University of Reading

Identification of the characteristic taste, aroma compounds and the corresponding precursors in pigmented rice wine

Submitted for the degree of Doctor of Philosophy

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Declaration

I confirm that the work presented in this thesis is my own and that the use of all the literature from other source has been properly and fully acknowledged.

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September, 2018

ABSTRACT

Rice wine (Sake) is an alcoholic beverage which is produced using glutinous rice, fungi and yeast. It has a pale yellow to clear colour, umami taste and fruity aroma. Moreover, pigmented rice wine has been consumed in Asian countries for a long time, and the taste and flavour are distinctly different to Sake. Whereas Sake is produced from high polished rice and shows more clear colour and pleasant fruity aroma, whole pigmented rice is used to produce pigmented rice wine and its taste and aroma profiles are less known.

In order to understand the differences in flavour and taste, commercial polished and unpolished pigmented rice wines were analysed for taste (sugars, organic acids, amino acids, phenolic acids, diketopiperazines (DKPs) and γ-glutamyl peptides) and aroma compounds. This study showed that compounds responsible for sweet, sour and metallic notes were found in commercial rice wines. These were attributed to glucose, acetic acid, cyclo(leucine-proline) and cyclo(isoleucine-proline). The aroma compounds in commercial rice wines were extracted using solid phase microextraction (SPME) and solid phase extraction (SPE) and analysed using gas chromatography-mass spectrometry (GC-MS) and GC-Olfactometry. Guaiacol and 4-vinylguaiacol were found to be the characteristic aroma compounds responsible for smoky-spicy note in commercial pigmented rice wine.

The brewing process for pigmented rice wine was investigated to develop a constant and reproducible protocol for brewing of pigmented rice wine which can be used to investigate the formation of taste and aroma compounds. The pigmented rice was cooked by steaming or pressure cooking. The cooked rice was saccharified using *Aspergillus oryzae*, and fermented using *Saccharomyces cerevisiae* at 25 °C or 30 °C. This study showed that steaming was suitable for cooking rice. The brewing that was selected was 2 days for

saccharification and 9 days for fermentation at 30 °C. These conditions were based on obtaining higher concentration of sugars and ethanol.

To study the formation of characteristic taste and aroma in pigmented rice wine, rice wines were brewed using unpolished (0%), 30% polished, 50% polished and 65% polished pigmented rice. They were analysed for taste and aroma compounds, and their precursors. The taste of lab-scale brewed rice wine was different from the commercial ones because astringent mouthfeel (gallic acid and protocatechuic acid) and umami were mainly found in lab-scale brewed rice wines, especially 0% RW. The bran increased the concentration of glutamic acid, phenolic acids and γ-glutamyl peptides (γ-glu-gly, γ-glu-his and γ-glu-tyr). The aroma compounds analysis also showed that guaiacol, 4-vinylguaiacol and vanillin were significantly and substantially increased in 0% RW. However, only guaiacol was found to be the characteristic aroma which contributed smoky-spicy note in 0% RW. This is consistent with sensory analysis which showed that a higher intensity of smoky-spicy note was found in 0% RW, compared to others.

This study also confirmed that phenolic acids were derived from the pigmented rice bran by fungi and yeast during brewing. Vanillic acid was decarboxylated to form guaiacol. Moreover, guaiacol was also formed from unpolished pigmented rice by steaming. The sensory analysis confirmed that guaiacol was the key aroma compound which contributed smoky-spicy note in pigmented rice wine.

Keywords: pigmented rice wine, characteristic aroma, smoky-spicy note, 4-vinylguaiacol, guaiacol

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

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CHAPTER 1: INTRODUCTION

1.1 Background

Rice wine is known differently depending on the Asian countries such as Sake (Japan), Jiu (China), Yakju (Korea), Tapuy (the Philippines), Ruou Nep Than (Vietnam), Tapai (Malaysia) and Sato, Krachae and Ou (Thailand) (Aidoo et al., 2005). They are produced using polished glutinous rice and starter cultures. For Chinese rice wine, wheat Qu which consists of wheat flour, fungi for saccharification, and yeast for subsequent fermentation, is used as the starter cultures (Chen et al., 2013a, Chen et al., 2013b, Zhao et al., 2015b), whereas Japanese rice wine is produced using rice Koji which consists of polished rice flour, fungi and yeast (Furukawa et al., 2006, Okuda et al., 2009b, Ito et al., 2016).

In order to produce a high quality Japanese rice wine, the bran is fully removed from the rice grain (Yoshizawa, 1999, Hashizume et al., 2007, Okuda et al., 2009b) because the excess of amino acids and proteins in the rice bran is believed to give an unpleasant aroma (Furukawa et al., 2006) and bitter taste to the rice wine (Maeda et al., 2011). In contrast, Xie et al. (2016) and Zhao et al. (2015b) showed that amino acids can be catabolised to the higher alcohols which contribute the pleasant aromas by the metabolism of the brewing microorganisms. During brewing, the polished glutinous rice is soaked in water to absorb moisture, and then steamed. The cooked rice is inoculated with the starter cultures (wheat Qu or rice Koji) for the fermentation (Liu et al., 2014a, Zhao et al., 2015b, Ito et al., 2016). After the fermentation is terminated, the rice wine is clarified and pasteurized (Japan Sake and Shochu Makers Assocoation and National Research Institute of Brewing, 2011)

The aroma in Chinese and Japanese rice wines have been investigated and almost 100 volatile compounds and semi-volatile compounds contribute the aroma in rice wine.

Among those compounds, esters and alcohols were the predominant aroma compounds (Chuenchomrat et al., 2008, Cao et al., 2010, Chen and Xu, 2010, Chen et al., 2013b, Jung et al., 2014, Yang et al., 2017a), which contribute solvent-like, sweet, fruity, buttery, and pungent aroma (Isogai et al., 2005, Chuenchomrat et al., 2008, Chen et al., 2013a, Chen et al., 2013b, Jung et al., 2014). Apart from these predominant aroma, Iizuka-Furukawa et al. (2017) showed that Japanese rice wine had grassy, bourbon-like and tonic-like aroma. The formation of this new characteristic aroma is caused by irregularity of yeast metabolism due to amino acid deficiency at the initial stage of the brewing. Moreover, Chen et al. (2013b) also showed that Huadiao rice wine from Zhejiang, China had herbal, smoky and Qu-like note (yeasty and mouldy) which might be derived from the wheat Qu.

Mimura et al. (2014) showed that lactic acid, succinic acid and malic acid are responsible for sour; nucleic acids are responsible for umami, and phenol derivatives are responsible for bitter. Moreover, Yu et al. (2015) also showed that isoleucine, valine, serine, aspartic acid, glycine, alanine and threonine contribute sweet whereas arginine and lysine contribute sweet/bitter in rice wines.

The pigmented rice which has the natural black, dark purple or brown-reddish colour is also used for the pigmented rice wine. This typical rice wine show rosé colour which is derived from the anthocyanins in the bran. As can be seen from the literature, the aroma and taste compounds in ordinary rice wines are widely published however there is much less known about pigmented rice wine. Therefore, this study identified the characteristic aroma and taste compounds of commercial pigmented rice wine and identified the corresponding precursors for the characteristic aroma in the pigmented rice wine.

1.2 Hypothesis

The bran of pigmented rice contains the precursors for the characteristic aroma compounds in the pigmented rice wine during the brewing process.

1.3 Objectives of the study

In order to validate the hypothesis, the objectives of the present work were:

a) Characterise and compare the characteristic aroma and taste compounds in commercial polished rice wine and commercial pigmented rice wine. (Chapter 3)
b) Optimise the brewing process for the pigmented rice wine, using parallel fermentation. (Chapter 4)
c) Directly compare and identify the characteristic aroma and taste compounds in rice wines which are brewed using pigmented rice polished to various degrees (0% for unpolished grain, 30%, 50% and 65% for bran fully removed). (Chapter 5)
d) Confirm the corresponding precursors for the characteristic aroma in the pigmented rice wine, using standard spiking technique. (Chapter 6)

1.4 Structure of the thesis

To fulfil the above objectives the study was divided into 6 chapters.

Chapter 1: General introduction

This chapter present a brief background to pigmented rice wine. The hypothesis and objective are also included in this chapter.

Chapter 2: Literature review

This section shows the information which is related to the manufacture of rice wine and the formation of aroma and taste compounds in rice wine during the brewing process.

Chapter 3: Taste and aroma compounds in rice wine: a comparison of polished rice wine and pigmented rice wine

This chapter shows the identification and comparison of the characteristic aroma and taste compounds in pigmented rice wine.

Chapter 4: The optimisation of pigmented rice brewing process

This chapter describes the effect of the rice cooking method and the brewing temperature on the saccharification and fermentation of pigmented rice wine. This section outlined the optimisation of pigmented rice brewing process for experiment in chapter 5.

Chapter 5: Influence of bran from pigmented rice on flavour formation in pigmented rice wine

The effect of the bran from pigmented rice on the aroma and taste compounds in the pigmented rice wine was investigated. The characteristic aroma and taste compound, and their corresponding precursors were identified.

Chapter 6: Confirmation of the precursors for the characteristic aroma compounds, smoky-spicy note in pigmented rice wine

The confirmation of precursors for guaiacol and 4-vinylguaiacol which contributed to smoky-spicy note in pigmented rice wine was shown in this chapter.

Chapter 7: General discussion and conclusion

This chapter summarised the optimisation of pigmented rice brewing, characteristic aroma and taste compounds in the pigmented rice wine and the corresponding precursors. Future work and the limitations were also discussed.

CHAPTER 2: LITERATURE REVIEW

2.1 Raw materials for the brewing of rice wine

2.1.1 Rice

Rice is an essential material for the production of rice wine. In Japanese Sake manufacture, rice is classified into two types, either rice for eating or rice for brewing. As seen in figure 2.1, the appearance and structure of rice for brewing is different from the table rice. It is described as having thick shape and chalky core in the grain (Furukawa, 2012).

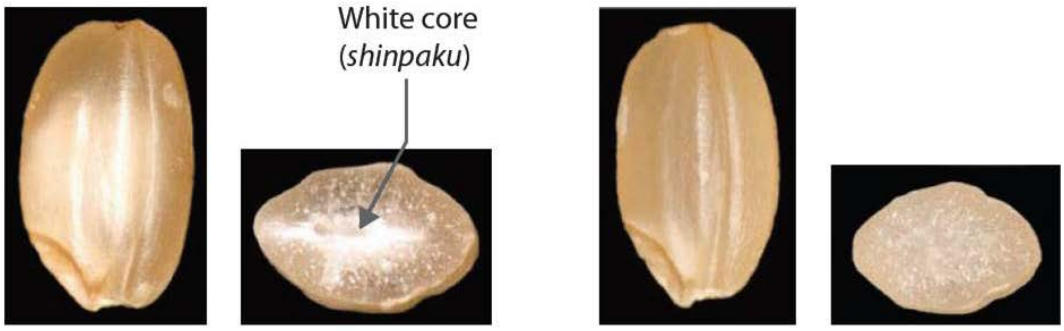

Figure 2-1: The appearance of Sake rice (left) and table rice (right).

From: Japan Sake and Shochu Makers Assocoation and National Research Institute of Brewing (2011)

The white core in Sake rice corresponds to the loose arrangement of starch granules and airspaces. The spaces between those scatter the light, and giving more opaque white colour to the rice grain. Due to the loose arrangement of starch granules in this chalky core, rice is easily inoculated and digested by Koji mycelia (Hashimoto et al., 2004). This information is in agreement with Anzawa et al. (2014) and Horigane et al. (2014) who showed that the loose morphological structure leads greater water absorption in Sake rice during soaking and steaming, thus resulting in a better fermentation. However, the price of high quality Sake rice is expensive (Yoshizawa, 1999). Thus, table rice can be used instead for the production of rice wine, but the quality of this rice wine is low (Furukawa, 2012, Anzawa et al., 2014).

Furthermore, pigmented rice is distinguished by the rice grain having red brown or dark purple colour in its covering layers (figure 2.2). The pigments in the aleurone layer of a rice grain consist of flavones, tannin, phenolics, sterols, tocols, γ-oryzanols (Deng et al., 2013) and especially anthocyanins, a component of reddish to purple water soluble flavonoids (Yodmanee et al., 2011, Deng et al., 2013). The pigmented rice is well known for having an enriched taste and typical colour. Thus, it has been consumed in China, Japan, and Korea for a long time (Deng et al., 2013). Moreover, pigmented rice is also used for the brewing of rice wine (Wang et al., 2014).

Figure 2-2: The appearance of pigmented rice which was used in this study.

Furukawa et al. (2006) and Furukawa (2012) showed that starch is the main component which constitutes up to 80% of unpolished rice. The abundant components in starch are amylose and amylopectin, which constitutes up to 98-99% of the dry weight of the starch granule (Tang et al., 2001, Tester et al., 2004, Chen et al., 2009). Both amylose and amylopectin consist of anhydrous glucose units and their chains, which are usually represented as $(C_6H_{12}O_6)$n, where n means the number of unit in the polymer (Schirmer et al., 2015).

A starch granule consists of semi-crystalline and amorphous regions that are packed in an alternating pattern (figure 2.3). The semi-crystalline form has an ordered structure of double helices of the amylopectin branches. This semi-crystalline region is also embedded in an amorphous region that contains the amylose and amylopectin branch points (Patindol et al., 2015). Starch is important for the production of rice wine because it is saccharified by fungi to produce glucose, which is subsequently fermented to produce ethanol by yeast (Uno et al., 2009, Anzawa et al., 2014).

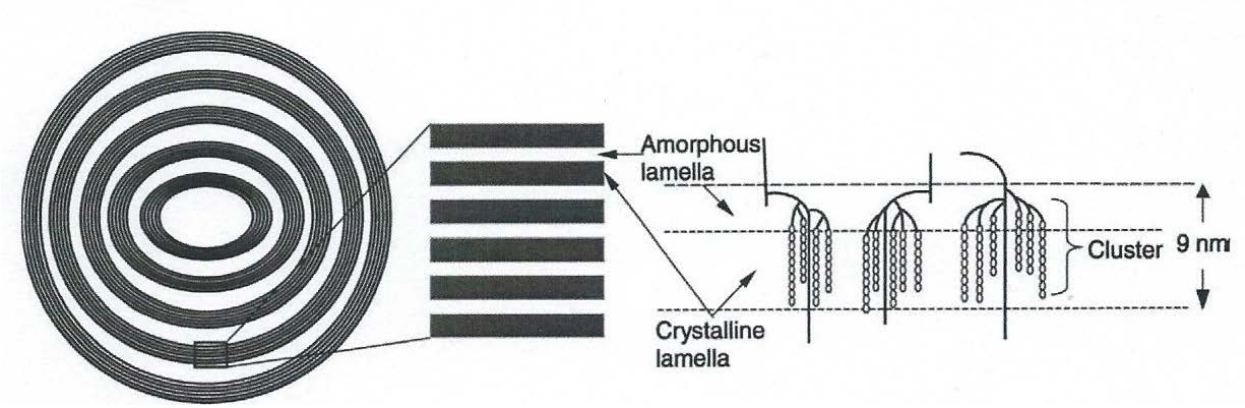

Figure 2-3: Nano structure of starch granule.

From: Patindol et al. (2015)

Proteins are the second most abundant component in rice (Furukawa et al., 2006, Furukawa, 2012), and are found at higher level in the outer layer of the rice grain, and decrease towards the centre of the rice kernel. Champagne (2004) showed that proteins in polished rice were in the range of 4-11% on a dry basis. Moreover, most proteins in rice are presented as discrete particles, namely protein bodies (PBs). They are classified into PB-I and PB-II, corresponding to prolamins and glutelins. The PB-I and PB-II comprise approximately 20% and 60% of the protein in polished rice. Furthermore, glutelins were mostly identified as having a negative effect on rice wine quality because they are derived from tyrosol which contributes to the Zatsumi (off-flavour or unpleasant bitter taste) (Hashizume et al., 2007, Okuda et al., 2016, Okuda et al., 2018). During brewing, proteins are degraded to small peptides and amino acids by enzymes from the starter culture. These liberated compounds are not only utilised as nutrients, but also converted to aroma and flavour in rice wine by brewing strains.

2.1.2 Starter for fermentation of rice wine

The fermentation starter for the brewing of rice wine consists of yeasts, fungi, and bacteria (Lv et al., 2015a, Lv et al., 2015b, Cai et al., 2018). This starter is known differently depending on the countries such as Koji (Japan), Nuruk (Korea), Banh Men (Vietnam) and Loog pang (Thailand). Moreover, the starter cultures are inoculated into a solid substrate such as rice flour and traditionally this may contain herbs (Cai et al., 2018), and in China the cultures are mixed into a wheat flour to produce wheat Qu (Yu et al., 2012, Chen et al., 2013a). The brewing starter for rice wine is not only used for the rice saccharification, but also used for the subsequent fermentation. Moreover, there are many studies which show that the microorganisms in a brewing starter have an effect on the generation of flavours in rice wine (Yu et al., 2012, Yang et al., 2017a, Cai et al., 2018). Briefly, the filamentous fungi in the starter culture degrade starch and proteins in rice or wheat flour, using amylolytic enzymes and proteolytic enzymes. The liberated compounds are further transformed to the corresponding flavour compounds in the rice wine (Carroll et al., 2017, Chung et al., 2017).

There are many studies which show that fungi, namely *Rhizomucor pusillus*, *Rhizopus oryzae, Absidia corymbifera*, *Aspergillus fumigatus*, *Aspergillus orgyze* and *Penicillium sp*. found in wheat Qu are used for the brewing of Chinese rice wine (Lim et al., 2006, Xie et al., 2007, Ohtsubo et al., 2008). Moreover, Dung et al. (2006) studied the diversity of fungi and yeast in Vietnamese brewing starters, and showed that *Amylomyces rouxii*, *Amylomyces* aff. *rouxii*, *Rhizopus oligosporus* and *Rhizopus oryzae* were the main fungi. These studies are also in agreement with Song et al. (2013), Bal et al. (2014) and Carroll et al. (2017) who showed that fungi, including *Aspergillus*, *Lichtheimia*, *Rhizopus*, *Rhizomucor*, and *Mucor* were commonly identified from Nuruk, Korean rice wine starter.

Both Gomes et al. (2005) and Khamkeaw and Phisalaphong (2016) showed that *Rhizopus spp*. and *Aspergillus spp*. were considered as important strains for the brewing of rice wine due to their high saccharification activity. Morales et al. (2008) also showed that these fungi produce α-amylase which breaks α-1,4 linkages of starch to yield dextrin, maltose, maltotriose, and maltopentose. Then, amyloglucosidase degrade these liberated compounds at α-1,4 and α-1,6 glucosidic linkages to yield glucose. This is in agreement with Dung et al. (2006) who showed that rice was saccharified to dextrin and maltose by using α-amylase and amyloglucosidase from fungi under aerobic solid state fermentation. Moreover, Cho et al. (2012), Saranraj and Stella (2013), Yang et al. (2013), de Oliveira et al. (2016) and Carroll et al. (2017) suggested filamentous fungi can produce both amylolytic and proteolytic enzymes which degrade starch and proteins in rice during the brewing process. This study is similar with Furukawa (2012) who showed that protease and carboxypeptidase from fungi degrade proteins in rice to amino acids and peptides, which have an important impact on yeast growth and Sake aroma and flavour.

