PRODUCTION AND ANTIOXIDANT ACTIVITY OF MONASCAL WAXY CORN

KITISART KRABOUN

A Thesis Submitted to the Graduate School of Naresuan University
in Partial Fulfillment of the Requirements
for the Doctor of Philosophy Degree
in Food Science and Technology (International Program)
September 2015

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Thesis entitled "Production and Antioxidant Activity of Monascal Waxy Corn"
by Kitisart Kraboun
has been approved by the Graduate School as partial fulfillment of the requirements
for the Doctor of Philosophy Degree in Food Science and Technology of
Naresuan University

Oral Defense Committee

.... Chair
(Assistant Professor Katekan Dajanta, Ph.D.)

.... Advisor
(Associate Professor Teeraporn Kongbangkerd, Dr. nat. techn.)

.... Co-Advisor
(Assistant Professor Worasit Tochampa, Ph.D.)

.... Co-Advisor
(Assistant Professor Wassana Chatdumrong, Ph.D)

.... Internal Examiner
(Associate Professor Kamonwan Rojsuntornkitti, M.S.)

Approved

....
(Panu Putthawong, Ph.D.)
Associate Dean for Administration and Planning
for Dean of the Graduate School

- 7 SEP 2015

ACKNOWLEDGEMENT

I would never have been able to finish my dissertation without the guidance of my committee members, help from friends, and support from my family.

I would like to express my deepest gratitude to my advisor and two co-advisors, Associate Professor Dr. Teeraporn Kongbangkerd, Assistant Professor Dr. Worasit Tochampa and Assistant Professor Dr. Wassana Chatdamrong for their excellent guidances, caring, patience, and providing me with an excellent atmosphere for doing research. Special thanks goes to Assistant Professor Dr. Katekan Dajanta, who was willing to participate in my final defense committee at the last moment.

I would like to thank the financial support from a scholarship under named Doctoral Research Scholarship from the Faculty of Agriculture, Natural Resources and Environment, Naresuan University. Finally, I would also like to thank my parents and three elder sisters. They were always supporting me and encouraging me with their best wishes.

Kitisart Kraboun

Title	PRODUCTION AND ANTIOXIDANT ACTIVITY OF MONASCAL WAXY CORN
Author	Kitisart Kraboun
Advisor	Associate Professor Teeraporn Kongbangkerd, Dr. Nat. Techn.
Co - Advisor	Assistant Professor Worasit Tochampa, Ph.D.
	Assistant Professor Wassana Chatdumrong, Ph.D.
Academic Paper	Thesis Ph.D. in Food Science and Technology (International Program), Naresuan University, 2015
Keywords	Waxy corn, Monascal waxy corn, Antioxidant activity

ABSTRACT

The principal aim of this research was to study the following four experiments: 1) different nitrogen levels of monosodium glutamate (MSG) and peptone; 2) 2-step fermentation process compared with the conventional method; 3) using the optimization of ultrasonic-assisted extraction (UAE) and shaking extraction; 4) stability of monascal waxy corn (MWC) during *in vitro* digestion the digestion of MWC on bioaccessibility, using a standardized *in vitro* gastrointestinal digestion which all experiments were focused on antioxidant activities, monacolin K and citrinin contents and pigment intensity of MWC fermented by *Monascus purpureus* TISTR 3090.

Firstly, the effects of different nitrogen contents of peptone and MSG, which are equivalent to 0.25, 0.50, 0.75 and 1.00% nitrogen content, on antioxidant activities, monacolin K and citrinin contents, *Monascus* pigmentation and growth in MWC products, were studied. The results showed that the addition of MSG or peptone to waxy corn affected the *Monascus* fermentation, leading to higher antioxidant activities, pigment intensity and glucosamine content, monacolin K and citrinin contents compared to the control (no nitrogen source added). The production of pigment at 25°C, monacolin K and citrinin contents and antioxidant activities were maximum on day 12 of fermentation while glucosamine content was maximum on day

8. The highest antioxidant activities were obtained using 12.08% MSG content, equivalent to 1.00% nitrogen content. Thus, MWC obtained from the treatment supplemented with 12.08% MSG content with fermentation for 12 days was selected to study the 2-step fermentation process compared with the conventional method. It was found that, after 8 days of fermentation, monacolin K content and pigment intensity of MWC from 2-step fermentation were 79.72 mg/kg dry weight and 3,500.00 unit/g dry weight, respectively, which were higher than those of the conventional method, while the residual reducing sugars were exhausted, giving the observed highest growth. The IC_{50} values of DPPH, ferric reducing/antioxidant power (FRAP) and chelating ability on Fe^{2+} assays of the 2-step fermentation were about 50% less than those of the conventional method. MWC from 2-step fermentation for 8 days gave the highest antioxidant activities and was selected to study the optimization of UAE and shaking extraction.

The optimization procedure using a central composition design (CCD) with two factors (ethanol concentration and temperature) was applied and a second-order polynomial model was used to describe the effect of these parameters on antioxidant activities, monacolin K and citrinin contents and the pigment intensity. It indicated that the effective extraction of UAE was better than that of shaking method because of higher antioxidant activities and monacolin K contents and pigment intensity but lesser extraction time. For UAE, the optimized conditions were 69.60%/49.15 °C (DPPH) and 70.66%/49.91°C (Chelating ability on Fe^{2}); 69.91 %/49.95°C (monacolin K); 85.00 %/60.00°C (citrinin).

For stability of monacolin K, citrinin and the antioxidant activities in MWC during *in vitro* digestion, after gastric digestion, monacolin K and citrinin contents, DPPH free radical scavenging ability and chelating ability on Fe^{2+} of MWC of the sample were increased 1.35, 8.69, 33.33 and 13.47 %, respectively, suggesting a release of antioxidants and pigments. After pancreatic digestion, however, monacolin K and citrinin contents and DPPH free radical scavenging ability and chelating ability on Fe^{2+} between soluble and insoluble MWC were decreased between 14.69 and 76.92 % which these values were lost during treatment with pancreatin. It indicated that pancreatic digestion causes reduced antioxidant activity between soluble/insoluble

fractions while the gastric digestion promoted more releasing the antioxidant activities.

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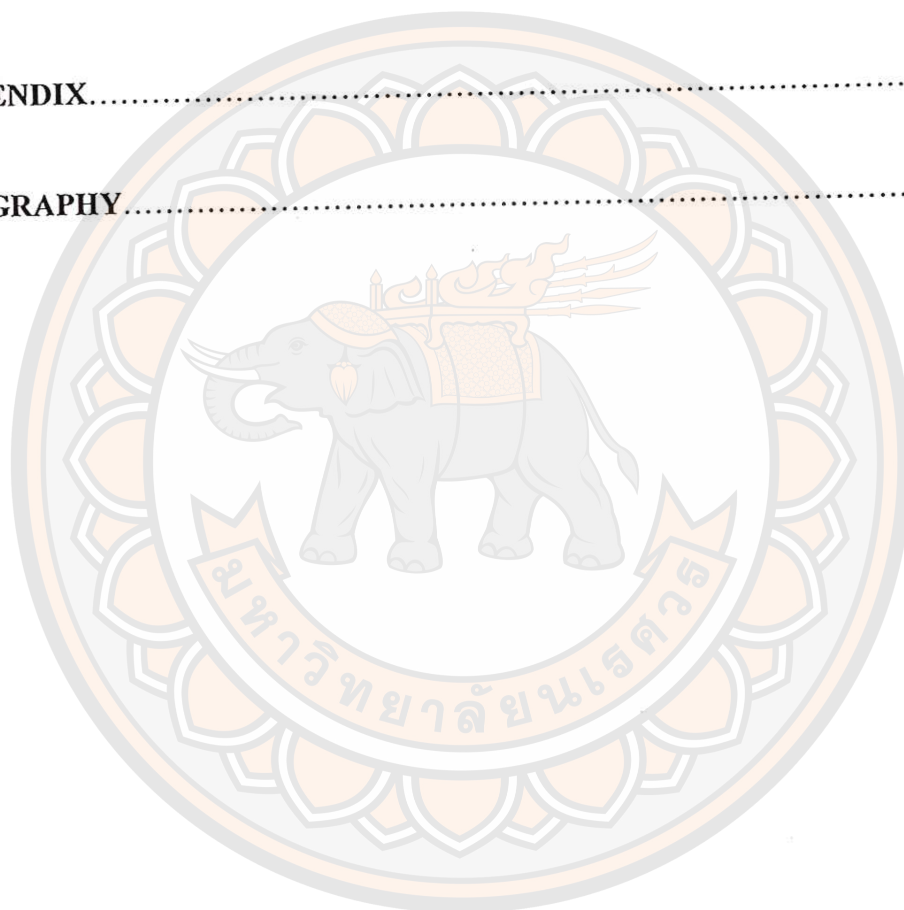

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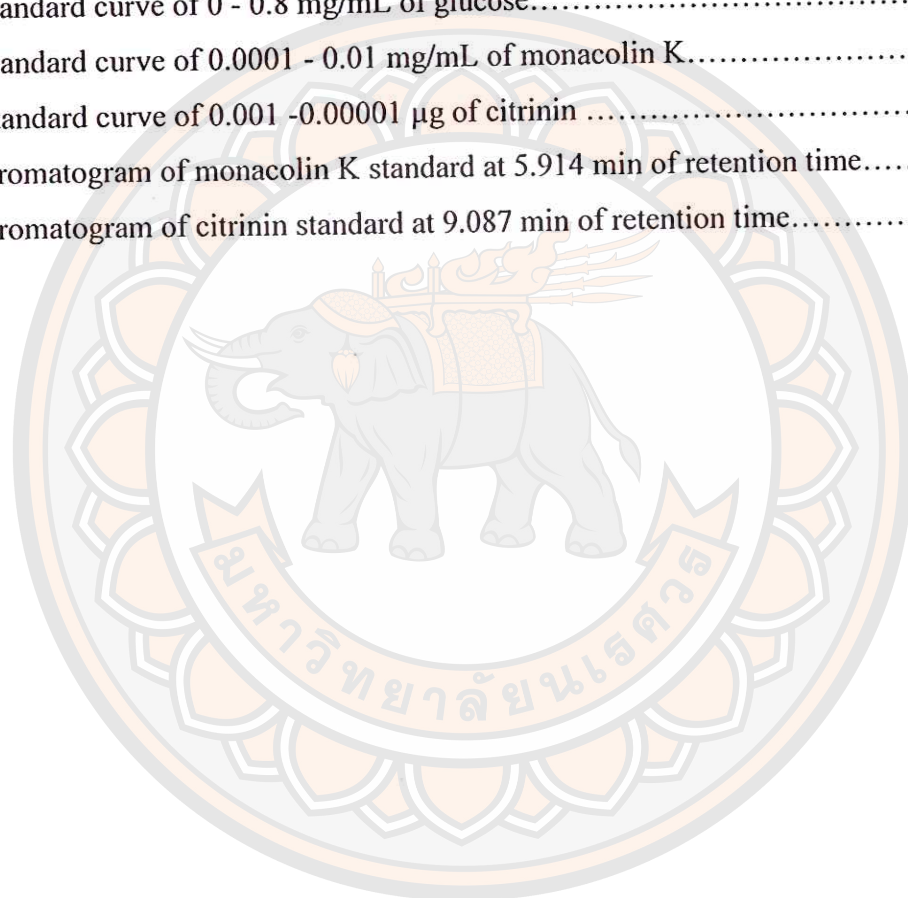
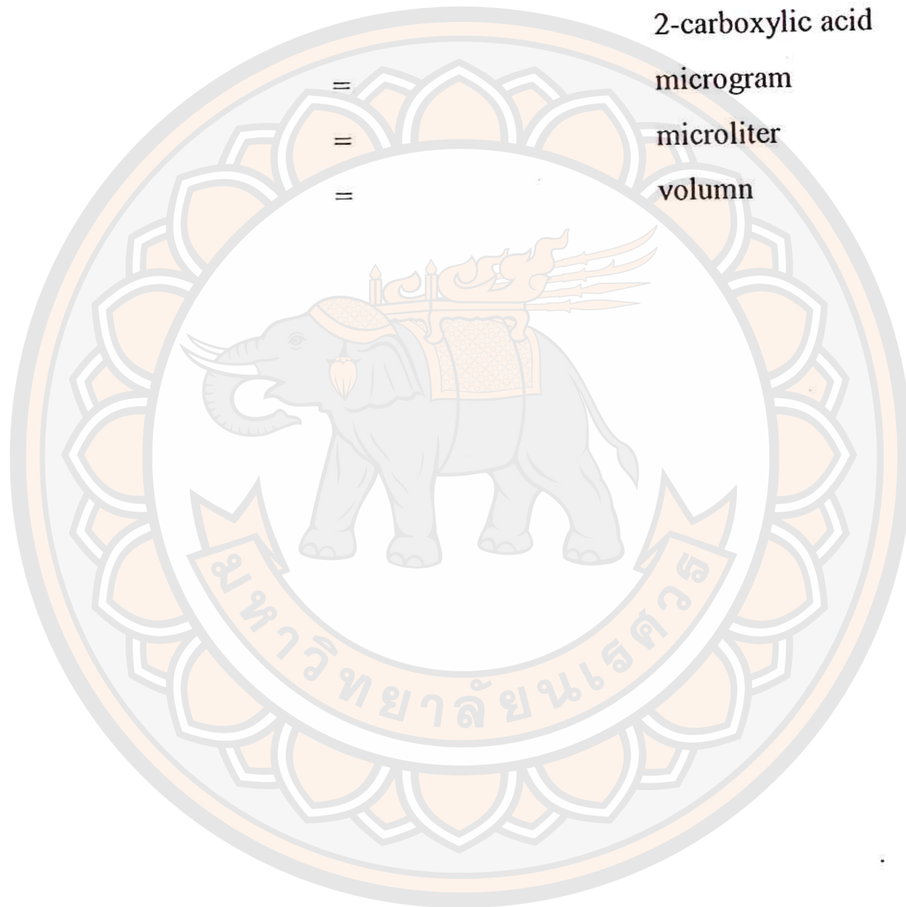


ABBREVIATIONS

atm	=	atmosphere
AU	=	astronomical unit
BHA	=	butylated hydroxyanisole
DMSO	=	dimethyl sulfoxide
Fe^{2+}	=	ferrous ion
g	=	gram
h	=	hour
K	=	kelvin
kg	=	kilogram
kHz	=	kilohertz
L	=	litre
LDL	=	low density lipoprotein
MFR	=	*Monascus*-fermented rice
mg	=	milligram
M	=	molar
MHz	=	megahertz
mL	=	milliliter
mM	=	millimolar
Mw	=	molecular weight
nm	=	nanometer
°C	=	degree celsius
P	=	probability value
pH	=	potential of hydrogen ion
ppb	=	part per billion
R^2	=	R-squared value
rpm	=	round per minute

ABBREVIATIONS (CONT.)

s	=	second
SSF	=	solid state fermentation
TPTZ	=	2,4,6-tripyridyl-s-triazine
Trolox	=	6-hydroxy-2,5,7,8-tetramethylchro-man-2-carboxylic acid
μg	=	microgram
μL	=	microliter
vol.	=	volumn



CHAPTER I

INTRODUCTION

Monascus sp., a fungus, can produce various significant antioxidants from its fermentation on rice solid substrates. During the fermentation of *Monascus* sp., carbohydrates of rice starch are utilized and changed into secondary metabolites, i.e., pigments which are significant antioxidants. The significant antioxidants of *Monascus* sp. consist of monacolin K, γ-aminobutyric acid (GABA), dimerumic acid (DMA) and flavonoids (Yang, et al., 2006). In an appropriate condition of monascal rice production, monacolin K content is highly produced, which demonstrates as a specific inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase in cholesterol biosynthesis (Alberts, et al., 1980; Endo, 1980). The key enzyme produces mevalonyl-CoA which is the important rate determining step to synthesize cholesterol (Hajjaj, Niederberger, Duboc, 2001; Su, Wang, Lin, 2003). Hence, it helps to decrease blood pressure (Li, et al. 2004; Wang, Lu and Chi, 1997). Yang, et al. (2006) reported that the *Monascus* pigment extract indicated high antioxidant activities such as inhibition of peroxidation, reducing power, scavenging ability on DPPH radicals and chelating ability on ferrous ions. Moreover, GABA in the aqueous extract of *Monascus*-fermented rice has shown decrease of *in vivo* blood pressure (Kohama, et al., 1978; Kushiro, et al., 1996) without affecting electrolyte metabolism or the activity of antiotesin-I-converting enzyme (Tsuji, et al., 1992).

Generally, polished rice or white rice has been used for a long time as a main solid substrate for monascal rice production. Meanwhile, this rice substrate is rich in carbohydrates but lacks of the germ which are embryo and bran parts. These parts consist of essential amino acids, vegetable oil and others (Black, Bewley and Hunter, 2006). The drawback of rice substrate is high cost so there is difficult application to industry degree. Waxy corn, *Zea mays* ceratina, contains high amylopectin but low amylose contents, while lipid and ash contents are higher than those of polished rice (Jiranuntakul, et al., 2011). Therefore, it would be used as an alternative substrate for

Monascus pigment production because of its various nutrients and the price is comparatively low.

Nitrogen sources are important for *Monascus* pigmentation involving the improvement of water soluble property which an oxygen atom will be replaced by nitrogen atom of amino group (amino acid, protein and peptide) in monascorubrine or rubropunctatine pigment, thus this replacement indicates to change the color from yellow to purple-red. This color is the reduced and oxidizing agents and also reacts with other substances. This color binds with an amino acid to complex forms, Glutamyl-monascorubine and Glutamyl-rubropunctatine which are quite dissolved in water (Dufosse, et al., 2005). Moreover, nitrogen is a nutritional source and is required for microbial growth (Broder and Koehler, 1980; Lin and Demain, 1991). Babitha, Soccol and Pandey (2007) reported the addition of 1 % monosodium glutamate (MSG) or peptone to jackfruit seed powder promoting *Monascus* growth and pigment production. Silveira, Daroit and Brandelli (2008) further reported MSG and peptone affected pigment production and *M. purpureus* growth by using grape waste broth as a substrate. From this standpoint, nitrogen source could clearly improve *Monascus* pigmentation while its effect on antioxidant activity of *Monascus* pigment from monascal waxy corn (MWC) has never been reported as using waxy corn as a substrate.

Extraction is an important tool for substrate separation to obtain significant substances. The extraction under the optimum condition leads to higher contents of selected substances. Normally, monascal rice was extracted by many solvents, e.g., methanol, hexane, chloroform, ethyl acetate and acetone. Presently, methanol is the best solvent for the extraction of pigments and monacolin K from the cell (Carvalho, et al., 2007). In the past, there were a lot of extraction methods for obtained *Monascus* pigments, e.g., ultrasonic wave, shaking, heating. Commonly, extraction of *Monascus* pigments and antioxidants from monascal rice by the shaking method has always been used which disadvantages of this method are time consumption about an hour but its extraction effectiveness is high, providing high pigment yield and maintaining antioxidant activity (Yang, et al., 2006). However, ultrasonic-assisted extraction (UAE) is more interesting method because of shorter time consumption, high pigment intensity and monacolin K content. For examples, Li, Peizheng and Aidong (2009)

reported the optimal extraction condition for red pigment with applying parameters as follows: dual-frequency (45, 28 kHz) alternately each 3 min, power 120 W, liquid-solid ratio 1.5 :1 (v:w), ultrasonic time 30 min, and under such conditions, the yield of the pigment could reach 92.84%. Yang, et al. (2005) reported that intermitted ultrasonic wave treatment promoted *Monascus* pigment and monacolin K content which were increased 29.74 % and 39.96%, respectively, as the following condition : frequency of 20 KHz and a power of 200 W for 2 min. Ultrasonic wave has been reported that it could induce the mutation of extracellular pigment at the basic pH values (>7.0-13.0), which the optimal ultrasonic treatment at 45 kHz for 2 min at 28°C was applied (Wongjewboot, Bowdang and Kongruang, 2012). Commonly, previous works on pigment extraction have been conducted using 'one-factor-at-a-time-technique'. This conventional approach frequently fails to locate optimal conditions due to its failure to clearly show possible effects of interactions between parameters (Kalil, Maugeri and Rodrigues, 2000). Central composite design (CCD) and response surface methodology (RSM) are important tools to determine the optimal process condition. This methodology has been successfully used in many areas of biotechnology, particularly to optimize the production and extraction of bioactive molecules (Cladera-Olivera, Caron and Brandelli, 2004; Thys, et al., 2006). The production optimization of pigment and GABA from *Monascus* fermentation using CCD and RSM has been published (Silveira, Daroit and Brandelli, 2008; Sun, et al., 2008). However, the extraction optimization using CCD and RSM for monacolin K and antioxidant activities from MWC has never been studied.

Generally, *Monascus* pigments have been only determined through *in vitro* antioxidant activity and they showed high *Monascus* antioxidant activities from monascal rice products (Yang, et al., 2006). Besides, its antioxidant activities after and/or during gastrointestinal digestion are doubted. Hence, the antioxidant activities of the pigments should be analyzed through mimicking *in vitro* gastrointestinal digestion. This model digestion benefit is a first approach to predict the *in vivo* antioxidant activity of substances. The *in vitro* gastrointestinal digestion is a starting point to predict the *in vivo* antioxidant power of substances, taking into account that the pigment potential is through the digestion approach (Emmons, Peterson and Paul,

1999; Zielinski and Kozlowska, 2000; Martinez-Tome, et al., 2004; Serpen, et al., 2007).

Therefore, this research conducts to the study of effects of nitrogen levels of monosodium glutamate (MSG) and peptone and 2-step fermentation on antioxidant activities, pigment intensity and *Monascus* growth of MWC. Moreover, the effects of ultrasonic-assisted extraction on antioxidant activities and pigment intensity and the stability during *in vitro* digestion of produced *Monascus* antioxidant were also discussed.

Objectives

1. To study effects of MSG and peptone on antioxidant activities of MWC
2. To study fermentation technique to enhance antioxidant activity of MWC production by using 2-step fermentation
3. To optimize the extraction method of monacolin K, antioxidant activity, pigment and citrinin contents from MWC produced by 2-step fermentation by using RSM
4. To study antioxidant stability of *Monascus* pigment from MWC via *in vitro* digestion

Scope of the study

Four experiments were conducted 1) different nitrogen levels of monosodium glutamate (MSG) and peptone; 2) 2-step fermentation process compared with the conventional method; 3) using the optimization of ultrasonic-assisted extraction (UAE) and shaking extraction; 4) stability of monascal waxy corn (MWC) during *in vitro* digestion the digestion of MWC on bioaccessibility, using a standardized *in vitro* gastrointestinal digestion which all studies were focused on antioxidant activities, monacolin K and citrinin contents and pigment intensity.

CHAPTER II

LITERATURE REVIEW

Meaning, history and traditional uses of Angkak

Monascal rice or angkak, also known as *Monascus*-fermented rice, anka or red koji, is a fermented cereal product with *Monascus* sp. (Bau and Mo, 1975). During the fermentation, *Monascus* sp. grows on cooked rice and hydrolyzes carbohydrates from the rice in order to produce various secondary metabolites, i.e., monacolin K, pigments and GABA, etc. Then appropriate condition, differently produced pigments such as red, orange and yellow pigments are gradually released, but the red pigment as the most produced pigment commonly affects whole dark-red seed (Lotong, 1992).

Angkak was used as an Asia traditional medicine over 100 years ago. As well, *Monascus* spp. has been already known in Europe and Indonesia more than a hundred years. For the countries of western world, *Monascus* sp. was known as a contaminated mold in cereal, starch and silage. Church (1920) reported that angkak has been produced in China over a long time and the mold from angkak was isolated to pure culture. This isolated mold was called "*Monascus purpureus*". Then, Palo, Vidal-Adeva, and Maceda (1960), Filipino scientists, who isolated *Monascus* sp. from traditional angkak to use directly as food color additive (Su and Wong, 1983). Lin (1973) was the first researcher, who studied different *Monascus* strains for optimal pigment production of submerged fermentation (Yoshimura, et al., 1975; Shepherd and Cerels, 1983; Yongsmith and Tabloka, 1984).

Morphology of *Monascus* sp.

Monascus spp. belongs to the group of Ascomycetes and particularly to the family of Monascaceae. The genus *Monascus* can be divided into four species: *M. pilosus*, *M. purpureus*, *M. ruber* and *M. froridanus*, which account for the majority of strains isolated from traditional oriental food (Sabater-Vilar, Maas and Fink-Gremmels, 1999). The common names of this fungal product are red yeast rice, red rice, angkak, red leaven, beni-koji (Japanese), hung-chu, hong qu, zhitai (Chinese),

rotschimmelreis (Europe) and red mould (USA). *M. purpureus* can be easily distinguished by its ascospores which appeared to be spherical in shape of 5 microns in diameter or slightly ovoid (6 x 5 microns). The mycelium is white in the early stage, however, it rapidly changes to a rich pink and subsequently to a distinctive yellow-orange color. The production of yellow-orange hyphae reflects the increased acidity of the medium. A deep crimson color is formed as the culture ages. Most of products could be used in powder form or pigment extracts for developing the color of products. It is difficult to determine the growth of *Monascus* by counting mycelia using electron microscope while an easier method could be performed using glucosamine analysis. This compound is a monomer of chitin, which is the main component in fungal cell wall and the growth of *Monascus* fungi is also regarded as a key indicator in the synthesis of pigments and other metabolites (Vignon, et al.,1986).

Life cycle of *Monascus*

Monascus exists widely in soil, starch, fresh graze, grain, rubber, dried fish, and surface sediments of river and root organ of pine tree (Iizuka and Lin, 1981). *Monascus* is homothallic and capable of sexual reproduction. The steps involved as follows;

1. Antheridia form an extended tube type multi-core cell.
2. While forming antheridia, ascogonium appear at the bottom of antheridia from hyphae cells.
3. The upper and lower part of ascogonium separate and form trichogyne.
4. After the antheridia and trichogyne fuse together, the core of antheridia enters trichogyne, and at the meantime, the cores existed in trichogyne disappear before the antheridia core enters.
5. Empty anteridia starts to wither and the core in trichogyne moves to ascogonium through small pores.
6. Ascogonium enlarged and the cores are in pairs and starts to form 11 ascogenous hyphae and generate small number of ascus.
7. Under sexual organ, there are peridial wall cells generates and uncovered ascogenous hyphae in one or two layers and formed a totally enclosed ascus. At this time, the ascus membrane and ascogenous hyphae are melted and disappeared. The

isolated ascospore deposit in ascogonium, and are released finally through the regeneration of peridial wall cells and starts their new life cycle. The life cycle of *Monascus anka* is shown in Figure 1.

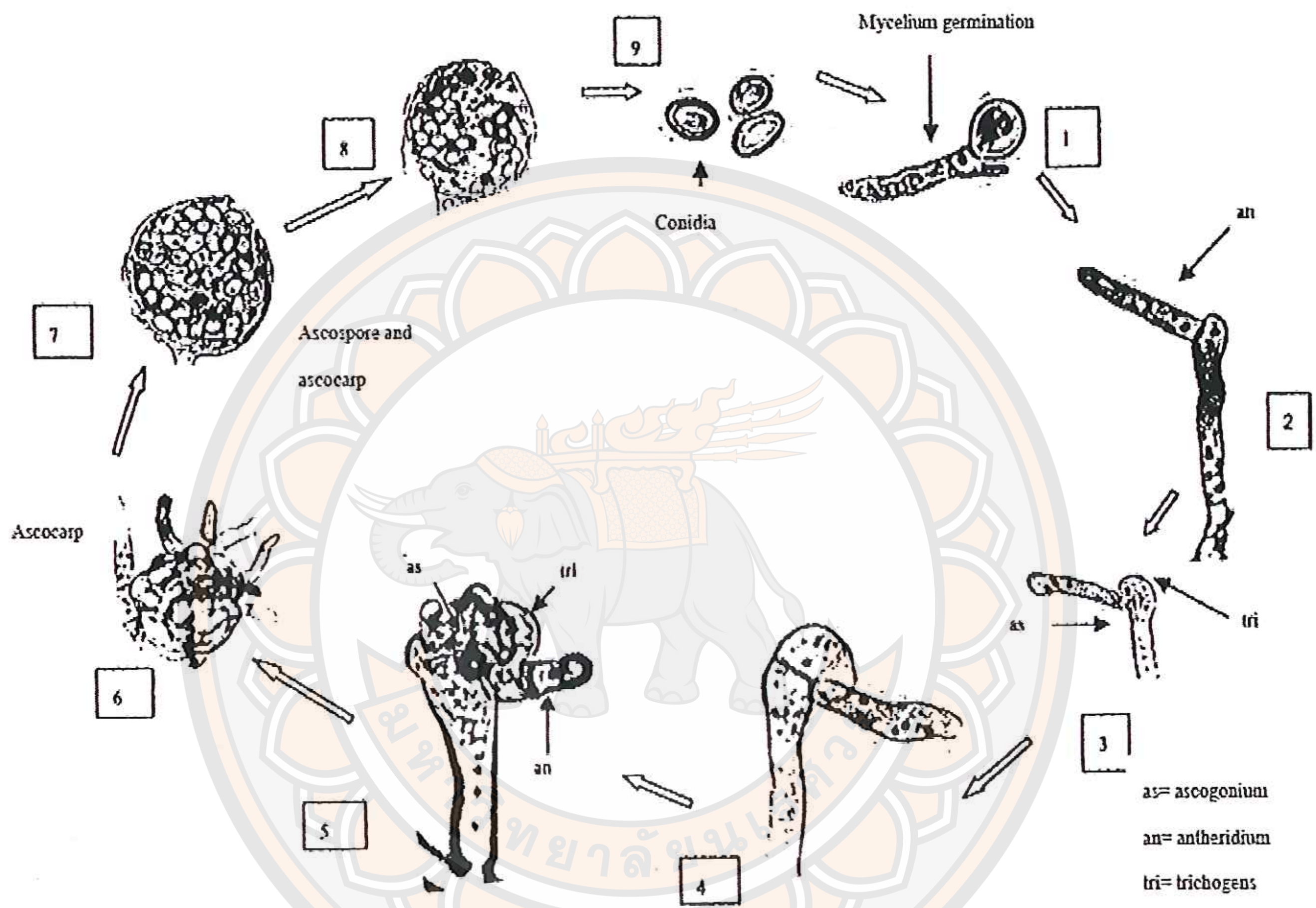


Figure 1 Life cycle of *Monascus* sp.

