the concentration of ammonium sulfate increased (Wang *et al.*, 2010). This result was in accordance with the research performed by Odabas and Koca (2021) which found that the anthocyanin content in upper phase increased when ammonium sulfate concentration increased from 17% to 19%.

In addition, Black Jasmine rice has been reported to be rich in bioactive compounds, which may be affected by processing conditions. These bioactive compounds have demonstrated anti-oxidation capacity. Therefore, the antioxidant activity of the extract obtained from different ATPS compositions was analyzed. The results are shown in Figure 2E and 2F. The ability to eliminate DPPH radicals at different ammonium sulfate concentrations showed a high value of elimination for DPPH radicals (87.36–82.61%) (Fig 2E). However, these extracts showed no significant difference in DPPH radical scavenging activity. The solvent was also measured for its ability to scavenge the DPPH radicals, and it was found that the solvent was able to remove 0.712% of the DPPH radicals. Therefore, the solvent did not affect the extract's ability to remove radicals or may have had very little effect.

Regarding Fe^{2+} chelating activity, it was found that the activity decreased as the concentration of ammonium sulfate increased, as shown in Fig 2F. It has been reported that an increase in the number of phenolic compounds leads to an increase in Fe^{2+}chelating activity (Sreerama *et al.*, 2012). This group of phenolic compounds plays an important role in preventing the formation of oxidizing intermediates of peroxidation, thereby inhibiting the activity of metals which contribute to the hydrogen transfer mechanism (Sreerama *et al.*, 2010).

4. Conclusion

Our study on the concentration of ethanol and the sample-to-solvent ratio for extracting biologically active substances from germinated Black Jasmine rice found that extraction using the ATPS at an ethanol concentration of 34% and sample-to-solvent ratio of 1:40 (w/v) yielded the highest amount of GABA. Subsequently, the concentration of ammonium sulfate was examined, and the results showed that an increase in ammonium sulfate concentration enhanced the extraction of GABA, phenolics, and flavonoids substances, although the anthocyanin content was negative affect. The ability to eliminate DPPH radicals of the extract obtained from different concentrations of ammonium sulfate ranged from 87.36–82.61%. Additionally, Fe^{2+} chelating activity decreased as the ammonium sulfate concentration increased.

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