The diversity of yeasts in traditional Thai rice wine starters was studied by Limtong et al. (2002). They showed that 43 yeast strains were found in 38 starter cultures for alcoholic sweetened rice, whereas 49 yeast strains were found in 19 starter cultures for rice wine. These yeast strains were identified as *Saccharomycopsis fibuligera*, *Pichia anomala*, *Pichia burtonii*, *Pichia fabianii*, *Pichia Mexicana*, *Pichia heimii*, *Candida rhagii*, *Candida glabrata*, *Torulaspora globose*, *Torulaspora delbrueckii*, *Issatchenkia orientalis*, *Trichosporon faecale*, *Rhodotorula philyla* and *Saccharomyces cerevisiae* (Aidoo et al., 2006). Moreover, Lv et al. (2013) studied the diversity of yeast in Chinese rice wine starters, they found different genera of yeasts such as *Cryptococcus*, *Rhodotorula*, *Sporobolomyces*, *Pichia*, *Saccharomyces*, *Candida*, *Rhodosporidium* and *Saccharomycopsis* in their samples. This study is in agreement

with Carroll et al. (2017) who showed these yeasts and *Kluyveromyces* and *Torulopsis* have been identified in the Korean starter culture.

According to the previous study, *Saccharomycopsis fibuligera* was reported as having a high amylolytic activity, but the capacity of ethanol production was low (Limtong et al., 2002). In contrast, *Saccharomyces cerevisiae* has been reported as having high ethanol production and ethanol tolerance (Dung et al., 2006, Cho et al., 2012, Yang et al., 2013, Carroll et al., 2017). During the brewing process, yeast produces ethanol from glucose (Furukawa et al., 2006, Okuda et al., 2009a, Uno et al., 2009, Anzawa et al., 2014) and other compounds, including higher alcohols and esters which have an effect on the quality and flavour of rice wine (Furukawa, 2012, Son et al., 2018).

2.2 Rice brewing process

2.2.1 Rice polishing

Rice kernels are covered by bran (pericarp and aleurone layer) and hull (figure 2.4). Normally, they are removed before using. The husk is easily removed because it is not rigidly packed within the rice kernel, whereas the bran is difficult to remove because it is tightly embedded in the rice kernel. The process of bran removal is called whitening, pearling or polishing. During the polishing, the intensive abrasion and thermal stresses result on the open of rice kernel (Afzalinia et al., 2004).

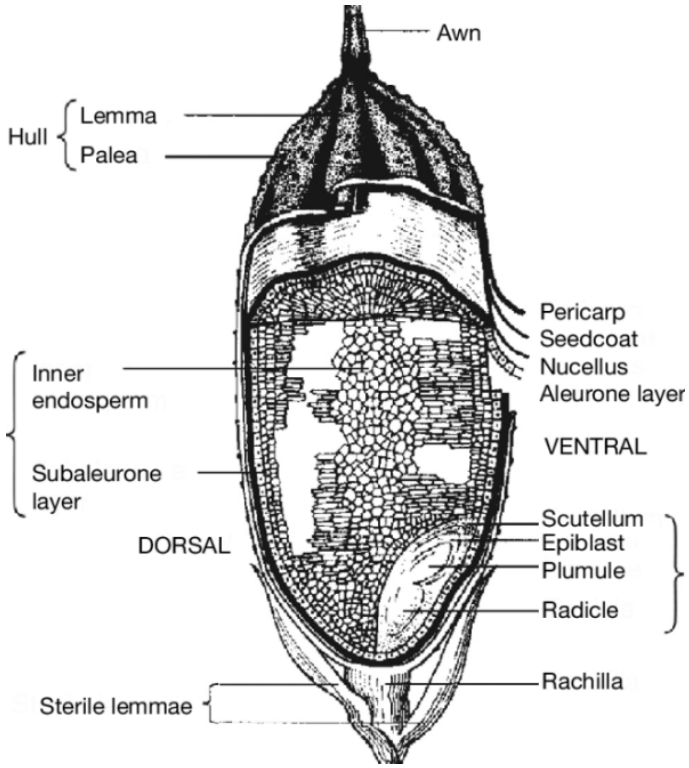

Figure 2-4: Longitudinal section of the rice grain.

From: Juliano (2016)

The polishing of rice grains is an essential process to remove excess lipids, proteins and nitrogen compounds in the aleurone layer of rice grains. Anzawa et al. (2014) and Liu et al. (2017) showed that these components have a negative effect on rice wine taste. Thus, they are removed by the polishing process for high quality Sake brewing. Normally, 30% of bran is removed from the Sake rice for regular rice wine, whereas 65% of bran is removed for high quality rice wine (Furukawa, 2012). This study is in agreement with Okuda et al. (2009b) and Okuda et al. (2016) who showed that highly polished rice grains are preferably used for premium quality Sake brewing (figure 2.5).

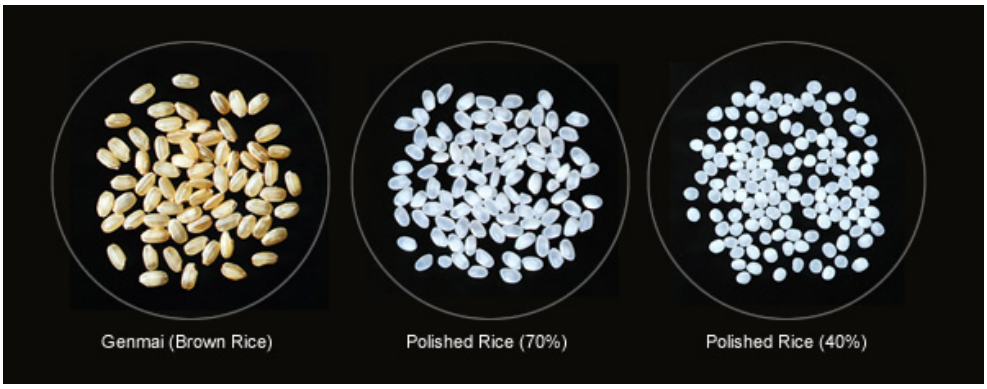

Figure 2-5: Rice for Sake brewing with various polishing yields.

From: Tatsuuma Honke Brewing (2011)

Paiva et al. (2014) studied the effect of polishing on the physicochemical and nutritional properties of pigmented rice. They found that increasing the degree of polishing resulted in a decrease of protein, lipid and ash in black rice and red rice grains. Moreover, flavonoids, anthocyanins, proanthocyanins, free phenolic acids and bound phenolic acids also decreased when the polishing process increased. The results confirmed that the bran of pigmented rice was enriched with these components. This study is in agreement with Liu et al. (2017) who showed that the polishing had an effect on the loss of protein, lipid and ash in ordinary rice. This study also showed that amino acids, thiamine, riboflavin, Cu, Fe and Se were evenly distributed in the bran and the outer endosperm, and decreased after 15% degree of polishing. However, phytic acid, Mg, Mn, Fe, and Pb likely accumulated in the outer layer of rice bran, thus they were removed at only 4-9% degree of polishing.

Kim et al. (2010) studied the effect of degree of polishing on the physicochemical characteristics and volatile compounds in glutinous rice wines. They found glucose and maltose were the abundant sugar in all rice wine samples. The increased degree of polishing resulted in a decrease in alcohol, amino acids and organic acids, whereas the soluble solids

and reducing sugars increased. They also showed that esters, alcohols and organic acids were the predominant volatiles in rice wines. The increased degree of polishing resulted in a decrease an ethyl succinate and 3-methylbutyl dodecanoate, whereas ethyl hexanoate and ethyl 2-octenoate increased. Therefore, this study concluded that different degree of polishing greatly affected the physicochemical and volatile characteristics of the glutinous rice wines.

Chun et al. (2012) also investigated the effects of the degree of polishing on the physicochemical and sensory characteristics of Sogokju, Korean rice wine. They found that increasing the degree of polishing resulted in a decrease in glucose, whereas total acid was increased. Moreover, the sensory evaluation showed that increasing the degree of polishing resulted in a decrease in consumer preference.

Park et al. (2015) investigated the quality characteristics of barley Makgeolli which was brewed from barley with various degree of polishing. Sugar and ethanol were not different between samples. However, pH, total acid and amino acids increased as the degree of polishing decreased. As the degree of polishing decreased, the rice wine went from a pale yellow to a deep purple colour.

2.2.2 Rice cooking

In order to produce the rice wine, the rice kernels are cooked to form gelatinised starch. This process increases the saccharification of cooked rice, which results in greater sugars and ethanol production. Rani et al. (1994) also showed that the hydrolysis of gelatinised starch was significantly higher than the corresponding raw starch. This study is in agreement with Srichuwong and Jane (2007) who found that starch gelatinisation is

necessary to increase the enzyme digestibility, because the raw starch is exceedingly resistant to enzyme hydrolysis. Moreover, López-Ulibarri and Hall (1997) shown that starch with high gelatinisation resulted a high glucoamylase activity during the saccharification process.

The starch gelatinisation is explained in figure 2.6. The starch granule is heated and absorbs more water. This physical action increases the hydration of the amorphous region, which subsequently disrupt the hydrogen bonds in the starch granule. The swollen granule induces the destabilisation and disruptive stress in the lamellar crystalline region. Further heating results in the melting of the starch crystals, an increase in starch solubility and the leaching out of amylose from the starch granule. The ordered structure of granular starch is disrupted. Therefore, the hydrolytic enzyme can more easily access these structures (Wang and Copeland, 2013). This is in agreement with Huang et al. (2005) who also showed that a high saccharification rate was found from the soluble starch solution because enzymes can easily access the soluble starch rather than the raw starch form. However, during the cooling the amorphous structure in amylose starts to recrystallize, followed by the recrystallization of amorphous regions in amylopectin (Schirmer et al., 2015). This physical action is also called retrogradation (Wang et al., 2015).

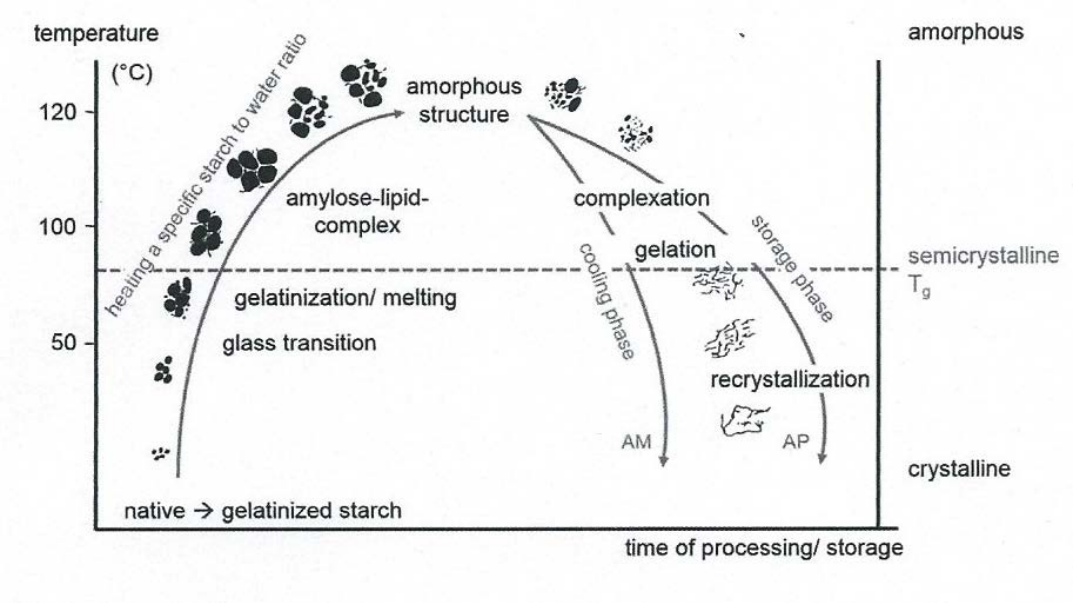

Figure 2-6: Phase diagram showing the state and phase transition of starch when applying a temperature profile (T_g, gelatinization temperature; AM, amylose; AP, amylopectin).

From: Schirmer et al. (2015)

In addition, the influence of water content on the starch retrogradation was studied by Zhou et al. (2011). They found the decrease of water content resulted in an increase in the starch retrogradation. Moreover, the effect of retrogradation on the starch digestibility was explained by Wang et al. (2015) who showed that retrograded starch starts to form the crystalline structure that is highly resistant to enzymatic digestion.

The amylose-lipid complex which comprises highly crystalline structures can be formed during the gelatinisation of starch in the presence of lipids. This complex is resistant to amylase digestion (Panyoo and Emmambux, 2017). Moreover, Wang et al. (2016) showed that the digestibility of starch-fatty acid complexes decreased, compared to its corresponding pure starch. This is also in agreement with Ai et al. (2014) and Kawai et al.

(2012) who showed that the decrease in hydrolysis of the starch-fatty acid complex can be explained by the fact that hydrolytic enzymes cannot bond with the substrate because the amylose-lipid complex has a compact structure, in which the α-(1-4)glycosidic bond is not exposed for hydrolytic enzyme action (Kawai et al., 2012).

Ashwar et al. (2016) showed that the resistant starch from rice was increased by using the autoclave at 120 °C for 30 min. This type of resistant starch is formed from the starch retrogradation process. This is consistent with Larsen et al. (2000) who also showed that the resistant starch in parboiled rice was increased by increasing the cooking pressure because a high pressure promoted the degradation of amylopectin which results in a long-term retrogradation. Moreover, moisture content influences the formation of resistant starch as described by Sievert and Pomeranz (1989). They showed that the decrease in water content resulted in the increase in resistant starch which was prepared using an autoclave. According to this, cooking methods and water content have an effect on the formation of resistant starch which subsequently influences the saccharification of rice starch because it is difficult to degrade by hydrolysis enzymes.

2.2.3 Fermentation of rice wine

2.2.3.1 Saccharification

The saccharification of rice starch by fungi is the most important process for the brewing of rice wine. The fungi from the brewing starter produce the enzyme for starch degradation, including α-amylase and amyloglucosidase, which break down rice starch into dextrin, maltose and mainly glucose (Dung et al., 2006, Saranraj and Stella, 2013). These fermentable sugars are utilised by yeast to produce ethanol during the subsequence or

parallel fermentation. According to figure 2.7, the α-amylase can be classified into endoamylase (intercept internal α-1,4 bonds), exoamylase (intercept α-1,4 or α-1,6 bonds of the terminal glucose residues), debranching enzymes (intercept α-1,6 bonds) and transferase (hydrolyse α-1,4-glycosidic bond of donor molecule and partly transfer the donor to glycosidic acceptor) (Zaferanloo et al., 2014).

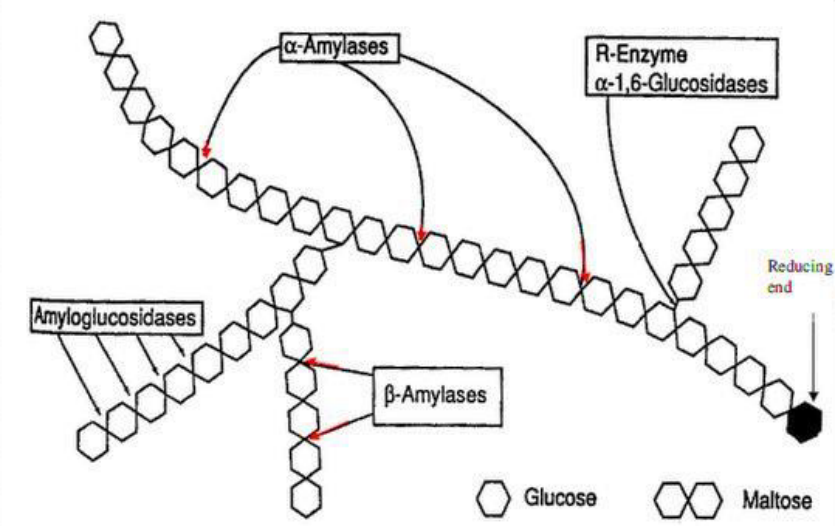

Figure 2-7: Mechanism of amylase activity.

From: Manners (1992)

In addition, amyloglucosidase hydrolyses α-1,4-glycosidic bond in oligosaccharides, and then invert the anomeric configuration of the resulting D-glucose. This enzyme successfully removes glucose from the non-reducing end of amylose or amylopectin. It has also been shown to hydrolyse α-1,6-glycosidic linkages of starch (Adeniran et al., 2010).

The α-amylase and glucoamylase have a large size, with limited access to the starch granule. Therefore, the swelling of the granule can increase its active site area. Li et al. (2014) studied the effects of granule swelling on the saccharification of corn starch by using

hydrolytic enzymes. They investigated the swelling of starch granules in the range of 40-90 °C. Their results showed that corn starch granules were greatly swollen at 60 °C. At this stage, the starch granule also lost the crystalline structure and birefringence. The swelling of starch granules were continuously increased until at 80 °C. The swelling of waxy corn starch was shown to be twice that of non-waxy corn starch at the same temperature. The amylopectin found in waxy corn starch promoted the swelling of the corresponding starch granule by absorbing more water in its structure during heating. Moreover, the saccharification of the swollen starch granules were 2 or 3-fold higher than the one without heat pre-treatment, because granule swelling provides greater enzyme access to the granule interior.

Najiah et al. (2017) studied the effect of saccharification conditions, including temperature, enzyme concentration, liquefaction temperature and the amount of substrate on the saccharification of sorghum starch for ethanol production. They found the temperature had a significant influence on the saccharification of starch. A high number of dextrin equivalents from sorghum starch was investigated at 45 °C by using α-amylase however it was reduced as the saccharification temperature increased (Aggarwal et al., 2001). This result is in agreement with Jha et al. (2017) who showed that an increase in saccharification temperature within the pH range of 4.5–7.5 resulted in the increase of reducing sugars in black rice. Moreover, Saranraj and Stella (2013) showed that the temperature had an effect on the amylase activity because it is related to the growth of microorganism. The suitable temperature for amylase activity was reported at 25-30 °C. This study is in agreement with Eriksen et al. (1998) who also showed that the optimum growth of *Aspergillus oryzae* was at 35 °C which corresponded to the maximum enzyme activity.

2.2.3.2 Alcoholic fermentation

The ethanol is produced via the Embden Meyerhof Parnas pathway (EMP), as shown in figure 2.8. According to this pathway, one molecule of glucose is metabolised to two pyruvate moieties which are then converted to two molecules of ethanol and two molecules of CO_2 under anaerobic condition (Madigan et al., 2000). The ethanol production occurs in parallel with the synthesis of adenosine triphosphate (ATP), the source of energy required for this bio-reaction. Thus, it can be said that the ethanol is the co-product which is produced by yeast during its growth. The biosynthesis of ethanol can be terminated by the lack of ATP, because phosphofructokinase (PFK), one of the most important regulation enzymes in the EMP pathway is interrupted (Bai et al., 2008).

