doi: 10.17221/154/2016-CJFS

Antioxidant Properties and Production of Monacolin K, Citrinin, and Red Pigments during Solid State Fermentation of Purple Rice (*Oryzae sativa*) Varieties by *Monascus purpureus*

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Abstract

Pengnoi P., Mahawan R., Khanongnuch C., Lumyong S. (2017): Antioxidant properties and production of monacolin K, citrinin, and red pigments during solid state fermentation of purple rice (*Oryzae sativa*) varieties by *Monascus purpureus*. Czech J. Food Sci., 35: 32–39.

This study aims to evaluate the effects of various purple rice varieties on the production of monacolin K, citrinin, and red pigments by *Monascus purpureus* CMU002U (UV-mutant strain) and their antioxidant properties. The lowest value of citrinin concentration (132 ppb) was found in the SSF of the Na variety, which passed the standards of Japan, Taiwan, and European Union. The high monacolin K (13 482 ppm) and red pigment (388.25 units/g) levels were obtained from the fermented Doi Muser variety. The lowest IC_{50} value of the DPPH assay was found in the extract of fermented Doi Saked variety. An investigation of temperature shifting of the SSF for the Doi Muser variety indicated that the incubation at 30°C for 5 days, followed by 25°C until 30 days, yielded the highest value of monacolin K (35 292 ppm) production. These results demonstrated that fermented purple rice has a high potential to be developed as a new food supplement.

Keywords: red mould rice; functional foods; health foods; temperature shifting; toxin

Rice (*Oryza sativa* L.) is a staple food for over half of the world population, especially for those living in Asia. The nutrient content of rice is based on the rice cultivar and nutrient quality of the soil. Brown rice is unpolished whole-grain rice that is produced by removing only the outermost layer, the hull of the rice kernel. It may be distinctly brown, reddish, or purplish and has a mild nutty flavour (Babu *et al.* 2009). In the past, it was rarely eaten because it has always been difficult to store and becomes rancid more quickly than polished rice. Nowadays, people have shown an increased interest in eating it and this has increased the demand. It contains a high amount of dietary fibre and is rich in B vitamins and minerals as well as protein (Oh & Oh 2004). It has also been reported that brown rice contains high amounts of phytic acid (antioxidant, anti-cancer) and polyphenols (Panlasigui & Thompson 2006).

Supported by the Royal Golden Jubilee Ph.D. Program (PHD/0160/2552), "Center of Excellence on Biodiversity (BDC), Office of Higher Education Commission (BDC-PG2-159011)", and the National Research University (NRU) Project under Thailand's Office of the Higher Education Commission, Chiang Mai University.

doi: 10.17221/154/2016-CJFS

It helps to decrease serum cholesterol levels, which assists in preventing the cardio-vascular disease, and it is considered as a low glycaemic index food (low starch and high complete carbohydrates which decrease the risk of type 2 diabetes). Any rice, including sticky rice, long-grain rice, or short-grain rice can be eaten as brown rice. The growing change in consumer preference for unpolished rice over polished rice as a staple food can help a large percentage of the world population maintain and promote the better overall state of health (Patil & Khan 2011).

In Thailand, purple rice is categorised as a glutinous type of rice, the genotype of which comprises local varieties. Glutinous rice is widely grown in different geographical areas. Purple rice varieties have been found to be a good source of nutrients, minerals, phytochemicals, and antioxidant compounds. The bran hull of purple rice has the unique characteristic of containing high levels of gamma oryzanol and anthocyanin antioxidants. These substances allow for purple rice to be used in traditional types of herbal medicine that could reduce plasma cholesterol, cholesterol absorption, and decrease early atherosclerosis, as well as decrease the *in vivo* inhibition of Lewis lung carcinoma cell growth (Chen *et al.* 2005).

Functional foods are foods that have the ability to provide physiological benefits and a potential to reduce the risk of chronic diseases. Red mould rice (RMR) is a product of the fermented rice by *Monascus purpureus* and has been regarded as a cholesterol-lowering functional food. The main components of RMR are carbohydrates (25–73%), proteins (14–31%), water (2–7%), and fatty acids (1–5%). During fermentation, many products of the secondary metabolism are formed including pigments, γ-aminobutyric acid (GABA), monacolin, glucosamine, lecithin, flavonoids, sterols, and a mycotoxin (Lin *et al.* 2008).