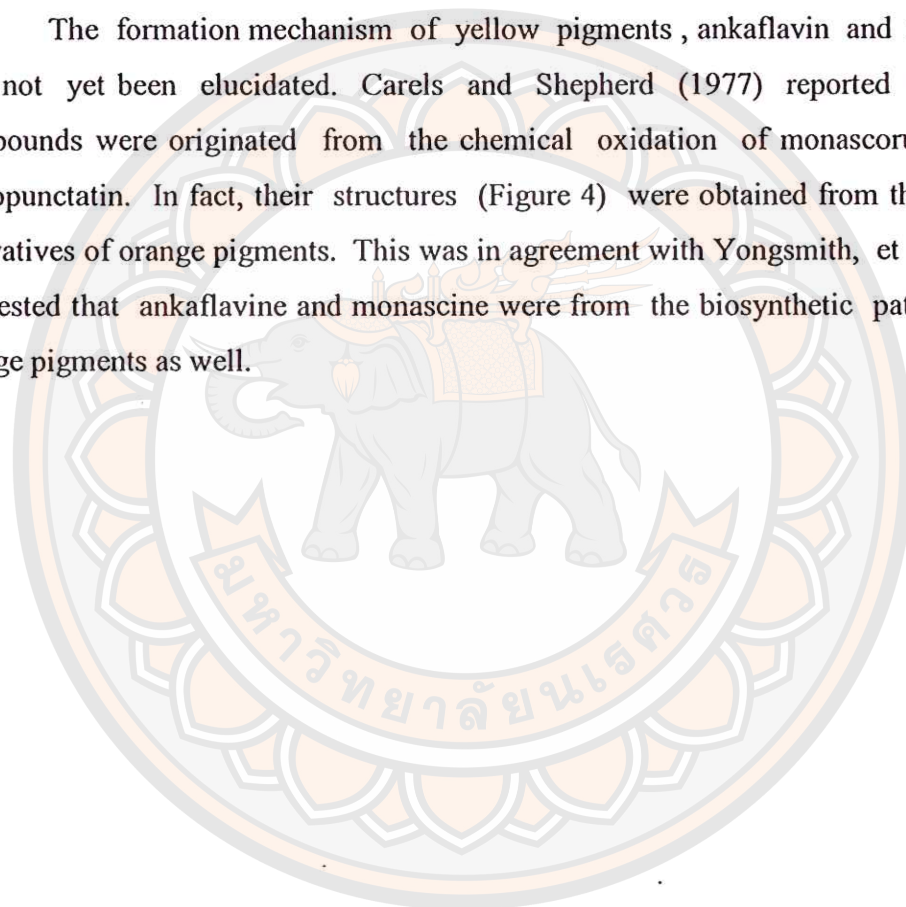
Note: an: antheridia, p: peridial wall cells, ag: ascogonium, a: ascus, tg: trichogyne, as: ascospore, ah: ascogenous hyphae, c: conidia, 1 and 2: ascospore forms vegetative hyphas, 3 to 7: Formation of reproductive organ and development of ascogenous hyphae, 8 and 9: matured ascogonium.

Source: Iizuka and Lin, 1981

Pigment synthesis

The orange pigments, monascorubrin and rubropunctatin (Figure 2), are synthesized in the cytosol from acetyl coenzyme A by the multienzyme complex of polyketide synthase I (Hopwood and Sherman, 1990). These pigments react with primary amino groups (so called aminophiles) (Figure 3), the complexes are changed to red pigments, monascorubramine and rubropunctamine yield the water-soluble (Blanc, et al., 1994).

The formation mechanism of yellow pigments , ankaflavin and monascin, has not yet been elucidated. Carels and Shepherd (1977) reported that these compounds were originated from the chemical oxidation of monascorubrin and rubropunctatin. In fact, their structures (Figure 4) were obtained from the reduced derivatives of orange pigments. This was in agreement with Yongsmith, et al. (1993) suggested that ankaflavine and monascine were from the biosynthetic pathways of orange pigments as well.



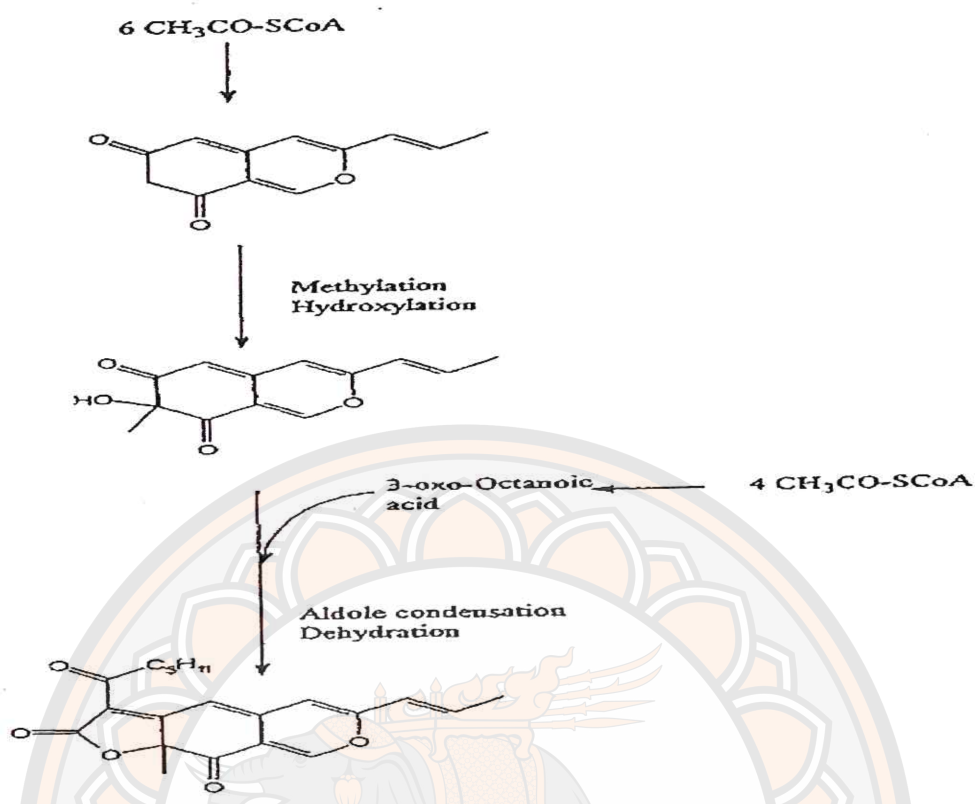


Figure 2 Probable mechanisms of the biosynthesis of rubropunctatin

Source: Hawksworth and Pitt, 1983

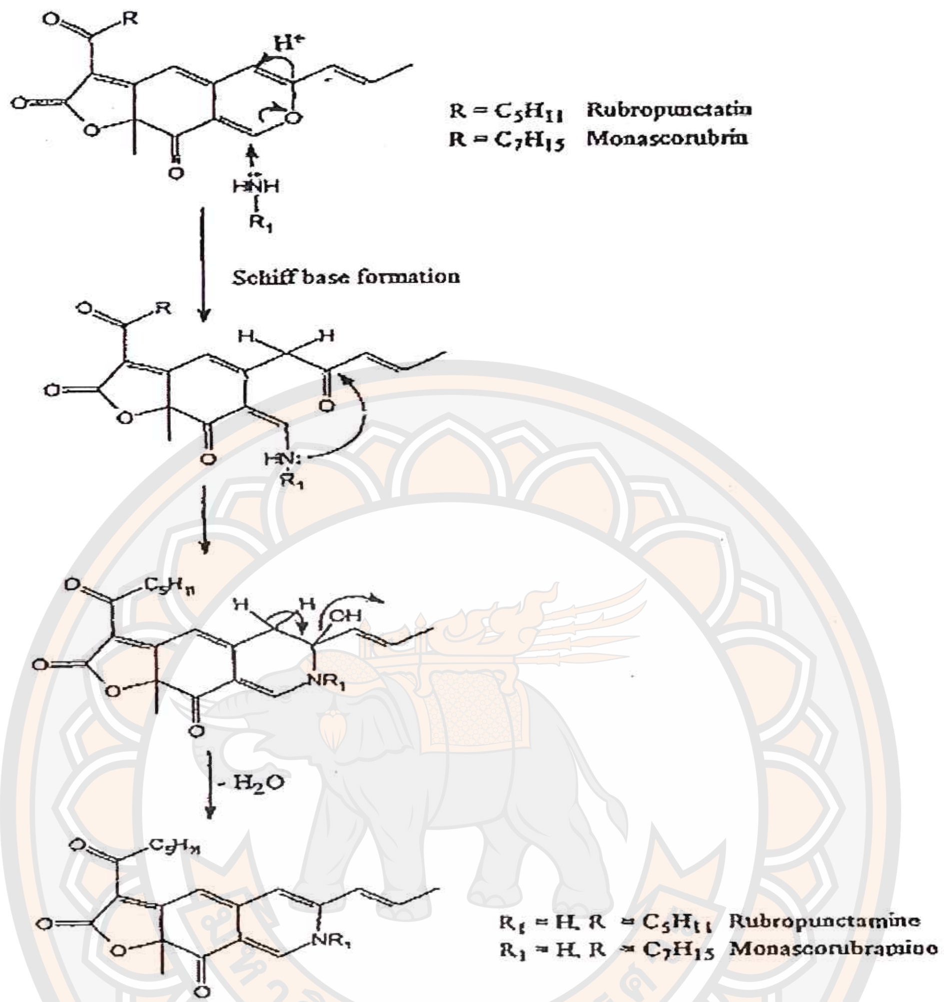
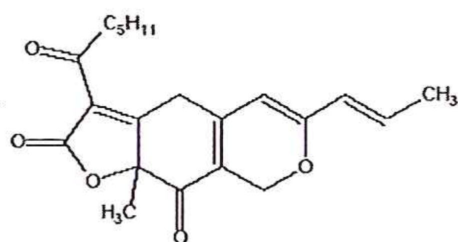
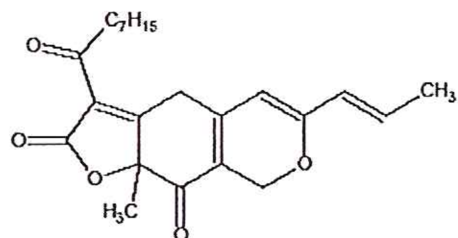


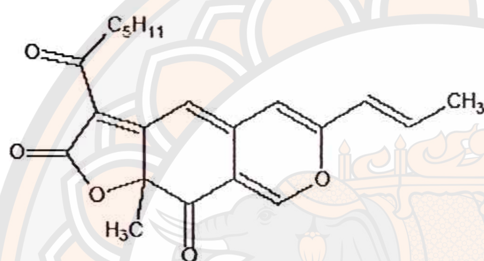
Figure 3 Formation of red pigments

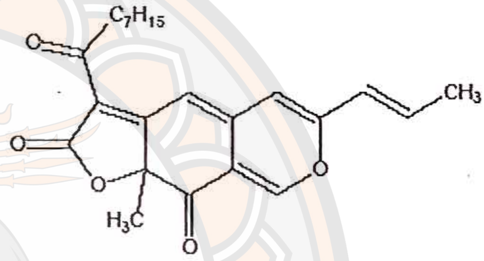
Source: Lin, et al., 1992



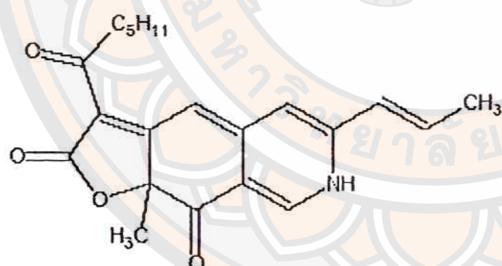


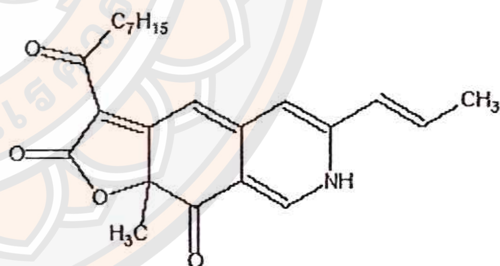
Yellow pigment structure





Orange pigment structure





Red pigment structure

Figure 4 Pigment structures from the isolated *Monascus* spp.

Source: Dufosse, et al., 2005

Secondary metabolites from *Monascus* sp.

During fermentation of *Monascus*, it can produce various enzymes such as amylase, acid protease, glucoamylase, maltase, pectinase, α-galactosidase and ribonuclease, etc, which are used as food enzymes (Lin and Lizuka, 1982). These enzymes can hydrolyze reducing sugars and hydrolyzed sugars are then changed into secondary metabolites, e.g., significant pigments and antioxidants. The step of secondary metabolites during the fermentation are as follows: 1. primary metabolic products, i.e., succinic acid, citric acid, gluconic acid, oxalic acid, ethanol alcohol, acid and ester compounds are produced during lag and log phases of *Monascus* fermentation (Kao, 2004); 2. secondary metabolic products, significant antioxidants and *Monascus* pigments are produced after primary metabolic products during stationary phase of *Monascus* fermetation (Kao, 2004).

Yongsmith (1997) explained that *Monascus* utilized carbon and nitrogen sources from the substrate during lag and log phases of the fermentation to produce primary metabolites, i.e., energy, CO_2 and water. Then, stationary phase of the fermentation, the products produced from former phases were changed to the secondary metabolites, such as pigment, citrinin and mevinolin. Therefore, the metabolites should be detected at stationary phase because the metabolites contents were relatively stable.

Monacolins, its related compounds and citrinin

The polyketide mevinolin (also referred to as lovastatin, monacolin K, mevacor, MB 530B, MK 803 or MSD 803) was produced not only by members of the genus *Monascus* but also by other filamentous fungi including *Aspergillus terreus*, some species of *Penicillium* (Alberts, 1988), *Hypomyces*, *Doratomyces*, *Phoma*, *Eupenicillium*, *Gymnoascus*, *Trichoderma* (Endo, et al., 1986) and *Pleurotus ostreatus* (Gunde-Cimerman, Plemenitas and Cimerman, 1993).

6-Demethylmevinolin (also referred to as compactin, such as ML-236B, CS 500, and mevastatin) was isolated from *Penicillium citrinum* and *P. brevicompacturn* by Endo and his colleagues at the Beecham Laboratories in 1976 (Alberts, 1988). Endo (1979) was the first scientist who reported mevinolin isolated from *M. ruber* and Alberts, et al. (1980) then found it from *Aspergillus terreus*.

The biosynthesis of mevinolin was detected in 17 strains from 124 *Monascus* strains (Negishi, et al., 1986). According to the new taxonomy, the active strains were *M. ruber*, *M. purpureus*, *M. pilosus*, *M. vitreus, M. pubigerus, M. vitreus*, *M. pubigerus* and *M. ruber* (Hawksworth and Pitt, 1983). All mevinolin-producing strains were inferior formation of red pigments.

The analysis of its biosynthesis was using *Aspergillus terreus* fungus (Chan, et al., 1983). The produced mevinolin (Figure 5) from this fungus contains two polyketide chains, C_{18} and C_4 synthesized from acetate units coupled to each other in head-to-tail fashion. The C_{18}-chain is cyclized while bound to the polyketide synthase or immediately after dissociation from the enzyme, oxidized at the 8-carbon atom and esterified by the side chain. The 6 α-methyl group and the methyl group on the side chain are derived from methionine. The methylations are sequential, the first one, on the 6α-carbon atom, occurs before the closure of the rings. The methylation from L-methionine is typical of the fungal metabolism whereas propionate incorporation is generally used by actinomyces. Fatty acids with three and more carbon atoms are not incorporated into mevinolin. The oxygen atoms on the main chain are introduced successively on a deoxygenated precursor.

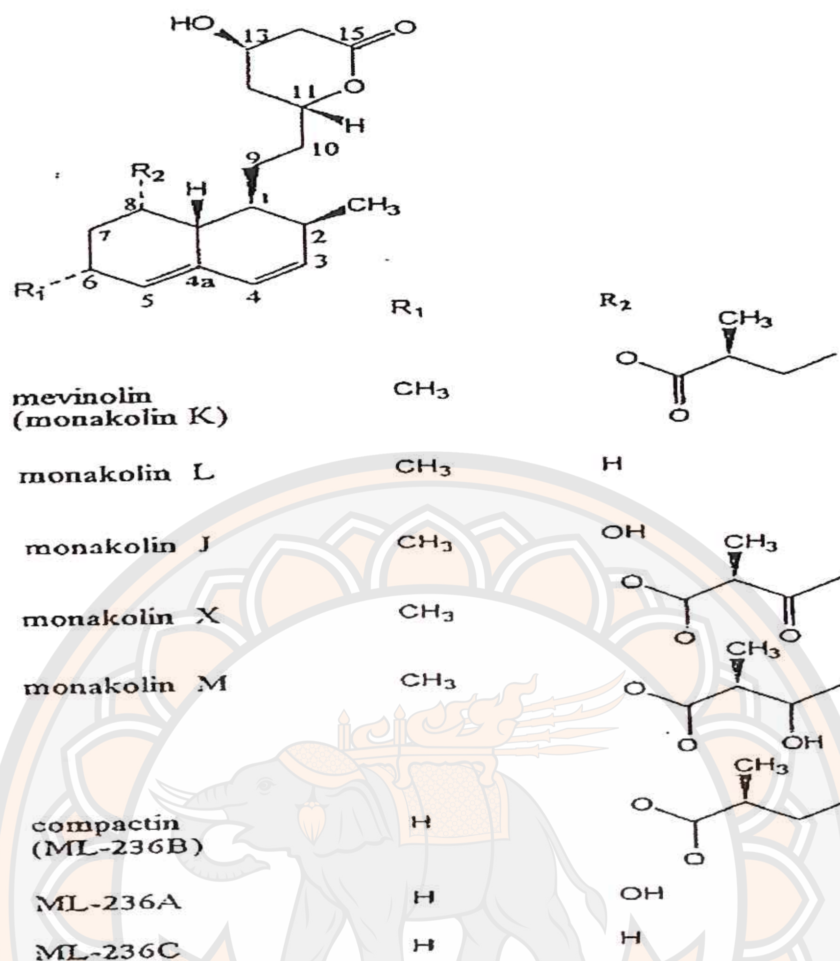


Figure 5 Structure of monacolin K , compactin and related compounds from *Monascus* sp.

Source: Juzlova, Martinkova and Kren, 1996

The biosynthesis of compactin or mevinolin by *Penicillium citrinum* and *Monascus ruber* proceeds in a similar way, i.e. the incorporation of acetate and methionine was observed, but not that of propionate. The enzymatic hydroxylation and subsequent esterification at the 8-carbon atom was also observed (Endo, 1985).

Mevinolin is produced as a mixture of a lactone and a free hydroxy acid (Alberts, et al., 1980). Mevinolin-related compounds (Figure 5) vary in composition of the C_4 side chain (monacolins J Endo, Hasumi and Negishi, 1985)

X (Endo, et al., 1985) and M (Endo, Komagata and Shimada, 1986) or lack this chain (monacolin L (Endo, Hasumi and Negishi, 1985) dihydromonacolin L (Endo, Hasumi and Negishi, 1985) and compactin derivative ML-236C (Endo, Kuroda and Tsujita, 1976). Growth experiments with *M. ruber* using 14C-labeled monacolin J or L suggested that both compounds are precursors of monacolin K (Endo, Hasumi and Negishi, 1985). The results of Komagata, et al. (1989) indicated that monacolin L is the precursor of monacolin J, which, in turn, can be converted to monacolin K (Kimura, et al., 1990), and that a monooxygenase is involved in this reaction.

Citrinin (Figure 6) was initially named as monascidin A and was regarded as an antibacterial component in the crude extract of *Monascus*. Monascidin A was then confirmed to be the same compound as citrinin (Blanc, et al., 1995).

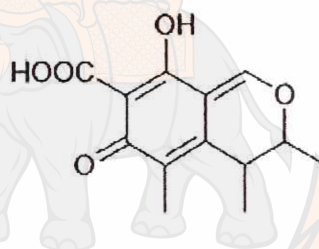


Figure 6 Chemical structure of citrinin from *Monascus* sp.

Source: Juzlova, Martinkova and Kren, 1996

Citrinin was found to be a hepatotoxic and nephrotoxic ingredient in *Monascus*-fermented rice (MFR). It adversely affected the function and ultrastructure of kidney in the canine model (Krejci, Bretz, and Koechel, 1996).

It also has negative effects on liver function and metabolism. A decrease in liver glycogen content and an increase in serum glucose were observed (Chagas, et al., 1992). Although the detailed molecular mechanism of the toxicity of citrinin is not well known, it has been demonstrated that citrinin mainly affects on mitochondria in cells. Citrinin permeated into the mitochondria, alters Ca^{2+} homeostasis (Chagas, et al., 1995), and interfered the electron transport system (Ribeiro, et al., 1997).

It is not a mutagen itself; however, if it is transformed by hepatocytes, it becomes mutagenic to NIH-3T3 cells. The content of citrinin dominated the mutagenicity of *Monascus* fermentation products in a dosage-dependent manner. Only the samples with higher citrinin content showed positive response in the *Salmonella*–hepatocyte assay. Citrinin has also been reported as a teratogenic agent in chicken embryos (Ciegler, Vesonder and Jackson, 1977). Although MFR can be purchased in the market without any restrictions, its adverse ingredient citrinin is still a concern. Therefore, efforts have been made to decrease the content of citrinin.

It is synthesized through the polyketide pathway which many secondary metabolites are synthesized, especially pigments. However, the synthesis of pigments and citrinin were not necessarily correlated (Wang, Ju and Zhou, 2005). Some of the *Monascus* strains produced pigments without citrinin (Pisareva, Savov and Kujumdzieva, 2005). However, the production of monacolin K without the existence of citrinin is not possible yet. Until now, citrinin can be only screened for the most suitable strain and maximize the parameters in production, to reduce the citrinin content to pass the statutory threshold. Currently, the statutory limit in the world is only legislated by the Japanese government.

The presence of citrinin should be lower than 0.2µg/g (200 ppb) of the *Monascus* pigments as used in food additives (Wang, Ju and Zhou, 2005). Most molecules decompose in a high-temperature environment, including citrinin, which decomposed and lost its cytotoxicity to HeLa cells after treated with 175°C dry air. Increase in moisture lowered the temperature required to deactivate the cytotoxicity of citrinin. In a moist environment (200µg citrinin/150µgH_2O), the deactivation temperature was lowered to 160 - 175°C (Kitabatake, Trivedi and Doi, 1991). Citrinin H_2, which is less toxic than citrinin, is considered the major product of citrinin decomposition (Hirota, et al., 2002). However, citrinin H_1, another identified product of citrinin pyrolysis, also formed and is tenfold more toxic than citrinin (on a weight basis; Bentrivedi, et al.,1993).

Mechanism of action and antioxidants activity of monacolin K and some substances from *Monascus* spp.

Monacolin K is a hypocholesteremic agent. Monacolin K has two different structures, β-hydroxy acid and lactone forms, which the ratio of the acid form to the lactone form depends on *Monascus* strains, pH, culture media, temperature and initial moisture content. Figure 7 elucidates that monacolin K competitively inhibits HMG-Co A reductase, as the first committed enzyme of the HMG-Co A reductase pathway. Therefore, the production rate of mevalonate is reduced, the next molecule in the cascade that eventually produces cholesterol, as well as a number of other compounds. By inhibiting HMG-Co A reductase, monacolin K blocks the pathway for synthesizing cholesterol in the liver. This is significant because most circulating cholesterol comes from internal manufacture rather than the diet. When the liver can no longer produce cholesterol, blood levels will fall. Cholesterol synthesis appears to occur mostly at night, so monacolin K with short half-lives are usually taken at night to maximize their effect. Studies have shown greater LDL and total cholesterol reductions in the short-acting simvastatin taken at night rather than the morning (Saito, et al., 1991; Wallace, Chinn, and Rubin, 2003), but have shown no difference in the long-acting atorvastatin. The other reports is reported responding to hyprocholester-olemic, liver-protective and antitumor effect (Endo, 1980).

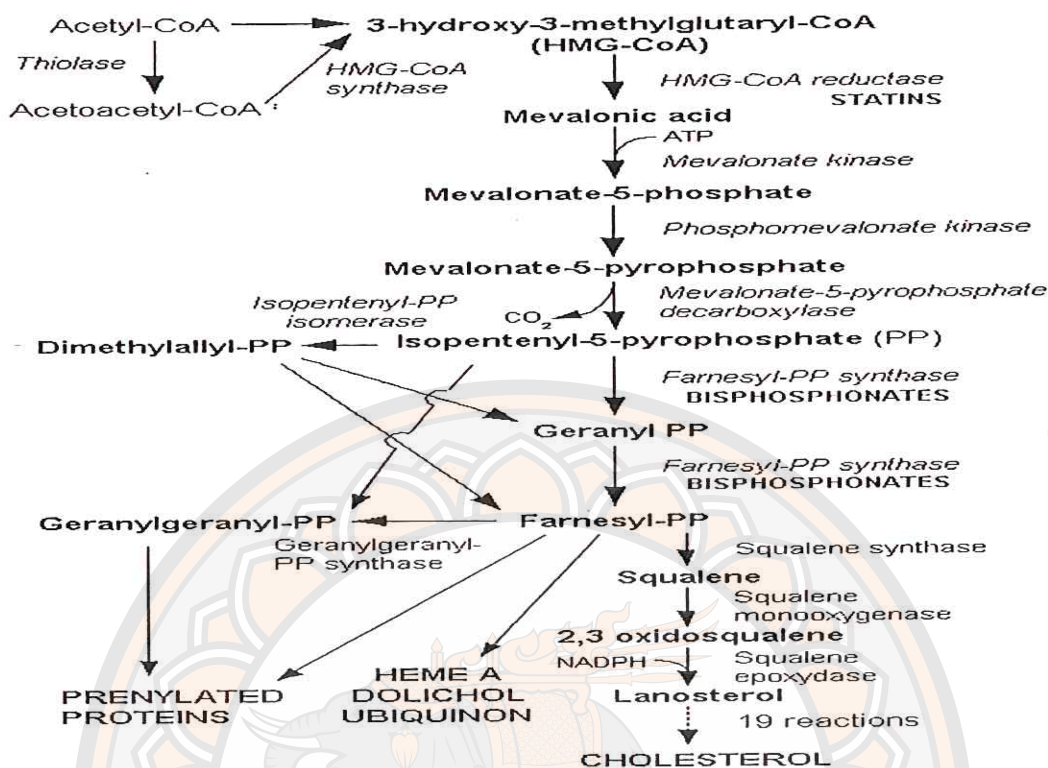


Figure 7 HMG-CoA reductase pathway is blocked by monacolin K via inhibiting the rate limiting enzyme HMG-CoA reductase

Source: Juzlova, Martinkova and Kren, 1996

In vitro evaluation of *Monascus* pigment, Yang, et al. (2006) studied antioxidant activity of methanolic extracts from monascal rice products. The methanolic extracts from monascal products indicated the activities of inhibition of peroxidation, reducing power, scavenging ability on DPPH radicals and chelating ability on ferrous ions. These abilities were increased together with the increased levels of diverse antioxidant components (e.g., ascorbic acid, β-carotene, α-tocopherol). Lee, Yang, and Mau (2008) found that the pigment extract of *Monascus* fermented soybean was not only found those antioxidant activities but also the scavenging ability on hydroxyl radicals. It is possible that low-molecular-weight viscous substance (Mw < 100,000) components in soybean consisted of the high contents of many total phenolic compounds, which played a role in high scavenging

ability on hydroxyl radicals. The extract appeared low-molecule-weight components showed higher antioxidant activities than BHA.