During the fermentation, yeast cells can suffer from the various stresses which are derived from environmental and cell metabolism such as nutrient deficiency, high temperature (> 35 °C), contamination and high ethanol content (> 15% v/v). These factors have a negative effect on yeast viability, which then results in the low ethanol production (Bai et al., 2008). Basso et al. (2011) also showed that *Saccharomyces cerevisiae* can produce the ethanol in the range of 8-12% v/v however the excess ethanol is a major stress that influences the yeast survival. Alexandre et al. (2001) and Ma and Liu (2010) showed that the ethanol can enter to the cytoplasm of yeast cell, where it then profoundly alters the membrane fluidity which consists of the lipids. As a result, a high level of some ions, especially H^+ pass through the membrane of a yeast cell, and then they dissipate the electro-chemical gradient across the membrane, which decreases the formation and maintenance of the proton driving force. As a result, the pH of the intracellular of yeast is decreased. Moreover, there are other effects on yeast cells that are caused by the ethanol such as growth inhibition and enzymatic inactivation.

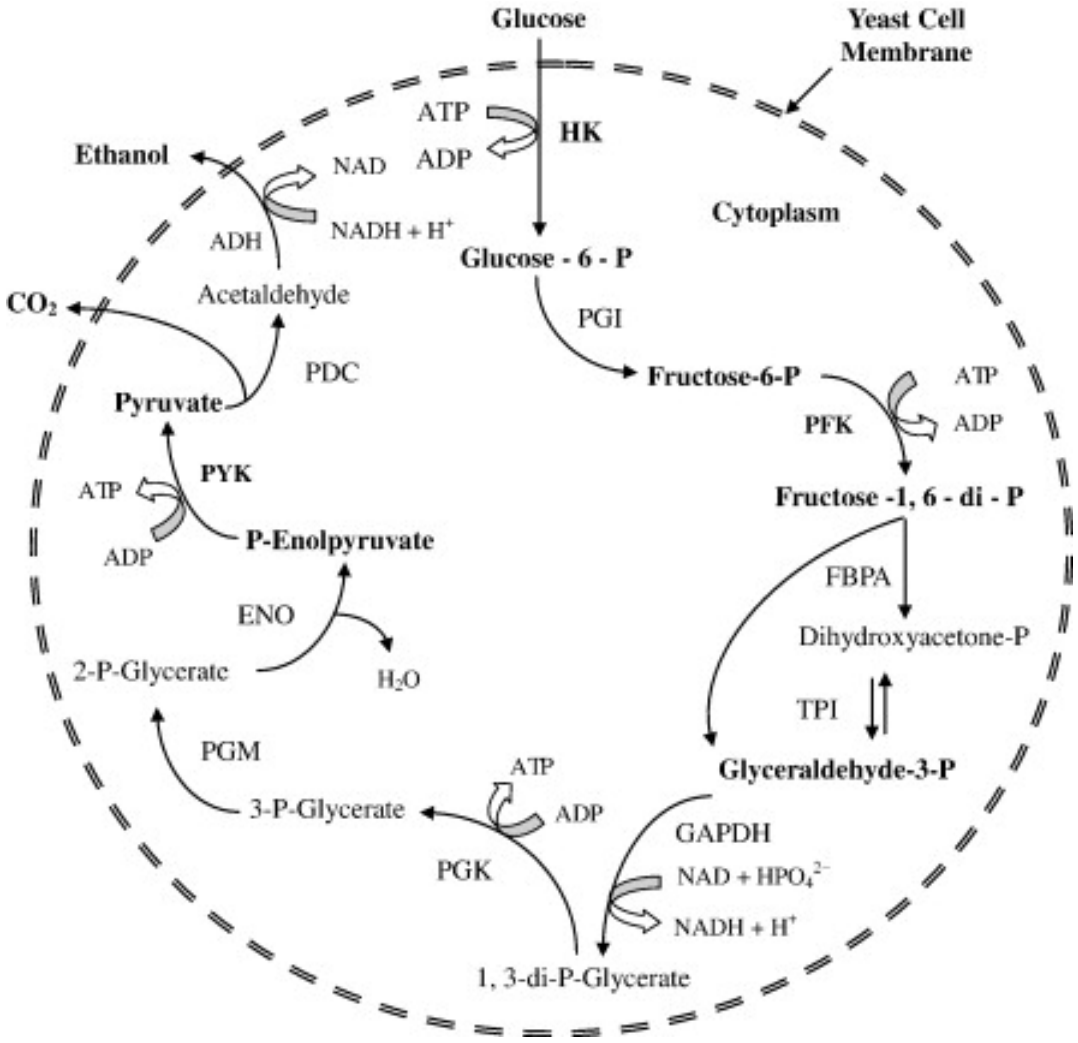

Figure 2-8: The EMP pathway for ethanol production by yeast, HK: hexokinase, PGI: phosphoglucoisomerase, PFK: phosphofructokinase, FBPA: fructose bisphosphate aldolase, TPI: triose phosphate isomerase, GAPDH: glyceraldehydes-3-phosphate dehydrogenase, PGK: phosphoglycerate kinase, PGM: phosphoglyceromutase, ENO: enolase, PYK: pyruvate kinase, PDC: pyruvate decarboxylase, ADH: alcohol dehydrogenase.

From: Bai et al. (2008)

High temperature has been investigated as the most important stress factor for the growth of microorganism (Cot et al., 2007). The range of suitable temperatures for *Saccharomyces cerevisiae* is 20-35 °C. The most suitable temperature for non-immobilised yeast cells is 30 °C, whereas for immobilised cells a slightly higher temperature is suitable because of its ability to transfer heat from particle surfaces to inside the cells (Liu and Shen, 2008). In addition, most enzymes, with regard to the microbial activity and fermentation process, are sensitive to high temperature because their tertiary structure might be denatured and inactivated. Therefore, the temperature should be controlled throughout the fermentation process (Phisalaphong et al., 2006).

Torija et al. (2003) also studied the effects of fermentation temperature on the growth rate of *Saccharomyces cerevisiae* and its fermentation activity. They showed that this species grows slowly at 15-20 °C, which resulted in a slower rate of fermentation. Moreover, the growth rate was increased at 25-30 °C with the yeast population was up to 10^8 cfu/ml. After fermentation for 8 days, the number of yeast cells was reduced to 10^6 cfu/ml. However, the yeast cells were further decreased to 10^2 cfu/ml from the fermentation at 35 °C for 6 days because of the yeast mortality at high temperature. The increase of temperature resulted in the decrease of ethanol, whereas acetic acid and glycerol increased.

Mohd Azhar et al. (2017) showed that pH and the concentration H^+ have an effect on the permeability of essential nutrients into the yeast cell. The range of suitable pH for the survival and growth of yeast was shown to be 2.75-4.25 (Fleet and Heard, 1993), whereas the suitable pH for the ethanol production by *Saccharomyces cerevisiae* was indicated as 4.00-5.00 (Lin et al., 2012). Moreover, Staniszewski et al. (2007) also showed that the fermentation might require a long time, if the pH was 4.00 however the

concentration of the ethanol was not reduced significantly. However, the high pH (>5.00) resulted in a decrease in ethanol.

2.3 Aroma compounds in rice wine

Similar to wine, the aroma in rice wine is mainly influenced by the metabolism of aroma from raw materials, the biosynthesis of flavour and aroma compounds from substrates during fermentation, the autolysis of yeast during post fermentation and the effect of spoilage bacteria (Fleet, 2003). However, the biosynthesis of flavour and aroma compounds is possibly the most important factor since many aroma active compounds in wine are derived during fermentation. Their formation is variable, depending on the yeast strain (Fleet, 2003). Moreover, Villamor and Ross (2013) showed that the aroma profile in wine is greatly complex, and most of them are volatile compounds which are found at very low concentration between 10^{-4} and 10^{-12} g/l (Guadagni et al., 1963). The volatile compounds in wine are classified into alcohols, esters, aldehydes, ketones, acids, terpenes, phenols, and sulfide compounds. Their concentrations vary with the change in viticulture and oenology (chemical compositions of grape, fermentation and post fermentation condition) (de Revel et al., 1999).

Grosch (2001) showed that the aroma compounds which have a high odour activity values (OAV, the ratio of the concentration to the corresponding threshold), especially esters, are the important compounds, because they contribute the fruity note in wine. Moreover, Guth (1998) studied the effect of ethanol on the aroma and taste compounds in Gewürztraminer. The results showed that the reduction of the initial ethanol in wine from 10 % to 9 % showed no effect on the aroma in the samples, compared to the control. The

decreases in ethanol from 10 % to 7 % resulted in an increase in fruity, floral and acid notes. However, the decrease in the ethanol to 3 % resulted in the low quality of wine because the characteristic flavour in wine was destroyed.

Chen et al. (2013b) studied chemicals, aroma compounds and aroma reconstitution in Chinese rice wines. The aroma compounds in rice wines were extracted using LiChrolut EN resins. The extracted aroma compounds in resins were separated into an acidic fraction and a neutral/basic fraction which was further separated into 16 fractions by silica gel normal phase liquid chromatography performed on a fast protein liquid chromatography (FPLC). The aroma from each fraction was analysed by gas chromatography-olfactometry (GC-O). The aroma compounds in samples were also extracted by static headspace (HS), solid phase extraction (SPE) and solid phase microextraction (SPME), and then analysed by gas chromatography-flame ionization detector (GC-FID), gas chromatography-pulsed flame photometric detection (GC-PFPD) and GC-MS. The quantitative results from the 4 different extraction methods showed that the main compounds were acids, esters, alcohols, aldehydes, vanillin, geosmin and γ-nonalactone, and their concentrations were higher than corresponding thresholds. This analysis showed that the most odour active compounds were vanillin, dimethyl trisulfide, 2-phenylethyl alcohol, guaiacol, geosmin, and benzaldehyde, and they might be responsible for the characteristic aroma in Chinese rice wine. Moreover, the odour active compounds were recombined and mixed into odourless rice wine model for aroma reconstitution study. The results showed that the aroma profile was not significantly different to the original rice wine.

Jung et al. (2014) studied the aroma compounds and sensory evaluation in Korean rice wines. The aroma compounds in samples were extracted using SPME and then analysed by gas chromatography coupled to time of flight mass spectrometry (GC-TOF/MS). The results showed 45 volatile compounds which mainly comprise esters and alcohols. The abundant compounds, including 2-phenylethanol, ethyl acetate, 3-methylbutanol, ethyl octanoate, ethyl decanoate, diethyl succinate, 2-phenethyl acetate, ethyl dodecanoate, ethyl hexadecanoate, ethyl 9-octadecenoate, ethyl tetradecanoate and ethyl 9,12-octadecadienoate were found in the samples. These compounds were similarly found in Sake. However, Makgeolli was reported as having a wide range of flavour due to the addition of herbs (Lee and Lee, 2008). The principle component analysis (PCA) showed that the contrast of fruity and yeast aroma was found. According to the sensory result, fruity and whiteness in Makgeolli were preferred. This result is in agreement with Lee and Lee (2008) who also showed that attributes, including ripe fruit and sweet grain were found in contrast to the herb-like and yeasty note. Jung et al. (2014) also studied the correlation ($r > 0.7$) between the sensory attributes and volatile compounds. The result showed the positive correlation between the alcohol note and ethanol content; sour and total acids; whiteness and b-value on the LAB colour scale indicating a more yellow colour.

Niu et al. (2017) extracted the volatile compounds in light aroma Chinese rice wine by solvent extraction technique. The aroma profile in the extract was analysed by GC-FPD, GC-MS and GC-O. The result showed that ethyl esters, including ethyl acetate, ethyl hexanoate, ethyl octanoate, 3-methylbutyl acetate and ethyl 2-methylpropanoate were found in rice wines. These compounds can impart the fruity aroma. Alcohols, namely 1-propanol, 2-methyl-1-propanol, 1-butanol, 3-methyl-1-butanol and 2-phenylethanol were detected in samples. These alcohols are known as the important compounds in the liquor

(Zhang et al., 2009). Organic acids, including acetic acid, 2-methylpropanoic acid, butanoic acid, 3-methylbutanoic acid, pentanoic acid, hexanoic acid and octanoic acid were the main acids with high flavour dilution factor. Compounds such as hexanoic acid, octanoic acid and other acids imparted sweaty, rancid and cheesy. Notably, 3-hydroxy 2-butanone was detected for the first time in light aroma Chinese rice wine. Two pyrazines, trimethylpyrazine and tetramethylpyrazine were also identified. A high level of pyrazines was found in the light aroma Chinese rice wine, compared to the strong aroma liquor. Pyrazines were formed through the Maillard reaction with the presence of saccharides and amino residues (López-Galilea et al., 2006). This chemical reaction can be found in the pasteurisation which is used for the rice wine processing. In addition, 3-hydroxy 2-butanon, thiazole, dimethyl trisulfide, diallyl disulfide, 2-hydroxy 1-ethanethiol, 4,5-dihydro-3(2H)-thiophenone, 3-thiophenecarboxaldoxime, 3-mercaptohexylacetate, 1-(3-methylthiophen-2-yl)ethanone, 3-methylthiophene-2-carboxaldehyde and 4-methyl-5-vinylthiazole were found in the light aroma Chinese rice wine for the first time. According to the sensory evaluation, seven attributes, namely alcoholic, grassy, fruity, acid, fermentation, sweet and floral were observed in light aroma Chinese rice wines. Moreover, 2-methylpropanoic acid, β-damascenone, ethyl hexanoate, 3-methylbutyl acetate and ethyl lactate were the key aroma compounds in light aroma Chinese rice wine.

Son et al. (2018) studied the volatile compounds in rice wine which was brewed by using rice, wheat Koji, *Aspergillus oryzae* and *Saccharomycopsis fibuligera*. The volatiles were extracted by SPME, and then analysed by GC-MS. The results showed that high levels of 2-methylpropanol, 2-methylbutanol, 3-methylbutanol and 2-phenylethanol were detected in rice wines. The formation of these compounds was also found in relation to their corresponding organic acids and aldehydes. Moreover, this study showed that the most

abundant esters, including 2-phenylethyl acetate, ethyl acetate, 3-methylbutyl acetate, ethyl hexadecanoate, ethyl octanoate and ethyl decanoate were found in all rice wines. The positive correlation between *Aspergillus oryzae* and the concentration of butanol, butanoic acid and their liberated esters was found. Moreover, 4-ethylphenol and 4-ethylguaiacol were found in rice wines. Their formations increased in rice wine that was brewed by using *Aspergillus oryzae*.

The literature of the aroma in pigmented rice wine is limited. However, one of useful information is published by Ueki et al. (1991). They showed that red rice wine contained a higher concentration of higher alcohols and esters which imparted fruity aroma, compared to polished rice wine.

2.4 The formation of the aroma compounds during the brewing of rice wine

The aroma and flavour compounds are mostly formed during the fermentation of rice wine by microorganisms. This section will show the main pathways of the aroma and flavour compounds which are found in rice wines.

2.4.1 Higher alcohols, aldehydes and organic acids from amino acids

The amino acids can be broken down to higher alcohols, aldehydes and organic acids via the Ehrlich pathway (Lilly et al., 2006, Hazelwood et al., 2008, Styger et al., 2011). Moreover, the important volatiles derived from amino acids are shown in table 2.1.

Table 2-1: Flavour formation from amino acid catabolism via Ehrlich pathway.

	aldehydes	higher alcohols	acids
Leu	3-methylbutanal	3-methylbutanol	3-methylbutanoic acid
Ile	2-methylbutanal	2-methylbutanol	2-methylbutanoic acid
Val	2-methylpropanal	2-methylpropanol	2-methylpropanoic acid
Phe	2-phenylethanal	2-phenylethanol	2-phenylacetic acid
Tyr	4-OH-phenylethanal	4-OH-phenylethanol	4-OH-phenylacetic acid
Trp	indole-3-acetaldehyde	tryptophol	indol-3-acetic acid
Met	3-methylthiopropanal	3-methylthiopropanol	3-methylthiopropionic acid

From: Styger et al. (2011) and Son et al. (2018)

The formation of higher alcohols, aldehydes and organic acids has been reported by Styger et al. (2011), Son et al. (2018) and Lilly et al. (2006). They showed that the amino acid is changed to α-ketoglutarate via the transamination reaction by aminotransferases,

and then subsequently converted to the corresponding α-keto acid and glutamate. The liberated α-keto acid is further decarboxylated to the corresponding aldehyde. This aldehyde is reduced to its corresponding higher alcohol via a NADH-dependent reaction by an alcohol dehydrogenase. This higher alcohol is further converted to organic acid using acetyltransferase (figure 2.9).

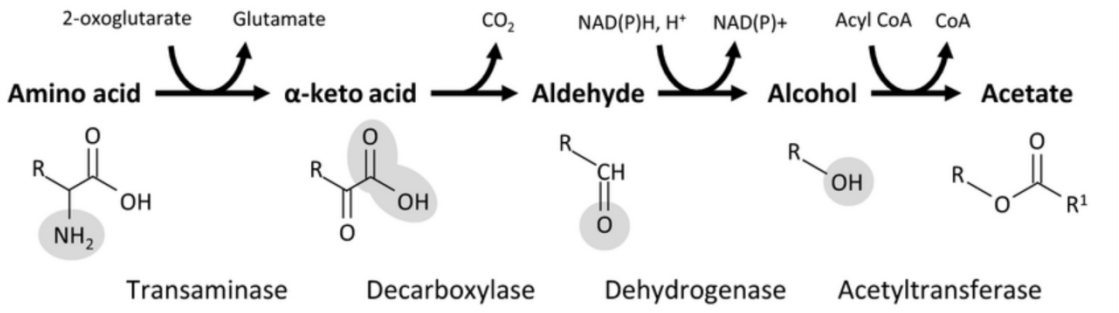

Figure 2-9: The Ehrlich pathway for the formation of amino acids-derived aldehydes, alcohols and organic acids.

From: Ravasio et al. (2014)

2.4.2 Esters

Saerens et al. (2010) and Son et al. (2018) explained that there are two main categories of volatile esters in fermented beverages, including acetate esters which are formed from acetic acid and alcohols, and medium-chain fatty acid ethyl ester (C_6-C_{12}) which formed from the corresponding medium chain fatty acids and ethanol. However, the acetate esters have been reported as the most important because they are easily produced at a high level. Son et al. (2018) showed that esters are formed by esterification between alcohols and acids. Moreover, Xu et al. (2015a) also showed that esters are formed via

biosynthesis, using alcohol acetyltransferase and substrates (alcohols and acetyl-CoA). A high concentration of medium chain fatty acids and the presence of acyl-CoA: ethanol *O*-acyltransferases (AEATases) promotes the formation of fatty acid ethyl esters (Saerens et al., 2008).

2.4.2.1 Acetate esters

The acetate ester, for example ethyl acetate, 3-methylbutyl acetate, 2-methylpropyl acetate and phenylethyl acetate have acetic acid as the parent acid and the parent alcohol is ethanol or higher alcohols. The formation of acetate esters is shown in the following chemical reaction: the acetate esters are formed from the parent alcohols and acetyl-CoA, using alcohol acetyltransferases (Saerens et al., 2010).

$$CH_3COCOOH + NAD^+ + CoA\text{-}SH \rightarrow CH_3CO\text{-}S\text{-}CoA + NADH + H^+ + CO_2 \quad (a)$$

$$CH_3CO\text{-}S\text{-}CoA + R_1OH \rightarrow CH_3COOR_1 + CoA\text{-}SH \quad (b)$$

This formation is in agreement with Nagasawa et al. (1998) and Verstrepen et al. (2003) who showed that alcohol acetyltransferases greatly affected the formation of acetate esters. Moreover, Lilly et al. (2006) studied the effect of alcohol acetyltransferases and esterase in *Saccharomyces cerevisiae* on the formation of acetate esters. The results showed that overexpression of the gene that is responsible for alcohol acetyltransferases in the yeast increased the formation of ethyl acetate, 3-methylbutyl acetate, 2-phenylethyl

acetate and ethyl acetate. However, the application of the gene that is responsible for esterase in yeast decreased the formation of those esters.