Monacolin K (lovastatin, mevinolin, and mevacor) is a secondary metabolite of the *Monascus* strain and is an inhibitor of the enzyme hydroxymethylglutaryl coenzyme A reductase (HMG-COA), which regulates and inhibits enzymes involved in cholesterol biosynthesis. Many studies have shown that some secondary metabolites of the *Monascus* species have improved hyperlipidaemia, hypertension, hyperglycaemia, Alzheimer's disease development, oxidative stress, and fatigue during exercise (Shi & Pan 2011). However, RMR may be contaminated with a mycotoxin known as citrinin. This mycotoxin has been investigated as an antibiotic and has been reported to be hepatotoxic and nephrotoxic in mammals, leading to a negative impact on the acceptability of RMR products (Betina 1989). Fermentation conditions and different fungal strains may lead to differences in citrinin levels. There have been many studies that have reported on possible ways to decrease citrinin. The culture conditions were improved by histidine addition by which red pigments increased 6-fold, while no citrinin was identified (Blanc *et al.* 1998).

In this study, we focused on the effects of various purple rice varieties on the production of monacolin K, citrinin, red pigment, and antioxidant properties. This is the first report that has focused on using local pigment glutinous rice as a substrate for monacolin K production.

MATERIAL AND METHODS

Microorganism and inoculum. *Monascus purpureus* CMU002U is a UV treated mutant strain that was obtained from the Sustainable Development of Biological Resources Laboratory, Faculty of Science, Chiang Mai University. This strain was prepared using the spore suspension treated with UV rays (18 W) for 20 to 30 minutes. The viable cells were harvested by centrifugation, washed with distilled water and suspended in a potato dextrose broth (PDB) at 30°C for 6 hours. Then, it was subcultured to potato dextrose agar (PDA) and incubated for 3 days. Each single colony with the survival rate from 0.1–1% was selected for the primary screening of citrinin production following the method described by Wang *et al.* (2004). The strain was cultivated on PDA at 30°C for 7 days.

Five mycelium plugs (5 mm in diameter) from the periphery of the growing colony on PDA at 30°C for 7 days were transferred to 50 ml PDB in a 250-ml Erlenmeyer flask. Cultivation was performed in the dark at 30°C with shaking at 150 rpm on a reciprocal shaker. After 5 days of incubation, the cultures were used as the inoculum.

Solid state fermentation. Six Thai purple glutinous rice varieties, Doi Muser, Doi Saked, Na, Nan, Phayao, and Hom CMU were used in this study. The SSF was conducted following the method described by Chairote *et al.* (2007) with some modifications. Each rice variety was soaked in water overnight, then 20 g of rice were placed in a 250-ml Erlenmeyer flask and autoclaved at 121°C for 15 minutes. The moisture content of each rice variety was adjusted to 60% (w/w) on a wet basis. After cooling, 1 ml of the

doi: 10.17221/154/2016-CJFS

liquid fungal inoculum (10^6 spores/ml) was added and the inoculated flasks were incubated at 30°C in the darkness. After 14 days, the fermented rice was collected and dried at 60°C overnight. Three replications were made for each rice variety.

Detection and quantification of monacolin K and citrinin. High performance liquid chromatography (HPLC) analysis was used to determine the levels of monacolin K and citrinin production using a method described by Wang *et al*. (2004) and Chairote *et al*. (2007). The presence of monacolin K and citrinin was confirmed by retention time and co-injection with standards (Sigma-Aldrich®, Germany). A standard curve was constructed with different levels of both standards. Monacolin K and citrinin in fermented substrates were quantified by correlating the peak area of the extracted sample and the calibration curve. Three replications were made in each sample.

Determination of red pigment. The RMR was extracted using the method described by Nimnoi and Lumyong (2011). The extracts were measured at 500 nm and pigment yield was expressed as OD per gram of dried RMR. Each sample was carried out in triplicate.