Factors affecting *Monascus* growth and secondary metabolites for solid state fermentation

1. Strains of *Monascus* sp.

Yongsmith (1997) reported that Van Tieghem (1884) divided the pure culture of *Monascus* into two species, i.e., *M. mucoroides*, *M. ruber*. Then, Went (1895) found that *M. purpureus* grew on Angkak. In present, *Monascus* species are classified more than 20 species (Table 1). Individual groups of *Monascus* are classified as follows: 1. Morphology; 2. Physiology; 3. Enzymology (Bridge and Hawksworth, 1985). However, *M. pilosus, M. purpureus, M.ruber* and *M, floridanus* differ in both physiology and enzymology. *M. floridanus* showed trypsinase activity except other species but did not show valine arylamidase except other species (Bridge and Hawksworth, 1985). As well, Nishikawa, et al. (1988) found that alkaline protein level obtained from *Monascus* sp. were higher than acid protease. Nevertheless, there are only a few species of *Monascus* producing both enzymes (Table 2). Generally, *Monascus* pigments were detected by a spectrophotometer at 420 and 500 nm. Some stains, pigment intensity from *M. purpureus* or *M. anka* showed higher 500 nm and 420 nm. Whereas, pigment intensity from *M. barkeri* or *M. kaoliang* showed higher 500 nm and at 370 nm (Yongsmith, 1997).

Table 1 ***Monascus*** **species discovered in present day**

Monascus species				
M.albidus	*M.albus*	*M.anka*	*M.araneosus*	*M.barkeri*
M.bisporus	*M.floridanus*	*M.fuliginosus*	*M.kaoliang*	
M.major	*M.mucoroides*	*M.olei*		
M.paxii	*M.pilosus*	*M.pubigerus*	*Mpurpureus*	*M.ruber*
M.rubiginosus	*M.rubropunctatus*	*M.serorubercens*	*M.vini*	*M.vitreus*

Source: Lizuka and Lin, 1981

Table 2 The types of enzyme activity in four species of *Monascus* sp.

Enzyme Activity	*M. floridanus*	*M. pilosus*	*M. purpureus*	*M. ruber*
Valine arylamidase*	-	+	+	+
Cystine arylamidase*	-	-	+	-
Trypsinase*	+	-	-	-
α-galactosidase*	-	+	-	+
β-galactosidase*	-	+	-	-
α-glucasidase*	-	+	-	-
Polypectase pH 6.0	-	-	+	-
Cellulose hydrolysis	-	-	-	+

Note: *API ZYM strip tests with *Monascus* cultivated after 14 days

Source: Bridge and Hawksworth, 1985

Monascus purpureus, one of popular stains, is used in many experiments because of higher production of the extracellular pigments and the antioxidants (Babitha, Soccol and Pandey, 2006; Babitha, Soccol and Pandey, 2007; Kraboun, et al., 2013; Wang, Lu, and Chi, 1997; Yang, et al., 2004; Yang, et al., 2006). Pattanagul, et al. (2008) reported that monacolin K content (25.03 mg/kg) from the adlay fermentation by *M. purpureus* DMKU could produce higher than that by *M. ruber* (15.33 mg/kg); however, monacolin K production indicated the citrinin production. This was in disagreement with Wang, Ju, and Zhou (2005), who studied citrinin production from different *Monascus* species on YES medium, the citrinin contents (between 73 and 386 mg/L) from *M. purpureus* were higher than those from *M. ruber* (between 65 and 160 mg/L). Those studies indicate that *Monascus* species and substrates influence on monacolin K and citrinin productions.

2. Substrates

White rice has been the most popular for pigment production since it could induce to high pigment intensity and antioxidant activities in many experiments such as Yang, et al. (2006) and Vidyalakshmi, et al. (2009). However, a few researches

indicated that the pigment intensity and antioxidant concentration from monascal waxy rice were higher than those from monascal rice. Chairote, et al. (2007) reported that monascal waxy rice, using Thai glutinous rice variety *Oryza sativa* L. cv. RD 6, from fermentation for 2 weeks showed 45.04 AU/g substrate of pigment intensity but monascal rice only produced 4.51 AU/g substrate. Chairote, et al. (2008) further reported that the highest contents of compactin and monacolin K were 21.98 and 33.79 mg/g, respectively, by using Thai glutinous rice variety *Oryza sativa* L. cv. RD 6.

Monascus growth and pigment intensity in monascal cereal products were better than those in monascal rice when optimum condition was occurred, i.e., optimum moisture content and final pH approaching neutral (Yongsmith, 1997). However, Tseng, et al. (2006) reported that the antioxidant activities of methanolic extracts from monascal adlay showed as same as those of monascal rice.

Carvalho, et al. (2007) suggested that the carbohydrate content of white rice is higher than that of other cereals whereas it is lower content of protein (Table 3). Presently, white rice may not be appropriate to be a substrate for *Monascus* fermentation in order to produce antioxidants and pigments because of high cost, inappropriate some compositions as well as hard application to industry scale.

Generally, waxy corn contains 2.06 % of amylose content less than rice, indicating just a little different to their contents but the contents of protein, lipid, ash and phosphorus are similar. However, it is cheaper than white rice (Jiranuntakul, et al., 2011). This information is an interesting because the difference in amylose content may affect produced metabolite content or antioxidant activity through *Monascus* fermentation.

Table 3 Biopigment production (AU/g dry substrate) and substrate compositions

Substrate	Approximate composition (g/kg dry basis)			Average specific absorbance (AU/g dry substrate)
	Carbohydrate	Protein	Phosphorus	
Rice	820	90	1.14	216
Wheat	770	140	3.63	79
Corn	780	130	3.19	60
Soy	330	400	6.00	13
Soy bran	400	480	7.00	22
TSP	-	-	-	12.60
Cassava	864	19	10.99	119.60
Cassava starch	900	20	3.30	38.50
Cassava flour	910	14	2.20	98.10
Cassava bagasse	660	11	-	15.70
Potato	800	100	9.60	4.70

Note: TSP = Textured Soy Protein, (-) indicates data not known

Source: Carvalho, et al., 2007

3. Nitrogen sources

Nitrogen sources has been used in *Monascus* fermentation in order to promote pigmentation and its yield which their sources appear both inorganic and organic substances. Inorganic sources, i.e., sodium nitrate supported the sporulation, limited growth and gave high pigment yield. Moreover, ammonium chloride resulted in a repression of conidiation and the sexual cycles and led to high pigment yield (Carels and Shepherd, 1977). Then, nitrogen organic sources are increasingly popular because they help to promote higher the pigmentation and the yield than inorganic one as the following study. Chen and Johns (1994) reported that peptone (organic source) used in *Monascus* fermentation promoted superior *Monascus* growth and pigment yield compared with sodium nitrate. Lin and Demain (1991) confirmed that MSG was the most favorable nitrogen source of *Monascus*.

Organic nitrogen sources such as MSG, peptone and yeast extract were often used as supplements. A previous study, the effect of yeast extract improved pigments yield in the broken rice fermentation compared with MSG and inorganic sources (Subhasree, et al., 2011). Dufosse, et al. (2005) further reported that MSG promoted the pigment production of *Monascus* in submerged culture. Vidyalakshmi, et al. (2009) reported both red and yellow pigments were higher pigment yield of 0.464 U/g and 1.314 U/g pigments, respectively when *Monascus* fermented rice was obtained from *Monascus* fermentation supplemented with MSG at 0.5 % concentration. Whereas, the medium supplemented with 20 – 22.5 g/l of peptone was optimal region of pigment production of *M. purpureus* with independent MSG level (Silveira, Daroit and Brandelli, 2008). For enhancement of monacolin K content, nitrogen sources from natural materials has been supplemented to the substrates in many researches. Chairote, et al. (2008) studied the addition of soybean milk to glutinous rice substrate, which indicating high productivity of monacolin K content. Kraboun, et al. (2013) further reported that the concentration of MSG and peptone equivalent to 1% nitrogen increased the pigmentation, growth and antioxidant activities as well. However, supplementation of inorganic nitrogen sources to *Monascus* substrate to produce monacolin K is less popular because of hard application to food products as residue of inorganic after the fermentation.

As well, the nitrogen source is a source which improves *Monascus* pigments solubility. The principal is the position of oxygen atom of monascorubrine or rubropunctatine pigment structure is replaced by nitrogen atom of amino group (amino acid, protein and peptide), so that this replacement indicate changes from yellow color to purple-red color. This color responded as the reduced and oxidized agents and also reaction with other substances, especially amino acid. This color binds with amino acid to complex form, Glutamyl-monascorubine and Glutamyl-rubropunctatine, as shown in Figure 8. The complex forms are isolated from submerge media (Dufosse, et al., 2005).

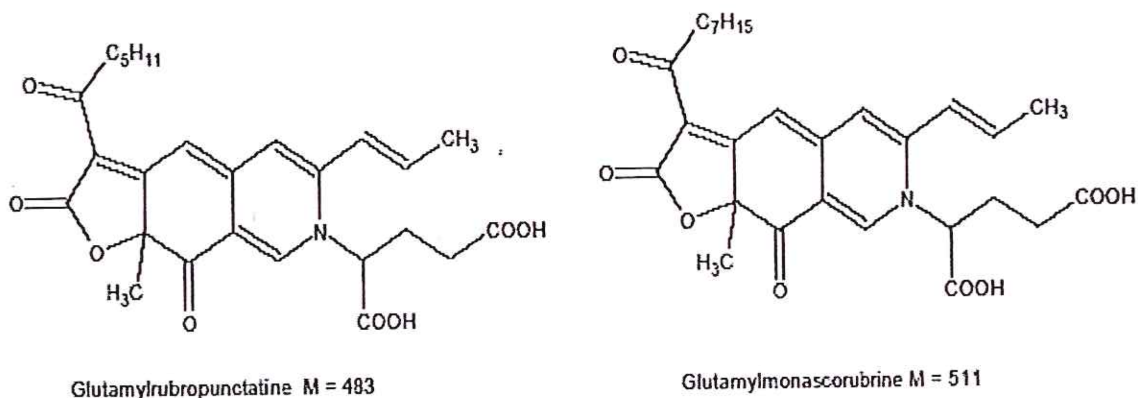


Figure 8 Glutamyl-rubropunctatine and Glutamyl-monascorubine

Source: Dufosse, et al., 2005

4. pHs

The optimum pH for *Monascus* pigment production is between 3.0 and 7.5 (Palo, Vidal-Adeva and Maceda, 1960; Yongsmith, 1997). This is in agreement with Babitha, Soccol and Pandey (2007) reported that the appropriate pH of substrates for *Monascus* pigment formation and its stability were in a wide range of pH 3.5 - 7.0.

5. Temperatures

Temperature is an important factor affecting metabolic activities and microbial growth (Babitha, Soccol and Pandey, 2007). Generally, the optimal temperature for *Monascus* pigment production is in the range of 27 - 37°C. To obtain high growth and glucoamylase production, proper temperature is between 35 and 37°C (Yongsmith, 1997). Babitha, Soccol, and Pandey (2007) further reported that the optimum pigment production is between 30 and 40 °C. Beyond 40°C, the pigment was decreased drastically. This study was similar to the finding of Ganrong, et al. (2000) reported that the optimum temperature for *Monascus* pigment was between 30 and 37°C. From those researches, the optimal temperature for *Monascus* pigmentation, growth and the involved enzyme is between 30 and 40°C.

6. Moisture contents

Palo, Vidal-Adeva, and Maceda (1960) reported that *Monascus* sp. could produce the highest pigment production when moisture content of the substrate was lower than 50 %. Whereas, Babitha, Soccol, and Pandey (2007) reported that 50 % of initial moisture content was optimum for pigment production of *Monascus* sp., beyond 50%, the pigment was decreased. Carrizales and Rodriguez (1981) noted that high initial moisture content in the substrate led to less pigment intensity because of reduced mass transfer (causing reduced solubility and low heat exchange), oxygen transfer and low availability of nutrients to the culture. For some strains of *M. purpureus*, *M. purpureus* TISTR 3080 favored initial moisture content of broken rice substrate between 30 and 31 % (Kraboun, et al., 2008).

Solid state fermentation (SSF) and 2-step fermentation

SSF is defined as any fermentation process performed on a non-soluble material that acts both as physical support and source of nutrients in absence of free flowing liquid (Pandey, 1992). SSF was used in many experiments to study optimization and secondary metabolites of the fermentation (Pandey, Soccol, and Mitchell, 2000). The advantages of SSF are simple technique, low cost, high productivity, good oxygen circulation, less processing downstream and low energy consumption. SSF offers numerous advantages for the production of significant chemicals and enzymes (Hesseltine, 1977). This process has been known since ancient times and leads to various food products (Table 4). Typical examples, the fermentation of rice by *Aspergillus oryzaeto* for the koji process and the cheese production from the fermentation of *Penicillium roquefortii*. In China, SSF has been extensively used to produce the brewed foods such as Chinese wine, soy sauce and vinegar since ancient time (Chen, 1992). In Japan, SSF has been commercially used to produce the industrial enzymes (Suryanarayan, 2003). From this standpoint, SSF should be applied to *Monascus* pigment production because of presented advantages. However, limitation of SSF is still presented primary metabolites after the fermentation, i.e., reducing sugars of monascal products are still appeared after the fermentation about 2,000 mg/kg substrate (Babitha, Soccol and Pandey, 2007). Hence, residual sugars

should be continuously utilized in the fermentation process by *Monascus* to enhance the pigment intensity and monacolin K content.

Two-step fermentation was then designed to enhance the effectiveness of the conventional fermentation and also used by many researches. For example, the first step, *Candida tropicalis* was inoculated onto sweet sorghum stalk under aerobic condition to study cell growth and protein content (PC) in the substrate, PC reached 26.3%. In the second-step, *Lactobacillus rhamnosus* was inoculated on the sterilized substrate harvested from the first step which was cultured under anaerobic condition. After *L. rhamnosus* fermented at 40°C for 80 h, the protein content in the product reached 35.7%. By the two-step fermentation, 200 tons of feed was produced from two tons of dry sweet sorghum stalk and PC in the product was 13.77%, higher than silage in sale. The two-step process, which was easy to operate, provided high protein feed from agricultural waste in a cost effective way and the optimization of fermentation conditions in the two-step fermentation provided theoretical guideline for plant-scale production (Hongzhang, Yumei and Shuhua, 2012).

Jin, et al. (2008) also reported that using a novel two-step fermentation process could enhance arachidonic acid (ARA) production by *Mortierella alpina* ME-1 in a 5 L fermentor. Agitation speed and aeration rate were adjusted from 180 to 40 rpm and from 0.6 to 1 vvm, respectively, after 5 days cultivation, to decrease physical damage to the mycelia and to extend the stationary phase. Moreover, 3% (w/v) and 2% (w/v) ethanol were fed after 5 and 7 days cultivation, respectively, to enhance ARA content of total lipid. Eventually, an ARA yield of 19.8 g/l was achieved, which was 1.7 times higher than that of a one-step fed-batch cultivation.

According to the previous 2-step fermentation to increase secondary metabolites, the experimental methods were different. The first experiment used 2 microorganisms for 2 steps of the fermentation while the second experiment, the second step of the fermentation was adjusted. This indicates that the design of 2-step fermentation of each experiment depends on substrates or appropriate conditions for important secondary metabolites. Therefore, definition of 2-step fermentation depends on designed experiment and appropriate condition to obtain the highest secondary metabolites.

Table 4 Main groups of microorganisms involved in SSF processes

Microflora	SSF process
Bacteria	
Bacillus sp.	Composting, natto, amylase
Pseudomonas sp.	Composting
Serratia sp.	Composting
Streptococcus sp.	Composting
Lactobacillus sp.	Ensiling, food
Clostridium sp.	Ensiling, food
Yeast	
Endomicopsis burtonii	Tape cassava, rice
Saccharomyces cerevisiae	Food, ethanol
Fungi	
Altemaria sp.	Composting
Aspergillus sp.	Composting, lignin degradation
Amylomyces rouxii	Tape cassava, rice
Aspergillus oryzae	Koji, food, citric acid
Rhizopus oligosporus	Tempeh, soybean, amylase, lipase, amylase
Aspergillus niger,	Feed, proteins citric acid
Pleurotus oestreatus , sajor-caju	Mushroom
Lentinus edodes	Shii-take mushroom
Penicilium notatum, roquefortii	Penicillin, cheese
Monascus sp.	Red koji

Source: Raimbault, 1998

Condition for *Monascus* pigment extraction and antioxidants

Extraction is an important tool for the separation of significant substances. According to the theory, the extraction removes the target compound from an impure matrix, the washing removes impurities from the target compound, i.e., water by extraction with saturated sodium chloride solution. Washing is also used as a step in the re-crystallization procedure to remove the impurity containing mother liquor adhering to the crystal surface. The optimum extraction condition leads to higher

contents of the target compounds. Many solvents have been used to extract monascal rice, e.g., methanol, hexane, chloroform, ethyl acetate and acetone. Methanol (CH_3OH) is better for the extraction of *Monascus* pigment from the cell, but it is a toxic solvent to humans. Table 5 indicates that methanol is the best solvent and DMSO and ethanol are followed. In present, ethanol (C_2H_5OH) is used often because of low price, non-harmful volatile, and non-toxic solvent. Hence, it was used in general experiments (Carvalho, et al., 2007). Physical properties of ethanol is reported in Table 6 (Howard, 1990; Merck, 1996).

Table 5 Specific absorbance of red pigments extracted using different solvents (solvents are tabulated in decreasing order of polarity)

Solvent	Relative absorbance (% of maximum extraction)
Water, (pH 1)	2.1
Water, (pH 3)	5.9
Water, (pH 7)	5.3
Water, (pH 11)	3.6
Water, (pH 12.3)	18.7
DMSO	94.9
Acetonitrile	67.2
Ethanol	91.6
Methanol	100.0
Ethyl ether	72.8
Hexane	7.9

Source: Carvalho, et al., 2007

Table 6 Physical properties of ethanol

Solvent	formula	Boiling point (°C)	Melting point (°C)	Density (g/mL)	Solubility in H_2O (g/100g)	Relative polarity	Eluant strength	Threshold limits(ppm)	Vapor pressure 20°C (hPa)
Ethanol	C_2H_6O	78.5	-114.1	0.789	M	0.654	0.88	100	59

Note: M = miscible

Source: Merck, 1996; Howard, 1990

Carvalho, et al. (2007) and He, Dandan and Xiong (2004) reported that effect of different temperatures in the range of 0 - 60°C on extraction of *Monascus* pigment showed no significant difference. Chunji, et al. (1997) further reported that using 70 – 80 % of ethanol promoted the pigment extraction rate reaching 90% (increased 5 times) when increased temperature affected an increase of the liquid concentration and low consumption of alcohol. However, the pigment has low water solubility, is sensitive to heat and fades with light. Therefore, temperature and solvent concentration should be appropriate for the extraction process.

Commonly, the antioxidant activities depend upon *Monascus* pigment yield obtained from the extraction. Yang, et al. (2006) reported that *Monascus* pigment yield indicated the antioxidant activities. Tseng, et al. (2006) further reported that the antioxidant activities such as scavenging abilities on DPPH radicals, chelating abilities on Fe^{2+} of *Monascus* pigment extracts increased with the pigment yield.

Theoretical aspects of ultrasound

During the past several years, ultrasound has been effectively applied as an emerging advanced oxidation process (AOP) for a wide variety of pollutants in wastewater treatment. It is proven to be an effective method for degrading organic effluents into less toxic compounds and able to mineralize the compounds completely in certain cases (Guo, et al., 2010). The ultrasound process does not require addition of oxidants or catalyst, and does not generate additional waste streams as compared to

adsorption or ozonation processes. Ultrasound process is also not affected by the toxicity and low biodegradability of compounds (Fu, et al., 2007). Besides, ultrasonic degradation is claimed to be a non-random process, with cleavage taking place roughly at the center of the molecule and with degrading rate faster with larger molecule (Gronroos, Pentti and Hanna, 2008).

Ultrasonic waves (occurs at frequencies above 20 kHz) are a branch of sound waves and it exhibits all the characteristics properties of sound waves. Basically, they are classified into four different categories (namely, longitudinal/compressional waves, transverse/shear waves, surface/Rayleight waves, and plate/Lamb waves) based on the mode of vibration of the particle in the medium, with respect to the direction of the propagation of the initial waves (Raj, Rajendran and Palanichamy, 2004). Depending on the frequency, ultrasound is divided into three categories, namely power ultrasound (20–100 kHz), high frequency ultrasound (100 kHz–1 MHz), and diagnostic ultrasound (1–500 MHz). Ultrasound ranging from 20 to 100 kHz is used in chemically important systems, in which chemical and physical changes are desired as it has the ability to cause cavitations of bubbles (Pilli, et al., 2011). Ultrasound ranging from 1 to 10 MHz is used for animal navigation and communication, detection of cracks or flaws in solids, and under water echo location, as well as diagnostic purposes (as shown in Figure 9) (Pilli, et al., 2011).

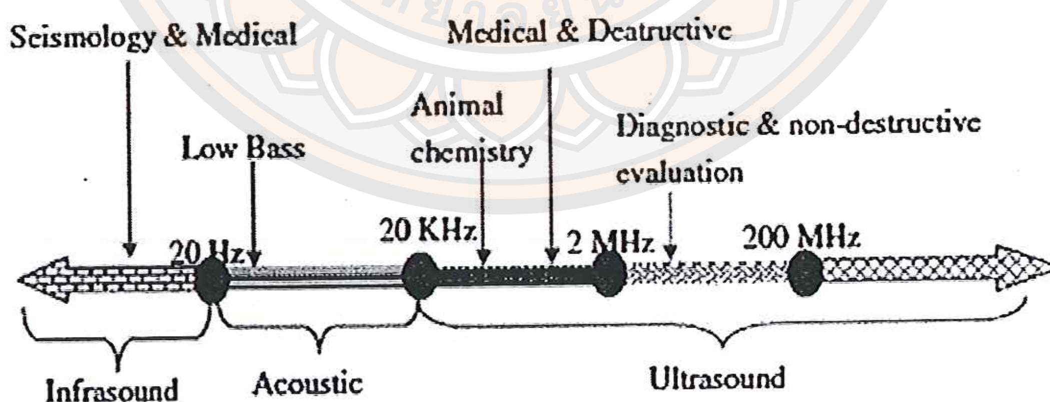


Figure 9 Diagram of ultrasound range

Source: Pilli, et al., 2011

When applied on liquid, ultrasound waves consist of a cyclic succession of expansion (rarefaction) and compression phases imparted by mechanical vibration (Tang, 2003). Compression cycles exert a positive pressure and push the liquid molecules together, while expansion cycles exert a negative pressure and pull the molecules apart (Vajnhandl and Marechal, 2005). When pressure amplitude exceeds the tensile strength of liquid in the rarefaction regions, small vapor-filled voids called cavitation bubbles are formed (Chen, 2012). Generally, pure liquids possess great tensile strengths and thus, available ultrasonic generators are unable to produce high enough negative pressures to cause cavitation. However, most of the liquids are usually impure and its tensile strength is reduced due to the presence of numerous small particles, pre-existing dissolved solids, and other contaminants. The impurities in liquid represent weak points in a liquid where nucleation of cavitation bubbles will occur (Vajnhandl and Marechal, 2005). For instance, when pure water is used, more than 1,000 atm of negative pressure would be required for cavitation whereas for tap water, only a few atmosphere of pressure would be sufficient to form bubbles (Chowdhury and Viraraghavan, 2009).

Once a bubble is created, two different cavitation phenomena which could take place in the liquid are: stable or transient cavitation. In stable cavitation, bubble wall couples with the acoustical field and oscillates about the equilibrium radius for several cycles. This occurs at low acoustic intensities, where the size of the bubble oscillates in phase with expansion and compression cycles and the bubbles grow slowly over many acoustical cycles (Thangavadivel, et al., 2012). Due to its small variation in bubble size changes, this process is of little significance in terms of chemical effects (Destaillats, Hoffmann and Wallace, 2003). The process is also called rectified diffusion as during expansion, water vapor, dissolved gases and organic vapor will enter the bubble and will leave during contraction because of the effect of bubble surface area (Thangavadivel, et al., 2012). When high intensity acoustic field is introduced, transient cavitation usually occurs. This causes growing cavitation bubble to eventually become unstable after a number of cycle and collapse during the compression cycle of ultrasonic wave. In this cavitation phenomena, the size of a bubble drastically increase from tens to hundreds of times the equilibrium radius before it collapses violently in less than a microsecond (Destaillats, Hoffmann and

Wallace, 2003; Vajnhandl and Marechal, 2005). Nevertheless, the classification of cavitation is vague as stable cavitation could lead to transient cavitation or transient cavitation could produce very small bubbles that undergo stable cavitation (Vajnhandl and Marechal, 2005). In summary, phenomenon of cavitation consists of the repetition of three distinct steps: formation (nucleation), rapid growth (expansion) during the cycles until it reaches a critical size, and violent collapse in the liquid as shown in Figure 10 (Pang, Abdullah and Bhatia, 2011).

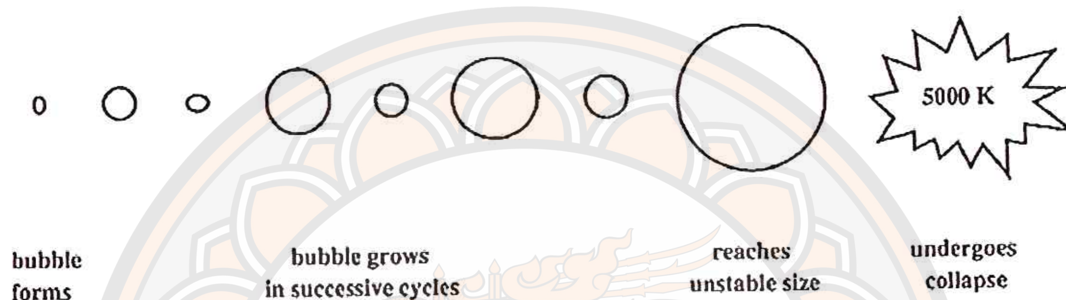


Figure 10 Cavitation bubble formation, growth and collapse.

Source: Pang, Abdullah and Bhatia, 2011

The produced cavitation serves as a mean to concentrate the diffused sound energy. Either in low or high intensity acoustic field, once a cavity bubble experienced rapid growth and could no longer absorb the energy efficiently, the liquid will rush in and the cavity will eventually implode (Suslick, 1990). Upon collapsing, each of the bubble would act as a hotspot, generating energy to increase the temperature and pressure up to 5,000 K and 500 atm, respectively, and cooling rate as fast as 10^9 K/s (Suslick, 1990). The formation and growth of the cavitation bubbles is shown in Figure 10. These collapsing bubbles create an unusual mechanism for high-energy chemical reactions due to enormous local temperatures and pressure (Suslick, 1990).

Ultrasonic-assisted extraction (UAE) and stimulation of bioactive compounds by ultrasonic wave

During the last decade, UAE has been widely used in food industry (Patist and Bates, 2008) since UAE could enhance yield of plant materials with short-term extraction (Toma, et al., 2001). Application of UAE for *Monascus* pigments extraction has also been studied. Li, Peizheng and Aidong (2009) reported the effect of extraction with different ultrasonic frequency and power on red pigment. The results showed that the factors affected the extraction of red pigment were in the following order: frequency, ultrasonic time, power and liquid-solid ratio whereas the order of ultrasonic factors influencing the color tone was liquid-solid ratio, frequency, ultrasonic time and power, respectively. The optimal extraction parameters for both the extraction of red pigment and color tone were as follows: dual-frequency (45, 28 kHz) alternately each 3 min, power 120 W, liquid-solid ratio 1.5 :1 (v:w), ultrasonic time 30 min, and under such conditions, extraction ratio could reach as high as 92.84%. Using ultrasonic wave with intermittent stimulation for the production of *Monascus* pigment and monacolin K during the fermentation has been reported, it was found that *Monascus* pigment and monacolin K content was increased 29.74 % and 39.96%, respectively, compared with the strain without the treatment. Those results were obtained from the optimal condition as follows: frequency of 20 KHz; power of 200 W; ultrasonic wave for 2 min every 6 h a day (Yang, et al., 2005). Furthermore, ultrasonic wave (45 kHz of frequency for 2 min at 28°C) could induce the mutation of extracellular pigment at the basic pH values (>7.0-13.0) (Wongjewboot, Bowdang and Kongruang, 2012).

UAE has been studied in other researches and could significantly improve juice extraction since it could adjust physicochemical properties of pineapple juice before the pectolytic treatment. The obtained yield was 4.5% higher than that of the control sample, which sonication time and a power were 60 sec and 225 W, respectively (Tran and Lee, 2011).

Obviously, the ultrasonic method indicated a lot of advantages such as the increased yield of *Monascus* pigment and monacolin K, improvement of some properties in the materials and short-term extraction.

Theory of response surface methodology (RSM) and central composite design (CCD)

1. Response surface methodology (RSM)

Response surface methodology (RSM) is a collection of mathematical and statistical techniques for empirical model building. By careful design of experiments, the objective is to optimize a response (output variable) which is influenced by several independent variables (input variables). An experiment is a series of tests, called runs, in which changes are made in the input variables in order to identify the reasons for changes in the output response (Montgomery, 1997).

The response can be represented graphically, either in the three-dimensional space or as contour plots that help visualize the shape of the response surface. Contours are curves of constant response drawn in the x_i, x_j plane keeping all other variables fixed. Each contour corresponds to a particular height of the response surface (Montgomery, 1997).