2.4.2.2 Medium chain fatty acid ethyl ester

The medium chain fatty acid ethyl esters, for example ethyl hexanoate and ethyl octanoate have the ethanol as the parent alcohol and the parent acid is a medium chain fatty acid. In order to form these compounds, the fatty acids are transferred to acyl-CoA derivatives, which then react with ethanol to form the corresponding esters by acyl-CoA: ethanol *O*-acyltransferases (AEATases), as can be seen in the following chemical reaction (Knight et al., 2014).

$$R_1COOH + ATP + CoA\text{-}SH \rightarrow R_1CO\text{-}S\text{-}CoA + AMP + PPi \quad (a)$$

$$R_1CO\text{-}S\text{-}CoA + CH_3CH_2OH \rightarrow R_1COOCH_2CH_3 + CoA\text{-}SH \quad (b)$$

Moreover, Saerens et al. (2010) studied the effect of AEATases in Saccharomyces cerevisiae on the formation of ethyl esters Their results showed that the deletion of the gene that is responsible for that formation of AEATases in yeast, resulted in a decrease of ethyl butanoate, ethyl hexanoate, ethyl octanoate and ethyl decanoate.

2.4.3 Organic acids

Rezaei et al. (2015) showed that the organic acids, mainly citrate, malate and succinate are produced by *Saccharomyces cerevisiae* via the tricarboxylic acid (TCA) cycle. These compounds are important in wine products due to their effect on the organoleptic properties (Camarasa et al., 2003). According to figure 2.10, their formation starts with the conversion of glucose to pyruvate in the glycolysis pathway, which is then converted to acetyl CoA. After that, the substrate goes through the TCA cycle. In order to generate the first organic acid, citric acid from this cycle, the acetyl CoA reacts with oxaloacetate to form citrate by citrate synthase. The citrate is further converted to its isomer, isocitrate by aconitase, and then reduced and decarboxylated to form α-ketoglutarate by using isocitrate dehydrogenase. In order to generate the succinic acid, α-ketoglutarate is dehydrogated and decarboxylated to form succinyl CoA by α–ketoglutarate dehydrogenase, which is then hydrolysed to succinate by succinyl CoA synthetase. The formation of the third acid, fumaric acid in this cycle goes through the oxidisation of succinate by succinate dehydrogenase. The fumarate is further converted to malic acid by fumarase, which is then oxidised to oxaloacetate by malate dehydrogenase.

Acetic acid is one of the most important organic acids found in wine. Fowles (1992) showed that acetic acid can be formed from the oxidation of acetaldehyde by acetaldehyde dehydrogenase (figure 2.10). The concentration of acetic acid can be increased by a longer fermentation, a higher concentration of sugar in the substrate and a lower fermentation temperature. Moreover, acetic acid is produced by the oxidation of ethanol by acetic acid bacteria under aerobic conditions.

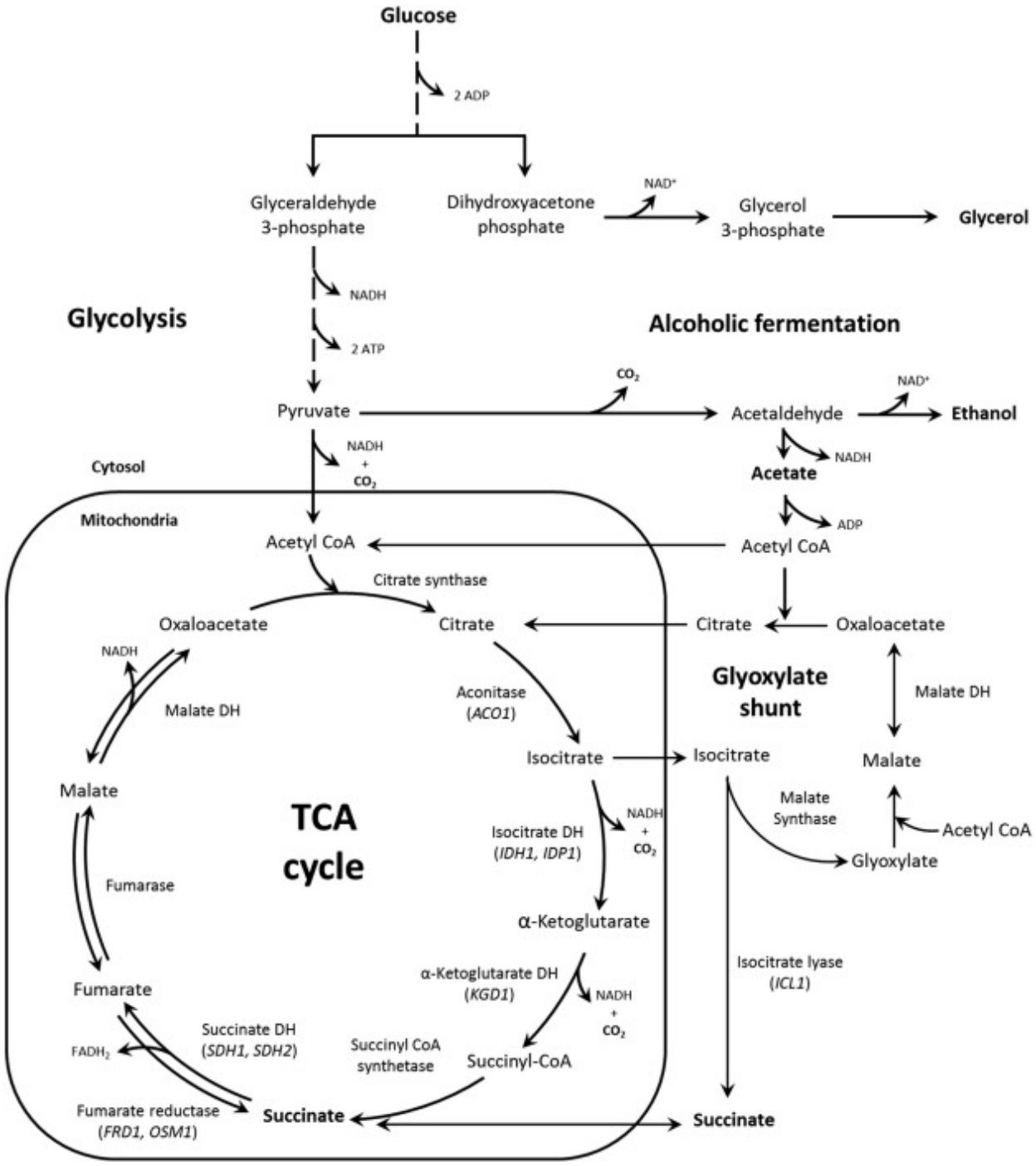

Figure 2-10: The TCA cycle for the formation of organic acids.

From: Rezaei et al. (2015)

Styger et al. (2011) and Lian and Zhao (2015) descripted the formation of lipid-derived organic acids that they are formed via fatty acid biosynthesis (FAB), as can be seen in figure 2.11.

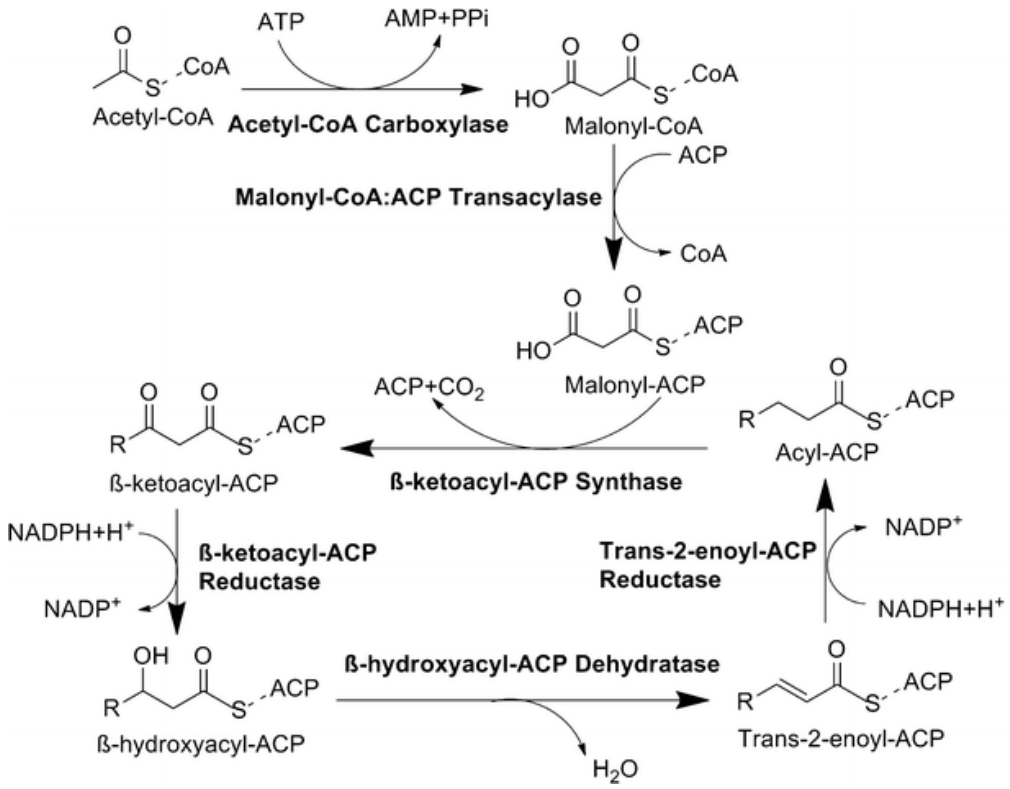

Figure 2-11: The fatty acid biosynthesis for the formation of fatty acids.

From: Lian and Zhao (2015)

This pathway starts with Acetyl-CoA being converted to malonyl-CoA by acetyl-CoA carboxylase (ACC), and then further converted to malonyl-ACP by malonyl-CoA:ACP transacylase. The formation of malonyl-ACP is very important because it is used as the elongation unit, bonding with acyl-ACP to form β-ketoacyl-ACP with two more carbon units. The extended β-ketoacyl-ACP is converted to β-hydroxyacyl-ACP, and then converted

to trans-2-enoyl-ACP by β-hydroxyacyl- ACP dehydratase. After that, the double bond in trans-2-enoyl-ACP is reduced to form acyl-ACP by trans-2-enoyl-ACP reductase with the presence of NADPH. The processed acyl-ACP from this elongation cycle results in the fatty acyl chains being extended by two carbon units. Therefore, more elongation cycles, the longer the fatty acid chain.

2.4.4 Volatile phenols

Phenolic acids which are derived from the degradation of lignin have been reported as the precursor of volatile phenols (Zhao et al., 2015a, Ito et al., 2016, Sunao et al., 2016). Ito et al. (2016) showed that vanillic acid can be decarboxylated to form guaiacol, and ferulic acid can be decarboxylated to form 4-vinylguaiacol by microorganisms (figure 2.12).

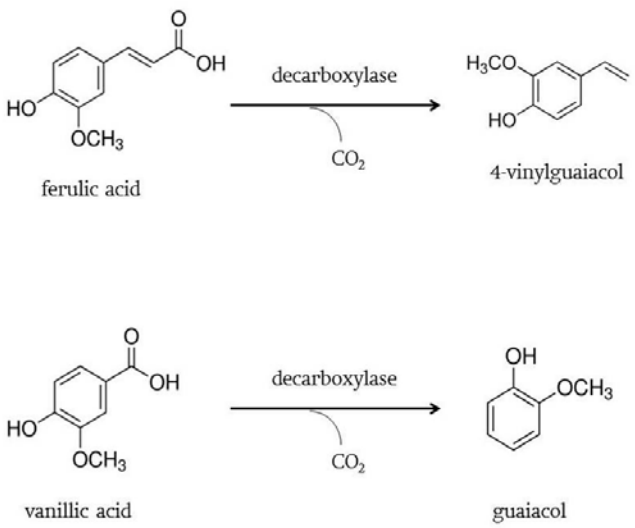

Figure 2-12: The formation of guaiacol and 4-vinylguaiacol from phenolic acids.

Modified from: Ito et al. (2016)

Moreover, Belda et al. (2017) also showed that 4-coumaric acid, ferulic acid and caffeic acid are decarboxylated to form 4-vinylphenol, 4-vinylguaiacol and 4-vinylcatechol, using phenolic acid decarboxylase. These volatile phenol compounds can be reduced to form 4-ethylphenol, 4-ethylguaiacol and 4-ethylcatechol using vinylphenol reductase, respectively (figure 2.13). These enzymes are produced from *Saccharomyces cerevisiae* and *Lactobacillus plantarum* (McKenna et al., 2014). Chang and Kang (2004) and Witthuhn et al. (2012) also showed that volatile phenol compounds contribute smoky note to wine, beer and apple juice.

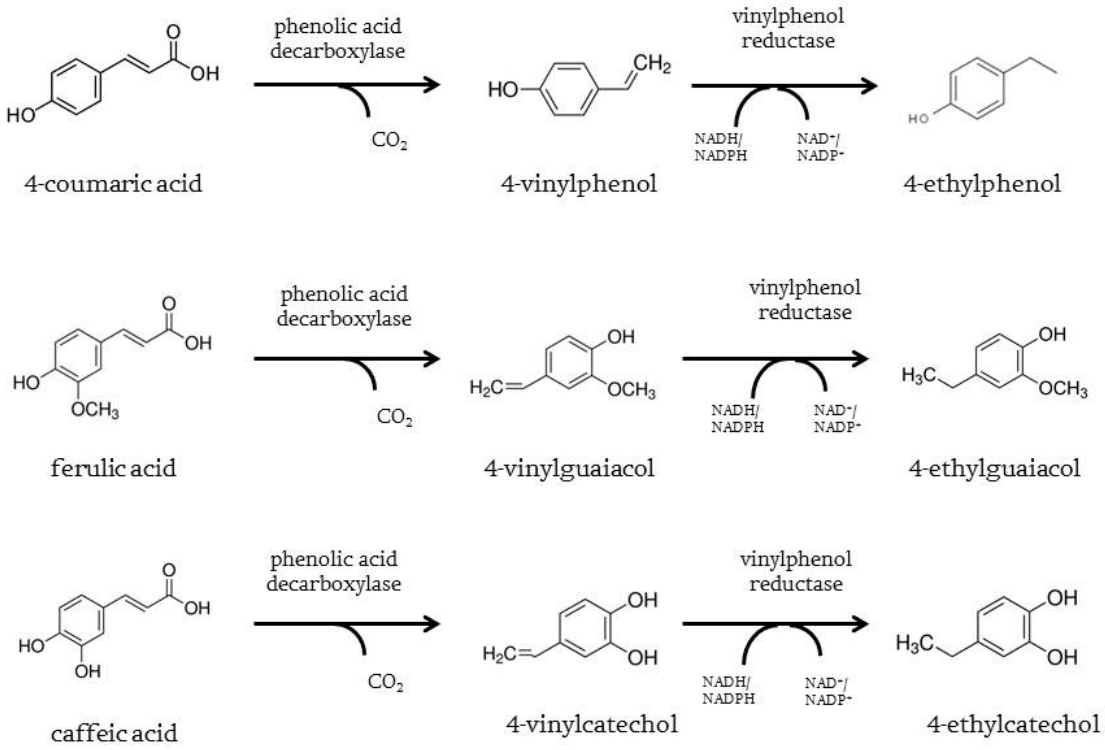

Figure 2-13: The formation of volatile phenol compounds from 4-coumaric acid, ferulic acid and caffeic acid by yeast.

Modified from: Belda et al. (2017)

2.4.5 Lactones

The formation of lactones (cyclic esters) in the fermentation process was shown by Berger et al. (1986), Vandamme and Soetaert (2002) and Romero-Guido et al. (2011). Briefly, fatty acids can be metabolised to form hydroxycarboxylic acids. The position of hydroxyl group in hydroxycarboxylic acids is moved to close to its carboxylic group via β-oxidation. After that, the bonding between the hydroxyl group of the hydroxycarboxylic acid chain and the hydroxyl group at the carboxylic end is done via the lactonisation (intramolecular esterification), as can be seen at figure 2.14. This intramolecular cyclisation mostly occur for γ or δ hydroxy acids, which form five or six ring lactones.

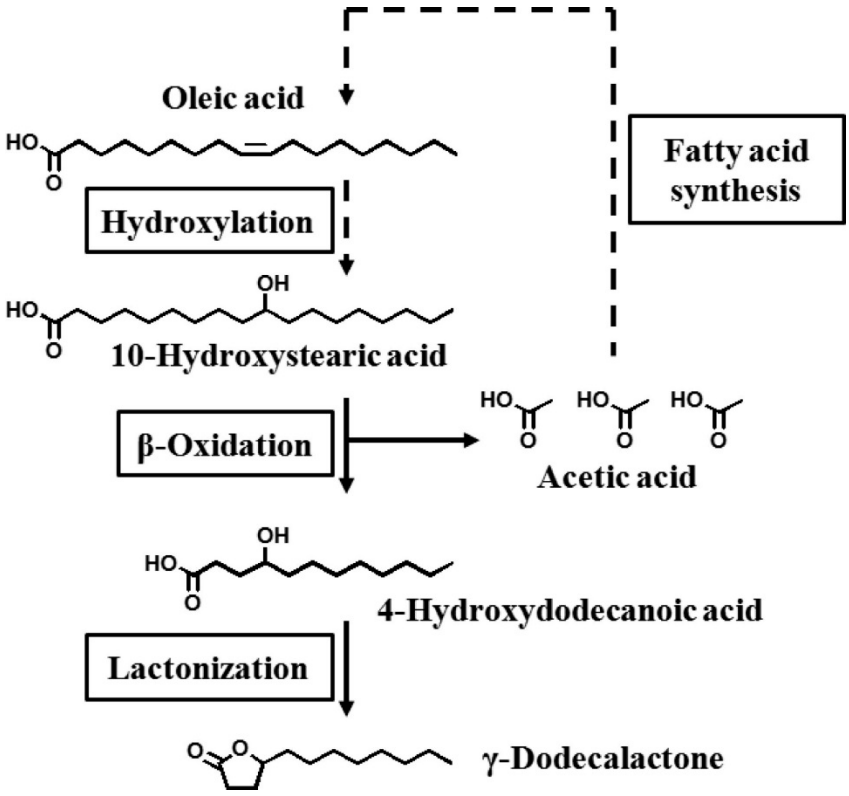

Figure 2-14: The formation of lactones, γ-dodecalactone by yeast via the lactonisation.