DPPH radical scavenging method. The evaluation of RMR extracts was carried out using dried products diluted with methanol at different concentrations (5.0, 2.5, 1.25, 0.63, and 0.32 mg/ml). Antioxidant activity was measured using the modified method described by Brand-Williams *et al*. (1995). One millilitre of RMR extracts was added to 2 ml of 0.004% DPPH (2,2-diphenyl-1-picrylhydrazyl) in methanol. The mixture was shaken and left to stand at room temperature (25°C) for 30 min in the dark. The solution was measured at 517 nm and calculated as the percentage of antioxidant activity using the following formula:

$$\%\ \text{antioxidant activity} = 100 - \{[(\text{sample absorbance} - \text{blank absorbance}) \times 100]\ \text{control absorbance}\}$$

[methanol + RMR extracts: blank, DPPH + methanol: control and butylated hydroxytoluene (BHT) was used as the standard]

Antioxidant activity was expressed in terms of the amount of antioxidants necessary to decrease the initial DPPH and IC_{50} value was determined by calculating the concentration of the sample that is required for a 50% reduction of antioxidant activity. All extracts were carried out in triplicate.

Production of monacolin K by temperature shifting. Suitable purple rice from the above experiment was selected and used in this experiment based on the highest ratio of monacolin K to citrinin production following the method of Kalaivani and Rajasekaran (2014). The SSF was carried out by a temperature shift from 30°C to 25°C. Different incubation times at 30°C followed by 25°C were evaluated and compared with unshifted temperature incubation experiments. All treatments were carried out in triplicate.

Statistical analyses. All the experimental data were analysed with SPSS program version 11.5 for Windows. All data were subjected to analysis of variance (ANOVA) by Tukey's test ($P < 0.05$).

RESULTS AND DISCUSSION

Red mould rice production. Red mould rice (RMR) was successfully prepared by solid state fermentation from various purple rice cultivars with mutant strains of *M. purpureus*. Generally, polished non-glutinous rice is used as a substrate for RMR production. In addition, brown rice, black rice both glutinous rice, and non-glutinous rice, cereal and agricultural waste can be used as the raw material for RMR production (Velmurugan *et al*. 2011). Several purple rice varieties, including Doi Muser, Doi Saked, Na, Nan, Phayao, and Hom CMU were used as the substrates for RMR production. Specifics of the grain characteristics included a hard texture and a dark purple colour, both of which affected the texture and the colour of products (Figure 1). The results showed that after being fermented, the grains had a soft texture resulting from amylase production and a

Figure 1. Purple rice varieties for red mould rice production: (a) Doi Muser; (b) Doi Saked; (c) Na; (d) Nan; (e) Phayao; and (f) Hom CMU (bar = 1 cm)

doi: 10.17221/154/2016-CJFS

Figure 2. Red mould rice from purple rice: (a) Doi Muser; (b) Doi Saked; (c) Na; (d) Nan; (e) Phayao; and (f) Hom CMU (bar = 1 cm)

pleasant odour resulting from the presence of esters and alcohols. The products had a dark red colour from the *Monascus* pigment and the grains retained their original shape (Figure 2). The yield percentage of recovered RMR from purple rice at 2 weeks of fermentation was similar to the results that had previously been described by Chairote *et al.* (2007). RMR from Nan and Hom CMU rice varieties gave the highest and the lowest percentages of yield at 33.3 and 27.5, respectively ($P < 0.05$).

Red pigment production. Doi Muser RMR and Na RMR displayed the highest and the lowest red pigment yield, respectively, and revealed a three-fold difference. The red pigment yields of RMR obtained from Doi saked, Nan, and Phayao were not found to be significantly different ($P > 0.05$). Pigments are secondary metabolites synthesised by *M. purpureus* and can be divided into three groups: orange (rubropunctain and monascorubrin), yellow (monascin and ankaflavin), and red (rubropunctamine and monascorubramine) (Campoy *et al.* 2006). These pigments have displayed a high stability towards pH and temperature changes, which has been identified as an interesting property when used as substitutes for synthetic dyes. For a long time in eastern Asia, the red pigment has been used as a food additive after fermentation (Fabre 1993). The advantages of using purple rice varieties as a substrate are that it contains higher values of protein, iron, and zinc when compared to both brown rice and white rice (Fernando 2013). Therefore, the process does not require any supplementation of sugar or nitrogen sources when compared with that of Chairote *et al.* (2007) and Nimnoi and Lumyong (2011). The contents of the pigments in RMR depend on the cultural conditions such as humidity and pH value as well as other nutrients and the supply of oxygen (Vidyalakshmi 2009). The red pigment yield can be obtained from liquid, submerged, and solid state fermentation processes. Solid state fermentation involves the cultivation of microorganisms under controlled conditions in the absence of free water, which makes up conditions that are similar to the natural habitats of fungi (Mienda *et al.* 2011).