RSM consists of a group of mathematical and statistical techniques that can be used to define the relationships between the response and the independent variables. RSM defines the effect of the independent variables, alone or in combination, on the processes. In addition to analyze the effects of the independent variables, this experimental methodology also generates a mathematical model. The graphical perspective of the mathematical model has led to the term Response Surface Methodology (Bas and Boyaci, 2007). The relationship between the response and the input is given in Equation (1):

$$\eta = f(x1, X2, \ldots\ldots\ldots, Xn) + \varepsilon \qquad (1)$$

where η is the response, f is the unknown function of response, $x_1, x_2, \ldots, x_n$ denote the independent variables, also called natural variables, n is the number of the independent variables and finally ε is the statistical error that represents other sources of variability not accounted for by f. These sources include the effects such as the measurement error. It is generally assumed that e has a normal distribution with mean zero and variance.

It is possible to separate an optimization study using RSM into three stages. The first stage is the preliminary work in which the determination of the independent parameters and their levels are carried out. The second stage is the

selection of the experimental design and the prediction and verification of the model equation. The last one is obtaining the response surface plot and contour plot of the response as a function of the independent parameters and determination of optimum points (Bas and Boyaci, 2007).

2. Central composite design (CCD)

A central composite design contains an imbedded factorial or fractional factorial design with center points that is augmented with a group of 'star points' that allow estimation of curvature. If the distance from the center of the design space to a factorial point is ±1 unit for each factor, the distance from the center of the design space to a star point is $\pm \alpha$ with $| \alpha | > 1$. The precise value of α depends on certain properties desired for the design and on the number of factors involved. The number of center point runs the design is to contain also depends on certain properties required for the design (Montgomery, 1997).

A second order polynomial, Equation (2), which included all interaction terms, was used to calculate the predicted response.

$$Y= \beta_0 +\sum \beta_i X_i +\sum \beta_{ii} Xi^2 +\sum \beta_{ij} X_i X_j \quad (2)$$

Where, Y represents response variable; β_0 is the interception coefficient; β_i is the coefficient of the linear effect; β_{ii} is the coefficient of quadratic effect and β_{ij} is the coefficient of interaction effect; Xi and Xj denote the coded levels of variable *Xi* and Xj investigated in the experiment. The variable Xi is coded as Xi according to the Equation (3)

$$x_i = \frac{X_i - X_0}{\Delta X} \quad (3)$$

Where, X_i is coded value of the variable X_i, X_0 is the real value of X_i at the center point (zero) level, and the X_i is the step change value.

To maintain rotatability, the value of α depends on the number of experimental runs in the factorial portion of the central composite design (Montgomery, 1997):

$$\alpha = [\text{number of factorial runs}]^{1/4}$$

If the factorial is a full factorial, then

$$\alpha = \left[2^k\right]^{1/4}$$

Use of CCD and RSM for production optimization of *Monascus* pigment and antioxidants

The existing one-variable-at-a-time technique is a method changing one parameter at a time in the practice while keeping the others at a constant level. This classical optimization method is not only time-consuming but also inefficient in depicting the complete effects of the parameters in the process and in considering the combined interactions between physico-chemical parameters. RSM is very useful to test multiple process variables because fewer experimental trials are needed compared to the study of one variable at a time. In addition, interactions between variables could be identified and quantified by such a technique. CCD was conducted in the optimum vicinity to locate the true optimum values of the multiple variables. In present, RSM and CCD are increasingly used for optimization in a number of the fermentation experiments (Chang, Lee and Pan, 2006).

A few studies on the production optimization of monacolin K and *Monascus* pigment were reported. Pigment production by *Monascus purpureus* in submerged fermentation using grape waste as a growth substrate was optimized by CCD and RSM techniques (Silveira, Daroit and Brandelli, 2008). RSM was employed to study the optimization of culture medium for monacolin K production in mixed solid-liquid state (or submerged) culture by *Monascus ruber* (Chang, et al., 2002). However, use of CCD and RSM for the extraction optimization of monacolin K has been never studied.

***In vitro* digestion models**

In vitro digestion models provide a useful alternative to animal and human models by rapidly screening food ingredients. The ideal *in vitro* digestion method would provide accurate results in a short time (Coles, Moughan and Darragh, 2005) and could thus serve as a tool for rapid screening foods or delivery systems with different compositions and structures. In practice, any *in vitro* method is inevitably going to fail to match the accuracy that can be achieved by actually studying a food *in vivo* due to the inherent complexity of the process (Coles , Moughan and Darragh, 2005). Consequently, some compromise is needed between accuracy and ease of utilization of any *in vitro* digestion model. During the past few years, food and animal scientists have utilized a number of *in vitro* digestion models to test the structural and chemical changes that occur in different foods under simulated gastrointestinal (GI) tract conditions, although none of these methods has yet been widely accepted.

Miller, et al. (1981) reported that *in vitro* digestion composed of two stages: gastric digestion and intestinal digestion. They represented *in* vitro digestion process. Briefly, pepsin plays a role in the digestion at 37°C under gastric digestion. Then, pancreatin and bile salts are cooperative for 2 h for small intestine digestion.

CHAPTER III

RESEARCH METHOLOGY

Microorganism

Lyophilized *Monascus purpureus* TISTR 3090 was purchased from the Thailand Institute of Scientific and Technological Research (TISTR). The strain was cultivated on potato dextrose agar (PDA) (Merck, Darmstadt, Germany) at 25°C for 7 days. After a pure culture was obtained, the mycelium was re-inoculated into PDA slant at 25°C for 7 days before being used for production of monascal waxy corn (MWC).

Materials

Waxy corn (*Zea mays* var. *ceratina*) was harvested between 67 and 70 days after planting in Sukhothai province, Thailand. It was peeled and cleaned and the seeds were removed and stored at -18°C prior to being used for *Monascus* fermentation.

Chemicals

Monacolin K

Citrinin

Monosodium glutamate (MSG)

Peptone

2,4,6-tripyridyl-s-triazine(TPTZ)

2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS), 2,2-diphenyl-1-picrylhy-drazyl (DPPH)

6-hydroxy-2,5,7,8-tetramethylchro-man-2-carboxylic acid (Trolox)

Methanol (CH_3OH)

Ethanol (C_2H_5OH)

Acetone ($(CH_3)_2CO$)

Ethyl acetate($C_4H_8O_2$)

Sodium hydroxide (NaOH)

Sulfuric acid (H_2SO_4)

Acetonitrile (C_2H_3N)

N-acetyl glucosamine

3,5-dinitrosalicylic acid

Rochelle salt, phenol

Potassium disulphide

Pepsin

Pancreatin

Bile salts

Sodium bicarbonate ($NaHCO_3$)

Milli-Q water

Polyethylenglycol

Hydrochloric acid (HCl)

All chemicals and solvents were obtained from Sigma – Aldrich (St. Louis, MO) except MSG obtained from Thaichuros band and they were analytical reagent grade.

Study on the effects of MSG and peptone on antioxidant activity of MWC

1. **MWC preparation**

The *Monascus* fermentation followed the modified method of Yang, et al. (2006). One hundred grams of cleaned waxy corn seeds with different contents of MSG or peptone were put into a flask. Peptone and MSG were used as nitrogen sources with different concentrations, equivalent to 0.25, 0.50, 0.75 and 1.00 % nitrogen. The calculated amounts of peptone and MSG were 2.08, 4.16, 6.25 and 8.33% (w/w) and 3.01, 6.04, 9.06 and 12.08% (w/w), respectively. The mixtures were sterilized in an autoclave at 121°C for 15 min and then left at ambient temperature. Spore suspension of *M. purpureus* was prepared from actively growing slants in sterile water and diluted to a concentration of 10^6 spores/ mL. A 5 mL aliquot of the spore

suspension was inoculated into sterilized waxy corn, and incubated at 25°C for 20 days. The fermented products were dried in an oven at 40°C for 24 h. A fine powder (20 mesh) was obtained using a mill (Retsch ultracentrifugal mill and sieving machine (Haan, Germany).

2. Sample extraction for antioxidant activity assay

The extraction method described by Yang, et al. (2006) was used with some modifications. A 10 g sample was extracted in a shaker with 100 mL of methanol at 170 rpm for 24 h and the solution was filtered through Whatman no.4 filter paper. The residue was then extracted with two additional 100 mL portions of methanol as described above. The combined methanolic extracts were then evaporated at 40°C to dryness. The dried product was used for analysis of antioxidant activities

3. Pigment intensity

One gram of MWC was extracted with 5 mL methanol using a rotary shaker at 170 rpm for 1 h. The extract was then filtered through Whatman no.4 filter paper to remove suspended solids and the supernatant was analysed by a spectrophotometer (Thermo spectrophotometer model Genesys 20) against a methanol blank. The pigment concentration was measured at 500 nm (Yongsmith, et al., 2000). Pigment intensity was calculated from the following Equation (4).

$$\text{Pigment intensity (Unit /g dry weight)} = \frac{\text{A500 X dilution factor X Volume of methanol}}{\text{Weight of sample (g)}} \quad (4)$$

4. Glucosamine content

The fungal growth was estimated by determining the amount of *N*-acetyl glucosamine released by acid hydrolysis of chitin, present in the mycelia cell wall. One gram of dried sample was washed with 50 mL of 5 M H_2SO_4 under agitation for 15 min. The mixture was rinsed twice with distilled water. For chitin hydrolysis to *N*-acetyl glucosamine, the washed sample was incubated with 10 mL of 10 M HCl at 20°C for 16 h. After dilution with 40 mL distilled water, the hydrolysis proceeded during autoclaving for 2 h at 130°C. The hydrolysate was neutralized to pH 7.0 with 10 M NaOH and subsequently with 0.5 M NaOH. The neutralized sample of 1 mL was

mixed with 1 mL acetyl acetone reagent and incubated in a boiling water bath for 20 min. After cooling, 6 mL of ethanol was added and followed by the addition of 1 mL of Ehrlich reagent and incubated at 65°C for 10 min. The optical density was read at 530 nm against the reagent blank. *N*-Acetyl glucosamine was used as a standard (Babitha, Soccol, and Pandey, 2007).

5. Trolox equivalent antioxidant capacity (TEAC)

For ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) assay, antioxidant activity of MWC extracts against $ABTS^{\cdot+}$ radical, was evaluated spectrophotometrically by a slightly modified method of Re, et al. (1999). The TEAC assay is based on the scavenging of $ABTS^{\cdot+}$ radical converting into a colourless product. The degree of decolorisation induced by a compound is related to that induced by Trolox, giving the ''TEAC value". The $ABTS^{\cdot+}$ radical was produced by the reaction between 2 mL of 7 mM ABTS solution and 40 μL of 2.45 mM potassium persulphate solution, stored in the dark at room temperature for 16 h. Before usage, the $ABTS^{\cdot+}$ solution was diluted to get an absorbance of 0.700 $\pm$ 0.025 at 734 nm with ethanol. For the assay, the resulting solution was mixed with 300 μL of sample of each MWC extract (1-20 mg/mL). The absorbance was read at 30°C after exactly 6 min. The obtained absorbance of samples was compared with a standard curve from the corresponding readings of Trolox (0.4-0.04 mM). The total antioxidant capacities (TAC) were estimated as Trolox equivalents (TEAC) by interpolation to 50% inhibition ($TEAC_{50}$).

6. Ferric reducing/antioxidant power (FRAP)

The procedure was adapted from Benzie and Strain (1996). This method is based on the reduction of a ferric-tripyridyltriazine complex to its ferrous, colored form in the presence of antioxidants. Briefly, the FRAP reagent contained 2.5 mL of a 10 mM TPTZ solution in 40 mM HCl plus 2.5 mL of 20 mM $FeCl_3$ and 25 mL of 0.3 M acetate buffer, pH 3.6. The reagents were freshly prepared and warmed at 37°C. Each aliquot (1-20 mg/mL) of 40 μL sample supernatant was mixed with 2.2 mL of distilled water and 1.8 mL of FRAP reagent. The absorbance of the reaction mixture at 593 nm was measured spectrophotometrically after incubation at 37°C for 10 min. IC_{50} value (mg extract/ mL) is the effective concentration at which the reducing power was 50% obtained by interpolation from linear regression analysis.

7. DPPH radical scavenging activity

The scavenging activity (H/e-transferring ability) against 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) was measured spectrophotometrically by following Velazquez, et al. (2003). Each 1-20 mg/mL of aliquot of 40 μL appropriately diluted extracts mixed with 200 μL of 0.02 mM DPPH solution and methanol 4 mL. Samples were kept for 15 min at room temperature and the absorbance was measured at 517 nm. The absorbance of a blank sample containing the same amount of solvent was also measured. The extent of decolourisation is calculated as percentage reduction of absorbance, and this is determined as a function of concentration and calculated relative to the 0.1-0.01 mM of equivalent Trolox concentration. The radical scavenging activity is expressed in mmol of equivalent Trolox per gram of sample (mmol Trolox /mL) with interpolation to 50% inhibition (IC_{50}).

8. Chelating ability on ferrous ions

Chelating ability on ferrous ions was evaluated spectrophotometrically by a slightly modified method of Kuo, et al. (2004). Three hundred μL of 2 mM $FeSO_4.H_2O$ were mixed with 1-20 mg/mL of each aliquot of 500 μL test samples before addition of 600 μL of 5 mM ferrozine. After the incubation at room temperature for 10 min, 5 mL of ethanol was added and the absorbance was measured at 562 nm. IC_{50} value (mg extract/mL) is the effective concentration at which ferrous ions were chelated by 50 % by interpolation from linear regression analysis.

9. Monacolin K analysis

An 0.5 g sample was extracted with 25 mL of 70% ethanol at 50°C for 2 h, followed by filtration through a 0.2 μm membrane (Chayawat, et al., 2009) and the extract was analysed by HPLC. The HPLC system consisted of Shimadzu LC-10AT VP Liquid Chromatograph, a FCV-10AL VP pump, an LDC Analytical SpectroMonitor 3100 detector set at 238 nm and an LDC Analytical CI-4100 integrator. A chromatography column Ascentis C18, 5μm, 250×4.6 mm was connected to a 20 μL loop injector. An isocratic mobile phase of acetonitrile:water in the ratio of 65:35 (by vol.) was used. The flow rate and temperature were 1.0 mL/min and 28°C, respectively (Friedrich, et al., 1995). Monacolin K dissolved in 70% ethanol was used as a standard.

10. Citrinin analysis

Citrinin analysis was described by Lim, et al. (2010). A 1 g sample was extracted with a solution (acetone : ethyl acetate = 1:1, v/v) at 65°C for 90 min under vigorous shaking. The supernatant was obtained by centrifugation at 1,600g for 10 min followed by filtration through a 0.45 µm PTEE filter unit (National Scientific, Rockwood, TN). The citrinin was determined by HPLC using a chromatography column Ascentis C18 column (4.6 x 250 mm). The mobile phase consisted of methanol/acetonitrile/ 0.1% phosphoric acid (3:3:4, v:v:v) and the analysis was performed with a fluorescence detector set at excitation and emission wavelengths of 330 and 500 nm, respectively. The flow rate was 0.6 mL/min and the sample was spiked to confirm the presence of citrinin.

11. Statistical analysis

All determinations were performed in triplicate and results were expressed as the mean ± standard deviation calculated using spreadsheet software Microsoft Excel. The data were analysed by an analysis of variance ($P \leq 0.05$) and means separated by Duncan's multiple range test. The relationship among the antioxidant capacity, antioxidant content, pigment intensity and glucosamine content of different MWC samples, as well as different antioxidant capacity assays, was analysed by Pearson correlation coefficients. The results were processed by SPSS 16.0 (SPSS Inc., Chicago, IL, USA) for Windows.

Study on fermentation technique to enhance antioxidant activity of MWC production by using 2-step fermentation

1. Conventional fermentation method of MWC

About 100 g of waxy corn seeds and 12.08 % MSG (equivalent to 1.00 % N_2 content) were transferred into a flask 500 mL. The mixture was sterilized by an autoclave at 121 °C for 15 min and then left until cool down. About 5 mL of 10^6 spores mL/spore suspension of *M. purpureus* obtained from actively growing slants in sterile water was inoculated into sterilized waxy corn and incubated at 25 °C for 12 days (Kraboun, et al., 2013). Then, the product was dried in an oven at 40 °C for 24 h.

A fine powder (20 mesh) was obtained using a mill (Retsch ultracentrifugal mill and sieving machine, Haan, Germany). Then, the samples were extracted and determined antioxidant activities, pigment intensity, monacolin K and citrinin contents and glucosamine content and statistical analysis following the methods above except reducing sugar content assay.

2. 2-step fermentation of MWC

MWC from the conventional method was reinoculated with 5 mL spore suspension of 10^6 spores mL of actively growing slants and continuously fermented with the same condition as the conventional method for another 12 days. Then, the samples were extracted and determined antioxidant activities, pigment intensity, monacolin K and citrinin contents and glucosamine content and statistical analysis following the methods above except reducing sugar content assay.

3. Reducing sugars

The dinitrosalicylic reagent was based on the method developed by Babitha, Soccol and Pandey (2006) and contained a 1:1:1:1 volumetric mixture of 3,5-dinitrosalicylic acid 1%, Rochelle salt 40%, phenol 0.2%, potassium disulphide 0.5%, all in sodium hydroxide 1.5%. Typically, to 100 µL sample mixture 100 µL DNS reagent were added. The microtiter plates were heated in the water bath mounted in a common microwave oven (Galanz D900ESL30R, Foshan, China, 1800 MHz) for 4 min, cooled to room temperature on an ice bath and the optical density of the samples was measured at 540 nm using a spectrophotometer (Thermo spectrophotometer model Genesys 20). Glucose dissolved in water was used as a standard.

Study on extraction optimization of monacolin K, antioxidant activity, pigment and citrinin content from MWC produced by 2-step fermentation by using RSM

1. Shaking extraction

The extraction method described by Yang, et al. (2006) was used with some modifications. A 10 g sample was extracted in a shaker with 100 mL of ethanol at 170 rpm for 24 h and the solution was filtered through Whatman no.4 filter paper. The ethanol concentration and temperature were depended on the design. The residue was then extracted with two additional 100 mL portions of methanol as described

above. The combined methanolic extracts were then evaporated at 40°C to dryness. The dried product was used for analysis of antioxidant activities, citrinin and monacolin K contents and pigment intensity as following the above methods .

2. Ultrasonic-assisted extraction (UAE)

UAE was performed in a sonication water bath (KH5200, Kunshan Ultrasonic Instrument Co., Ltd., Jiangsu, China). UAE was described by Deng, et al. (2010) with some modifications. The working frequency and power were fixed at 40 kHz and 200 W. The temperature and time of extraction were controlled from a panel. The ground powder of 1 g of the selected MWC from the 2-step fermentation was extracted in a 250 mL volumetric flask with the volume of 10 mL of the ethanol concentration and temperature depended on the design. The flask was sealed by plastic film to avoid loss of solvent. The extraction was controlled at 30 min. Then, the extract was obtained from filtration through Whatman no.4 filter paper. All of the measurements were carried out in triplicate. The extract was evaporated at 40°C to dryness. The dried extract was used for the analyses of antioxidant activities, citrinin and monacolin K contents and pigment intensity as following the above methods.

3. Central composite design (CCD)

RSM was used to determine the optimum levels of ethanol concentration (%) and temperature (°C) on five responses namely, monacolin K and citrinin contents, DPPH• scavenging ability and chelating ability on Fe^{2+} and pigment intensity in MWC. The influence of ethanol concentration (X_1) and temperature (X_2) was evaluated using CCD (2^2) with four star points (α =1.41) and five replicates at the centre points, resulting in a total number of 13 runs (Table 7 and 8). The experiments were performed in triplicate. A second order polynomial, equation (2), which included all interaction terms, was used to calculate the predicted response.

$$Y= \beta_0 +\sum \beta_i X_i +\sum\beta_{ii} Xi^2 +\sum\beta_{ij} X_i X_j$$

(2)

Where, represents response variable; β_0 is the interception coefficient; β_i is the coefficient of the linear effect; β_{ii} is the coefficient of quadratic effect and β_{ij} is the

coefficient of interaction effect; Xi and Xj denote the coded levels of variable Xi and Xj investigated in the experiment.

The quality of fit of the second-order model equation was expressed by the coefficient of determination R^2, and its statistical significance was determined by an F-test. All experimental designs and statistical data were analyzed by using software Design-Expert® 7.0.0 (Stat-Ease Inc., Minneapolis, MN, USA).

The central composite design (CCD) was conducted in the optimum vicinity to locate the true optimum extraction of UAE and shaking methods for monacolin K and citrinin contents, DPPH• scavenging ability, chelating ability on Fe^{2+} and pigment intensity. A 2^2 CCD with four star points (α =1.41) and five replicates at the centre points, resulting in a total number of 13 experiments, was carried out according to Table 7. The experimental design is shown in Table 8. The experiments were performed in triplicate.

Table 7 Variables and their levels for the CCD

Independent variables	-1.4142	-1	0	1	1.4142
% ethanol (X_1)	54.82	60	72.5	85	90.18
Temperature (min) (X_2)	35.86	40	50	60	64.14

Table 8 Experimental design of the CCD for extraction optimization

Number	% ethanol	Temperature (°C)
1	54.82	50
2	60	40
3	60	60
4	72.50	35.86
5	72.50	64.14
6	85	40
7	85	60
8	90.18	50
9	72.50	50
10	72.50	50
11	72.50	50
12	72.50	50
13	72.50	50

Study on antioxidant stability of *Monascus* pigment from MWC via *in vitro* digestion

In vitro digestion of MWC, the technique of Miller, et al. (1981), modified to our requirements, was followed. It comprised two stages: gastric and intestinal digestions. Shortly before use, 0.4 g of pepsin was dissolved in 2.5 mL of 0.1 M HCl. For intestinal digestion, 0.1 g of pancreatin and 0.625 g of bile salts were dissolved in 25 mL of 0.1 M $NaHCO_3$. One gram of MWC with a final volume of 10 mL of milli-Q water was put together. pH of the mixture was adjusted to 2 with HCl 6 N and a pepsin solution was added at a proportion of 0.05 g of pepsin/g of sample. The mixture was incubated at 37°C in a shaking water bath at 110 oscillations/min for 2 h for the gastric digestion. For the intestinal digestion, the pH of the digest was raised to pH 6 with 1 M $NaHCO_3$dropwise, and 2.50 mL of pancreatin + bile salts mixture was added. The pH was then adjusted to pH 7.5 with 1 M $NaHCO_3$, and samples were incubated at 37°C at 110 oscillations/min for 2 h. After gastrointestinal digestion, the digestive

enzymes were inactivated by heat treatment for 4 min at 100°C in a polyethylenglycol bath. The samples were then cooled by immersion in an ice bath and centrifuged at 3200g for 60 min at 4°C (CS-6R centrifuge, Beckman) to separate soluble and non-soluble fractions. In both of them, moncolin K and citrinin contents as well as the antioxidant activities were measured as following the above methods.

CHAPTER IV

RESULTS AND DISCUSSION

Study of MSG and peptone on antioxidant activity of MWC

1. Pigment intensity and glucosamine content

Pigment intensity and glucosamine content of MWC supplemented with different contents of MSG and peptone are shown in Figures 11 and 12, respectively. Both pigment intensity and glucosamine content increased with increasing peptone and MSG contents. This indicated that nitrogen sources promote the growth and pigment production of fungi (Vidyalakshmi, et al., 2009). On day 12, pigment intensity of every treatment reached a maximum and was stable afterwards. Meanwhile, glucosamine content also reached a maximum on day 8 and then decreased until the end of fermentation. The result affirmed that the pigment is a secondary metabolite since it was increasingly produced following the growth of fungus. These results were similar to the finding of Babitha, Soccol, and Pandey (2007).

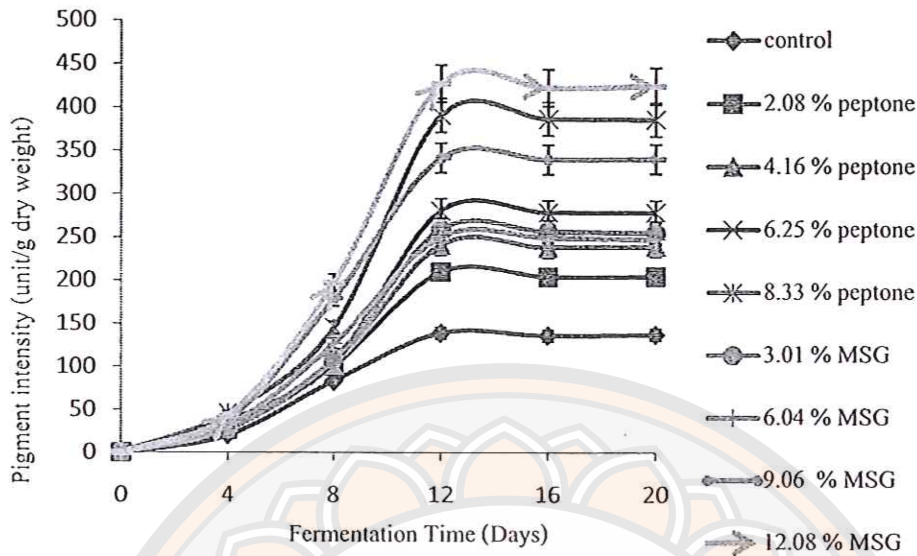


Figure 11 Pigment intensity of MWC during *Monascus* fermentation with different contents of MSG and peptone. Each value is expressed as mean ± standard deviation (n = 3)

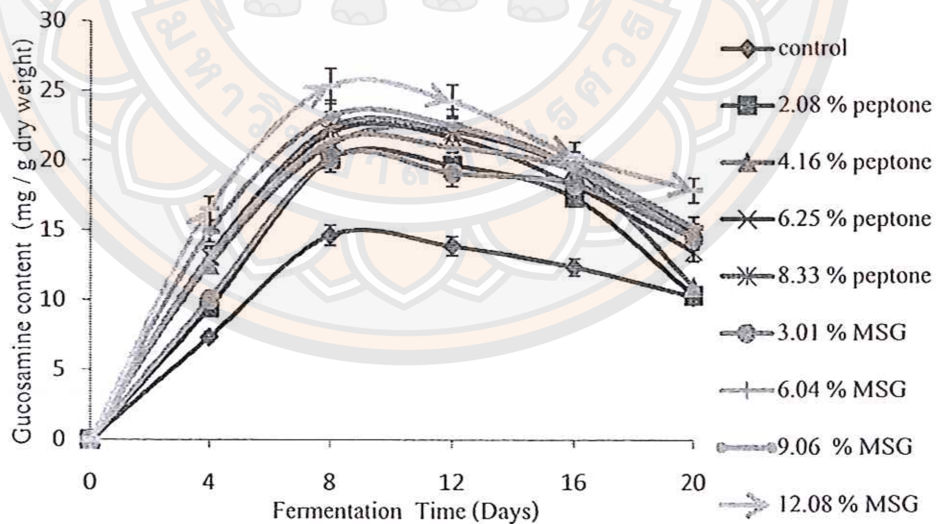


Figure 12 Glucosamine contents of MWC during *Monascus* fermentation with different contents of MSG and peptone. Each value is expressed as mean ± standard deviation (n = 3)

An increased content of MSG and peptone resulted in increasing pigment production and glucosamine content compared with the control (no nitrogen source added). The addition of 12.08% (w/w) MSG (1.00% of nitrogen) provided maximum pigment intensity and glucosamine of 427.3 unit/ g dry weight and 25.34 mg /g dry weight, respectively. Compared with the same nitrogen content from peptone (8.33% (w/w)), pigment intensity and glucosamine contents were 390.07 unit/g dry weight and 22.12 mg/g dry weight, respectively, less than those of MSG. Vidyalakshmi, et al. (2009) reported that monascal rice supplemented with 0.50 % (w/w) MSG fermented with *Monascus ruber* contained higher pigment yield than those supplemented with peptone, yeast extract and ammonium nitrate. Furthermore, Mukherjee and Singh (2011) suggested that the pigment production and growth of *M. purpureus* in submerged fermentation supplemented with 5.00% (w/v) MSG were higher than those with other nitrogen sources, such as peptone ,KNO_3, NH4NO_3, NH_4Cl, $NaNO_3$ and $(NH_4)_2SO_4$. The obtained results confirmed that MSG was the most appropriate additive for pigment production and growth of *M. purpureus* because MSG is stable under heating conditions and is not easily destroyed at high temperatures (Yamaguchi and Ninomiya, 1998), whereas the amino acids of peptone are partially lost during sterilization (Purcell and Walter, 1982). Therefore, the more remaining nitrogen contents of MSG could be better utilized by *M. purpureus* to produce higher levels of pigment and glucosamine. The lower pigmentation in the product supplemented with peptone was caused by a high glucose concentration which leads to lower growth rates, pigment synthesis and considerable ethanol production (Chen and Johns, 1994). The glucose when consumed is first metabolited to acetyl CoA, which could be channelled into pigment production on entry to the TCA cycle (Wang and Hesseltine, 1979). This suggests that the *Monascus* pigment could serve as a carbon sink by incorporating the carbon of acetyl CoA when glucose is present in excess concentration (Lee, et al., 2001).