From: An et al. (2013)

2.5 Taste compound in rice wine

Taste and mouthfeel are important factors contributing to consumer preference and acceptability of wines (Niu et al., 2012). There are many studies on the aroma in rice wines, including direct nasal or retronasal perception and flavour profiling (Chen and Xu, 2010, Chen et al., 2013b, Chen and Xu, 2013, Su et al., 2016, Niu et al., 2017). However, the study of the effect of taste and mouthfeel on the overall quality, and the development of taste active compounds in wines has been studied more. Hufnagel and Hofmann (2008b) also showed that sourness, sweetness, bitterness, and astringency impart the important orosensory quality in red wine. However, the other attributes in wine samples were also investigated, for example velvety astringency (finely astringent mouthfeel), puckering astringency (reflexive action which released in an attempt to lubricate mouth surfaces) and mouthfulness or body were described as a full oral-sensation that was perceived during wine consumption.

Niu et al. (2017) studied the taste compounds in wine which produced from cherry. The sensory evaluation was carried out, and the results compared with the chemical analysis, including sugars, organic acids, amino acids, tannic acid and phenolic acids. The sensory results revealed that sour, sweet and bitter taste were the most important attributes, whereas astringent sensation was the second most important attribute in cherry wine. However, Jung et al. (2014) showed that the commercial rice wine had bitter, sour, sweet, salty and umami taste however the characteristic note, including astringency and pungency can be found from the samples.

Iwano et al. (2004) studied the effect of amino acids on the taste of Sake. The results showed that alanine, arginine, glutamic acid and aspartic acid were found to be the important amino acids in Sake. This study also showed that alanine imparted sweet taste, arginine imparted bitter taste, glutamic acid and aspartic acid correspond to acidity, astringency, and added flavour.

Correlation between the sensory test and the chemical analysis in rice wine showed that tartaric acid, methionine and proline contribute sour taste; sucrose, glucose and fructose contribute sweet taste; asparagine, serine, glycine, threonine, phenylalanine, leucine, gallic acid and chlorogenic acid contribute bitter taste; vanillic acid, arginine and tannic acid contribute astringent mouthfeel (Niu et al., 2012). However, Yu et al. (2015) also showed that fucose, arabinose, lactic acid, glutamic acid, arginine, isoleucine, valine, threonine and lysine were found to be the additional taste compounds in Chinese rice by using HPLC. The correlation between the taste-active compounds and the sensory attributes also showed that fucose and arabinose contributed to the harmony attribute; lactic acid, glutamic acid and arginine contributed sour taste; isoleucine, valine, threonine and lysine contributed sweet taste.

Mimura et al. (2014) also studied the effect of non-volatile compounds on the taste of rice wine. The result showed that amino acids were positively correlated to the dull aftertaste and were considered to contribute to the body of rice wine. Their high level can increase the Zatsumi sensation which is an unpleasant bitter taste (Mimura et al., 2014, Takahashi et al., 2016). The sensory score of sour taste which corresponded to succinic acid was correlated to the pungent/smoothness attribute. The compounds including peptides, amino acids, phenol derivatives, and sugar derivatives, responsible for bitter taste were also

found in rice wine. However, Jung et al. (2014) showed that sour and bitter taste were the negative factors in rice wine samples.

Polyphenolic compounds, including anthocyanins, flavonols, phenolic acids and flavanol monomers and some oligomers have been found in wines (Lesschaeve and Noble, 2005, Preys et al., 2006, Hufnagel and Hofmann, 2008a, Gambuti et al., 2012). The phenolic compounds in Chinese rice wine, Japanese Sake, and Korean were studied by Huang et al. (2015) they showed that phenolic acids including gallic acid, protocatechuic acid, vanillic acid, syringic acid, caffeic acid, ferulic acid, 4-coumaric acid, sinapic acid, chlorogenic acid, catechin, epicatechin, quercetin, and rutin were found in the samples. This is consistent with Xu et al. (2015b). However, Wang et al. (2014) extracted the phenolic acids and polyphenolic acids in black rice wines by SPE, and they were analysed by HPLC-MS/MS. The result showed that cyanidin-3,5-*O*-diglucoside, cyanidin-3-*O*-glucoside, cyanidin-3-*O*-rutinoside and peonidin-3-*O*-glucoside were found, apart from non-colour phenolic compounds. The phenolic compounds which have the molecular weight below 500 Da such as flavan-3-ol monomers , flavan-3-ol dimers and trimers and hydroxybenzoic acids elicited astringent sensation in wine due to the precipitation of glycoproteins in saliva, generating a loss of lubrication in mouth (Lesschaeve and Noble, 2005, Hufnagel and Hofmann, 2008b). However, some phenolic compounds such as catechin, epicatechin, procyanidin and phenolic acid ethyl esters contribute bitterness rather than astringency (Hufnagel and Hofmann, 2008a, Hufnagel and Hofmann, 2008b)

Diketopiperazines (DKPs) are produced by the degradation of polypeptides in several foods and beverages (Borthwick and Da Costa, 2017). Takahashi et al. (2016) showed that cyclo(leu-phe) and corresponding amino acids were generated in rice wine by the metabolism of the microorganism, or the thermal process during the pasteurisation.

Borthwick and Da Costa (2017) showed that cyclo(ala-pro), cyclo(ala-leu), cyclo(pro-val), cyclo(ile-pro), cyclo(pro-pro), cyclo(leu-pro), cyclo(ile-leu), cyclo(ala-phe), cyclo(phe-phe), cyclo(leu-phe) and cyclo(phe-pro) were found in alcoholic beverages such as beer, wine and Awamori spirit. This is in agreement with Oruna-Concha et al. (2015) who showed that cyclo(leu-leu), cyclo(pro-leu), cyclo(pro-ile), cyclo(pro-met), cyclo(pro-val), cyclo(pro-pro) and cyclo(val-ala) were detected in sherry wine. Moreover, they can contribute astringent, salty, grainy, metallic and bitter note to food products.

γ-glutamyl peptides, responsible for mouthfulness, complexity and continuity taste by acting with CaSR calcium channels on the tongue, leading to a release of intracellular Ca^{2+} in the surrounding taste cells (Toelstede et al., 2009, Hillmann et al., 2016). Hillmann et al. (2016) showed that γ-glutamyl peptide compounds, including γ-glu-lys, γ-glu-his, γ-glu-gln, γ-glu-phe, γ-glu-glu, γ-glu-met, γ-glu-thr, γ-glu-gly, γ-glu-val, γ-glu-leu, γ-glu-asp, γ-glu-trp, γ-glu-tyr and γ-glu-gln were found in parmesan cheese by liquid chromatography tandem mass spectrometry (LC-MS/MS). Although Hillmann et al. (2016) found 14 different γ-glutamyl peptide in cheese, only γ-glu-val-gly was found in beer at concentrations in the range of 0.08–0.18 mg/l (Miyamura et al., 2015) and none γ-glutamyl peptides have been reported in pigmented rice wine.

2.6 The formation of typical taste compounds in rice wine

2.6.1 Amino acids

Ljungdahl and Daignan-Fornier (2012) showed that the amino acids are produced by yeast during the fermentation. Their formation starts with the formation of glutamate from α-ketoglutarate by NADPH-dependent glutamate dehydrogenase with the incorporation of ammonia (reaction 1). Then, glutamate is converted to glutamine by glutamine synthetase (reaction 2). The presence of glutamate and glutamine is important due to their involvement in transamination reactions required for the synthesis of each amino acid, as can be seen from figure 2.15 (Magasanik and Kaiser, 2002). The amino acids which were produced by this pathway can be classified into glutamate family (glutamate, glutamine, arginine, proline, and lysine); the aromatic family (phenylalanine, tyrosine, and tryptophan); the serine family (serine, glycine, cysteine, and methionine); the aspartate family (aspartate, asparagine, threonine, cysteine and methionine); and the pyruvate family (alanine, valine, leucine, and isoleucine). Moreover, Valero et al. (2003) showed that yeast has the ability to store amino acids in its vacuole. This action can protect the amino acids from high metabolic regulatory purposes. However, the amino acids are released from yeast by autolysis during wine production (Feuillat and Charpentier, 1982, Martínez-Rodríguez and Polo, 2000). Moreover, amino acids in Chinese rice wine are also liberated from the hydrolysis reaction of proteins and microorganism by protease and carboxypeptidase during the fermentation (Shen et al., 2010).

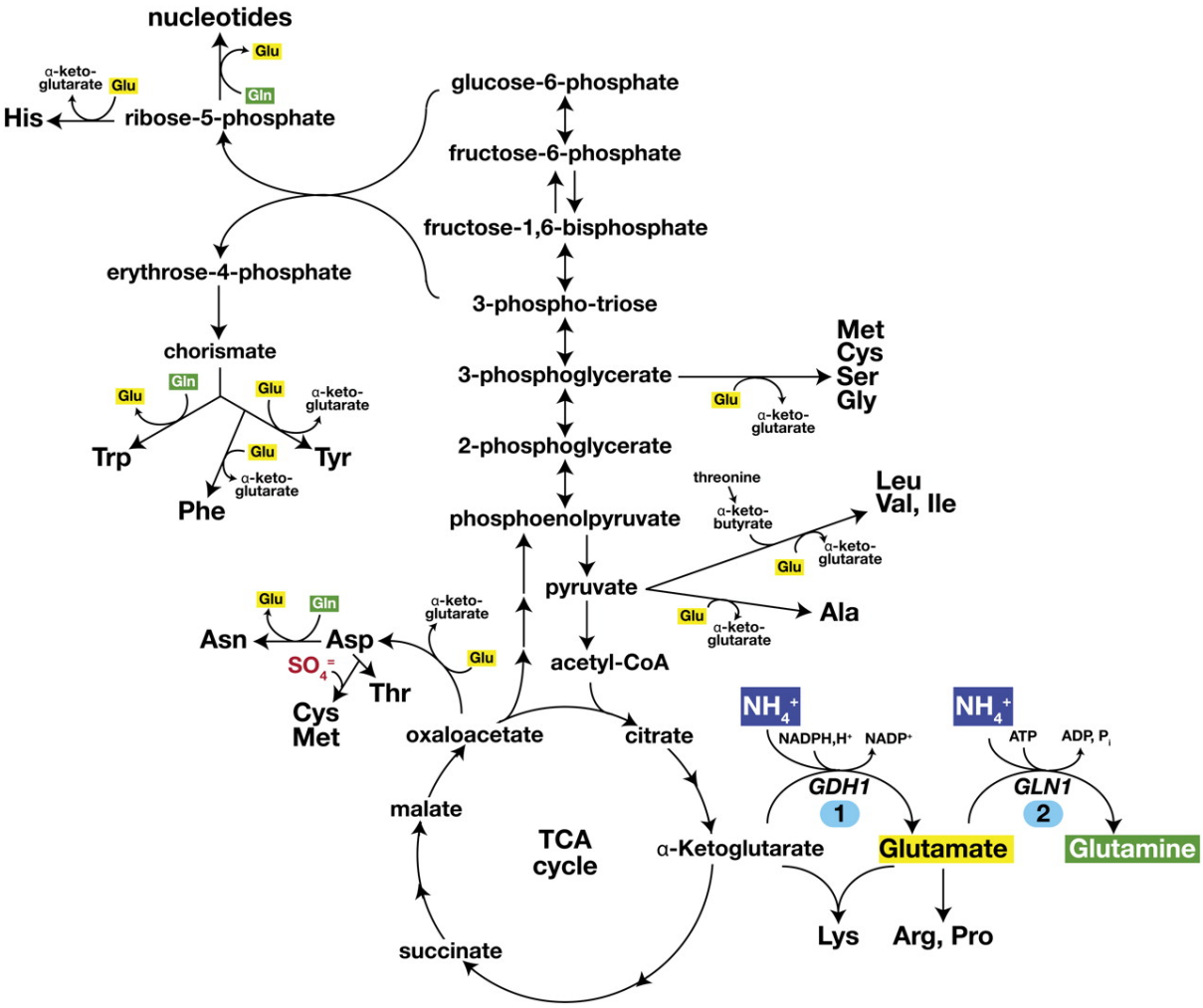

Figure 2-15: The formation of amino acids from the protein synthesis by yeast.

From: Ljungdahl and Daignan-Fornier (2012)

2.6.2 Phenolic acids

The phenolic compounds can be formed from the hydrolysis or degradation of lignin (Cai et al., 2014, Rasmussen et al., 2014, Lee et al., 2015), Normally, the enzymes for lignin degradation, including lignin peroxidase, manganese peroxidase, LiP-MnP hybrid versatile peroxidase and laccase are produced by fungi (Guerriero et al., 2015). The mechanism of lignin degradation starts with the degradation of nonphenolic recalcitrant aromatic substrates by lignin peroxidase. Furthermore, the manganese peroxidase can oxidise Mn^{2+}

to Mn^{3+}, which releases an electron from the low redox substrates. After that, Mn^{3+} reacts with dicarboxylic acid to form a complex compound, which penetrates through the inside of partly degraded lignin to elicit its oxidative degradation (Wong, 2009). The degradation of lignin releases the monomeric units, including 4-hydroxyphenyl, guaiacyl and syringyl groups (de Gonzalo et al., 2016), as can been seen from figure 2.16.

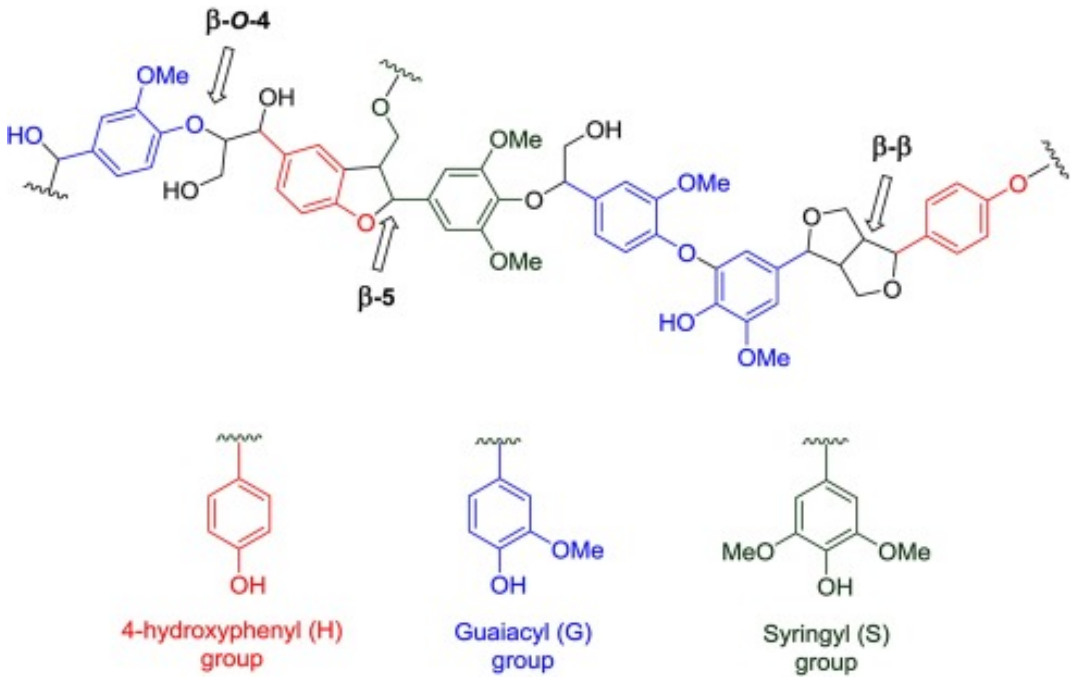

Figure 2-16: The formation of monomers from the degradation of lignin.

From: de Gonzalo et al. (2016)

The LiP-MnP hybrid versatile peroxidase has been considered as the supporting enzyme, which accompanies the lignin peroxidase and manganese peroxidase to increase the high redox activity (Wong, 2009). In addition, laccases or phenol oxidases are copper containing enzymes found in bacteria, fungi, and plants. These enzymes are involved in many response, especially lignin biosynthesis, lignin degradation and detoxification of

phenolic compounds released from the lignin degradation (Wong, 2009, Abdel-Hamid et al., 2013).

2.6.3 Diketopiperazines (DKPs)

DKPs consist of two amino acids backbone form, which 2 molecules of water have been removed. Mishra et al. (2017) showed that they can be formed by the condensation of two amino acids (figure 2.17) however Borthwick and Da Costa (2017) showed that they cannot be produced from the heating of an equimolar amounts of amino acids.

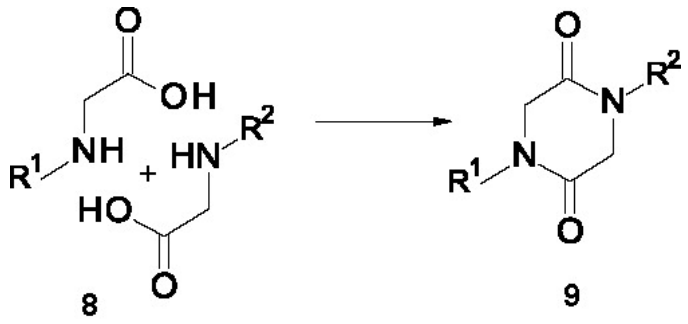

Figure 2-17: The condensation of two amino acids to form DKPs.

From: Borthwick and Da Costa (2017)

Another formation of DKPs was showed by Borthwick and Da Costa (2017). They described that the heating of the acyclic tripeptides with the presence of acidic conditions did produce the corresponding cyclic dipeptides (figure 2.18).

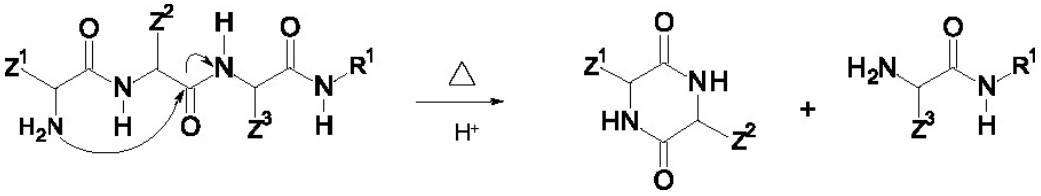

Figure 2-18: The formation of DKPs from acyclic tripeptides.

From: Rizzi (1989)

Moreover, approximately 90% of DKPs can be generated by microorganism rather than the thermal process (Giessen and Marahiel, 2014, Falorni et al., 2000). This is in agreement with Oruna-Concha et al. (2015) who showed that the formation of DKPs in sherry wines might be produced from flor or film of yeast during the fermentation. Mauricio et al. (2001) showed that flor yeast was likely grown under a high concentration of ethanol and acidity, thereby establishing a strongly reducing wine medium. During this stage, flor yeast maintains their intracellular redox balance by minimising the NAD(P)H. Thus, this compound can react with glutamate to form proline which is the most abundant amino acids in grape wine. This proline can be condensed with other amino acids to form proline-based DKPs, as described by Borthwick and Da Costa (2017)

2.6.4 γ-glutamyl peptides

The formation of γ-glutamyl peptides was showed by Toelstede et al. (2009) and Hillmann et al. (2016), as can be seen in figure 2.19. They described that γ-glutamyl donor amino acid L-glutamine (1) is merged with γ-glutamyl transferase (GGT) to form covalent γ-glutamyl-enzyme conjugate (2). After that, this intermediate can react with one more L-glutamine to form the homotranspeptidation product, γ-Glu-Gln (3), or react with other amino acids to form the heterotranspeptidation products, γ-Glu-X (4). Alternatively, the hydrolytic cleavage of intermediate can produce free amino acid L-glutamate (5).