Monacolin K and citrinin production. Purple rice can be used as a material for the synthesis of monacolin K or other secondary metabolites through the RMR process. The effects of purple rice varieties on monacolin K productivity are shown in Table 1. SSF on Phayao and Doi Muser purple rice achieved high monacolin K levels, at 13 496 ppm and 13 482 ppm, respectively. The lowest yield of monacolin K was found in SSF of Na purple rice (2118 ppm), which was 6.37-fold less than that of Phayao purple rice. This result was related to the low level of red pigment yield detected during the previous phases of

Table 1. Percentage of yield, red pigment, monacolin K, citrinin production, and ratio of monacolin K and citrinin of RMR from purple rice

Purple rice variety	Yield (%)	Red pigment (unit/g)	Monacolin K (ppm)	Citrinin (ppb)	Ratio of monacolin K and citrinin
Doi Muser	28.53 ± 0.45[ab*]	388.25 ± 23.61[a]	13 482 ± 1 932[a]	633 ± 29[bc]	21 299
Doi Saked	28.93 ± 4.75[ab]	270.83 ± 16.22[ab]	10 804 ± 1 898[a]	769 ± 24[bc]	14 049
Na	29.47 ± 0.14[ab]	120.50 ± 7.89[c]	2 118 ± 619[b]	132 ± 7[c]	16 045
Nan	33.28 ± 1.02[a]	331.75 ± 26.48[ab]	11 277 ± 2 619[a]	882 ± 35[a]	12 786
Phayao	31.42 ± 0.71[ab]	318.17 ± 15.53[ab]	13 496 ± 659[a]	1 125 ± 78[a]	11 996
Hom CMU	27.47 ± 1.11[b]	229.17 ± 12.55[bc]	9 435 ± 2 318[a]	751 ± 33[bc]	12 563

Values are expressed as mean ± SD of three replications; different letters in the same column indicate significant differences ($P < 0.05$)

doi: 10.17221/154/2016-CJFS

this study. The research of Chiu *et al.* (2006), who studied the liquid state fermentation (LSF) process for monacolin K production on rice material, showed that monacolin K was produced at a lower level (46.5–53.5 ppm). Different factors of SSF included the raw materials used, temperature, initial moisture, the strain of the fungus used and cultivation times, all of which affected the secondary metabolite production (Carvalho *et al.* 2007). SSF is the only type of fermentation that could improve the yield of the products and has a low energy requirement, which reduced the production costs. SSF was conducted by incubation at a similar temperature like that used by Su *et al.* (2003), who stated that the highest yield of monacolin K was obtained at 30°C. However, the production of RMR is frequently contaminated with citrinin. Contamination with citrinin is a problem that influences acceptability because it is a mycotoxin that damages the liver and kidneys of mammals. *Monascus purpureus* used in this study has been improved through mutation by UV radiation. This strain produced fewer toxins than the parental strain. The highest citrinin level was found in RMR from Na purple rice (1125 ppb) and the lowest citrinin level was found in Phayao rice (132 ppb). The monacolin K produced in our study was higher than that which was obtained in other RMR research studies. SSF on Indian rice by *M. purpureus* MTCC1090 under optimal conditions (supplemented with fructose, sodium nitrate, and acetic acid) produced monacolin K at only 37 ppm (Rajasekaran & Kalaivani 2012). In addition, monacolin K can be produced by other strains of *Monascus*. Xu *et al.* (2005) studied the optimal conditions for monacolin K production using *M. ruber* on rice. Their results showed that the addition of soybean powder increased monacolin K from 3446 to 4020 ppm. In their research work Park *et al.* (2014) used Korean rice varieties as raw materials for RMR production. Their results showed that the highest monacolin K content of only 117 ppm from *M. ruber* KCCM60141 cultures occurred in the Sangjuchalbyeo rice variety.