2. Monacolin K and citrinin contents

The results of monacolin K and citrinin contents in MWC supplemented with peptone and MSG at different contents presented in Tables 9 and 10, respectively. Monocolin K and citrinin are secondary metabolites from *Monascus* sp. and citrinin is known as a toxic substance to humans and animals. Monacolin K

content of every treatment was increased during fermentation as well as increasing peptone and MSG contents. The highest monacolin K contents between 1.30 – 16.83 mg/kg dry weight , were produced on day 12 of fermentation and after that the values were not significantly different (P>0.05) until the end of fermentation. Hence, it could be said that the suitable concentration of nitrogen as a supplement for monacolin K production was 12.08% (w/w) MSG which equated to 1.00% of nitrogen. Chairote et al. (2008) reported that monascal product from glutinous rice cv. Kam by *M. purpureus* incubated at 30°C for 3 weeks contained 3.13 mg/g of monacolin K which was slightly more than that of commercial monascal rice (about 2.00 mg/g). Even though the amylopectin content in glutinous rice is as high as waxy corn (Jiranuntakul, et al., 2011), monacolin K content in MWC was yet less than that of monascal glutinous rice according to less starchy substrate of waxy corn compared to glutinous rice. On the other hand, Pattanagul, et al. (2008) elucidated that monacolin K content from monascal adlay produced from *M. purpureus*, incubated at 32-35°C for 28 days, was 14.97 mg/kg.

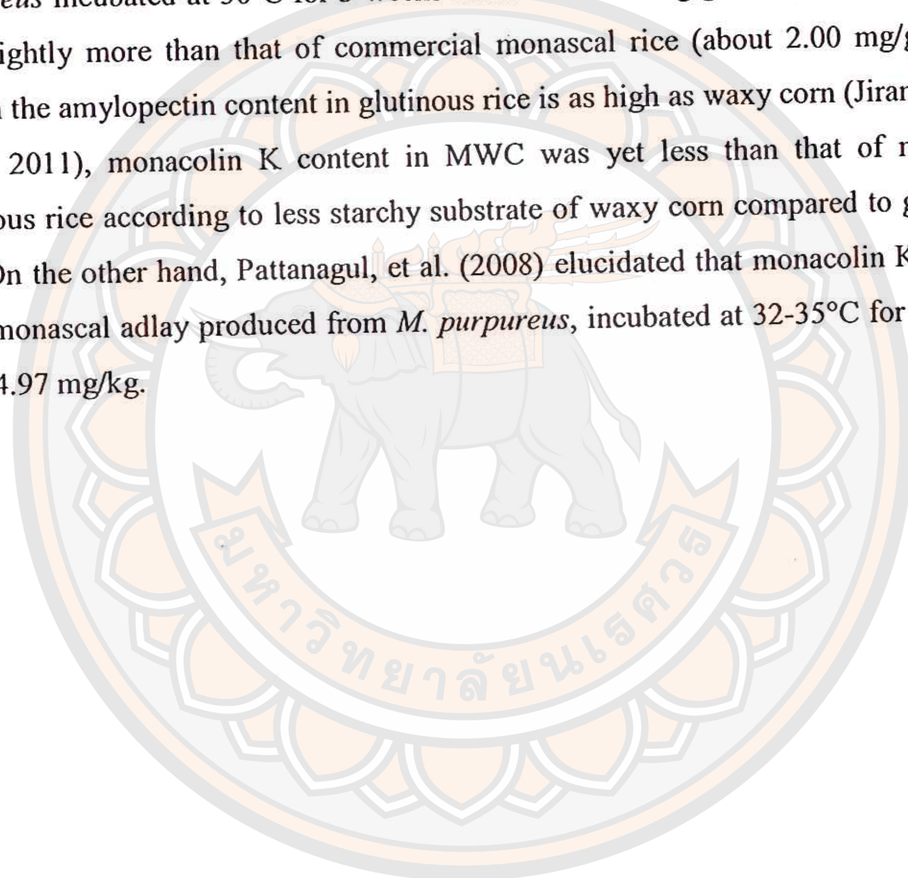


Table 9 Monacolin K contents of MWC during *Monascus* fermentation with different contents of MSG and peptone

Treatments	Monacolin K contents (mg/kg dry weight) at different incubation time (day)				
	4	8	12	16	20
*control**	$^{a}0.32\pm0.01^{a}$	$^{b}0.39\pm0.02^{a}$	$^{c}1.21\pm0.04^{a}$	$^{c}1.21\pm0.00^{a}$	$^{c}1.18\pm0.00^{a}$
2.08% peptone	$^{a}0.41\pm0.02^{b}$	$^{a}0.43\pm0.01^{b}$	$^{b}1.30\pm0.02^{a}$	$^{b}1.31\pm0.00^{a}$	$^{b}1.30\pm0.01^{a}$
4.16 % peptone	$^{a}0.42\pm0.03^{b}$	$^{b}0.46\pm0.03^{c}$	$^{c}2.32\pm0.03^{b}$	$^{c}2.30\pm0.02^{b}$	$^{c}2.30\pm0.02^{b}$
6.25% peptone	$^{a}0.47\pm0.06^{c}$	$^{b}0.48\pm0.01^{c}$	$^{c}3.21\pm0.06^{c}$	$^{c}3.18\pm0.02^{c}$	$^{c}3.18\pm0.04^{c}$
8.33% peptone	$^{a}0.50\pm0.01^{d}$	$^{b}0.66\pm0.03^{d}$	$^{c}5.12\pm0.05^{d}$	$^{c}5.11\pm0.01^{d}$	$^{c}5.09\pm0.01^{d}$
3.01 % MSG	$^{a}0.51\pm0.01^{d}$	$^{b}1.23\pm0.01^{e}$	$^{c}10.12\pm0.21^{e}$	$^{c}10.13\pm0.04^{e}$	$^{c}10.11\pm0.05^{e}$
6.04 % MSG	$^{a}0.53\pm0.03^{e}$	$^{b}1.58\pm0.01^{f}$	$^{c}12.79\pm0.04^{f}$	$^{c}12.78\pm0.00^{f}$	$^{c}12.70\pm0.21^{f}$
9.06 % MSG	$^{a}0.63\pm0.01^{f}$	$^{b}2.00\pm0.01^{g}$	$^{c}13.12\pm0.03^{g}$	$^{c}13.13\pm0.02^{g}$	$^{c}13.12\pm0.01^{g}$
12.08 % MSG	$^{a}1.23\pm0.01^{g}$	$^{b}3.43\pm0.01^{h}$	$^{c}16.83\pm0.12^{h}$	$^{c}16.80\pm0.02^{h}$	$^{c}16.80\pm0.01^{h}$

*Different letters in front of means within a row are significantly different ($P \leq 0.05$)

**Different letters behind means within a column are significantly different ($P \leq 0.05$)

Citrinin was increased with incubation time and increasing contents of peptone and MSG until day 12 and then it was stable. The citrinin contents in peptone supplemented monascal product were in the range of 0.34 – 0.67 μg/kg dry weight, whereas those supplemented with MSG were 1.54 – 3.55 μg/kg dry weight. Japan has issued an advisory limit of 200 μg/kg of citrinin in agricultural products for sale. The limit set by the Chinese Food and Drug Administration (FDA) is 20 μg/kg, while the European Union has recommended a citrinin limit of 100 μg/kg (Shi and Pan, 2011). Obviously, the citrinin contents of products in Table 10 were not over the maximum

allowance for sale according to Chinese FDA. Furthermore, citrinin contents between 0.20 – 3.55 µg/kg were presented in the control and nitrogen supplemented products, which were less than those produced from monascal rice and monascal adlay products which were 100–300 and 0.26 – 14.64 mg/kg, respectively (Pattanagul, et al., 2008).

Table 10 Citrinin contents of MWC during *Monascus* fermentation with different contents of MSG and peptone

Treatments	Citrinin contents (µg/kg weight) at different incubation time (day)				
	4	8	12	16	20
*control**	$^{a}0.01\underline{+}0.00^{a}$	$^{a}0.02\underline{+}0.00^{a}$	$^{b}0.20\underline{+}0.01^{a}$	$^{b}0.21\underline{+}0.02^{a}$	$^{b}0.20\underline{+}0.00^{a}$
2.08 % peptone	$^{a}0.01\underline{+}0.00^{a}$	$^{b}0.03\underline{+}0.00^{b}$	$^{c}0.34\underline{+}0.02^{b}$	$^{c}0.33\underline{+}0.02^{b}$	$^{c}0.33\underline{+}0.02^{b}$
4.16 % peptone	$^{a}0.03\underline{+}0.00^{b}$	$^{a}0.04\underline{+}0.00^{b}$	$^{b}0.39\underline{+}0.01^{c}$	$^{b}0.39\underline{+}0.02^{c}$	$^{b}0.39\underline{+}0.01^{c}$
6.25 % peptone	$^{a}0.03\underline{+}0.00^{b}$	$^{b}0.05\underline{+}0.00^{c}$	$^{c}0.55\underline{+}0.04^{d}$	$^{c}0.55\underline{+}0.04^{d}$	$^{c}0.05\underline{+}0.02^{d}$
8.33 % peptone	$^{a}0.04\underline{+}0.00^{b}$	$^{b}0.07\underline{+}0.00^{d}$	$^{c}0.67\underline{+}0.01^{e}$	$^{c}0.67\underline{+}0.04^{e}$	$^{c}0.67\underline{+}0.03^{e}$
3.01 % MSG	$^{a}0.04\underline{+}0.00^{b}$	$^{b}0.08\underline{+}0.00^{d}$	$^{c}1.54\underline{+}0.04^{f}$	$^{c}1.54\underline{+}0.02^{f}$	$^{c}1.54\underline{+}0.01^{f}$
6.04 % MSG	$^{a}0.05\underline{+}0.00^{b}$	$^{b}0.12\underline{+}0.02^{e}$	$^{c}1.72\underline{+}0.05^{g}$	$^{c}1.72\underline{+}0.01^{g}$	$^{c}1.72\underline{+}0.01^{g}$
9.06 % MSG	$^{a}0.07\underline{+}0.00^{c}$	$^{b}0.13\underline{+}0.01^{e}$	$^{c}2.45\underline{+}0.02^{h}$	$^{c}2.45\underline{+}0.03^{h}$	$^{c}2.45\underline{+}0.02^{h}$
12.08 % MSG	$^{a}0.13\underline{+}0.01^{d}$	$^{b}0.29\underline{+}0.03^{f}$	$^{c}3.55\underline{+}0.02^{i}$	$^{c}3.56\underline{+}0.01^{i}$	$^{c}3.56\underline{+}0.02^{i}$

*Different letters in front of means within a row are significantly different ($P \leq 0.05$)

**Different letters behind means within a column are significantly different ($P \leq$ 0.05)

3. Antioxidant activities of MWC

In ABTS assay, $TEAC_{50}$ values of MWC are shown in Table 11. $TEAC_{50}$ values of every treatments were minimum on day 12 and slightly decreased until the

end of fermentation (P>0.05). The lower $TEAC_{50}$ values were obtained from the increasing contents of peptone and MSG. The lowest $TEAC_{50}$ value of 0.02 mmol trolox / mL was found in the fermented product supplemented with 12.08% (w/w) MSG (1% of nitrogen) during day 12 - 20. However, $TEAC_{50}$ value of the control was higher than those of other treatments throughout the fermentation. This implied that the extracts from waxy corns supplemented with nitrogen might contain more phenolic hydroxyl groups obtained from phenolic compounds of MWC (Yang, et al., 2006) , which show high electron/hydrogen donors antioxidant activities (Jayaprakasha and Patil, 2007).

Table 11 $TEAC_{50}$ of ABTS assay of MWC during *Monascus* fermentation with different contents of MSG and peptone

Treatments	$TEAC_{50}$ values (mmol Trolox/ mL) at different incubation time (day)				
	4	8	12	16	20
control	$^{d}0.18 \pm 0.01^{e}$	$^{c}0.16+0.01^{i}$	$^{a}0.08 \pm 0.01^{c}$	$^{a}0.09 \pm 0.01^{c}$	$^{b}0.10 \pm 0.01^{c}$
2.08 % peptone	$^{c}0.15 \pm 0.03^{de}$	$^{b}0.10 \pm 0.02^{f}$	$^{a}0.03 \pm 0.00^{b}$	$^{a}0.04 \pm 0.00^{ab}$	$^{a}0.04 \pm 0.00^{b}$
4.16 % peptone	$^{c}0.13 \pm 0.02^{cd}$	$^{d}0.09 \pm 0.01^{e}$	$^{a}0.03 \pm 0.00^{b}$	$^{b}0.04 \pm 0.00^{b}$	$^{c}0.04 \pm 0.00^{b}$
6.25 % peptone	$^{c}0.10 \pm 0.04^{bc}$	$^{d}0.12 \pm 0.02^{h}$	$^{a}0.03 \pm 0.00^{b}$	$^{a}0.03 \pm 0.00^{ab}$	$^{b}0.04 \pm 0.00^{b}$
8.33 % peptone	$^{c}0.08 \pm 0.00^{ab}$	$^{b}0.07 \pm 0.00^{c}$	$^{a}0.03 \pm 0.00^{b}$	$^{a}0.03 \pm 0.00^{ab}$	$^{a}0.03 \pm 0.00^{b}$
3.01 % MSG	$^{c}0.61 \pm 0.01^{f}$	$^{b}0.12 \pm 0.01^{g}$	$^{a}0.03 \pm 0.00^{b}$	$^{a}0.03 \pm 0.00^{ab}$	$^{a}0.04 \pm 0.01^{b}$
6.04 % MSG	$^{b}0.07 \pm 0.00^{ab}$	$^{b}0.07 \pm 0.00^{d}$	$^{a}0.04 \pm 0.00^{b}$	$^{a}0.04 \pm 0.00^{ab}$	$^{a}0.04 \pm 0.00^{b}$
9.06 % MSG	$^{d}0.06 \pm 0.00^{a}$	$^{c}0.04+0.00^{a}$	$^{ab}0.03 \pm 0.00^{b}$	$^{a}0.03 \pm 0.00^{ab}$	$^{b}0.04+0.00^{b}$
12.08 % MSG	$^{d}0.05 \pm 0.00^{a}$	$^{c}0.05 \pm 0.00^{b}$	$^{a}0.02 \pm 0.00^{a}$	$^{b}0.02 \pm 0.00^{a}$	$^{b}0.02 \pm 0.00^{a}$

The IC_{50} of DPPH assay of MWC are shown in Table 12. IC_{50} values of every treatments tended to decrease after day 4 and were minimum on day 12. The lower IC_{50} values were due to the increased contents of peptone and MSG. The lowest IC_{50} value of 0.02 mmol Trolox/mL was obtained from the product supplemented with 12.08 % (w/w) MSG. The IC_{50} value of the control was higher than those of MSG and peptone at each time point. However, the IC_{50} value of the control was less than that of Yang, et al. (2006), which showed that EC_{50} value of scavenging ability on DPPH radicals of monascal polished rice was 0.59 mmol Trolox/mL. These data suggested that addition of nitrogen promoted antioxidant production of monascal rice (Su, Wang and Lin, 2003), which possessed more effective hydrogen donation to oxidants.

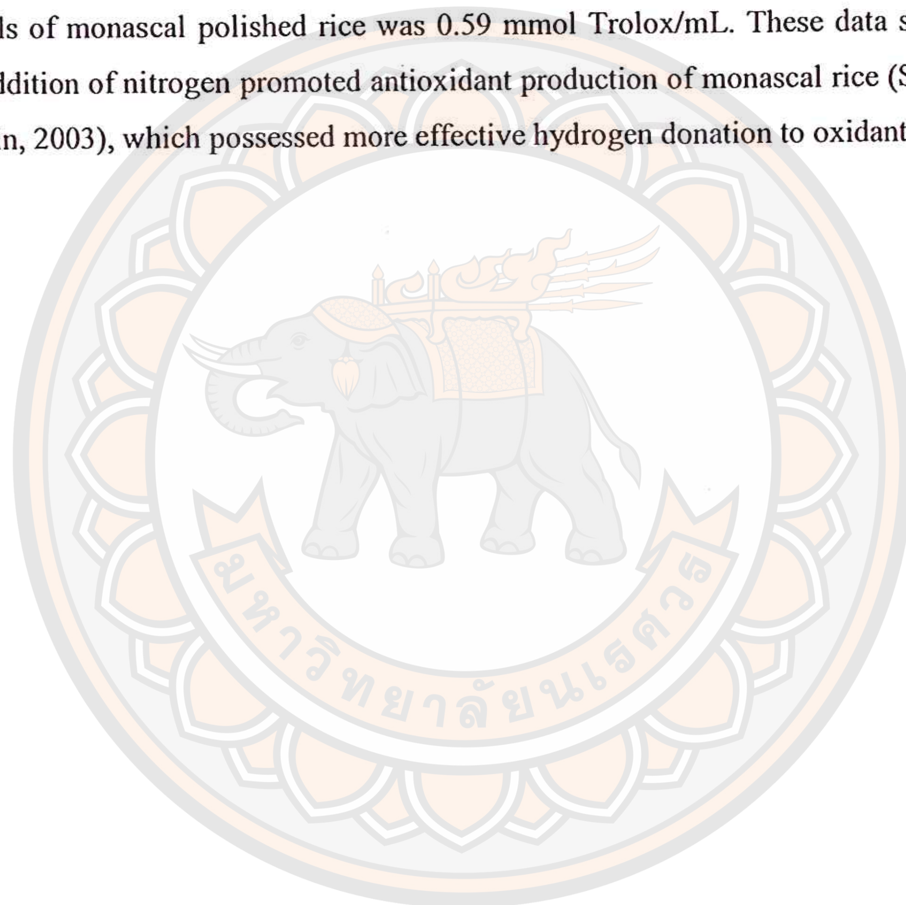


Table 12 IC_{50} of DPPH assay of MWC during *Monascus* fermentation with different contents of MSG and peptone

Treatments	*IC_{50} value** (mmol Trolox / mL) at different incubation time (day)				
	4	8	12	16	20
control	$^{b}0.33\pm0.01^{h}$	$^{a}0.25\pm0.02^{f}$	$^{a}0.20\pm0.02^{g}$	$^{a}0.18\pm0.02^{g}$	$^{a}0.17\pm0.01^{g}$
2.08 % peptone	$^{b}0.28\pm0.01^{f}$	$^{b}0.15\pm0.03^{e}$	$^{a}0.13\pm0.01^{f}$	$^{a}0.13\pm0.07^{f}$	$^{a}0.13\pm0.00^{f}$
4.16 % peptone	$^{b}0.24\pm0.01^{e}$	$^{a}0.13\pm0.05^{d}$	$^{a}0.11\pm0.00^{e}$	$^{a}0.10\pm0.00^{de}$	$^{a}0.1\pm0.02^{de}$
6.25 % peptone	$^{b}0.21\pm0.07^{d}$	$^{a}0.09\pm0.04^{c}$	$^{a}0.09\pm0.00^{d}$	$^{a}0.09\pm0.01^{cd}$	$^{a}0.09\pm0.00^{d}$
8.33 % peptone	$^{b}0.15\pm0.01^{c}$	$^{a}0.05\pm0.00^{a}$	$^{a}0.04\pm0.00^{b}$	$^{a}0.05\pm0.00^{b}$	$^{a}0.04\pm0.00^{b}$
3.01 % MSG	$^{c}0.31\pm0.03^{g}$	$^{a}0.12\pm0.00^{d}$	$^{a}0.10\pm0.00^{de}$	$^{a}0.11\pm0.00^{de}$	$^{a}0.11\pm0.02^{e}$
6.04 % MSG	$^{c}0.26\pm0.04^{e}$	$^{b}0.09\pm0.02^{c}$	$^{a}0.07\pm0.01^{c}$	$^{a}0.07\pm0.00^{bc}$	$^{a}0.07\pm0.01^{c}$
9.06 % MSG	$^{c}0.13\pm0.02^{b}$	$^{b}0.07\pm0.00^{b}$	$^{a}0.06\pm0.00^{c}$	$^{a}0.06\pm0.00^{b}$	$^{a}0.06\pm0.00^{c}$
12.08 % MSG	$^{c}0.10\pm0.01^{a}$	$^{b}0.04\pm0.00^{a}$	$^{a}0.02\pm0.00^{a}$	$^{a}0.02\pm0.00^{a}$	$^{a}0.02\pm0.02^{a}$

*Different letters in front of means within a row are significantly different ($P \leq 0.05$)

**Different letters behind means within a column are significantly different ($P \leq 0.05$)

In FRAP assay, IC_{50} values of MWC are shown in Table 13. A similar trend was observed between IC_{50} values of FRAP and DPPH assays (P>0.05). The IC_{50} values were lower with increasing MSG and peptone contents. Comparing the same nitrogen content between MSG and peptone, average IC_{50} value of MSG supplemented products was lower than that of peptone throughout the fermentation. The lowest IC_{50} value of 0.09 mg extract/ mL was also obtained from MWC supplemented with 12.08% (w/w)

MSG. IC_{50} value of the control on day 12 was about 100 times higher than that of 12.08% (w/w) MSG. EC_{50} values of reducing power of monascal polished rice were 0.79 mg extract/mL (Yang, et al., 2006) and 0.78 mg extract/ mL from monascal polished adlay (Tseng, et al., 2006), hence, our products presented higher potential in reducing Fe (III)/tripyridyltriazine complex.

Table 13 IC_{50} of FRAP assay of MWC during *Monascus* fermentation with different contents of MSG and peptone

Treatments	*IC_{50} values** (mg extract/ mL) at different incubation time (day)				
	4	8	12	16	20
control	$^{c}11.11\pm0.11^{i}$	$^{b}10.20\pm0.54^{i}$	$^{a}9.73\pm0.22^{h}$	$^{a}9.43\pm0.17^{h}$	$^{a}9.33\pm0.15^{h}$
2.08% peptone	$^{c}5.69\pm0.12^{h}$	$^{b}5.21\pm0.41^{h}$	$^{a}4.90\pm0.66^{g}$	$^{a}4.70\pm0.17^{g}$	$^{a}4.80\pm0.35^{g}$
4.16% peptone	$^{c}5.47\pm0.21^{g}$	$^{b}3.70\pm0.01^{g}$	$^{a}2.13\pm0.12^{e}$	$^{a}2.43\pm0.43^{e}$	$^{a}2.21\pm0.12^{e}$
6.25% peptone	$^{c}4.53\pm0.02^{f}$	$^{b}2.22\pm0.10^{d}$	$^{a}1.23\pm0.02^{c}$	$^{a}1.24\pm0.55^{c}$	$^{a}1.19\pm0.56^{c}$
8.33% peptone	$^{c}3.16\pm0.26^{c}$	$^{b}1.55\pm0.27^{c}$	$^{a}1.21\pm0.38^{c}$	$^{a}1.22\pm0.15^{c}$	$^{a}1.23\pm0.57^{c}$
3.01 % MSG	$^{c}4.24\pm0.60^{e}$	$^{b}3.24\pm0.19^{f}$	$^{a}3.10\pm0.12^{f}$	$^{a}3.11\pm0.71^{f}$	$^{a}3.12\pm0.39^{f}$
6.04 % MSG	$^{c}3.40\pm0.51^{d}$	$^{b}2.43\pm0.23^{e}$	$^{a}1.50\pm0.10^{d}$	$^{a}1.56\pm0.34^{d}$	$^{a}1.46\pm0.67^{d}$
9.06 % MSG	$^{c}2.72\pm0.03^{b}$	$^{b}1.37\pm0.18^{b}$	$^{a}0.97\pm0.19^{b}$	$^{a}0.93\pm0.15^{b}$	$^{a}0.98\pm0.14^{b}$
12.08 % MSG	$^{c}1.89\pm0.14^{a}$	$^{b}0.67\pm0.05^{a}$	$^{a}0.09\pm0.03^{a}$	$^{a}0.09\pm0.02^{a}$	$^{a}0.08\pm0.00^{a}$

*Different letters in front of means within a row are significantly different ($P \leq 0.05$)

**Different letters behind means within a column are significantly different ($P \leq 0.05$)

IC_{50} values of MWC from the chelating ability on ferrous ions assay are shown in Table 14. IC_{50} values tended to decrease until day 12 of fermentation ($P \leq 0.05$). The lower IC_{50} values obtained from the increasing contents of MSG and peptone. The lowest IC_{50} value of 2.89 mg extract/ mL was presented in the product supplemented with 12.08% (w/w) MSG. The IC_{50} value of the control on day 12 was about 3 times higher than that of 12.08% (w/w) MSG. EC_{50} values of chelating ability on ferrous ions of monascal rice and monascal adlay were 3.92 mg extract/ mL and 2.91 mg extract/ mL, respectively (Tseng, et al., 2006; Yang, et al., 2006). Generally, ferrous ions are the most effective pro-oxidants in the food system and also are quenched by effective Fe^{2+} chelating antioxidant (Yamaguchi, et al., 1988). Our results showed stronger chelating ability on Fe^{2+} for inhibition of lipid oxidation than the previous studies.

Table 14 IC_{50} of Chelating ability on ferrous ions assay of MWC during *Monascus* fermentation with different contents of MSG and peptone

Treatments	IC_{50} values (mg extract / mL) at different incubation time (day)				
	4	8	12	16	20
control	$^{c}14.57\pm1.43^{h}$	$^{b}11.2\pm0.34^{i}$	$^{a}9.73\pm0.61^{i}$	$^{a}9.68\pm0.36^{i}$	$^{a}9.66\pm0.36^{i}$
2.08% peptone	$^{c}11.83\pm0.03^{g}$	$^{b}9.83\pm0.09^{h}$	$^{a}7.9\pm0.69^{g}$	$^{a}7.83\pm0.99^{g}$	$^{a}7.88\pm0.55^{g}$
4.16% peptone	$^{c}11.47\pm1.00^{f}$	$^{b}8.7\pm0.12^{f}$	$^{a}7.7\pm0.01^{f}$	$^{a}7.67\pm0.48^{f}$	$^{a}7.54\pm0.27^{f}$
6.25% peptone	$^{c}10.53\pm0.31^{e}$	$^{b}7.32\pm0.07^{d}$	$^{a}6.22\pm0.11^{d}$	$^{a}6.23\pm0.46^{d}$	$^{a}6.2\pm0.38^{d}$
8.33% peptone	$^{c}10.16\pm0.66^{d}$	$^{b}5.55+0.46^{b}$	$^{a}4.5\pm0.66^{b}$	$^{a}4.45\pm0.03^{b}$	$^{a}4.43\pm0.66^{b}$
3.01 % MSG	$^{c}10.24\pm0.76^{d}$	$^{b}8.94\pm0.28^{g}$	$^{a}8.00\pm1.21^{h}$	$^{a}7.9\pm0.60^{h}$	$^{a}7.9\pm0.76^{h}$
6.04 % MSG	$^{c}9.4\pm0.44^{c}$	$^{b}7.43\pm0.38^{e}$	$^{a}6.8\pm0.34^{e}$	$^{a}6.75\pm0.19^{e}$	$^{a}6.77\pm0.26^{e}$
9.06 % MSG	$^{c}8.87\pm0.01^{b}$	$^{b}6.37\pm1.66^{c}$	$^{a}5.97\pm0.77^{c}$	$^{a}5.94\pm1.26^{c}$	$^{a}5.93\pm0.59^{c}$
12.08 % MSG	$^{c}5.89\pm0.12^{a}$	$^{b}4.67\pm0.09^{a}$	$^{a}2.89\pm0.18^{a}$	$^{a}2.86\pm0.76^{a}$	$^{a}2.85\pm0.68^{a}$

4. Pearson's correlation amongst antioxidant activities, pigment intensity, glucosamine content, monacolin K and citrinin contents

The correlation among antioxidant activities, pigment intensity, glucosamine, monacolin K and citrinin contents was analysed on day 12 because that was when the highest antioxidant activities and monacolin K and citrinin contents were obtained (Table 15 and 16). Good positive correlation was found between pigment intensity and monacolin k, between pigment intensity and citrinin contents and between pigment intensity and glucosamine content. On the other hand, good negative correlation was found between pigment intensity and inhibition of DPPH radical, between pigment intensity and chelating ability on ferrous ions and between monacolin K content and chelating ability on ferrous ions of both products supplemented with peptone and MSG. The results showed that Pearson's correlation coefficients of the product supplemented with MSG were higher than those of peptone. IC_{50} values obtained from different antioxidant activity assays decreased when increasing pigment intensity. This indicated that *Monascus* pigment containing high content of monacolin K could effectively scavenge DPPH radical (r^2 between -0.904 and -0.985) and chelate Fe^{2+}(r^2 between -0.967 and -0.988) and it was well related to the growth of *M. purpureus* in waxy corn product supplemented with both MSG and peptone (r^2 between 0.949 and 0.977).