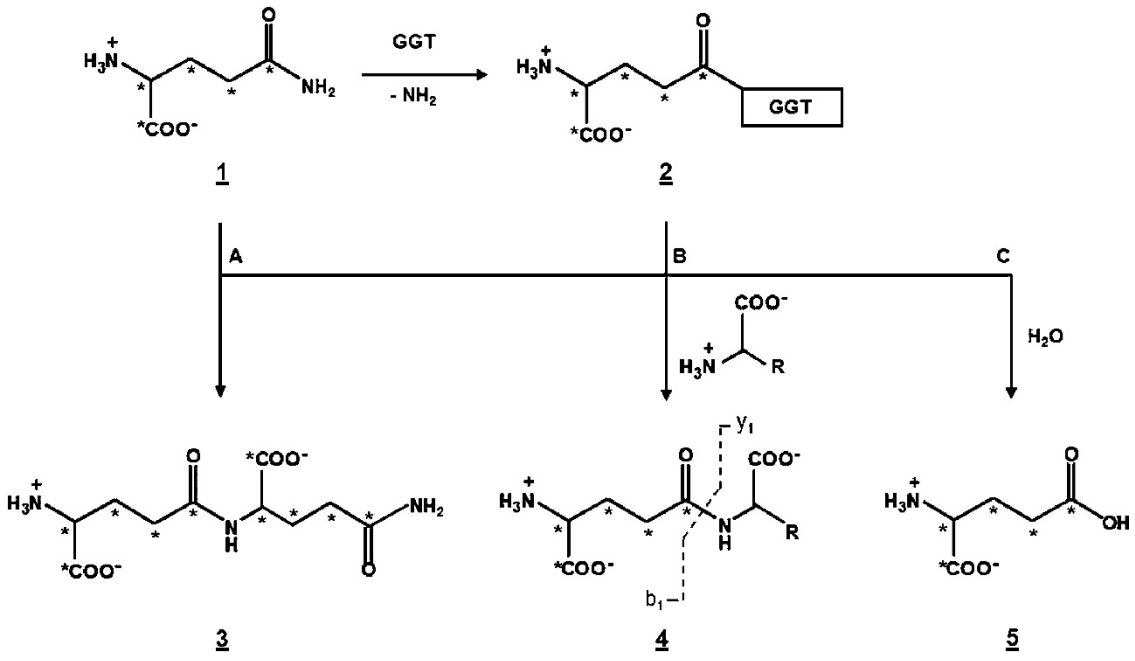

Figure 2-19: The formation of γ-glutamyl peptides by γ-glutamyl transferase (GGT).

From: Hillmann et al. (2016)

Moreover, it can be seen that γ-glutamyl peptides were preferably produced from neutral amino acids, compared with acidic and basic amino acids (Toelstede et al., 2009). The GGT in the formation of γ-glutamyl peptides can be found from fungi, including *Aspergillus*, *Bacillus* and *Lactobacillus* (Hillmann et al., 2016).

2.7 Concluding remarks

In order to produce rice wine, the brewing process consists of saccharification and a parallel alcoholic fermentation. During the saccharification, the starch in rice is mainly degraded to sugars, including maltose and glucose. These fermentable sugars are further converted to ethanol and flavour compounds by the metabolism of the brewing microorganisms, fungi and yeast. Moreover, the moisture content, degree of gelatinisation and brewing temperature have been reported as having an effect on the quality of rice wine. The selected brewing process for rice wine is normally determined by a high level of ethanol, whereas the acetic acid content is low.

The aroma compounds are formed during the brewing process by microorganisms. According to the review, esters, alcohols, acids and aldehydes are the main aroma compound in the rice wine. Moreover, characteristic aroma compounds, especially the volatile phenols are also found. Fruity and floral were reported as the basic aroma in rice wine however a characteristic aroma, smoky and herb-like aroma were found in rice wine that was brewed by using typical materials (wheat Qu and/or whole grain). Phenolic acids which are found in these typical materials promote the formation of volatile phenols. Furthermore, the five basic taste compounds in rice wine, including sugars, organic acids, amino acids and phenolic acids can be liberated by the catabolism of brewing strains

however DKPs and γ-glutamyl peptides are generated by the anabolism of the brewing strains.

The aim of this thesis is firstly to identify the typical aroma and taste compounds in commercial pigmented rice wines then develop a suitable and consistent lab-scale brewing process, and finally to study the effect of bran from pigmented rice on the characteristic aroma and taste compounds and confirm their precursors in pigmented rice wine by using standard spiking technique.

CHAPTER 3: TASTE AND AROMA COMPOUNDS IN RICE WINE: A COMPARISON OF POLISHED RICE WINE AND PIGMENTED RICE WINE

Abstract

Rice wine can be produced from both polished or unpolished (or pigmented) rice. The latter gives a reddish-brown colour and unique flavour to the wine. The rice wines were analysed for the taste compounds, including sugars, organic acids, amino acids, phenolic acids, diketopiperazines (DKPs) and γ-glutamyl peptides. The volatile and semi-volatile compounds in 3 commercial rice wines (two polished and one pigmented rice wine) were extracted, using solid-phase microextraction (SPME) and solid phase extraction (SPE), and analysed by gas chromatography-mass spectrometry (GC–MS) and gas chromatography–olfactometry (GC–O). A higher concentration of phenolic acids and DKPs (cyclo(val-pro), cyclo(ala-val), cyclo(ile-pro), cyclo(leu-pro) and cyclo(gly-leu)) were found in pigmented rice wine ($p<0.05$). The taste compounds which were presented at concentrations above the reported thresholds were glucose, cyclo(ile-pro) and cyclo(leu-pro) in all rice wines, and acetic acid in only pigmented rice wine. Ethyl ester and the higher alcohols were the most abundant compounds detected in rice wine samples. The analysis of GC–O showed 16 aroma active compounds were detected in the headspace of commercial rice wines, however guaiacol and 4-vinylguaiacol were the characteristic aroma which was only found in pigmented rice wine.

Keywords: pigmented rice wine, diketopiperazines, γ-glutamyl peptides, volatile compound, characteristic aroma

3.1 Introduction

The aroma of rice wine has been widely reported as being floral note, fruity note, sweety note, vinegar, solvent-like note, cheesy note and vegetable-like note (Kim et al., 2010, Chen et al., 2013b, Chen et al., 2013a), however a smoky aroma also was detected in Chinese rice wine which is produced by using wheat Qu (Chen et al., 2013b). These aromas are derived from the abundance of volatile compounds such as esters, higher alcohols, aldehydes, organic acids and phenols (Kim et al., 2010, Chen and Xu, 2010, Chen et al., 2013a, Yang et al., 2017a), which are generated during fermentation from raw materials (mostly rice starch) and the fermentable products of microbial metabolism (Yang et al., 2017a).

In addition to aroma, some non-volatile compounds also are important and influence flavour, taste and oral sensation (Breslin, 2001). Non-volatile compounds in the wine matrix likely influence the aroma release, aroma intensity and aroma perception (Sáenz-Navajas et al., 2012), and they also contribute to taste and mouthfeel. Similar to wine, previous research carried out investigating the taste characteristics of rice wine has described it as being sweet, sour, harmonious, mellow, and fresh attribute (Yu et al., 2015, Shen et al., 2010), however bitter, umami and salt taste characteristics were also found (Jian-guo, 2004).

The research to date has tended to focus on the aroma and taste compounds of non-pigmented rice wine rather than pigmented rice wine. The aim of this study is (i) to identify the characteristic taste and aroma compounds in pigmented rice wine, and (ii) to characterise and compare the taste and aroma profile of commercial polished rice wines and pigmented rice wines.

3.2 Materials and methods

3.2.1 Materials

Four rice wines, RW1 (Kikusui Shuzo Co., Ltd, Niigata, Japan), RW2 (Oenon Holdings Inc., Tokyo, Japan), PRW1 (Kiuchi Brewery, Ibaraki, Japan) and PRW2 (Mae Sai Winery, Thailand) were purchased from market in UK, Japan and Thailand. Samples were stored at -20°C until analysis. Prior to analysis, rice wines were filtered through Minisart® 0.22 µm polyethersulfone (PES) syringe filter from Sartorius (Goettingen, Germany), and then diluted as appropriate.

3.2.2 Chemicals

The following chemicals were purchased from Sigma-Aldrich (Dorset, UK): ethanol, methyl acetate, diethyl ether, >98% 4-coumaric acid, 98% epicatechin, > 97% vanillic acid, >98% sinapic acid, >97% protocatechuic acid, >99% ferulic acid, >95% syringic acid, >98% caffeic acid, 98% catechin, 97% gallic acid, 99% 4-hydroxybenzoic acid, 99.5% glucose, >99% fructose, 99% maltose, 95% maltotriose, >99% malic acid, 98% lactic acid, 99% citric acid, 99% sodium succinate, 99% sodium tartrate, 1,2-dichlorobenzene, saturated alkane standard C_5–C_{30} and C_7–C_{40} and all aroma standards. Cyanidin-3-glucoside (>96%) was purchased from Extrasynthese (Genay, France). Analytical grade sulfuric acid, HPLC grade methanol, >99% acetic acid, Optima™ 0.1% formic acid and Pierce® acetonitrile were purchased from Fisher Scientific (Loughborough, UK). Analytical grade formic acid was purchased from BDH (Poole, UK). The EZfaast™ amino acid analysis kit was purchased from Phenomenex (Macclesfield, UK). The standard of DKPs, >99% cyclo(proline-valine), >99% cyclo(alanine-valine), >99% cyclo(isoleucine-proline), >99% cyclo(leucine-proline), >98% cyclo(proline-

proline), >99% cyclo(alanine-proline) and standard of γ-glutamyl peptides including >99% γ-glutamyl tyrosine (γ-glu-tyr), >99% γ-glutamyl phenylalanine (γ-glu-phe), >99% γ-glutamyl histidine (γ-glu-his), >99% γ-glutamyl methionine (γ-glu-met), >99% γ-glutamyl glutamic acid (γ-glu-glu), >99% γ-glutamyl leucine (γ-glu-leu), >99% γ-glutamyl valine (γ-glu-val), >99% γ-glutamyl alanine (γ-glu-ala) and >99% γ-glutamyl glycine (γ-glu-gly) were purchased from Bachem (Bubendorf, Switzerland).

3.2.3 Analysis of taste compounds in rice wine

3.2.3.1 Sugars, ethanol and organic acids

The analytical method was adapted from Zeppa et al. (2001). The analyses were performed on an Agilent series 1100 HPLC system (Waldbronn, Germany) with diode array detector (DAD) and reflective index (RI) detector, series ERC-7515A from Polymer laboratories (Shropshire, UK). The sample (50 µl) was injected into an Aminex HPX-87H column (300 mm x 7.8 mm, 9 µm particle size) from Bio-Rad (Hertfordshire, UK) which was thermostatically maintained at 45 °C. The compounds were eluted with 5mM sulfuric acid, and the flow rate was controlled at 0.6 ml/min. Chromatograms were analysed at 210 nm for organic acids, whereas the RI detector was used for sugars and ethanol analysis. The identification of compounds was based on retention times by comparison with standards of both organic acids and sugars. Quantification was carried with the external calibrations using the corresponding standards in the following concentrations: 10, 25, 50, 70 and 100 mg/l for organic acids, and 50, 125, 250, 350 and 500 mg/l for sugars respectively, $R^2 > 0.99$ for all analytes.

3.2.3.2 Free amino acids

Free amino acids in samples were derivatised by using the EZ:faast™ amino acid kit from Phenomenex (CA, USA). The derivatised samples were analysed by GC-MS, using GC series 7890A and MS series 5975C from Agilent (CA, USA) as described by Elmore et al. (2005). In order to quantify the compounds, calibration curves were came out using the corresponding standard compounds in the range of 25-200 µM, $R^2 > 0.99$.

3.2.3.3 Free phenolic acids

The phenolic acids were analysed using HPLC series 1200 from Agilent (Waldbronn, Germany). This analytical method was modified from Seal (2016). Briefly, the pre-filtered sample (5 µl) was injected into a Nova-Pak® C18 column (250 mm x 4.6 mm, 4 µm particle size) from Waters (Dublin, Ireland) which was thermostatically controlled at 40 °C. The mobile phase was composed of 0.1% formic acid (solvent A) and methanol (solvent B) with a flow rate of 0.8 ml/min. The gradient elution was changed from 5% to 10% B in 13 min, from 10% to 25% B in 25 min, from 25% to 35% B in 30 min, from 35% to 20% B in 32 min, from 20% to15% B in 35 min, from 15% to 10% B in 40 min and from 10% to 5% B in 50 min, then the gradient back to initial condition (solvent A: solvent B: 95: 5) in 60 min before the next injection. Compounds were detected by using DAD detector at 280 and 320 nm for phenolic acids, whereas the wavelength at 520 nm was used for cyanidin-3-glucoside analysis. Quantification was carried out by the external calibration method using commercially available standards in the following concentrations: 0.5, 1, 5, 10 and 50 mg/l for phenolic acids, and 50, 125, 250, 350 and 500 mg/l for anthocyanins, respectively ($R^2 > 0.99$).

3.2.3.4 DKPs

Analysis of DKPs was carried out as described by Oruna-Concha et al. (2015). Briefly, internal standard 50 µl (1,2-dichlorobenzene, 100 mg/l) was added into 15 ml of rice wine. Samples containing 0.33 mg/l of internal standard were passed through the SPE cartridge (Strata-X 33 µm polymeric reversed phase giga tube) from Phenomenex (CA, USA), HPLC water and methyl acetate were used for washing and elution, respectively. The eluent was concentrated by flushing with N_2, and then injected into the GC-MS, using GC series 7890A and MS series 5975C from Agilent (CA, USA), which was equipped with a DB-WAX Ultra Inert column (30 m x 0.25 mm id, 0.25 µm) from Agilent (CA, USA). For the quantification of DKPs, external calibration curves of the corresponding DKP standards were prepared relative to 1,2-dichlorobenzene (internal standard) ($R^2 > 0.90$).

3.2.3.5 γ-glutamyl peptides

The analysis of γ-glutamyl peptides was modified from Toelstede et al. (2009). The sample (500 µl) containing 0.2 mg/l of γ-glu-met (internal standard) was filtered through an Amicon® Ultra, 0.5 ml centrifugal filter, MWCO 3kDa, from Sigma-Aldrich (Steinheim, Germany) using Minispin centrifuge from Eppendorf (Ontario, Canada) at 12,045 g for 10 minutes. The sample (5 µl) was analysed by liquid chromatography-tandem mass spectrometry (LC-MS/MS), series 6410 from Agilent (CA, USA) equipped with a reversed phase Zorbax C18 column (2.1 mm x 100 mm, 1.8 µm) from Agilent (CA, USA). The compounds were separated by using a mobile phases which comprised of 0.1% formic acid and 0.1% formic acid in acetonitrile, and the flow rate was controlled at 0.2 ml/min. The gradient elution was changed from 0% to 10% B in 20 min, from 10% to 100% B in 45 min,

and then back to initial condition (solvent A: solvent B: 100: 0) in 50 min before the next injection. The LC-MS/MS was operated in the positive electrospray ionization mode. The detection of γ-glutamyl dipeptides was performed in Dynamic MRM mode. The ion spray voltage was set at 4000 eV, and nitrogen served as curtain gas (35 psi). The compounds were quantified using the optimised mass transitions as follows: γ-glu-glu (m/z 277.1→84.1), γ-glu-met (m/z 279.1→150.1), γ-glu-his (m/z 285.1→110.1), γ-glu-ala (m/z 219.1→44.1), γ-glu-gly (m/z 205.1→84.1), γ-glu-leu (m/z 261.2→84.1), γ-glu-val (m/z 247.1→84.1), γ-glu-tyr (m/z 311.1→182.1) and γ-glu-phe (m/z 295.1→120.1), respectively. For quantification, the peak area of the compound of interest was compared to the corresponding standard curve which was prepared in the range of 0.01-5 mg/l, ($R^2 > 0.93$).

3.2.4 Aroma analysis for rice wines

3.2.4.1 Volatile compounds

The volatile compounds were extracted by SPME based on a method by Chen and Xu (2010). The sample (5 g) was spiked with 50 µl of internal standard (1,2-dichlorobenzene, 1 mg/l). After that, the sample containing 0.01 mg/l of internal standard was incubated at 40 ºC for 10 min to equilibrate. A 50/30 µm fibre (DVB/CAR/PDMS) from Supelco (PA, USA) was exposed to the headspace for 30 min, and the extract was analysed by GC-MS, using GC series 7890A and MS series 5975C from Agilent (CA, USA). The compounds were separated on a Zebron™ ZB-5MSi column (30 m × 250 µm internal diameter, 1 µm film thickness) from Phenomenex (Cheshire, USA). Helium was used as the mobile phase at a flow rate of 0.9 ml/min. The oven temperature was set at 40 °C for 5 min, and then increased to 220 °C for 5 min at a heating rate of 5 °C/min. The extract was also analysed on Stabilwax®-DA

column (30 m × 250 µm internal diameter, 0.5 µm film thickness) from Restek (PA, USA) for compound confirmation. Mass spectra were recorded in the electron-impact mode at an ionisation voltage of 70 eV and source temperature of 230 °C, with a scan range of 20–280 m/z. Linear retention indices (LRIs) were determined using a series of standard alkanes C_5-C_{30} under the same chromatographic conditions. Identification of aroma compounds was based on comparison of their mass spectrum and LRIs with authentic standards. The relative quantity of each compound in rice wine was calculated by comparing the peak area with peak area of internal standard, using a response factor of 1. The Quantification of guaiacol and 4-vinylguaiacol was carried out using their calibration curves which were prepared in the range of 0.01-5 mg/l and 0.002-2 mg/l respectively, $R^2 > 0.99$.

3.2.4.2 Semi-volatile compounds

The semi-volatile compounds were extracted using SPE. This procedure was modified from Lignou et al. (2013). Briefly, 50 µl internal standard (1,2-dichlorobenzene, 100 mg/l) was added to15 ml of rice wines. Samples containing 0.33 mg/l of internal standard were passed through the SPE cartridge (Strata-X 33 µm polymeric reversed phase giga tube) from Phenomenex (CA, USA). Methanol and water were used to condition the cartridge, and methyl acetate was used for compound elution. After that, the eluents were dried using N_2, and reconstituted in methyl acetate to 1 ml. The extract (2 µl) was injected on GC-MS, using GC series 7890A and MS series 5975C from Agilent (CA, USA). The compounds were separated using a Stabilwax®-DA column (30 m x 250 µm internal diameter, 0.5 µm film thickness) from Restek (PA, USA). The inlet temperature was 250 °C and detector temperature was 280 °C. Helium was used as carrier gas with the flow rate was 1.4 ml/min. The oven temperature was started at 50 °C for 1 min, and increased to 200

°C at a rate of 6 °C/min, and then increased to 250 °C for 30 min at a rate of 30 °C/min. The extract was also analysed on Zebron™ ZB-5MSi column (30 m × 250 µm internal diameter, 1 µm film thickness) from Phenomenex (CA, USA) for compound confirmation. Mass spectra were recorded in the electron impact mode at an ionisation voltage of 70 eV and source temperature of 150 °C, with a scan range of 10–300 m/z. Linear retention indices (LRIs) were determined using a series of standard alkanes C_7-C_{40} under the same chromatographic conditions. Identification of aroma compounds was based on comparison of their mass spectrum and LRIs with authentic standards. The relative quantity of each compound in rice wine was calculated by comparing the peak area with peak area of internal standard, using a response factor of 1.