The results of a comparative study of the ratio of monacolin K to citrinin production are shown in Table 1. RMR from Doi Muser rice varieties showed the highest ratio and monacolin K production was 21 299-fold higher than citrinin production (13 482 ppm of monacolin K and 633 ppb of citrinin). The levels of citrinin detected in RMR were in accordance with the standards of Japan and Taiwan, which have imposed citrinin concentration limits to 200 and 2000 ppb, respectively (Chung *et al.* 2009). In 2014, Commission Regulation (EU) set the maximum levels of citrinin in rice fermented with *M. purpureus* at 2000 ppb in the European Union (European Commission 2014). Our fermented products from the Na rice variety have passed the standards of Japan, Taiwan, and the EU but could not pass those standards of Korea and the US FDA since the acceptable levels must be less than 50 and 20 ppb, respectively (Chung *et al.* 2009; Le Bloc'h *et al.* 2015). In Thailand, there have been many reports about mycotoxin contamination in raw agricultural commodities and processed food, which can be identified as aflatoxin. However, there is no set regulation limit of citrinin contamination (Songsermsakul 2015).

RMR is commonly used as a food supplement in many countries including the USA, China, and Taiwan for the treatment of high cholesterol. Journoud and Jones (2004) demonstrated that RMR seems to be safe when compared with other available statins (cholesterol-lowering drugs), as the incidence of adverse side effects is fairly low. However, the US FDA issued a consumer warning stating that consumers should avoid RMR products because some products contained unauthorised drugs (monacolin K) (Childress *et al.* 2013). The RMR has no clear position on the use of this substance in food supplements due to the medical regulation (EC No. 2001/83) which states that the level of monacolin K in products can range from 10 mg to 20 mg. Therefore, its regulatory status in the EU depends mainly on the dosage of monacolin K in consumer products (Le Bloc'h *et al.* 2015).

DPPH radical scavenging. The antioxidant activity of BHT was used as a comparative standard. The initial concentration of all RMR products at 5 mg/ml, as well as 1 mg/ml of BHT could scavenge more than

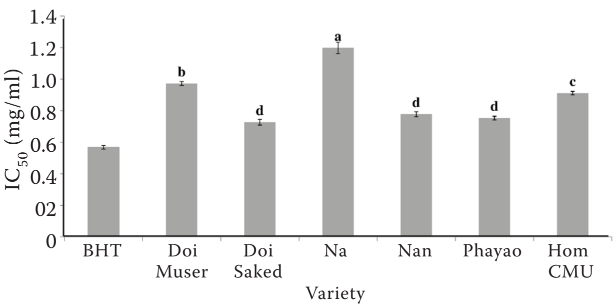

Figure 3. IC_{50} value of BHT and red mould rice extracts. Data are presented as means of three replicates

The error bar at each point indicates ± SD; different letters above each bar for the extract indicate the significant differences ($P < 0.05$)

doi: 10.17221/154/2016-CJFS

Table 2. DPPH radical scavenging performance of the RMR extracts compared to BHT

Purple rice variety	Compared to BHT
Doi Muser	0.581
Doi Saked	0.776
Na	0.471
Nan	0.725
Phayao	0.749
Hom CMU	0.619

90% of DPPH radicals. The antioxidant activity of the RMR extracts and BHT standard was expressed as IC_{50} values and is presented in Figure 3. Doi Saked RMR had the lowest IC_{50} value, which means that the concentration of RMR at 0.727 mg/ml reduced free radical contents to 50%, which represented 0.776 equivalency to BHT. A comparison of the DPPH radical scavenging activity of RMR products to the BHT standard is shown in Table 2. Antioxidant activity from our RMR products displayed a greater level of efficiency when compared to Chairote *et al.* (2009). IC_{50} value from fermented purple glutinous rice was calculated at a value of 0.315 equivalency to BHT. Other substrates including Mali 106 (non-glutinous rice), RD6, and Sanpatong glutinous rice showed IC_{50} values of 0.426, 0.333, and 0.222 equivalency to BHT, respectively. RMR is one of the dietary foods that possess antioxidant properties. Al-Shahrani *et al.* (2013) studied the amounts of antioxidants in nutraceutical food products and also demonstrated that RMR has antioxidant properties. They reported that their RMR had 1.067 Mesfer units (1 Mesfer unit = antioxidant activity of 500 mg of ascorbic acid), which was more than it is reportedly contained in concentrated green tea and curcuminoid material that contain only 0.426 and 0.372, respectively.