Table 15 Pearson's correlation coefficients of pigment intensity and antioxidant activity values in MWC supplemented with different peptone contents on day 12

	Pigment	Glucosamine	ABTS	DPPH	FRAP	Chelate Fe^{2+}	Monacolin K
Glucosamine	0.977**						
ABTS	-0.705	-0.961**					
DPPH	-0.985**	-0.846	0.749				
FRAP	-0.838	-0.981**	0.922*	0.901*			
Chelate Fe^{2+}	-0.988**	-0.784	0.737	0.970**	0.857		
Monacolin K	0.968**	0.643	-0.525	-0.951*	-0.740	-0.948*	
Citrinin	0.979**	0.796	-0.731	-0.974**	-0.878*	-0.995**	0.951*

*Correlation is significant at the 0.05 level (2-tailed)

**Correlation is significant at the 0.01 level (2-tailed)

Table 16 Pearson's correlation coefficients of pigment intensity and antioxidant activity values in MWC supplemented with different MSG contents on day 12

	Pigment	Glucosamine	ABTS	DPPH	FRAP	Chelate Fe^{2+}	Monacolin K
Glucosamine	0.949*						
ABTS	-0.874	-0.937*					
DPPH	-0.904*	-0.807	0.635				
FRAP	-0.875	-0.977**	0.969**	0.669			
Chelate Fe^{2+}	-0.967**	-0.923*	0.808	0.967**	0.831		
Monacolin K	0.922*	0.989**	-0.967**	-0.766	-0.987**	-0.903*	
Citrinin	0.995**	0.965**	-0.890*	-0.911*	-0.898*	-0.980**	0.947*

Study on fermentation technique to enhance antioxidant activity of MWC production by using 2-step fermentation

The 2-step fermentation was started from the conventional *Monascus* fermentation for 12 days. Then, the spore suspension was added and the MWC was continuously fermented for another 12 days. The addition of spore suspension on day 12 after the conventional fermentation since the residual reducing sugar content was still high and *Monascus* pigment production was stable (Figure 11), indicating its stationary phase and the death phase of *Monascus* was then noticed which it was confirmed by the reduction of glucosamine content (Figure 12); the monacolin K and citrinin contents and antioxidant activities were also stable after day 12 until the end of the fermentation.

1. Pigment intensity, glucosamine and reducing sugar contents

The *Monascus* pigment was continuously produced during the 2-step fermentation, which was simultaneously formed with the growth of *M. purpureus* after addition of the *Monascus* spore suspension for the second step fermentation (Figures 13 and 14). It was shown that the maximum pigment intensity and glucosamine content were obtained from the 2-step fermentation on day 8, which were 3,500.00 unit/g substrate and 480.00 mg glucosamine/g substrate, respectively, about 4 and 19 times higher than those of the conventional method. Babitha, Soccol, and Pandey (2007) reported that reducing sugars in monascal jackfruit seed were about 2 mg/g after the fermentation, while in our research, reducing sugar contents were still remained at 8.00 mg/g dry weight after the conventional fermentation. The addition of *Monascus* spores led to more effective utilization of the rest of reducing sugars, which was continuously hydrolyzed from the conventional method. After 2nd step fermentation, it is noted that reducing sugars were rapidly hydrolyzed until absent after 8 days, which related to the maximum growth of *M. purpureus*, as indicated by the highest glucosamine content (Figure 15). Moreover, decrease in reducing sugar content might be due to the presence of two hydrolyzing amylase enzymes, α-amylase and glucoamylase after the 2nd step fermentation (Babitha, Soccol and Pandey, 2007).

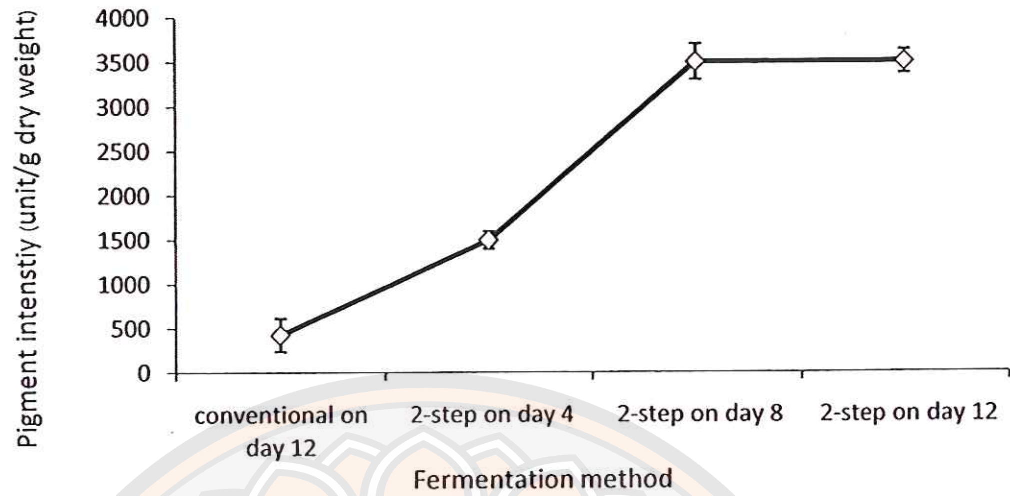


Figure 13 Pigment intensity of MWC obtained from conventional method and 2-step fermentation with different incubation period. Each value is expressed as mean ± standard deviation (n = 3)

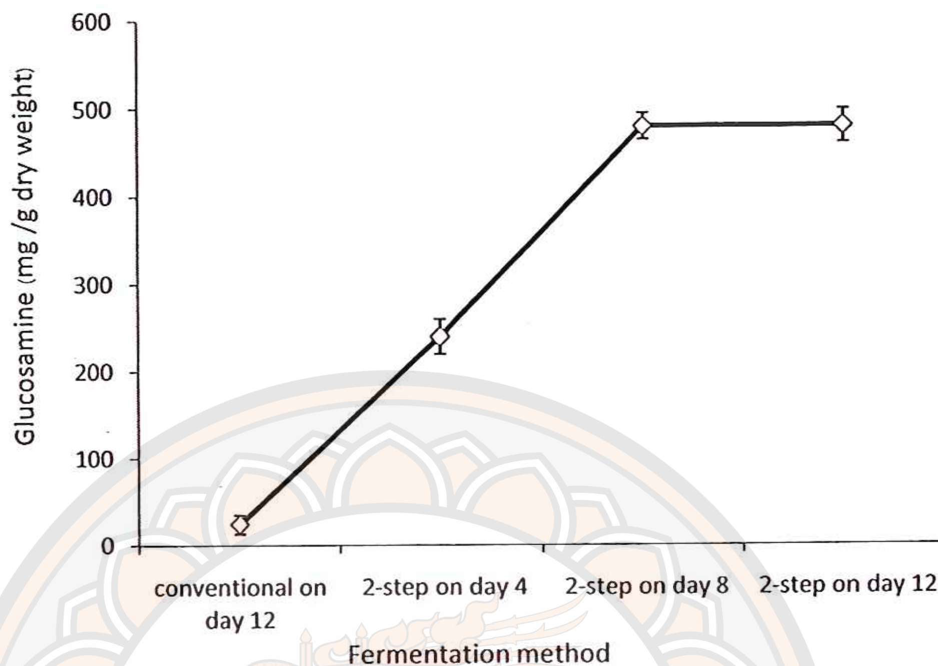


Figure 14 Glucosamine contents of MWC obtained from conventional method and 2-step fermentation with different incubation period. Each value is expressed as mean ± standard deviation (n = 3)

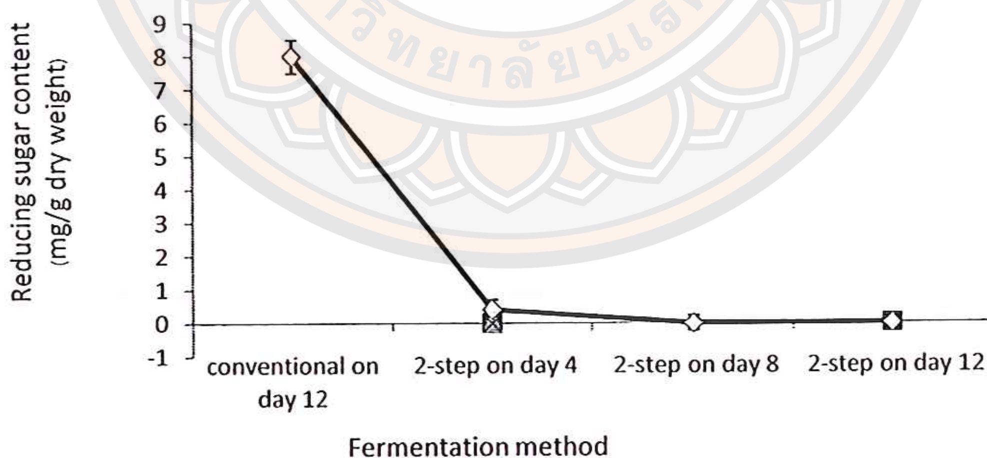


Figure 15 Reducing sugar contents of MWC obtained from conventional method and 2-step fermentation with different incubation period. Each value is expressed as mean ± standard deviation (n = 3)

2. Monacolin K and citrinin contents

The results of monacolin K and citrinin contents of MWC in conventional and 2-step fermentation are shown in Table 17.

Table 17 Monacolin K and citrinin contents, $TEAC_{50}$ of ABTS assay and IC_{50} values of antioxidant activities of MWC via conventional method and 2-step fermentation at different incubation period

Conventional* and 2-step fermentation	Monacolin K (mg/kg dry weight)	Citrinin (μg/kg dry weight)	$TEAC_{50}$ABTS (mmol Trolox/mL)	IC_{50} DPPH (mmol Trolox/mL)	IC_{50} FRAP (mg extract/mL)	IC_{50} Chelate Fe^{2+} (mg extract/mL)
conventional on day 12	$16.83 \pm 0.32^{a*}$	3.55 ± 0.04^{a}	0.02 ± 0.00^{NS}	0.04 ± 0.00^{a}	0.09 ± 0.00^{a}	2.89 ± 0.28^{a}
2-step on day 4	26.13 ± 0.20^{b}	6.11 ± 0.10^{b}	0.02 ± 0.00^{NS}	0.02 ± 0.00^{b}	0.08 ± 0.00^{a}	2.37 ± 0.08^{b}
2-step on day 8	79.72 ± 0.57^{c}	10.57 ± 0.53^{c}	0.02 ± 0.00^{NS}	0.02 ± 0.00^{b}	0.05 ± 0.00^{b}	1.54 ± 0.16^{c}
2-step on day 12	79.52 ± 0.22^{c}	10.57 ± 0.42^{c}	0.02 ± 0.00^{NS}	0.02 ± 0.00^{b}	0.05 ± 0.00^{b}	1.53 ± 0.18^{c}

*Different letters behind means within a column are significantly different ($P \leq 0.05$).
^{NS}Not significantly different

Monacolin K and citrinin contents of 2-step fermentation were simultaneous with pigment production and growth of *M. purpureus*. Pattanagul, et al. (2008) suggested that mevinolin (monacolin K) and citrinin synthesis depended upon *Monascus* mycelial growth, while, Jirasatid, et al. (2013) reported that the secondary metabolites from *Monascus* such as monacolin K and citrinin were formed together during yellow pigment production. The contents of monacolin K and citrinin in the conventional fermentation were 16.83 mg/ kg dry weight and 3.55 μg/kg dry weight, respectively and the maximum monacolin K and citrinin contents were produced by the 2-step fermentation after 8 days, which were 79.72 mg/ kg dry weight and 10.57

μg/kg dry weight, respectively. This indicated that the monacolin K and citrinin contents were enhanced by the 2-step fermentation, which were about 4 and 3 times higher than those of the conventional method. The obtained monacolin K and citrinin contents from our study were much less than those of Jirasatid, et al. (2013). The maximum monacolin K content of 5,900 mg/kg and the minimum citrinin content of 0.26 mg/kg from the optimisation study were achieved in the medium containing 2% glycerol, 0.14% methionine, and 0.01% sodium nitrate at 25°C for 16 days of cultivation, showing that the appropriate enrichment medium provided higher monacolin K and citrinin contents. Chairote, et al. (2008) have reported that the production of monacolin K obtained from *Monascus* fermentation using glutinous rice (high amylopectin substrate) as substrate was found in the range of 3.13 – 33.54 mg/kg. Pattanagul, et al. (2008) also reported that mevinolin content of 14.97 – 25.03 mg/kg was observed in monascal adlay, as a substrate with high amylopectin (Lakkham, Wangsomnuk and Aromdee, 2009). Comparing their results with the results achieved in our work, that is from using the 2-step fermentation method, it has been demonstrated that applying a substrate with high amylopectin content increased monacolin K and citrinin contents. This showed that residual reducing sugars were more utilized to produce higher monacolin K and citrinin levels (Figure 15 and Table 17). Hongzhang, Yumei and Shuhua (2012) reported that the 2-step fermentation of sweet sorghum stalk to produce protein feed, a twice-inoculated microorganism fermentation process, led to increase utilization of residual reducing sugars after the end of fermentation than those of one step fermentation. In addition, Jin, Huang and Xiao (2008) indicated that the modified fermentation of *Mortierella alpine*, called 2-step fermentation, could improve arachidonic acid (ARA) production for commercial production of ARA. Concerning the citrinin content during the fermentation, although it was increased along with an increasing of monacolin K, it was still under the maximum allowance level in red fermented rice. Japan has issued an advisory limit of 200 μg/kg of citrinin in commercially agricultural products. The limit set by the Chinese Food and Drug Administration is 20 μg/kg, while the European Union has recommended a citrinin limit of 100 μg/kg (Shi and Pan, 2011).

The incubation temperature of 25 °C in this experiment was the optimal temperature inducing the mycelial growth, monacolin K and citrinin synthesis. A

report showed that temperatures between 25 to 35°C was good for production of red pigment and monacolin K by *M. purpureus* (Endo, 1979). This result was similar to the findings of Chiu, et al. (2006) that monacolin K and citrinin contents of koji produced using a modified Nagata type at temperature of 26°C was higher than those of 30 and 34°C. In this experiment, waxy corn supplemented with MSG as a nitrogen source seems to give a more satisfactory amount of monacolin K. Similarly, Pinthong, Bipong and Raviyan (2004) indicated that soybean milk used as a natural nitrogen source for monascal waxy rice production could improve monacolins synthesis. Therefore, the optimal temperature and nitrogen source may support the 2-step fermentation for enhancement of monacolin K level.

3. Antioxidant activities

Antioxidant activities of the conventional method and the 2-step fermentation are shown in Table 17.

$TEAC_{50}$ values of ABTS assay in the conventional method and the 2-step fermentation were 0.02 mmol Trolox/mL with no statistically significant difference ($P > 0.05$), showing that there was no difference in the inhibition of ABTS radical formation providing the same ability to quench free radical ($ABTS^{o+}$). No difference of the activity between the conventional and 2-step fermentation methods was observed. Monacolin K and/or total phenols (reported by Yang, et al., 2006) formed during the 2-step fermentation may be similar in molecular weight, numbers of aromatic ring, and nature of hydroxyl group substitutions as observed in the conventional fermentation (Hagerman, et al., 1998).

The IC_{50} value of DPPH assay observed in the conventional fermentation method was 0.04 mmol troxlox/mL, while 0.02 mmol trolox/mL was found constantly in the 2-step fermentation. The IC_{50} value of MWC derived from the 2-step fermentation method was about 2 times higher than that of the conventional fermentation method. The higher antioxidant potential of MWC from the 2-step fermentation method may be due to the presence of the glycone part from phenolic compounds which masks hydrogen donation property of MWC, indicating an important feature for free radical scavenging (Bhanja, et al., 2008).

Reducing capacities (IC_{50}) by FRAP assay in the conventional method was 0.09 mg extract/ mg, and 0.08 and 0.05 mg extract /mL were observed in the 2-

step fermentation after 4 and 8 days, respectively, and the value was stable until the end of the fermentation (Table 17). The lowest IC_{50} value (0.05 mg extract/mL) from the 2-step fermentation method was 1.8 times higher than the value from the conventional method. As well, Yang, et al. (2006) have reported that the EC_{50} values from monascal rice were between 6.48 and 22.86 mg extract/ mL. The results from these experiments clearly indicated that the lowest IC_{50} value of reducing capacities from the 2-step fermentation method possessed a higher reducing capacity of between 129 and 457 times compared with those of monascal rice. Tseng, et al. (2006) further reported that EC_{50} values in reducing power from monascal adlay were in the range of 3.34 - 5.15 mg extract/ mL. Yen and Chen (1995) suggested that the higher reducing capacity indicated that the antioxidant compounds are greater electron donors and can reduce more oxidized intermediates, so that they can act as better primary and secondary antioxidants.

The IC_{50} value of chelating ability on Fe^{2+} assay in the conventional method was 2.89 mg extract/ mL and the minimum IC_{50} value was 1.54 mg extract/ mL observed in 2-step fermentation after 8 days, showing the highest ability of chelating ferrous ion activity, which was about 1.87-time less than that of the conventional method. Compared to the previous work of Yang, et al. (2006), The IC_{50} values of MWC of both fermentation methods were less than those of monascal rice products (in the range of 3.78 - 7.09 mg extract/mL) , indicating that chelating abilities of MWC products were better than those of the monascal rice products. As shown in Table 17, the IC_{50} values of each antioxidant activities decreased with increased monacolin K contents in each time point during the fermentation. The presence of monacolin K indicated to enhance these antioxidant abilities. As well, the lower antioxidant activities presented in previous studies (Tseng, et al., 2006; Yang, et al., 2006) may be related to the less antioxidant contents, i.e., monacolin K, other antioxidants. A few studies reported that the antioxidants found in monascal rice contained total phenols, ascorbic acid, α-tocopherol, γ-tocopherol and δ-tocophenol and GABA (Sun, et al., 2008; Yang, et al., 2006). Aniya, Ohtani and Higa (2000) and Ho, et al.(2011) further reported that DMA, as a potential antioxidant, isolated from *Monascus*-fermented products showed better antioxidant ability than other antioxidants, including silymarin, curcumin, and resveratrol. These data suggest that

synergistic effects between monacolin K and other compounds are a significant reason to increase their antioxidant properties. Gordon (1990) suggested that synergistic effects between phenolic compounds and non-phenolic compounds were effective metal-chelating agents. Furthermore, the enzymatic hydrolysis of conjugated phenolics can lead to an increase of free phenolic content with enhanced antioxidant potential, improving the nutraceutical value of the products and can be exploited as value-added food products (Shetty and McCue, 2003). However, total phenolic content and DMA content as well as these synergistic effects were not determined in our study.

4. Pearson's correlation among antioxidant activities, pigment intensity, glucosamine content, monacolin K and citrinin contents and reducing sugar content

A correlation was done among antioxidant activities, pigment intensity, glucosamine, monacolin K and citrinin contents and reducing sugar content of both conventional and 2-step fermentations at day 8 (Table 18).

Significantly positive correlations between monacolin K contents and pigment intensity (r^2 = 0.984, $P \leq 0.05$), and between monacolin K and glucosamine contents (r^2 = 0.955, $P \leq 0.05$), and between IC_{50} FRAP and IC_{50} chelating Fe^{2+}(r^2= 0.992, $P \leq 0.01$) were obtained. These results showed that the *Monascus* pigment exhibited acting roles in both primary (FRAP assay) and secondary antioxidants (Chelating on Fe^{2+}assay) (Gordon, 1990) due to high relationship values of both the Ferric reducing/antioxidant power and chelating on Fe^{2+}. On the other hand, significantly negative correlations were found between monacolin K content and IC_{50} FRAP (r^2 = -0.996, $P \leq 0.01$), and between monacolin K and IC_{50} Chelating on Fe^{2+} (r^2= -0.979, $P \leq 0.05$). This could clearly support the correlations of the IC_{50} values of each antioxidant activities and monacolin K content in each time point during the fermentation. Moreover, an ideal negative correlation (r^2 = -1.000) was presented between pigment intensity and chelating ability on Fe^{2+}, indicating that the pigment intensity could be a good indicator of chelating ability on Fe^{2+},since Fe^{2+} is the most effective pro-oxidants in the food system (Yamaguchi, et al., 1988).

Table 18 Pearson's correlation coefficients of monacolin K, antioxidant activities (ABTS, DPPH, FRAP and chelating ability on Fe^{2+}), pigment intensity, glucosamine and citrinin in MWC via conventional method and 2-step fermentation

	Monacolin K	Citrinin	ABTS	DPPH	FRAP	Chelate Fe^{2+}	Pigment	Glucosamine
Citrinin	0.993**							
ABTS	-0.482	-0.374						
DPPH	-0.661	-0.750	0.333					
FRAP	-0.996**	-0.999**	0.404	0.727				
Chelate Fe^{2+}	-0.979*	-0.996**	0.293	0.803	0.992**			
Pigment	0.984*	0.998**	-0.319	-0.787	-0.995**	-1.000**		
Glucosamine	0.955*	0.984*	-0.201	-0.856	-0.977*	-0.995**	0.992**	
Reducing sugar	-0.700	-0.781	-0.288	0.999**	0.752	0.831	-0.816	-0.88

*Correlation is significant at the 0.05 level (2-tailed)

**Correlation is significant at the 0.01 level (2-tailed)

Study on extraction optimization of monacolin K, antioxidant activity, pigment and citrinin content from MWC produced by 2-step fermentation by using RSM

1. Fitting the models

Monacolin K content, DPPH• scavenging ability, Chelating ability on Fe^{2+}, pigment intensity and citrinin content in the pigment extract of MWC after ultrasonic-assisted extraction (UAE) and shaking extraction with different ethanol concentrations and temperatures are shown in Tables 19 and 20, respectively.

Both *F*-test and *P*-value statistical parameter are used to confirm the significance of factors studied. The obtained results from UAE and shaking method demonstrated that the extraction was more significantly affected by ethanol concentration ($P \leq 0.05$) presented in Tables 21 and 22. The larger the regression coefficient in a model with significant *P*-value indicates a more significant effect on the respective response variables (Yang, Liu, and Gao, 2009). The

ANOVA of the regression model demonstrated that each model is highly significant due to the evident from the calculated F values and a very low probability value ($P > F = < 0.00$). The results indicated that ethanol concentration had statistically significant on all responses ($P \leq 0.05$). Higher percentages of ethanol concentration promoted increase in UAE and shaking extraction for monacolin K and citrinin contents, pigment intensity and both antioxidant activities. However, the ethanol concentration reached 90.17 % affected those decreased responses for both extraction methods. The effect of temperature was not statically significant ($P > 0.05$) on all responses, which showing that only 35.86°C was enough for both extraction methods. This was agreement with Carvalho, et al. (2007), who reported that 2, 22, 32, 39 and 58°C did not significantly affect extraction of *Monascus* pigment. Dufosse, et al. (2005) suggested that *Monascus*-pigments coordination compounds containing pigments and monacolin K would be destroyed when the extraction temperature was suboptimal. The interactions between % ethanol and temperature were statically significant at a 95% confidence level except monacolin K for UAE and shaking methods and except Chelating ability on Fe^{2+} for shaking method. The fit of all models is checked by the coefficient of determination r^2. The r^2 values of monacolin K, pigment, DPPH free radical scavenging ability, Chelating ability on Fe^{2+} and citrinin for UAE were calculated to be 0.924, 0.994, 0.962, 0.985 and 0.973, respectively, and for shaking method were calculated to be 0.985, 0.989, 0.967, 0.949 and 0.966, respectively. They indicated that not less than 92.40 and 94.90 % of the variability in the response could be explained by the models for UAE and shaking methods, respectively.

Table 19 Experimental design and results of the CCD with observed experimental data from UAE

	Independent values		Dependent values (Responses)				
Run	Ethanol concentration X_1 (%)	Temperature X_2 (%)	Y_1	Y_2	Y_3	Y_4	Y_5
1	54.82	50	250.21	1.53	61.34	2,600	4.22
2	60	40	250.97	1.5	63.35	2,400	5.45
3	60	60	267.87	1.57	74.44	3,100	8.77
4	72.5	35.86	265.56	1.6	72	2,967	7.45
5	72.5	64.14	258.66	1.55	63.43	2,844	5.77
6	85	40	249.67	1.47	56.34	2,231	3.54
7	85	60	220.56	0.14	50	1,650	2.33
8	90.18	50	100.33	0.04	49.35	978	1.08
9	72.5	50	374.00	2.60	95.44	4,050	11.80
10	72.5	50	354.12	2.66	95.89	4,010	12.88
11	72.5	50	303.00	2.55	96.19	4,022	11.78
12	72.5	50	345.00	2.78	97.00	4,064	11.99
13	72.5	50	355.00	2.31	95.22	4,013	12.45

Note: Y_1 : Monacolin K content (mg/kg dry weight); Y_2: DPPH• scavenging ability (mmol Trolox/mL); Y_3: Chelating ability on Fe^{2+} (%); Y_4: Pigment intensity (unit/g dry weight); Y_5: Citrinin content (µg/kg dry weight)

Table 20 Experimental design and results of the CCD with observed experimental data for shaking extraction

Run	Independent values		Dependent values (Responses)				
	Ethanol concentration X_1 (%)	Temperature X_2 (%)	Y_1	Y_2	Y_3	Y_4	Y_5
1	54.82	50	187.11	0.31	55	1,687	3.89
2	60	40	172.33	0.3	50	1,420	2.23
3	60	60	200.23	0.39	68	2,100	7.55
4	72.5	35.86	193.18	0.38	67	1,927	6.23
5	72.5	64.14	186.45	0.33	60	1,824	5.55
6	85	40	152.39	0.27	20	1,234	2
7	85	60	144.32	0.04	15	650	1.11
8	90.18	50	96.31	0.01	13	538	1.06
9	72.5	50	257.32	0.44	76	2,400	10.56
10	72.5	50	259.67	0.42	77	2,530	11.37
11	72.5	50	253.98	0.48	79	2,520	9.96
12	72.5	50	255.45	0.52	75	2,564	11.16
13	72.5	50	260.68	0.41	75	2,489	10.36

Note: Y_1 : Monacolin K content (mg/kg dry weight); Y_2: DPPH• scavenging ability (mmol Trolox/mL); Y_3: Chelating ability on Fe^{2+} (%); Y_4: Pigment intensity (unit/g dry weight); Y_5: Citrinin content (µg/kg dry weight)

Table 21 Regression coefficients for UAE for pigment intensity, monacolin K and citrinin contents, DPPH free radical scavenging ability and chelating ability on Fe^{2+}

Factor	Regression coefficient	Std. err	*F*-value	*P*-value
Monacolin K content				
Mean	346.20	11.91	17.37	< 0.00*
% ethanol (A)	-32.57	9.42	11.95	0.01*
Temperature (B)	-2.75	9.42	0.08	0.77
A^2	-78.32	10.1	66.24	< 0.00*
B^2	-34.90	10.1	14.75	< 0.00*
AB	-11.50	13.32	0.74	0.51
Pigment intensity				
Mean	4010	43.96	250.1	< 0.00*
% ethanol (A)	-489.10	34.76	197.98	< 0.00*
Temperature (B)	-6.86	34.76	0.03	0.84
A^2	-1111	37.27	888.3	< 0.00*
B^2	-552.75	37.27	219.88	< 0.00*
AB	-320.25	49.15	42.44	< 0.00*
DPPH free radical scavenging ability				
Mean	2.66	0.08	59.27	< 0.00*
% ethanol (A)	-0.44	0.06	48.54	< 0.00*
Temperature (B)	-0.16	0.06	6.75	0.07
A^2	-0.94	0.06	187.6	< 0.00*
B^2	-0.54	0.06	63.06	< 0.00*
AB	-0.35	0.09	14.95	< 0.00*
Chelating ability on Fe^{2+}				
Mean	95.89	1.33	94.65	< 0.00*
% ethanol (A)	-6.05	1.05	32.89	< 0.00*
Temperature (B)	-0.92	1.05	0.76	0.41
A^2	-20.39	1.13	325.04	< 0.00*
B^2	-14.21	1.13	157.8	< 0.00*
AB	-4.35	1.49	8.53	0.02*
Citrinin content				
Mean	12.18	0.40	51.57	< 0.00*
% ethanol (A)	-1.60	0.32	25.39	< 0.00*
Temperature (B)	-0.033	0.32	0.011	0.91
A^2	-4.67	0.34	188.14	< 0.00*
B^2	-2.69	0.34	62.36	< 0.00*
AB	-1.13	0.45	6.37	0.03*

*Significant factors $P \leq 0.05$

Table 22 Regression coefficients for shaking extraction for pigment intensity, monacolin K and citrinin contents, DPPH free radical scavenging ability and chelating ability on Fe^{2+}

Factor	Regression coefficient	Std. err	*F*-value	*P*-value
Monacolin K content				
Mean	257.42	3.74	95.27	< 0.00*
% ethanol (A)	-25.53	2.95	74.66	0.00*
Temperature (B)	1.29	2.96	0.19	0.67
A^2	-57.45	3.17	328.82	< 0.00*
B^2	-33.42	3.17	111.20	< 0.00*
AB	-8.99	4.18	4.63	0.07
Pigment intensity				
Mean	2500	42.69	129.23	< 0.00*
% ethanol (A)	-407.59	33.75	145.89	< 0.00*
Temperature (B)	-6.21	33.75	0.034	0.85
A^2	-729.62	36.18	406.62	< 0.00*
B^2	-348.44	36.20	92.65	< 0.00*
AB	-316.00	47.73	43.84	< 0.00*
DPPH free radical scavenging ability				
Mean	0.45	0.016	41.26	< 0.00*
% ethanol (A)	-0.10	0.013	61.01	< 0.00*
Temperature (B)	-0.026	0.013	4.19	0.08
A^2	-0.15	0.014	116.33	< 0.00*
B^2	-0.051	0.014	13.86	< 0.00*
AB	-0.080	0.018	19.32	< 0.00*
Chelating ability on Fe^{2+}				
Mean	76.36	3.23	26.22	< 0.00*
% ethanol (A)	-17.81	2.55	48.61	< 0.00*
Temperature (B)	0.42	2.56	0.027	0.87
A^2	-23.72	2.74	75.01	< 0.00*
B^2	-9.08	2.74	10.95	0.01*
AB	-5.84	3.61	2.61	0.15
Citrinin content				
Mean	10.56	0.43	39.95	< 0.00*
% ethanol (A)	-1.33	0.34	15.65	< 0.00*
Temperature (B)	0.43	0.34	1.65	0.23
A^2	-4.28	0.36	140.23	< 0.00*
B^2	-2.58	0.36	50.72	<0.00*
AB	-1.55	0.48	10.60	< 0.01*

*Significant factors $P \leq 0.05$

2. RSM of monacolin K content

Ethanol concentration has an effect on monacolin K content with good regression coefficients for UAE (r^2 = 0.924) and shaking methods (r^2 = 0.985). The relationship between the monacolin K and the UAE and shaking method parameters are shown in Equation (5) and (6), respectively as follows:

$$\text{Monacolin K} = 346.20 - 32.57 X_1 - 2.75 X_2 - 78.32 X_1^2 - 34.90 X_2^2 - 11.50 X_1X_2 \quad (5)$$

$$\text{Monacolin K} = 257.42 - 25.52 X_1 + 1.29 X_2 - 57.45 X_1^2 - 33.42 X_2^2 - 8.99 X_1X_2 \quad (6)$$

Ethanol concentration only had significant ($P \leq 0.05$) and no significant interaction effect between ethanol concentration and temperature was observed in UAE and shaking methods ($P > 0.05$) (Tables 21 and 22). Figures 16 and 17 illustrate response surface plot of ethanol concentration and temperature on monacolin K content. The optimal ethanol concentration for both extraction methods was in a range of 66.25 to 78.75 %. However, higher and lower ethanol concentration than this range, monacolin K decreased with temperature. At 72.50 % ethanol concentration, temperature was increased from 45 – 55°C, higher monacolin K content was detected before showing signs of decreasing. In order to obtain high monacolin K, the ethanol concentration plays a more critical role in temperature reduction. Therefore, high monacolin K could be obtained through both extraction methods at moderate temperature (40 – 55°C) with ethanol concentration of 66.25 – 78.75 %. Even though pattern of the responses for both extraction methods were the same way, the results from UAE was higher than those from shaking method (Tables 19 and 20). The means of monacolin K content at the central point of both extraction methods are much higher than those reported by Su, Wang, and Lin (2003), Manzoni, et al. (1999) and Yim, et al. (2012). Carvalho, et al. (2007) reported that higher extract content was obtained as appropriate ethanol concentration was compatible polality, affecting the properties of the solvent e.g. density and dielectric of solutes and solvents (Deng, et al., 2010). Use of extraction heating can improve the mass transfer because of

resulting from the increase solubility of monacolin K and the decrease viscosity of the solvent (Deng, et al., 2010). Moreover, ultrasonic wave increases more effective extraction for monacolin K because cavitation phenomenon generates the number of acoustic cavitation bubbles created (Yang, et al., 2005). However, shaking effectiveness is not enough for the extraction compared to UAE for destroying cell wall of the fungus so that leaked monacolin K is lesser from the cell.