3.2.4.3 GC-Olfactometry

The analytical method was adapted from Lignou et al. (2013). The samples were extracted using SPME and SPE, and then analysed by gas chromatography-olfactometry (GC-O), using an HP5890 GC from Agilent (Waldbronn, Germany), and an ODO 2 series II from SGE (Buckinghamshire, UK). The compounds were separated using a Zebron™ ZB-5MSi column (30 m × 250 µm internal diameter, 1 µm film thickness) from Phenomenex (CA, USA). Two assessors (one female and one male) were used for the detection and verbal description of the aroma active compounds of the extracts. The assessors had at least 2 years of experience in recognising odorants by GC-O. The intensity of aromas was scored in the range of 1-9, where 1 means very weak and 9 means very strong. To confirm the result, the extracts were analysed using a Stabilwax®-DA column (30 m × 250 µm internal diameter, 0.5 µm film thickness) from Restek (PA, USA).

3.2.5 Statistical analysis

IBM SPSS Statistics for Windows, Version 22.0 software (IBM Corp., Armonk, NY, USA) was used for the statistical analysis of experimental data. The difference between the mean values was considered at 95% ($p < 0.05$), 99% ($p < 0.01$) or 99.9% ($p < 0.001$) confidence interval, using the analysis of variance (ANOVA) with the post hoc Duncan test.

3.3 Results and discussions

3.3.1 Taste compounds in rice wine

3.3.1.1 Sugars, ethanol and organic acids

The maltose, glucose and fructose in the commercial rice wines are presented in table 3.1. Fructose was only found in PWR2 at a high concentration, whereas glucose was the second most abundant sugar in all commercial rice wines. The highest concentration of glucose was also found in PRW2, with a value of twenty, six and five times higher compared to PRW1, RW1 and RW2 respectively. Moreover, the concentration of glucose in all rice wine samples was above the reported threshold, so it will contribute to the sweet taste. The concentration of maltose and glucose in RW1, RW2 and PRW1 are in agreement with Shen et al. (2011) who showed that Chinese rice wine contained maltose and glucose in the range of 11–543 mg/100 ml and 224–1,626 mg/100 ml, respectively. Notably, a high concentration of fructose in PWR2 is well in access of that reported by Niu et al. (2008) and Shen et al. (2011) who showed that maltose and glucose are the main sugar in rice wine. In addition, Das et al. (2014) showed that fructose was found in rice beer at only 20 mg/l.

The percent of ethanol in commercial rice wines was also investigated. The results showed that the highest concentration of ethanol was found in RW1 and RW2, whereas PRW2 had the lowest ethanol content ($p < 0.05$). Higher ethanol content in RW1 and RW2 corresponds to higher glucose content, compared to PRW1. The concentration of ethanol in RW1, RW2 and PRW1 is in agreement with Furukawa (2012) who showed that Japanese rice wines have the ethanol around 15 % (v/v), however the ethanol content of PRW2 was rather low.

The organic acids, including citric acid, malic acid, succinic acid and lactic acid were investigated in all commercial rice wines. Among them, succinic acid, malic acid, lactic acid and acetic acid were found to be the most abundant organic acids. The PRW2 showed the highest concentration of malic acid and acetic acid, with a value of ten and thirty times higher than the reported thresholds, whereas the concentration of succinic acid was lower than others. Moreover, the concentration of acetic acid in PRW1 was five times above the threshold. The lactic acid was also found in PRW1 at the highest concentration. The concentration of citric acid, malic acid, lactic acid in RW1, RW2 and PWR1 were lower than those found by Yu et al. (2015) and Das et al. (2014). The concentration of succinic acid in all rice wine samples was higher than its reported threshold. Moreover, the concentration of malic acid and acetic acid in PWR2 were also higher than those published reports. The lower pH in commercial rice wines corresponded to a higher concentration of organic acids.

The formation of sugars in rice wine was explained by Shen et al. (2011) and Das et al. (2014) who showed that maltose and glucose comes from the hydrolysis of starch in rice during the fermentation. The glucose is converted to acetyl CoA, and further converted to ethanol via the Embden Meyerhof pathway (Lin and Tanaka, 2006). Glucose is also

converted to organic acids via the tricarboxylic acid pathway (West, 2017). However, lactic acid and acetic acid are formed via the hetero-fermentative catabolism of yeasts (Pardali et al., 2017) and the oxidation of ethanol by *Acetobacter* (Bartowsky and Henschke, 2008). Notably, the highest concentration of glucose and fructose in PRW2 were not likely to correspond with the concentration of ethanol, however they were likely to correspond with the highest concentration of acetic acid in that sample.

Table 3-1: Sugars, ethanol and organic acids content in commercial rice wines and their reported thresholds.

compounds	†threshold	concentration				§Sig
		RW1	RW2	PRW1	PRW2	
ethanol	-	15.5 ± 0.1^{d}	15.2 ± 0.1^{c}	12.3 ± 0.1^{b}	5.2 ± 0.1^{a}	***
pH	-	4.2 ± 0.1^{c}	4.3 ± 0.1^{d}	3.6 ± 0.1^{b}	2.7 ± 0.1^{a}	***
sugars (mg/ 100 ml)						
maltose	-	383 ± 9^{b}	324 ± 10^{a}	387 ± 1^{b}	423 ± 3^{c}	***
glucose	324	1327 ± 6^{b}	1631 ± 1^{c}	434 ± 1^{a}	$8{,}864 \pm 98^{d}$	***
fructose	183	nd	nd	nd	$13{,}408 \pm 470$	-
organic acids (mg/ 100 ml)						
citric acid	50	6.6 ± 1.3^{a}	8.7 ± 0.9^{b}	9.8 ± 1.4^{b}	nd	**
tartaric acid	44	nd	nd	8.4 ± 1	nd	-
malic acid	49	21.6 ± 0.5^{c}	13.8 ± 2.8^{b}	6.8 ± 0.6^{a}	558 ± 6^{d}	***
succinic acid	106	378 ± 2^{b}	557 ± 1^{c}	381 ± 3^{b}	109 ± 7^{a}	***
lactic acid	139	3.1 ± 0.1^{a}	7.4 ± 0.1^{b}	113 ± 1^{d}	21.4 ± 0.9^{c}	***
acetic acid	12	nd	nd	60.5 ± 3.7^{a}	401 ± 13^{b}	***

Values with the same letter superscripts within each row are not significantly different ($p = 0.05$).

Data are presented as mean ± standard error, n=3. nd = not detected.

†Taste threshold in bottled water was obtained from Hufnagel and Hofmann (2008b).

§Sig = Probability, as obtained from ANOVA, that there is a difference between means; **Significant at the 1% level ($0.001 < p \leq 0.01$) and ***Significant at the 0.1% level ($p \leq 0.001$).

3.3.1.2 Free amino acids

Of seventeen amino acids identified (table 3.2), the predominant ones were alanine, glycine, proline, valine and leucine. This amino acid profile is similar to that reported by Shen et al. (2010) and Das et al. (2014). The results also showed very low concentrations of amino acids were found in PRW2 ($p < 0.05$). Contrary to expectations, this study did not find a significant difference in most amino acids between RW1 and RW2 which were produced from the polished rice (proteins are almost removed), compared to PRW1 which was produced from pigmented rice (unpolished rice). According to the results, the amino acids in the commercial rice wines are varied due to the manufacturing process rather than the type of rice (polished rice or pigmented rice). This results are in disagreement with Xie et al. (2016) who showed that amino acids in Chinese rice wines are derived from the degradation of proteins in rice, and they have a positive correlation with the total proteins in rice grain, $r = 0.92$.

Hufnagel and Hofmann (2008b) showed that amino acids contribute to the taste of wine, which vary from sweet (ala, gly, thr, ser and pro), umami (glu, asn and asp) and bitter (val, leu, ile, phe, lys, tyr and his). This is in agreement with Zhao et al. (2016) who showed that amino acids contribute sweet (met, ala, gly, pro and ser), umami (glu, tyr and asp), and bitter (his, lys, val, try, tyr, phe, ile and leu) in foods. However, their concentrations in these commercial rice wines were lower than the reported thresholds.

Table 3-2: Amino acids identified in commercial rice wines and their threshold values.

compounds	taste	†threshold (μM)	concentration (μM)				§Sig
			RW1	RW2	PRW1	PRW2	
alanine	sweet	12,000	1030 ± 43^{c}	1092 ± 16^{d}	940 ± 15^{b}	39 ± 4^{a}	***
glycine	sweet	25,000	1020 ± 51^{b}	1211 ± 21^{c}	973 ± 27^{b}	27 ± 1^{a}	***
threonine	sweet	35,000	236 ± 14^{b}	484 ± 3^{d}	277 ± 12^{c}	13 ± 6^{a}	***
serine	sweet	25,000	504 ± 40^{a}	972 ± 11^{c}	719 ± 27^{b}	nd	***
cysteine	sweet	-	55.2 ± 1^{b}	94 ± 6^{c}	25 ± 10^{a}	nd	***
proline	sweet	25,000	749 ± 32^{c}	796 ± 18^{d}	655 ± 12^{b}	18 ± 2^{a}	***
glutamic acid	umami	1,200	531 ± 79^{b}	853 ± 45^{c}	536 ± 22^{b}	10 ± 1^{a}	***
asparagine	umami	50,000	448 ± 40^{c}	725 ± 4^{d}	268 ± 8^{b}	20 ± 4^{a}	***
aspartic acid	umami	20,000	361 ± 23^{b}	685 ± 10^{d}	618 ± 23^{c}	153 ± 9^{a}	***
valine	bitter	20,000	479 ± 29^{b}	786 ± 5^{d}	641 ± 33^{c}	20 ± 1^{a}	***
leucine	bitter	11,000	709 ± 47^{b}	923 ± 19^{c}	692 ± 26^{b}	22 ± 9^{a}	***
isoleucine	bitter	10,000	232 ± 13^{b}	384 ± 3^{d}	320 ± 14^{c}	10 ± 1^{a}	***
phenylalanine	bitter	45,000	207 ± 15^{b}	385 ± 4^{d}	286 ± 10^{c}	14 ± 1^{a}	***
lysine	bitter	80,000	339 ± 31^{b}	880 ± 30^{c}	317 ± 25^{b}	29 ± 2^{a}	***

Table 3-2: Amino acids identified in commercial rice wines and their threshold values (continued).

compounds	taste	[†]threshold (μM)	concentration (μM)				[§]Sig
			RW1	RW2	PRW1	PRW2	
tyrosine	bitter	4,000	337 ± 26^{b}	487 ± 8^{c}	353 ± 16^{b}	18 ± 1^{a}	***
histidine	bitter	45,000	158 ± 9^{b}	311 ± 15^{c}	39 ± 2^{a}	nd	***
ornithine	-	-	109 ± 8^{a}	335 ± 18^{c}	264 ± 18^{b}	nd	***

Values with the same letter superscripts within each row are not significantly different ($p = 0.05$).

Data are presented as mean ± standard error, n=3.

nd = not detected.

[†]Taste threshold in bottled water was obtained from Hufnagel and Hofmann (2008b).

[§]Sig = Probability, as obtained from ANOVA, that there is a difference between means; ***Significant at the 0.1% level ($p \leq 0.001$).

3.3.1.3 Free phenolic acids

According to table 3.3, gallic acid, protocatechuic acid, 4-hydroxybenzoic acid and syringic acid were found in all commercial rice wines, however vanillic acid, 4-coumaric acid and ferulic acid were only detected in PRW1. This results is in agreement with Que et al. (2006) who showed that gallic acid, catechin, vanillic acid, caffeic acid, syringic acid, epicatechin, 4-coumaric acid, ferulic acid, rutin and quercetin were found in rice wines, however the concentration of vanillic acid and ferulic acid in PRW1 was higher than those samples. The results also showed that PRW2 had only gallic acid and 4-hydroxybenzoic acid, and their concentrations were much lower than the others.

Most of the free phenolic acids were significantly higher in pigmented rice wine, PRW1 ($p<0.05$). This finding was also reported by Wang et al. (2014) who showed that the phenolic compounds in black rice wine were higher than in polished rice wine, because the precursors of phenolic acids were mainly found in the outer layer of rice grains, and is lost by polishing from whole rice to 90% polished rice (Zhou et al., 2004).

The formation of phenolic acids was reported by Lambert et al. (2014) who showed that ferulic acid, vanillic acid and protocatechuic acid were generated from lignin in plants by microbial enzymes. Thus, they might be released from rice grain during fermentation. Moreover, Hufnagel and Hofmann (2008b) showed that phenolic acids contributed bitter and astringent in wine. However, their concentration in commercial rice wines was lower than the reported thresholds.

Table 3-3: Phenolic acids and anthocyanins identified in commercial rice wines and their reported thresholds.

compounds	†threshold (mg/l)	concentration (mg/l)				§Sig
		RW1	RW2	PRW1	PRW2	
gallic acid	49	10.8 ± 0.1^{b}	18.1 ± 0.1^{d}	17.2 ± 0.5^{c}	5.1 ± 0.2^{a}	***
protocatechuic acid	31	2.6 ± 0.1^{a}	3.2 ± 0.1^{b}	19.2 ± 0.4^{c}	nd	***
4-hydroxybenzoic acid	92	13.5 ± 0.1^{b}	15.1 ± 0.1^{c}	16.4 ± 0.1^{d}	3.3 ± 0.1^{a}	***
vanillic acid	53	nd	nd	3.3 ± 0.1	nd	-
syringic acid	52	1.1 ± 0.0^{a}	nd	1.8 ± 0.1^{b}	nd	**
4-coumaric acid	23	nd	nd	0.5 ± 0.0	nd	-
ferulic acid	13	nd	nd	3.3 ± 0.4	nd	-

Values with the same letter superscripts within each row are not significantly different ($p = 0.05$).

Data are presented as mean ± standard error, n=3.

nd = not detected.

†Taste threshold in bottled water was obtained from Hufnagel and Hofmann (2008b).

§Sig = Probability, as obtained from ANOVA, that there is a difference between means; **Significant at the 1% level ($0.001 < p \leq 0.01$) and ***Significant at the 0.1% level ($p \leq 0.001$).

3.3.1.4 DKPs

Several DKPs were detected in commercial rice wines (table 3.4). However, the concentration of cyclo(val-pro), cyclo(ala-val), cyclo(ile-pro), cyclo(leu-pro), and cyclo(gly-leu) were significantly higher in pigmented rice wines, PRW1 ($p < 0.05$). The results also showed that DKPs in commercial rice wine were proline-based. This result is in agreement with Borthwick and Da Costa (2017) who showed that proline-based DKPs are found widespread in foods and beverages. Moreover, Gautschi et al. (1997) also showed that proline-based DKPs which were cyclo(ala-pro), cyclo(val-pro), cyclo(ile-pro), cyclo(leu-pro), cyclo(met-pro), cyclo(phe-pro), and cyclo(pro-pro) were found in beer.

The highest concentration of DKPs in PRW1 was not related with the concentration of the corresponding amino acids (table 3.2). Therefore, the amino acids might not be their precursors. Moreover, DKPs in commercial rice wines, except in PRW2 were likely formed under the acidic condition. This is in agreement with Borthwick and Da Costa (2017) who showed that DKPs are more formed by the intramolecular cyclization of acyclic peptides with the presence of heating under acidic condition. However, Mishra et al. (2017) showed that 90% of DKPs in fermented foods was formed by microorganism.

It has been shown that DKPs contribute the astringent, salty, grainy and metallic in food products (Borthwick and Da Costa, 2017). Moreover, the concentration of cyclo(ile-pro) and cyclo(leu-pro) in RW1, RW2 and especially PRW1 were above their thresholds.

Table 3-4: Diketopiperazines (DKPs) identified in commercial rice wines and their reported thresholds.

compounds	[†]TC (bitter)	[†]TC (metallic)	concentration (mg/l)				[§]Sig
			RW1	RW2	PRW1	PRW2	
cyclo(val-pro)	251	63	4.9 ± 0.1^{c}	3.9 ± 0.5^{b}	9.2 ± 0.3^{d}	0.04 ± 0.0^{a}	***
cyclo(ala-val)	250	69	0.37 ± 0.0^{b}	0.03 ± 0.0^{a}	0.87 ± 0.1^{c}	nd	***
cyclo(ile-pro)	101	25	42.7 ± 1.1^{c}	36.2 ± 5.1^{b}	60.3 ± 1.3^{d}	1.1 ± 0.1^{a}	***
cyclo(leu-pro)	250	25	43.5 ± 1.3^{c}	36.6 ± 5.2^{b}	70.4 ± 1.5^{d}	0.48 ± 0.0^{a}	***
cyclo(pro-pro)	501	147	10.5 ± 1.6^{a}	10.8 ± 1.1^{a}	12.8 ± 1.7^{a}	nd	***
cyclo(gly-leu)	100	90	0.81 ± 0.0^{b}	0.57 ± 0.1^{a}	1.4 ± 0.1^{c}	nd	***

Values with the same letter superscripts within each row are not significantly different ($p = 0.05$).

Data are presented as mean ± standard error, n=3.

nd = not detected.

[†]Taste threshold in bottled water (mg/l) was obtained from Stark and Hofmann (2005).

[§]Sig = Probability, as obtained from ANOVA, that there is a difference between means; ***Significant at the 0.1% level ($p \leq 0.001$).

3.3.1.5 γ-glutamyl peptides

This investigation showed the presence of γ-glutamyl peptides in pigmented rice wine (PRW1) for the first time (table 3.5). The γ-glu-gly and γ-glu-his were the most abundant compound in RW1, RW2 and PRW1. Moreover, the concentrations of γ-glu-val, γ-glu-leu, γ-glu-glu, γ-glu-phe and γ-glu-tyr were higher in PRW1, compared to polished rice wine (RW1 and RW2). The formation of the most γ-glutamyl peptides is likely corresponded parent amino acids in the pigmented rice. However, the concentrations of γ-glu-gly and γ-glu-his in polished rice wines were higher than pigmented rice wines. This can be explained by the fact that the polished rice wines contained the more of corresponding amino acids (glu, gly and his), compared to PRW1 and PRW2.

Toelstede et al. (2009) showed that γ-glutamyl peptides are formed from γ-glutamyl donor amino acid L-glutamine, which further react with another amino acids to form the corresponding γ-glutamyl peptides, using γ-glutamyl transferase (GGT) from *Aspergillus*, *Bacillus* and *Lactobacillus* (Hillmann et al., 2016).

Miyamura et al. (2015), Zhao et al. (2016) and Shibata et al. (2017) also showed that γ-glutamyl peptides contribute kokumi taste which is responsible for long-lasting mouth fullness. However, their concentrations in the commercial rice wines were lower than the reported thresholds. Although no single compound was above threshold for the kokumi taste in commercial rice wines, the effect of the overall concentration of these compounds on the kokumi taste should be further studied.