Production of monacolin K by temperature shifting. The highest ratio of monacolin K and citrinin was found in the SSF of Doi Muser purple rice. Therefore, this rice variety was used in the experiments. The optimal temperature for the growth of *M. purpureus* is 30°C (Subsaendee 2014); the mycelium covered all rice grains and changed the substrate colour to dark red more rapidly than when incubated at 25°C for 30 days. However, higher values of monacolin K were found when the SSF process was conducted at 25°C. The fermentation with temperature shifting is a simple technique that has been used for the improvement of product yields (Ansorge & Kula 2000; Tsukahara *et al.* 2009). In this study, the temperature shifting was modified for SSF. The highest ratio of monacolin K and citrinin was found in the SSF of Doi Muser purple rice. Therefore, this rice variety was used in this experiment. The monacolin K contents in SSF of Doi Muser purple rice at 25 and 30°C were found to be 23 832 and 12 344 ppm, respectively. The monacolin K content at 25°C increased to 93.07%, compared to incubation at 30°C. The initial temperature of fermentation was 30°C and then it was shifted to 25°C after various periods and the specimens were incubated for 30 days. In this part of the experiment, it was necessary to increase the fermentation time from 14 to 30 days in order to allow the fungal growth to cover all grains. The results are shown in Table 3. In treatment B, fermentation for 5 days at 30°C followed by 25 days at 25°C revealed the highest monacolin K (35 292 ppm) and citrinin (1769 ppb) (19 950 : 1) values. The monacolin K and citrinin values were 186 and 1.695% higher, respec-

Table 3. Monacolin K and citrinin production by temperature shifting experiment

Treatment	Incubation period (day)		Monacolin K (ppm)	Citrinin (ppb)	Ratio (Monacolin K : citrinin)
	30°C	25°C			
A	2.5	27.5	29 040 ± 1167	1 116 ± 123	26 022 : 1
B	5	25	35 292 ± 991	1 769 ± 82	19 950 : 1
C	7.5	22.5	32 258 ± 870	960 ± 35	33 602 : 1
D	10	20	16 778 ± 459	779 ± 13	21 538 : 1
E	15	15	16 156 ± 382	854 ± 12	18 918 : 1
F	20	10	17 672 ± 456	861 ± 18	20 525 : 1
G	25	5	17 089 ± 427	344 ± 23	49 677 : 1
H	30	0	12 344 ± 402	99 ± 16	124 687 : 1
I	0	30	23 832 ± 817	856 ± 30	27 841 : 1

The values of monacolin K and citrinin are expressed as mean ± SD of three replications

doi: 10.17221/154/2016-CJFS

tively, when compared with treatment H (constant incubation at 30°C). Treatment G (fermentation for 25 days at 30°C followed by 5 days at 25°C) was found to be optimal for the highest ratio of the values of monacolin K and citrinin (49 677 : 1). The levels of monacolin K and citrinin production were 17 089 and 344 ppb, respectively. This treatment produced 163% and 37% more monacolin K and citrinin, respectively, than treatment H. Moreover, treatment G was applied to the SSF of white Sanpatong glutinous rice. The results showed that the ratio of monacolin K and citrinin was 29 780 : 1 (monacolin K 22 097 ppm and citrinin 742 ppb). The findings of our research work agree with those of Tsukahara *et al*. (2009), who studied the effects of temperature shifting. Temperature shifting from 30°C to 23°C increased monacolin K production when compared to cultivation at a constant temperature and our results agreed with Feng *et al*. (2014), who studied monacolin K production by temperature shift (30°C 2.5 days and 24°C 14 days) on non-glutinous rice and soybean flour that could result in higher levels of monacolin K at 18 733 ppm.

CONCLUSIONS

Our study shows that the production of monacolin K, citrinin, red pigments, and antioxidant properties in SSF by *M. purpureus* were dependent on the purple rice varieties. The SSF of the Doi Muser variety showed a high level of monacolin K and red pigment production. Interestingly, the fermented product of the Na variety showed the lowest citrinin concentration, which passed the standards of Japan, Taiwan, and the EU. The extracts of the fermented products acquired from Doi Saked revealed the highest DPPH scavenging activity. Moreover, the temperature shifting treatment by fermentation for 5 days at 30°C followed by 25 days at 25°C on the SSF of the Doi Muser variety yielded higher values of monacolin K (35 292 ppm) and citrinin (1769 ppb). Hence, our findings suggest that fermented purple rice has a high potential to be developed as a new food supplement.

Acknowledgements. The author wish to thank Dr. Jaturong Kumla for helping improve the manuscript.

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Received: 2016–04–27
Accepted after corrections: 2016–11–29