The maximal monacolin K contents for UAE and shaking method predicted by response surface analysis (RSA) were 349.59 and 260.34 mg/kg dry weigh, respectively, which the optimum ethanol concentration and extraction temperature for UAE and shaking methods were 69.91 %/49.95°C and 69.88 %/50.50°C, respectively.

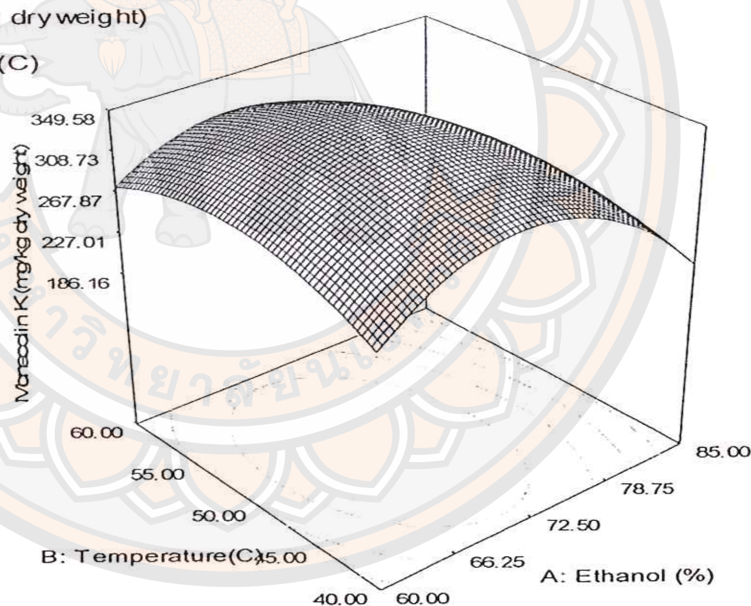


Figure 16 Response surface of monacolin K for UAE as a fraction of ethanol concentration (%) and temperature (°C)

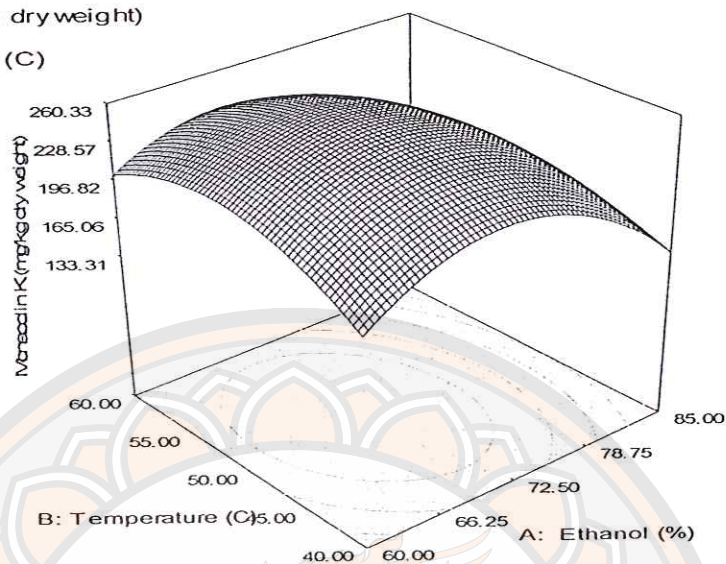


Figure 17 Response surface of monacolin K for shaking method as a fraction of ethanol concentration (%) and temperature (°C)

3. RSM of DPPH˙ scavenging ability and Chelating ability on Fe^{2+}

For UAE, The relationships between DPPH˙ scavenging ability and extraction parameter (r^2 = 0.962) and between chelating ability on Fe^{2+} and extraction parameter (r^2 = 0.985) are good regression coefficients, and Equations (7) and (8) show the relationship as follows:

$$DPPH = 2.66 - 0.44 X_1 - 0.16X_2 - 0.94 X_1^2 - 0.54 X_2^2 - 0.35 X_1X_2 \quad (7)$$

$$Chelate\ Fe^{2+} = 95.89 - 6.05 X_1 - 0.92X_2 - 20.39 X_1^2 - 14.21 X_2^2 - 4.35 X_1X_2 \quad (8)$$

For shaking method, r^2 values = 0.967 and 0.949 are the relationships between DPPH• scavenging ability and extraction parameter and between chelating ability on Fe^{2+} and extraction parameter, respectively, which are good regression

coefficients (Tables 21 and 22), and Equations (9) and (10) show the relationship as follows:

$$DPPH = 0.45 - 0.10X_1 - 0.026X_2 - 0.15X_1^2 - 0.051X_2^2 - 0.080X_1X_2 \quad (9)$$

$$\text{Chelate } Fe^{2+} = 76.36 - 17.81X_1 + 0.42X_2 - 23.72X_1^2 - 9.08X_2^2 - 5.84X_1X_2 \quad (10)$$

Ethanol concentration showed significant effect on $DPPH^{\bullet}$ scavenging ability and chelating ability on Fe^{2+} except temperature for both extraction methods, whereas significant interaction effect on DPPH• scavenging ability response but no effect on chelating ability on Fe^{2+} response was observed between extraction time and temperature ($P \leq 0.05$) (Tables 21 and 22). Both responses of both extraction methods had similar pattern of response surface plots. Figures 18, 19, 20 and 21 show the response surface plots of the effects of ethanol concentration and temperature on DPPH• scavenging ability and Chelating ability on Fe^{2+}, respectively, indicating that the four responses increased with the increase of ethanol concentration from 60 to 72.50 %, but they decreased with ethanol concentration beyond 72.50 % and temperature beyond 50°C. Likewise, it was noted that increasing extraction temperature from 40°C to 50°C affected higher both antioxidant activities of the extracts. From 50°C to 60°C, these responses of UAE fell slightly as increased extraction time but the responses from shaking method were not different. Extraction favors increased working temperature, thus enhancing both the solubility of solute and the diffusion coefficient, but beyond the antioxidants could be denatured (Yim, et al., 2012). Compound stability may be affected due to chemical and enzymatic degradation and/or lost by thermal decomposition. This may be the main mechanism for less stability in DPPH• scavenging ability and Chelating ability on Fe^{2+} as reported by Kiassos, et al. (2009). At this point, if temperature is suboptimal concentration, it will cause their activities reduced. From the results, ultrasonic wave can efficiently destroy the cell wall of fungus so that important substances, i.e., monacolin K, α-aminobutyric acid (GABA), phenolic compounds, can

be better released from the cell, which affecting higher antioxidant activities. Yang, et al. (2005) reported that cavitation mechanism obtained from ultrasonic wave has been used as a stimulating tool to enhance monacolin K content. However, total phenolic and GABA contents were not determined in our study. Comparing UAE and shaking methods, the results of DPPH• scavenging ability and Chelating ability on Fe^{2+} for UAE were higher than those of shaking method which their means at the center point were 2.126 mmol Trolox/mL and 19.59 % higher than those from shaking method. This indicated that the results of from both antioxidant assays increased with monacolin K contents

The optimal predicted DPPH• scavenging ability and chelating ability on Fe^{2+} by RSA for UAE were 2.64 mmol Trolox/mL (69.60 % of ethanol/49.15◦C of extraction temperature) and 96.40 % (70.66 % of ethanol/49.91◦C of extraction temperature), respectively, and for shaking methods were 0.47 mmol Trolox/mL (68.24 % of ethanol/50.08°C of extraction temperature) and 79.85 % (67.00 % of ethanol/51.65°C of extraction temperature), respectively.

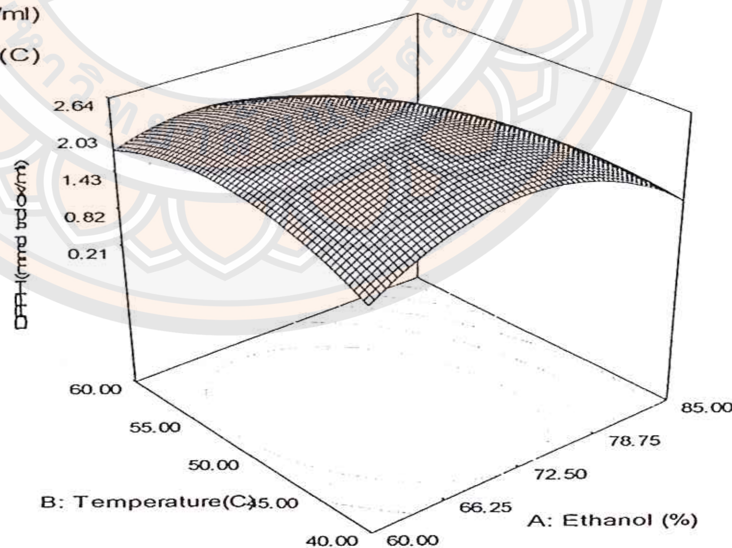


Figure 18 Response surface of DPPH free radical scavenging ability for UAE as a fraction of ethanol concentration (%) and temperature (°C)

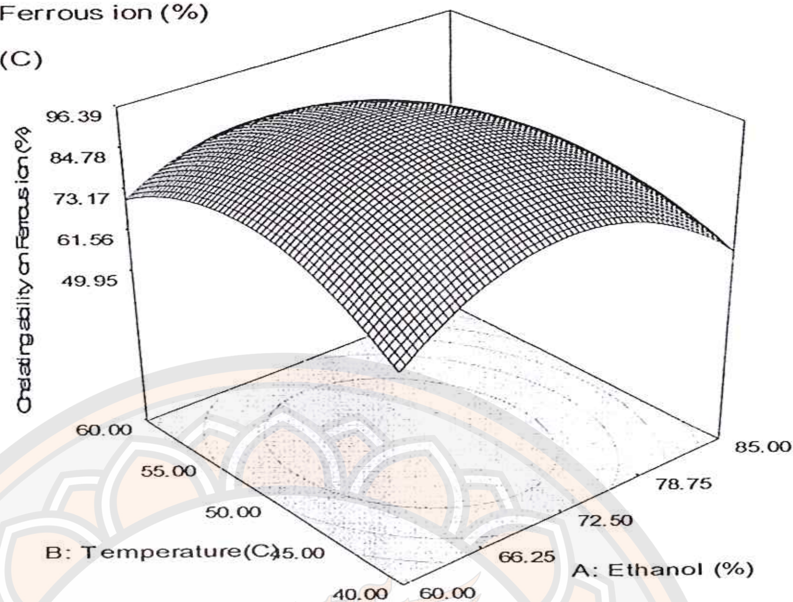


Figure 19 Response surface of chelating ability on Fe^{2+} for UAE as a fraction of ethanol concentration (%) and temperature (°C)

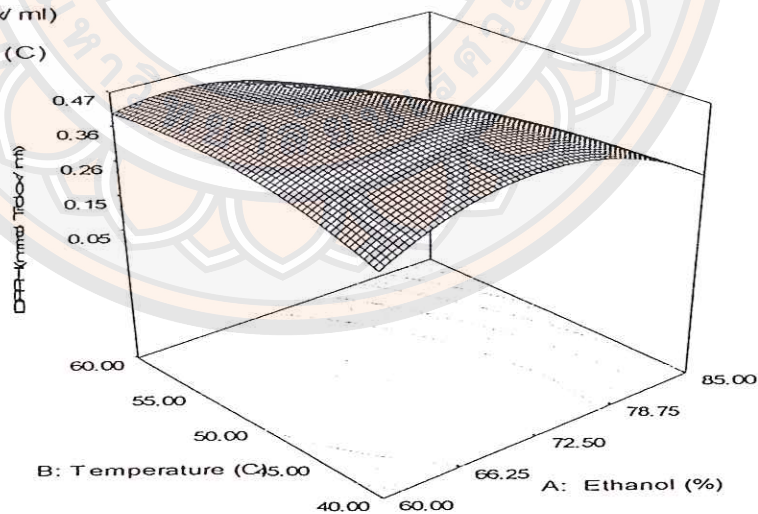


Figure 20 Response surface of DPPH free radical scavenging ability for shaking method as a fraction of ethanol concentration (%) and temperature (°C)

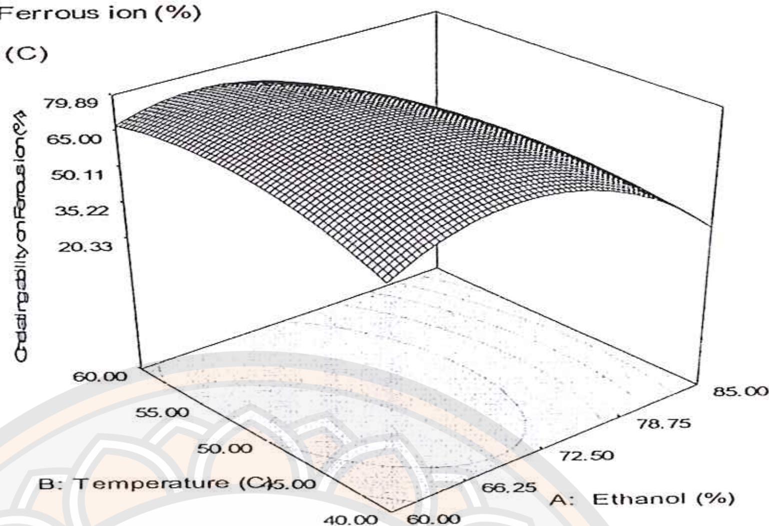


Figure 21 Response surface of chelating ability on Fe^{2+} for shaking method as a fraction of ethanol concentration (%) and temperature (°C)

4. RSM of pigment intensity

The relationships between pigment intensity and extraction parameter (r^2 = 0.994) for UAE and between pigment intensity and extraction parameter (r^2 = 0.989) for shaking methods have good regression coefficients and Equaltions (11) and (12) show the relationship of parameters for UAE and shaking methods respectively as follows:

$$\text{Pigment} = 4{,}010.00 - 489.10\,X_1 - 6.86X_2 - 1{,}111.00\,X_1^2 - 552.75\,X_2^2 - 320.25\,X_1X_2 \quad (11)$$

$$\text{Pigment} = 2{,}500.60 - 407.59\,X_1 - 6.21X_2 - 729.62\,X_1^2 - 348.44\,X_2^2 - 316.00\,X_1X_2 \quad (12)$$

From the results of pigment intensity between both extraction methods, ethanol concentration had significant ($P \leq 0.05$) but temperture did not affect pigment intensity, which significant interaction effect ($P \leq 0.05$) was found between ethanol

concentration and extraction temperature. Figures 22 and 23 show response surface plots of pigment intensity which indicating that it was drastically increased with temperature 40°C - 50°C of temperature and 60.00 to 72.50 % of ethanol concentration. At 72.50 % of ethanol concentration and 50°C of extraction temperature, the highest pigment intensity was observed in UAE and shaking methods. The results were disagreement with Carvalho, et al. (2007), who indicated maximum *Monascus* pigment obtained when using of 60% ethanol as a solvent. However, differences in the polarity of the extracting solvents could result in a wide variation in the pigment extraction (Carvalho, et al., 2007). Obviously, pattern of the responses for UAE and shaking processes were similar but the results of UAE were higher than that shaking method which the mean at the center point for UAE was 37.97 % higher than that for shaking method.

The optimal extraction condition for UAE was predicted to be ethanol concentration of 69.45 % and temperature of 49.78°C and for shaking methods was predicted to be ethanol concentration of 68.67 and temperature 51.30°C. The optimal pigment extraction predicted by RSA for UAE and shaking methods were 4,082.40 and 2,562.88 unit/g dry weight, respectively.

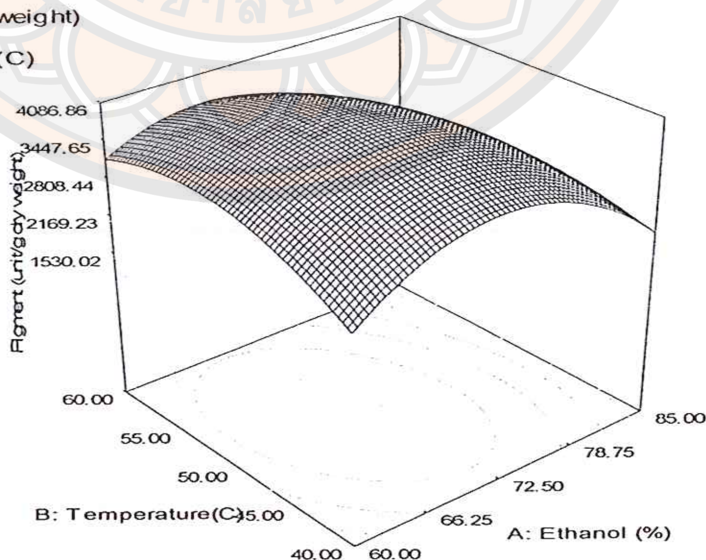


Figure 22 Response surface of pigment for UAE as a fraction of ethanol

concentration (%) and temperature (°C)

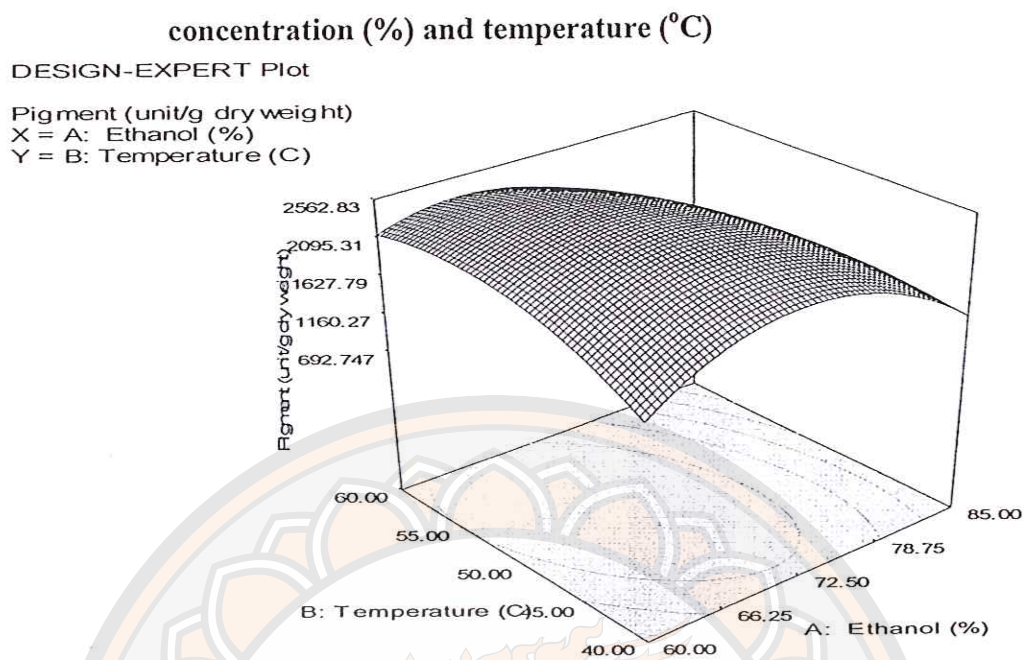


Figure 23 Response surface of pigment for shaking method as a fraction of ethanol concentration (%) and temperature (°C)

5. RSM of citrinin content

The coefficients of determination (r^2) for UAE and shaking methods for citrinin extraction were calculated to be 0.973 and 0.966, respectively and Equations (13) and (14) showed the relationship between citrinin content and extraction parameters of ethanol concentration and temperature.

$$\text{Citrinin content} = 12.18 - 1.60 X_1 - 0.033X_2 - 4.67X_1^2 - 2.69X_2^2 - 1.13X_1X_2 \quad (13)$$

$$\text{Citrinin content} = 10.56 - 1.33 X_1 - 0.43X_2 - 4.28X_1^2 - 2.58X_2^2 - 1.55X_1X_2 \quad (14)$$

The significant effect was ethanol concentration but temperature was not significant, and significant interaction between ethanol concentration and temperature was found (Tables 21 and 22). Figures 24 and 25 show response surface plots of citrinin contents of each extraction methods which they gradually increased from 66.25 % to 72.50 % of ethanol concentration, and beyond 72.50 % of ethanol

concentration, they were descended. Extraction temperature between 40°C and 50°C led to increase citrinin contents and beyond 50°C affected their contents were slightly decreased since the applied temperatures were suboptimal. Therefore, to obtain low citrinin content, ethanol concentration and extraction temperature suggested were higher than 72.5 % and 50°C, respectively. The increased level of ethanol concentration and extraction temperature could enhance both the solubility of solute and the diffusion coefficient, but beyond a certain extend significant substances could be denatured (Yim et al., 2012). From these figures, pattern of the responses obtained from UAE and shaking methods is similar. However, the values of UAE was higher than those of shaking method and the mean of the citrinin content at central point for UAE was 0.86 μg/kg dry weight higher than that for shaking method.

The minimal citrinin contents predicted by response surface analysis (RSA) for UAE and shaking methods were 2.06 and 1.25 μg/kg dry weight, respectively, which their contents of both methods were obtained from 85.00 % ethanol and 60°C of temperature.

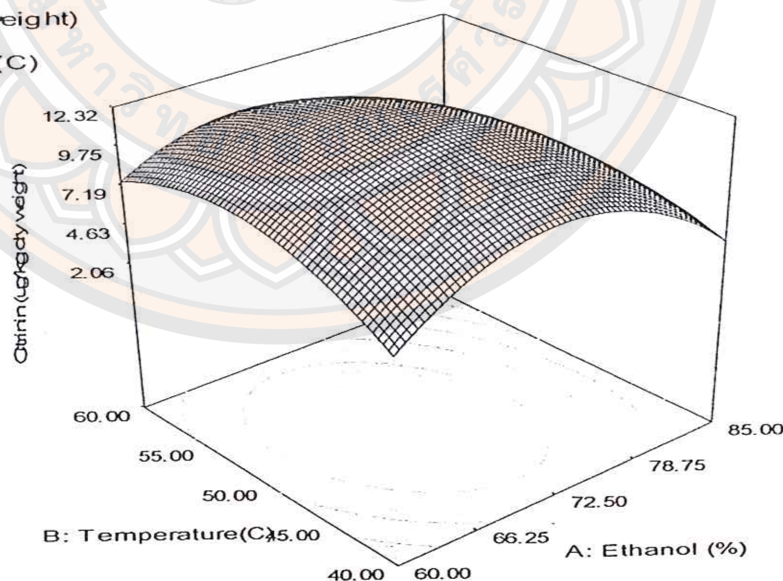


Figure 24 Response surface of citrinin for UAE as a fraction of ethanol concentration (%) and temperature (°C)

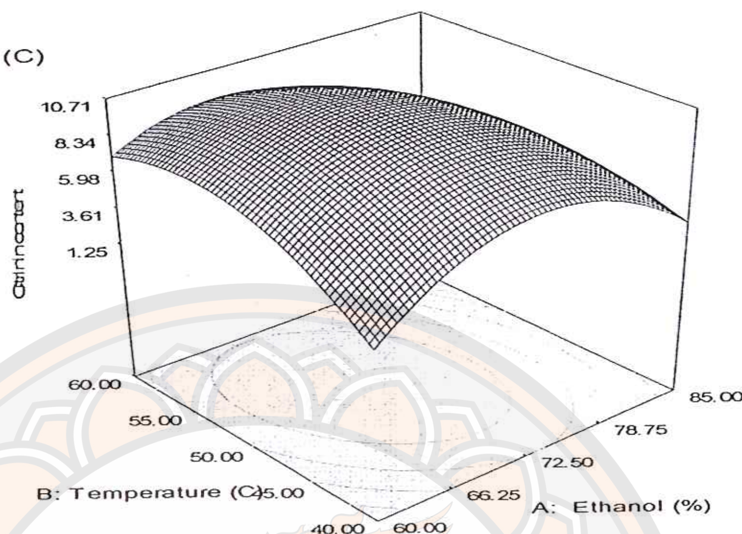


Figure 25 Response surface of citrinin for shaking method as a fraction of ethanol concentration (%) and temperature (°C)

6. Verification of predictive model

Yim, et al. (2012) suggested that verification step was done to ensure that the predicted results were not biased towards the practical value with the objective of each response to obtain maximum yield using deduced optimal condition. Tables 23 and 24 indicate five individual verification experiments for Y_1 : Monacolin K content (mg/kg dry weight); Y_2: DPPH• scavenging ability (mmol Trolox/mL); Y_3: Chelating ability on Fe^{2+} (%); Y_4: Pigment intensity (unit/g dry weight); Y_5: Citrinin content (µg/kg dry weight) were carried out under respective optimal extraction conditions within the experimental range. The experimental values of monacolin K content, DPPH• scavenging ability, Chelating on Fe^{2+}, pigment intensity and citrinin content for UAE were 348.72 mg/kg dry weight, 2.65 mmol Trolox/mL, 96.76 %, 4,072.45 unit/g dry weight and 2.04 µg/kg dry weight, respectively, and for shaking method were 258.72 mg/kg dry weight, 0.45 mmol Trolox/mL, 76.76 %, 2,542.45 unit/g dry weight and 1.24 µg/kg dry weight, respectively. The CV values for UAE were between 0.06 and 0.97 % but for shaking were between 0.62 and 4.25 % . It indicated that the results of experiment from

verification of predicted parameters for UAE and shaking methods gave less CV, showing that the predicted parameters were appropreate. CV value is calculated with the difference between the predicted and experimental values.

Table 23 Experimental data of the verification of predicted UAE parameters

Dependent variables[a]	Ethanol concentration (%)	Temperature (°C)	Predic value	Experimental value[b]	%Different (CV)
Y_1	69.91	49.95	349.59	348.72 ± 5.23	0.25
Y_2	69.60	49.15	2.64	2.65 ± 0.03	0.37
Y_3	70.66	49.91	96.40	96.76 ± 7.76	0.38
Y_4	69.45	49.78	4,082.40	4,072.45 ± 10. 64	0.06
Y_5	85.00	60.00	2.06	2.04 ± 0.18	0.97

Note: Y_1 : Monacolin K content (mg/kg dry weight); Y_2: DPPH• scavenging ability (mmol Trolox/mL); Y_3: Chelating ability on Fe^{2+} (%); Y_4: Pigment intensity (unit/g dry weight); Y_5: Citrinin content (μg/kg dry weight) [b] Results were expressed as mean ± standard deviation (*n* = 3)

Table 24 Experimental data of the verification of predicted shaking method parameters

Dependent variables[a]	Ethanol concentration (%)	Temperature (°C)	Predic value	Experimental value[b]	%Different (CV)
Y_1	69.88	50.50	260.34	258.72 ± 3.23	0.62
Y_2	68.24	50.08	0.47	0.45 ± 0.01	4.25
Y_3	67.00	51.65	79.85	76.76 ± 4.26	3.86
Y_4	68.67	51.30	2562.88	2,542.45 ± 11. 34	0.79
Y_5	85.00	60.00	1.25	1.24 ± 0.02	0.80

Study on antioxidant stability of *Monascus* pigment from MWC via *in vitro* digestion

1. MWC after *in vitro* gastric digestion

This research is performed a simulated stomach digestion of MWC. The determination of stability of monacolin K, DPPH free radical scavenging ability, chelating ability on Fe^{2+} and citrinin under the incubation condition was observed. Mean monacolin K and citrinin contents, DPPH free radical scavenging ability and chelating ability on Fe^{2+} of original MWC and MWC after *in vitro* gastric digestion studied are given in Tables 25. Gastric-simulated digestion of MWC with pepsin-HCl (pH 2.0) for 2 h had a substantial effect on monacolin K and citrinin contents and both antioxidant activities in MWC. The levels of monacolin K and citrinin in MWC after the stomach treatment were slightly increased in the range of 1.35 – 8.69 %. The increased percentages of MWC after the *in vitro* gastric digestion were in the descending order of citrinin > monacolin K. This indicated the high stability under *in vitro* stomach condition of monacolin K and citrinin. Our results on the stability of monacolin K and citrinin under the *in vitro* acidic condition are in agreement with those reported for raspberry (McDougall, et al., 2005) or pomegranate (Perez-Vicente, et al., 2002) anthocyanins. We and others (Perez-Vicente, Gil-Izquierdo, and Garcia-Viguera, 2002) reported a small increase in the antioxidants after *in vitro* stomach digestion. However, our results were disagreement with Bermudez-Soto, Tomas-Barberan, and Garcıa-Conesa. (2007), which showed that chokeberry juice after gastric-simulated digestion had a decreased effect on any of the major phenolic compounds in chokeberry juice.

DPPH free radical scavenging ability in digestive fraction of MWC was increased 33.33 % and chelating ability on Fe^{2+} was increased 13.47 %. It was possible that the antioxidant activities of MWC could be further enhanced by gastrointestinal pH during colonic digestive processes (Baublis, et al., 2000). Rufian-Henares and Delgado-Andrade (2009) reported that after the digestion process the overall antioxidant activity statistically increased up to four times. Liyana-Pathirana and Shahidi (2005) found that, after a simulated gastric conditions, the antioxidant activity of flour measured by the DPPH method increased from 1.85 to 12.30 mmol trolox/kg

of sample. Baublis, et al. (2000) demonstrated that gastrointestinal pH conditions caused a dramatic increase in antioxidant activities of aqueous extracts, suggesting that acid condition causes alterations in antioxidant activities and compositions as well as concentration of water-soluble antioxidants. These studies stated that it could be possible that acid hydrolysis influence monacolin K level by causing the release of monacolin K from MWC. Therefore, higher antioxidant activities indicated that the digestive process had some activity on pigment and monacolin K.

Table 25 Mean monacolin K and citrinin contents, DPPH free radical scavenging ability and chelating ability on Fe^{2+} in digestive fraction after gastric digestion of MWC

Compounds and antioxidant activities	Raw material	Digested MWC	% Increase*
Monacolin K content (mg/kg dry weight)	79.52 ± 1[a]	80.61 ± 9[a]	1.35
Citrinin content (μg/kg dry weight)	10.50 ± 0.05[a]	11.50 + 1.28[a]	8.69
DPPH (IC_{50} mmol Trolox/mL)	0.03 ± 0.007[a]	0.02 ± 0.004[a]	33.33
Chelating ability on Fe^{2+} (%)	82.20 ± 4.90[a]	95.67 ± 9.03[b]	13.47

Note: *Percentage of total increase at the end of the digestion process, [a,b]Different letters within the same column indicate statistical differences (one-way ANOVA and Duncan test, $P \leq 0.05$). Values are mean ± S.D of triplicate determinations

2. Soluble and insoluble MWC after *in vitro* gastric and pancreatic digestions

Following the stomach incubation, soluble and insoluble MWC was then subjected to a 2 h digestion under conditions mimicking those of the duodenum. Results are also shown in Table 26. Overall, monacolin K and citrinin contents and DPPH free radical scavenging ability and chelating ability on Fe^{2+} between soluble and insoluble MWC were between 14.69 and 76.92 % which these values were lost

during treatment with pancreatin. Rufian-Henares and Delgado-Andrade (2009) confirmed that dietary antioxidants were highly sensitive to the mild alkaline conditions in the small intestine and suggest that, during digestion in the duodenum, a proportion of the compounds may be transformed into different structural forms with different chemical properties. When examining the effects of *in vitro* digestion on antioxidants, light and O_2 are two important factors to consider as they can alter the structures and properties of monacolin K, due to oxidative degradation (Jorgensen et al., 2004) and polymerization reactions (Talcott and Howard, 1999). The antioxidant activities and monacolin K and citrinin contents in insoluble fractions from MWC were less than those in soluble factions in MWC. It is possible that acid and enzymatic hydrolysis influenced soluble concentration of monacolin K and phenolic compounds by causing the release of them from MWC which this experiment was agreed with Ohta, et al. (1994) reported the increase between the antioxidants solubility from food matrix by alkaline hydrolysis.

Table 26 Mean monacolin K and citrinin contents, DPPH free radical scavenging ability and chelating ability on Fe^{2+} in soluble and insoluble fractions after gastric and pancreatic digestions of MWC

Compounds and antioxidant activities	Raw material	Soluble MWC	Insoluble MWC	% loss*
Monacolin K content (mg/kg dry weight)	79.52 ± 6.23^{b}	54.61 ± 6.00^{a}	13.23 ± 5.43^{a}	14.69
Citrinin content (ug/kg dry weight)	10.50 ± 0.01^{b}	5.50 ± 1.20^{a}	1.45 ± 0.00^{a}	64.54
DPPH(IC_{50} mmol Trolox/mL)	0.03 ± 0.01^{a}	0.04 ± 0.00^{a}	0.09 ± 0.00^{b}	76.92
Chelating ability on Fe^{2+} (%)	82.20 ± 3.00^{c}	65.56 ± 5.67^{b}	2.43 ± 0.41^{a}	17.29

Note: *% Total digested MWC, ^{a,b}Different letters within the same column indicate statistical differences (one-way ANOVA and Duncan test, $P \leq 0.05$). Values are mean ± S.D of triplicate determinations

Chapter V

Conclusions

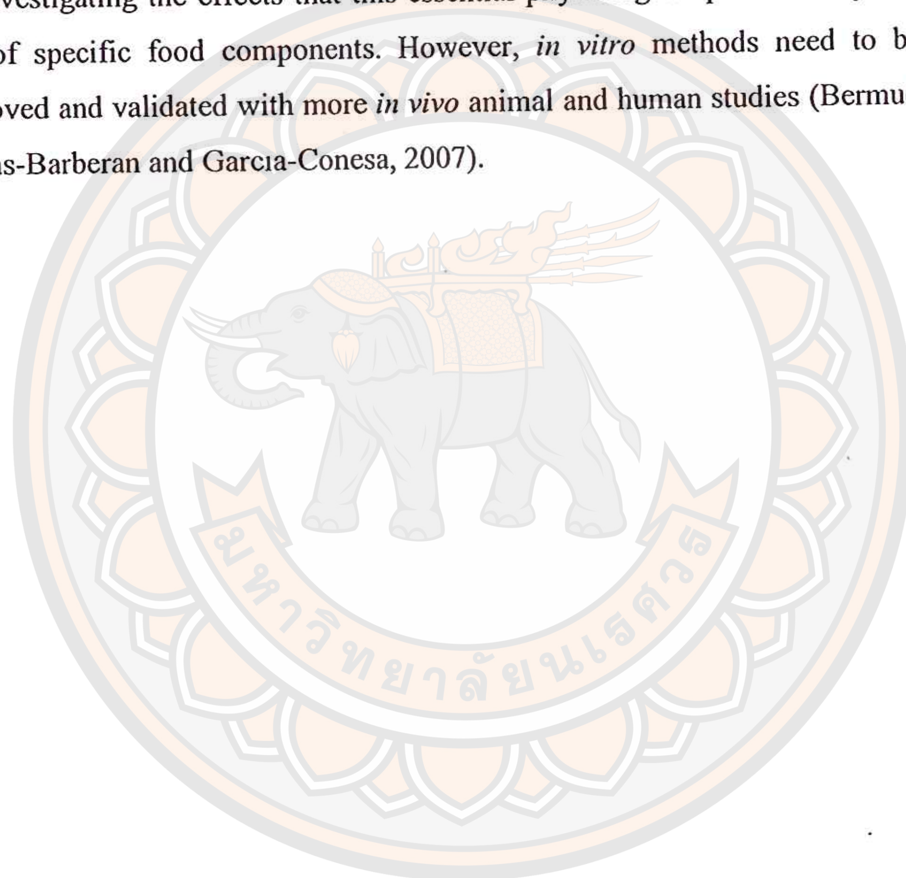
The MWC obtained from *M. purpureus* (TISTR 3090) supplemented with nitrogen presented high potential antioxidant activity. The increase of antioxidant activities and monacolin K was influenced by nitrogen sources and concentrations. The highest antioxidant activities and monacolin K values were found in monascal product supplemented with 12.08 % (w/w) MSG with the fermentation for 12 days. Furthermore, the citrinin contents in MWC were less than the maximum allowed level in red fermented rice legislated by the Chinese FDA. Pigment intensity could be a reliable indicator for monacolin K production and antioxidant activities.

The significant outcomes of the 2-step fermentation of MWC were higher monacolin K content, pigment intensity and antioxidant activity compared with the conventional step, which could be an effective utilisation of the rest reducing sugars from the conventional fermentation. After 2-step fermentation, the reducing sugar content was totally exhausted by *Monascus* on day 8, indicating the highest monacolin K content and pigment intensity as well as the maximum antioxidant activities. From the results of our study, the 2-step fermentation for the production of MWC could be an alternative process to enhance antioxidant activity and pigment production.

The optimization of UAE and shaking extraction conditions for MWC with maximum of monacolin K content, antioxidant activities and pigment intensity, but minimal citrinin content was carried out and adequately performed by using RSM. The values of monacolin K and citrinin contents and antioxidant activities from UAE were much higher than those from shaking method. Nevertheless, all response surface plots of UAE having the patterns were similar to shaking method. Comparing individual verification tables, the values from experimental and predicted values were very close.

For its stability after gastric digestion, digestive process allowed the increase of 1.35 % of monacolin K content, 8.69 % of citrinin content and DPPH free radical scavenging ability and chelating ability on Fe^{2+} of digested MWC increased in the

range of 13.47 - 33.33 %. After pancreatic digestion, it has been demonstrated that monacolin K and citrinin contents and DPPH free radical scavenging ability and chelating ability on Fe^{2+} decreased both soluble and insoluble MWC in the range of 14.69 - 76.92 %, indicating that a mimic pancreatic digestion causes these antioxidant activities reduced. With all the limitations of *in vitro* models, and all caution when interpreting the results derived from them, methods mimicking *in vivo* human conditions, such as those simulating the gastrointestinal digestion remain a useful tool for investigating the effects that this essential physiological process may have in the fate of specific food components. However, *in vitro* methods need to be further improved and validated with more *in vivo* animal and human studies (Bermudez-Soto, Tomas-Barberan and Garcıa-Conesa, 2007).



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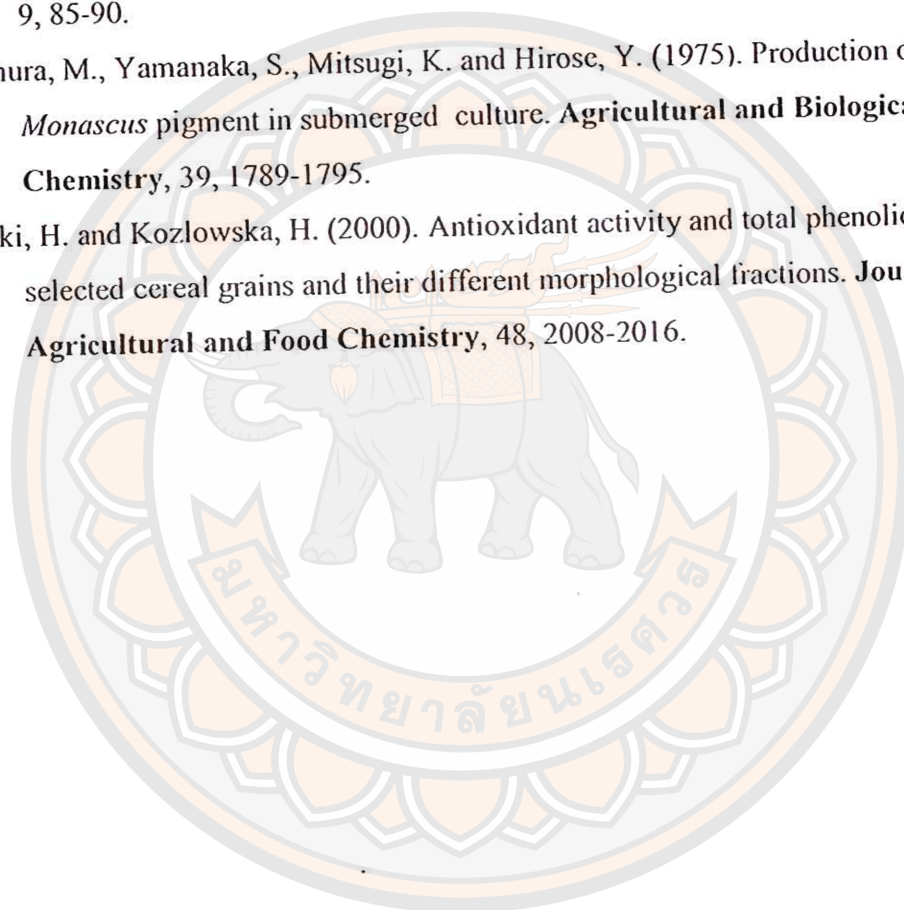

APPENDIX

Calculation of $TEAC_{50}$ and IC_{50}

Total antioxidant capacities of ABTS and DPPH assays showing $TEAC_{50}$ and IC_{50} values, respectively, were estimated as Trolox equivalents by interpolation to 50% inhibition while IC_{50} values of pigment extract for chelating ability on Fe^{2+} and FRAP assays were estimated as interpolation to 50% inhibition of extract concentration with the following calculation.

1. Absorbance of the mixture (oxidant and pigment extract 1-20 mg/mL) was expressed to decreased percentage of each extract compared to the blank.

2. Data of decreased percentage compared to extract concentration of each extract were used to calculate an equation of linear regression which indicates interpolation to 50% inhibition of radicals. The obtained concentration was reported in mg extract / mL for FRAP and chelating ability on Fe^{2+} assays.

3. Known extract concentration of interpolation to 50% inhibition of radicals was compared to absorbance of standard curve of ABTS and DPPH assays to report mmol Trolox / mL

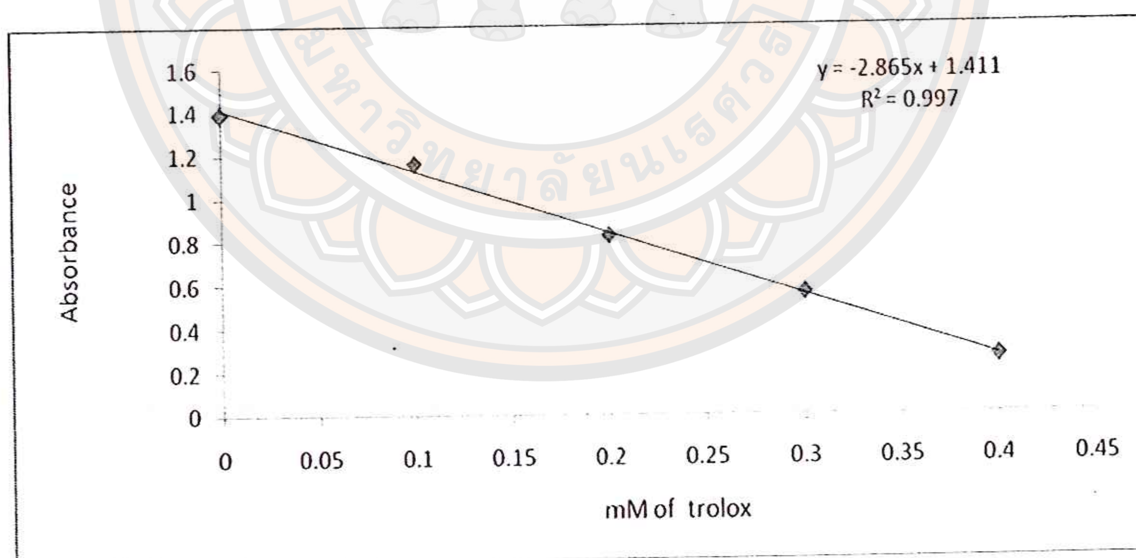

Figure 26 Standard curve of 0 – 0.4 mM of Trolox for ABTS assay

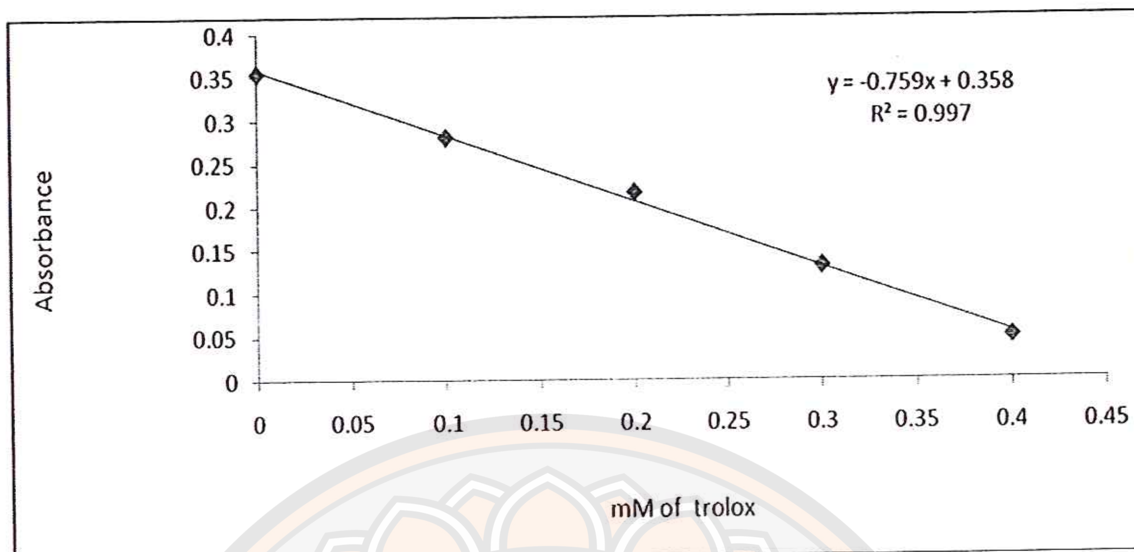

Figure 27 Standard curve of 0 – 0.4 mM of Trolox for DPPH assay

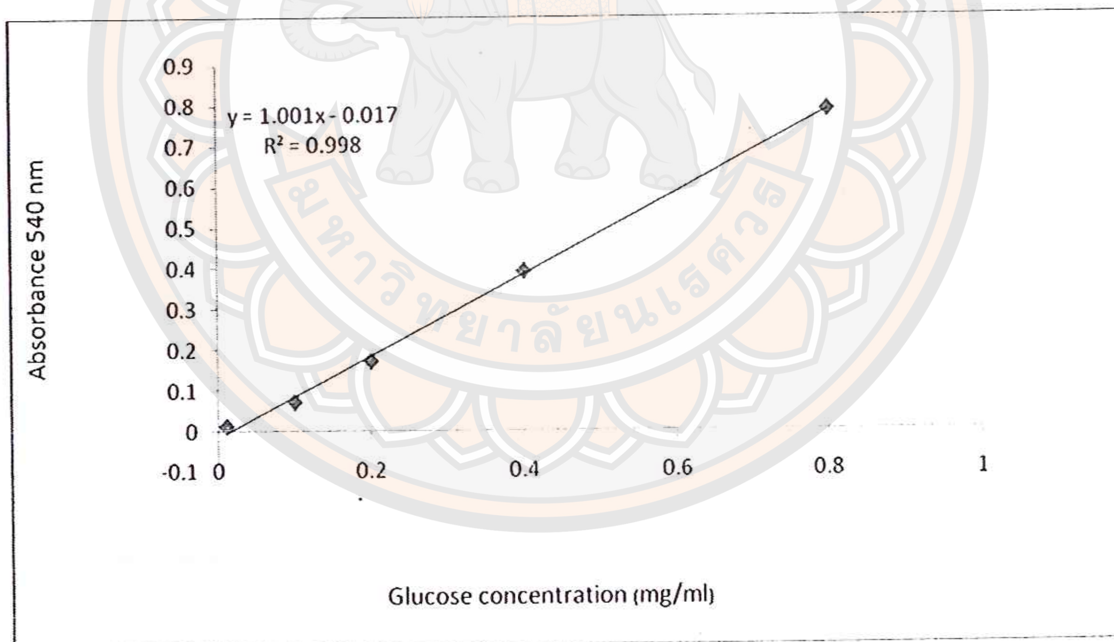

Figure 28 Standard curve of 0 - 0.8 mg/mL of glucose

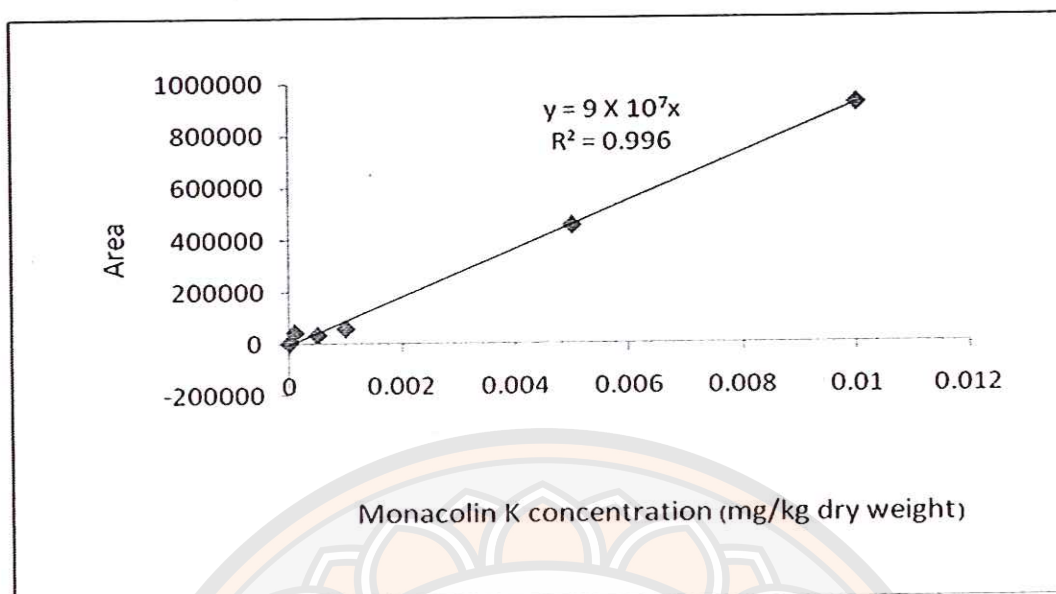

Figure 29 Standard curve of 0.0001 - 0.01 mg/mL of monacolin K

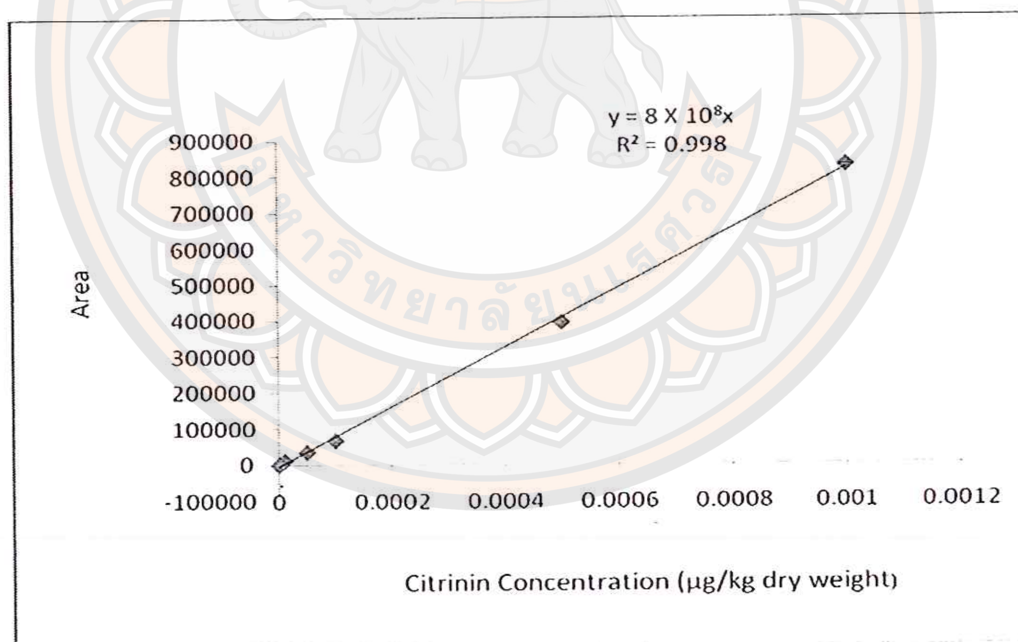

Figure 30 Standard curve of 0.001 -0.00001 µg of citrinin

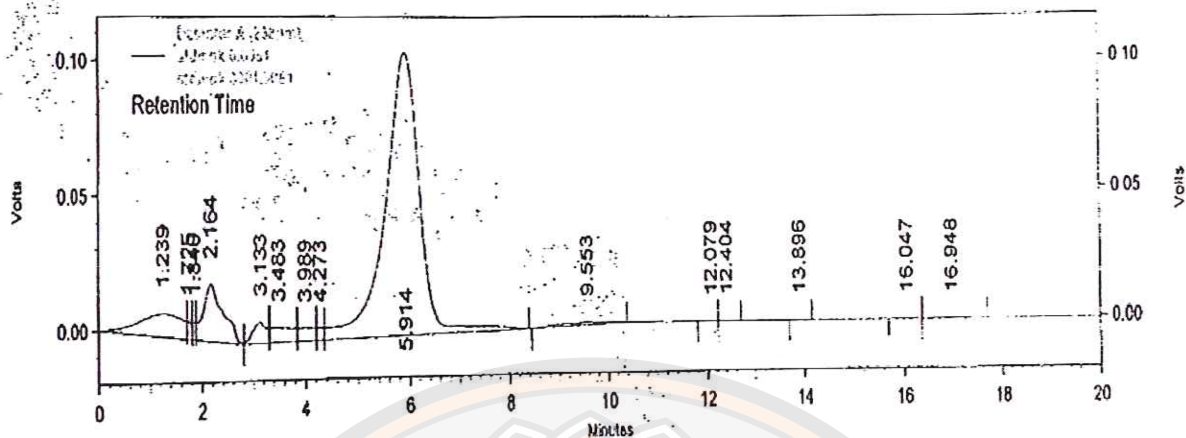

Figure 31 Chromatogram of monacolin K standard at 5.914 min of retention time

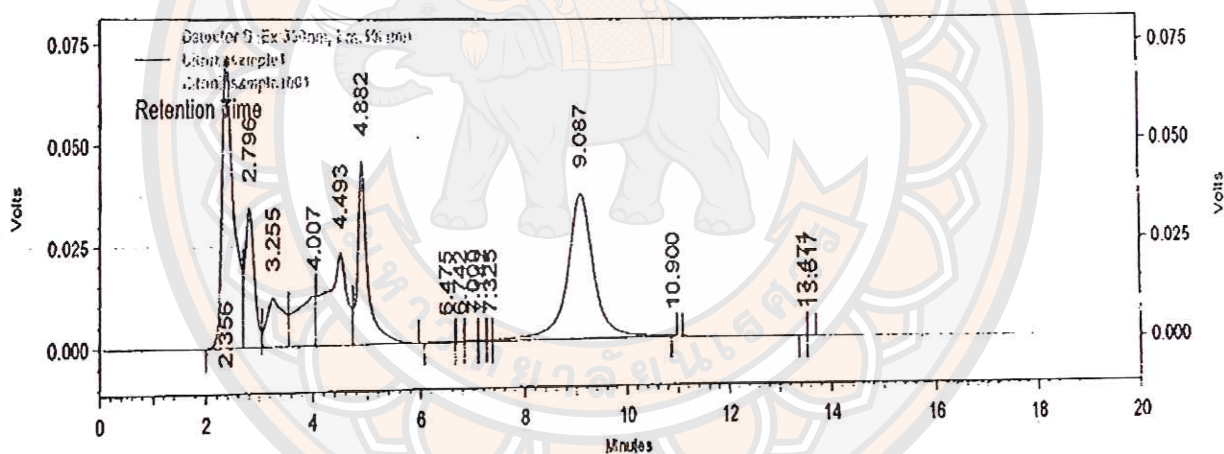

Figure 32 Chromatogram of citrinin standard at 9.087 min of retention time