Table 3-5: γ-glutamyl peptides identified in commercial rice wines and their reported thresholds.

compounds	†threshold (mg/l)	concentration (mg/l)				§Sig
		RW1	RW2	PRW1	PRW2	
γ-glu-gly	3.6	0.29 ± 0.01^{b}	0.68 ± 0.01^{c}	0.08 ± 0.02^{a}	nd	***
γ-glu-val	0.7	nd	nd	0.05 ± 0.0	nd	-
γ-glu-leu	1.3	0.02 ± 0.0^{a}	0.02 ± 0.0^{a}	0.06 ± 0.0^{b}	nd	***
γ-glu-glu	4.9	nd	nd	0.04 ± 0.0	nd	-
γ-glu-his	2.8	0.38 ± 0.6^{b}	0.66 ± 0.1^{c}	0.09 ± 0.0^{a}	nd	***
γ-glu-phe	0.8	0.02 ± 0.0^{a}	0.02 ± 0.0^{a}	0.05 ± 0.0^{b}	nd	***
γ-glu-tyr	1.5	nd	nd	0.07 ± 0.0	nd	-

Values with the same letter superscripts within each row are not significantly different ($p = 0.05$).

Data are presented as mean ± standard error, n=3. nd = not detected.

†Taste threshold of γ-glutamyl peptides was obtained from Zhao et al. (2016), except γ-glu-phe and γ-glu-tyr were obtained from Shibata et al. (2017)

§Sig = Probability, as obtained from ANOVA, that there is a difference between means; ***Significant at the 0.1% level ($p \leq 0.001$).

3.3.2 The analysis of aroma in the commercial rice wines

3.3.2.1 Volatile compounds

The commercial rice wine samples were analysed for volatile compounds which contribute to aroma. As results from the analysis of taste compounds, PRW2 did not look like a genuine rice wine due to the highest concentrations of fructose, glucose and acetic acid were found in that sample (table 3.1).

The results of the volatile analysis are shown in table 3.6. They have been classified into eight groups, including esters, alcohols, aldehydes, volatile acids, volatile phenols and lactones. Among them, esters and alcohols were the most abundant volatiles in the commercial rice wine samples. These results are in agreement with the work published by Chen and Xu (2013), Jung et al. (2014) and Son et al. (2018) who showed that the volatile compounds in rice wines which had been brewed from polished rice are predominantly esters and alcohols. For esters, ethyl acetate, ethyl hexanoate and 2-methylbutyl acetate were the most abundant esters in the commercial rice wines. Notably, (E)-2-decenyl acetate in PRW1, and ethyl 2-furancarboxylate and ethyl 2-hydroxy 4-methylpentanoate in PRW2 were also found. These compounds have not been reported before in rice wine by Chen et al. (2013b) and Son et al. (2018). The results also showed that PRW1 and PRW2 contained the lipid derived esters at a high concentration. These lipid derived esters are likely to be formed from the corresponding fatty acids, which are liberated from the bran of unpolished rice. Sandhu et al. (2018) also showed that the bran of rice is the source of proteins, lipids and minerals, and they were reduced by the polishing process. Moreover, Lee et al. (2018) showed that a high concentration of fatty acids and the corresponding volatile compounds in fermented rice are formed by the degradation of lipid in its bran. The

acetate esters were found in PRW2 at a higher concentration possibly due to a higher concentration of acetic acid was found in that sample.

The higher alcohols, including propanol, 2-methylpropanol, 3-methylbutanol and 2-methylbutanol were found in RW2 at a high concentration, and their formations may be related to the high concentration of the corresponding amino acids which are val, leu and ile in that sample. This is in agreement with Jung et al. (2014) and Zhang et al. (2015) who showed that 2-methylpropanol, 3-methylbutanol and 2-phenylethyl alcohol were found in rice wine samples. These amino acids-derived alcohols are formed from the corresponding amino acids via the Ehrlich pathway (Chen et al., 2013b). Moreover, the lipid-derived alcohols which are hexanol, octanol and 2-decenol were mostly found in PRW1. These compounds are likely to be formed from the corresponding fatty acids, which are derived from the degradation of lipid in the bran of unpolished rice. This is in agreement with Wanyo et al. (2016) who showed that rice bran contains more fatty acids especially linoleic acid.

Strecker aldehydes which are 3-methylbutanal and 2-phenylethanal were detected in commercial rice wines, and their concentration was higer in pigmented rice wine. These Strecker aldehydes are formed from the corresponding amino acids which are leu and phe in commercial rice wines via the Ehrlich pathway. Moreover, the highest concentration of 2-furfural was found in PRW2. This compound is formed from the degradation of hexose sugars (fructose or glucose) in the presence of heating (pasteurisation) and acid condition (Pereira et al., 2011). This is consistent with the highest concentrations of fructose, glucose and acetic acid in PRW2 (table 3.1).

Phenolic acids-derived aroma compounds were detected in pigmented rice wines (table 3.6). Guaiacol was found in PRW1, whereas 4-ethylphenol and 4-ethylguaiacol were found in PRW2. Moreover, Belda et al. (2017) showed that guaiacol, 4-ethylphenol and 4-ethylguaiacol are likely to be formed from vanillic acid, 4-coumaric acid and ferulic acid, and their formation pathway was shown in table 2.13. These corresponding phenolic acids are derived from the degradation of lignin, which are found in rice bran at a high concentration (Zhou et al., 2004).

Table 3-6: Selected aroma compounds detected in commercial rice wines using SPME.

volatile compounds	†LRI		‡ID	approximate concentration (µg/l)				§Sig
	ZB-5MSi	WAX-DA		RW1	RW2	PRW1	PRW2	
ethyl esters								
ethyl acetate	615	900	A	23.7 ± 0.7^{a}	22.7 ± 2.4^{a}	46 ± 1.8^{a}	479 ± 46^{b}	***
ethyl propanoate	714	-	B	0.01 ± 0.0^{a}	nd	0.02 ± 0.0^{b}	nd	***
ethyl 2-methylpropanoate	762	973	A	0.13 ± 0.0^{a}	nd	0.18 ± 0.1^{a}	1 ± 0.3^{b}	***
ethyl butanoate	802	1046	A	1.2 ± 0.1^{c}	0.59 ± 0.1^{b}	0.36 ± 0.0^{a}	0.63 ± 0.1^{b}	***
ethyl 2-methylbutanoate	852	1062	A	nd	nd	0.29 ± 0.0^{a}	0.56 ± 0.1^{b}	***
ethyl 3-methylbutanoate	855	1078	A	nd	nd	0.01 ± 0.0^{a}	0.6 ± 0.1^{b}	***
ethyl hexanoate	1001	1237	A	65 ± 2.0^{d}	1.6 ± 0.1^{b}	11.5 ± 0.7^{c}	0.91 ± 0.1^{a}	***
ethyl 2-furancarboxylate	1057	1636	A	nd	nd	nd	0.1 ± 0.0	-
ethyl 2-hydroxy 4-methylpentanoate	1061	1553	A	nd	nd	nd	0.27 ± 0.0	-
ethyl heptanoate	1097	1341	A	nd	nd	0.11 ± 0.0^{b}	0.05 ± 0.0^{a}	***
ethyl benzoate	1180	1684	A	0.03 ± 0.0^{a}	nd	0.08 ± 0.0^{b}	1 ± 0.0^{c}	***
ethyl octanoate	1196	1444	A	1.3 ± 0.0^{b}	0.04 ± 0.0^{a}	15.6 ± 0.3^{d}	2.1 ± 0.1^{c}	***
ethyl phenylacetate	1252	1798	A	0.06 ± 0.0^{a}	nd	0.16 ± 0.0^{b}	2.3 ± 0.1^{c}	***
ethyl decanoate	1395	1646	A	nd	nd	0.34 ± 0.0^{a}	0.68 ± 0.0^{b}	***
acetate esters								
2-methylpropyl acetate	776	1023	A	0.29 ± 0.0^{a}	0.27 ± 0.0^{a}	nd	6.4 ± 1.3^{b}	***
2-methylbutyl acetate	878	1129	A	0.33 ± 0.0^{a}	0.72 ± 0.1^{b}	0.2 ± 0.0^{a}	1.2 ± 0.3^{c}	***
3-methylbutyl acetate	880	-	B	8.2 ± 0.4^{d}	4.1 ± 0.4^{b}	0.52 ± 0.0^{a}	5.4 ± 1.1^{c}	***

Table 3-6: Selected aroma compounds detected in commercial rice wines using SPME (continued).

volatile compounds	†LRI		‡ID	approximate concentration (µg/l)				§Sig
	ZB-5MSi	WAX-DA		RW1	RW2	PRW1	PRW2	
2-phenethyl acetate	1265	1831	A	0.03 ± 0.0^{b}	0.02 ± 0.0^{a}	0.53 ± 0.0^{d}	0.17 ± 0.0^{c}	***
(2E)-2-decenyl acetate	1408	1746	A	nd	nd	7.1 ± 0.9	nd	-
other esters								
butyl butanoate	996	1226	A	nd	nd	nd	0.2 ± 0.0	-
3-methylbutyl hexanoate	1251	-	B	0.03 ± 0.0	nd	nd	nd	-
alcohols								
propanol	595	1051	A	6.8 ± 0.5^{a}	12.4 ± 1.1^{b}	12.4 ± 3.4^{b}	nd	***
2-methylpropanol	627	1110	A	9.6 ± 3.1^{ab}	18.4 ± 0.7^{b}	5.4 ± 0.7^{a}	43 ± 9.0^{c}	***
3-methylbutanol	737	1218	A	8.6 ± 0.5	8.24 ± 1.3	6.5 ± 0.0	6.8 ± 1.7	ns
butanol	664	1157	A	nd	nd	nd	8.8 ± 1.6	-
2-methylbutanol	740	1215	A	3 ± 0.2^{b}	3.7 ± 0.3^{c}	2.7 ± 0.0^{b}	1.4 ± 0.3^{a}	***
2-furfuryl alcohol	867	1670	A	nd	nd	nd	0.16 ± 0.0	-
hexanol	870	1362	A	nd	nd	0.1 ± 0	nd	-
phenylmethyl alcohol	1043	1890	A	nd	nd	nd	0.86 ± 0.0	-
octanol	1071	1566	A	nd	nd	0.62 ± 0.2	nd	-
phenylethyl alcohol	1124	1925	A	12 ± 0.3^{c}	10.6 ± 0.2^{b}	14.8 ± 0.9^{d}	3.3 ± 0.1^{a}	***
(E)-2-decenol	1271	1824	A	nd	nd	0.62 ± 0.1	nd	-
aldehydes								
3-methylbutanal	654	928	A	0.36 ± 0.0^{c}	0.04 ± 0.0^{a}	0.06 ± 0.0^{b}	nd	***

Table 3-6: Selected aroma compounds detected in commercial rice wines using SPME (continued).

volatile compounds	†LRI		‡ID	approximate concentration (µg/l)				§Sig
	ZB-5MSi	WAX-DA		RW1	RW2	PRW1	PRW2	
2-furfural	837	1483	A	0.58 ± 0.0^{a}	0.84 ± 0.0^{c}	0.67 ± 0.0^{b}	9.2 ± 0.1^{d}	***
2-phenylethanal	1054	1664	A	1.1 ± 0.1^{c}	0.56 ± 0.03^{b}	1.2 ± 0.03^{d}	0.2 ± 0.0^{a}	***
volatile phenols								
¦guaiacol	1098	1875	A	nd	nd	0.07 ± 0.02	nd	-
4-ethylphenol	1168	2213	A	nd	nd	nd	0.22 ± 0.0	-
4-ethylguaiacol	1291	2043	A	nd	nd	nd	0.1 ± 0.0	-
others								
acetic acid	661	1469	A	nd	nd	5.2 ± 0.8^{a}	63 ± 13.3^{b}	***
2,3-butanediol	789	-	B	3.1 ± 0.8^{b}	2.5 ± 0.6^{ab}	0.8 ± 0.0^{a}	3.1 ± 0.8^{b}	**
2,3-dimethylpyrazine	925	1366	A	nd	nd	nd	0.09 ± 0.0	-
2-acetylpyrazine	1031	1649	A	nd	nd	nd	0.75 ± 0.0	-

Values with the same letter superscripts within each row are not significantly different ($p = 0.05$).

Data are present as mean ± standard error, n=3. nd = not detected.

†Linear retention index calculated from a linear equation between each pair of straight chain alkanes (C_5–C_{30}).

‡ID, mass spectrum and LRI agree with those of authentic compound; A agreement on both column and B agreement on just one column.

¦Compound was quantified in mg/l, using the corresponding standard calibration curve.

§Sig = Probability, as obtained from ANOVA, that there is a difference between means; ns, no significant difference between means ($P > 0.05$), **Significant at the 1% level ($0.001 < p \leq 0.01$) and ***Significant at the 0.1% level ($p \leq 0.001$).

3.3.2.2 Semi-volatile compounds

The semi-volatile compounds in the commercial rice wines were extracted using SPE. Notably, PRW2 was excluded from this experiment due to the colour which was eluted from the SPE cartridge during the extraction. The anthocyanins were not found in this sample, so that colour might be derived from an alternative source. Additional aroma compounds in rice wine samples were detected, using SPE. It can be seen that the volatile esters were reduced, whereas organic acids were increased. This is in agreement with Pietrogrande and Basaglia (2007) and Mendes et al. (2012) who showed that the SPE is widely used for the extraction of volatile and semi-volatile compounds in wine. It is suitable for the extraction of semi-volatiles, non-volatiles, non-polar, polar, and ionised compounds, and less suitable for non-polar volatile compounds.

According to the table 3.7, the lactic acid-derived esters were mostly found in PRW1. The formation of these compounds was likely to be related to a high concentration of lactic acid in that sample (table 3.1). Moreover, PRW1 showed a higher concentration of medium chain lipid-derived esters which were ethyl 2-hydroxybutanoate, ethyl 3-hydroxybutanoate and ethyl 2-hydroxyhexanoate, compared to others. They are likely to be formed from the corresponding fatty acids, which are derived from the degradation of lipid in the rice bran (Wanyo et al., 2016).

Organic acids were found to be the most abundant semi-volatiles in commercial rice wine samples. They were classified to amino acids-derived acids (2-methylpropanoic acid, 2-methylbutanoic acid and 3-methylbutanoic acid) which were likely to be formed from valine, isoleucine and leucine (table 3.2), and fatty acids which were hexanoic acid, octanoic acid, 2-octenoic acid and decanoic acid.

4-vinylguaiacol was found in PRW1 only. This compound is likely to be formed from ferulic acid, which is derived from the degradation of lignin (Belda et al., 2017). This is in agreement with Mo and Xu (2010) who showed that 4-vinylguaiacol is the most abundant volatile phenol compound in alcoholic beverages, which is also formed from the thermal decarboxylation of ferulic acid during rice cooking or sterilisation. However, Sunao et al. (2016) showed that 4-vinylguaiacol in rice wine is formed by spoilage bacteria (*Bacillus spp.* or *Staphylococcus spp.*) through the enzymatic decarboxylation of ferulic acid during the brewing process.

Table 3-7: Additional aroma compounds detected in commercial rice wines using SPE.

volatile compounds	†LRI		‡ID	approximate concentration (mg/l)			§Sig
	WAX-DA	ZB-5MSi		RW1	RW2	PRW1	
Esters							
ethyl lactate	1350	817	A	26 ± 3.8[a]	30 ± 4.0[a]	153 ± 37[b]	***
ethyl 2-hydroxybutanoate	1409	910	A	nd	nd	0.05 ± 0.0	-
2-methylpropyl lactate	1461	968	A	nd	nd	0.64 ± 0.1	-
ethyl 3-hydroxybutanoate	1524	935	A	0.79 ± 0.2[a]	0.6 ± 0.0[a]	4.7 ± 0.5[b]	***
ethyl 2-hydroxyhexanoate	1545	1059	A	nd	nd	0.11 ± 0.0	-
3-methylbutyl lactate	1573	1072	A	nd	nd	4.9 ± 0.5	-
ethyl succinate	1680	1182	A	0.3 ± 0.1[a]	0.34 ± 0.0[a]	2.7 ± 0.3[b]	***
ethyl malate	2042	1270	A	0.17 ± 0.1[a]	0.1 ± 0.0[a]	0.27 ± 0.0[b]	*
3-methylbutyl dodecanoate	2066	-	B	0.18 ± 0.1	0.12 ± 0.0	0.06 ± 0.0	ns
Alcohols							
3-ethoxypropanol	1377	843	A	0.23 ± 0.0[a]	0.38 ± 0.0[ab]	0.48 ± 0.1[b]	**
homovanillyl alcohol	2842	1537	A	nd	nd	0.07 ± 0.0	-
4-hydroxybenzeneethanol	3009	1428	A	23.7 ± 5.9	21.3 ± 4.1	26.7 ± 6.4	ns
Aldehydes							
benzaldehyde	1548	961	A	0.18 ± 0.0	0.04 ± 0.0	0.07 ± 0.0	ns
5-hydroxymethylfurfural	2497	-	B	nd	0.1 ± 0.0	0.09 ± 0.0	ns
vanillin	2562	1402	A	0.14 ± 0.0[a]	0.07 ± 0.0[a]	0.44 ± 0.1[b]	**
4-hydroxybenzaldehyde	2958	-	B	0.24 ± 0.1[a]	0.05 ± 0.0[a]	0.63 ± 0.0[b]	***

Table 3-7: Additional aroma compounds detected in commercial rice wines using SPE (continued).

volatile compounds	[†]LRI		[‡]ID	approximate concentration (mg/l)			[§]Sig
	WAX-DA	ZB-5MSi		RW1	RW2	PRW1	
organic acids							
2-methylpropanoic acid	1570	-	B	0.34 ± 0.1	0.3 ± 0.0	0.37 ± 0.0	ns
butanoic acid	1630	775	A	0.56 ± 0.1^{a}	0.49 ± 0.0^{a}	1.8 ± 0.2^{b}	***
3-methylbutanoic acid	1671	839	A	0.13 ± 0.0	0.1 ± 0.0	0.19 ± 0.0	ns
2-methylbutanoic acid	-	847	B	0.02 ± 0.0	0.02 ± 0.0	0.03 ± 0.0	ns
hexanoic acid	1846	1000	A	8.2 ± 2.8^{b}	0.74 ± 0.0^{a}	3.7 ± 0.4^{ab}	**
octanoic acid	2058	-	B	0.33 ± 0.1^{b}	0.06 ± 0.0^{b}	2.5 ± 0.0^{a}	***
2-octenoic acid	2183	-	B	nd	nd	0.13 ± 0.0	-
decanoic acid	2269	-	B	0.2 ± 0.1^{ab}	0.47 ± 0.2^{b}	0.07 ± 0.0^{a}	**
homovanillic acid	2340	1468	A	nd	nd	0.46 ± 0.1	-
dodecanoic acid	2479	-	B	0.42 ± 0.2	0.29 ± 0.1	0.14 ± 0.1	ns
benzeneacetic acid	2556	1250	A	0.37 ± 0.1	0.34 ± 0.1	0.28 ± 0.1	ns
hexadecanoic acid	2903	-	B	0.35 ± 0.1^{a}	0.36 ± 0.0^{a}	0.62 ± 0.1^{b}	*
octadecanoic acid	3116	-	B	nd	0.77 ± 0.3^{b}	0.27 ± 0.1^{a}	**
4-hydroxybenzenacetic acid	3697	-	B	0.13 ± 0.0^{a}	0.16 ± 0.0^{a}	0.36 ± 0.1^{b}	**
volatile phenols							
phenol	2008	-	B	0.01 ± 0.0^{a}	0.01 ± 0.0^{a}	0.04 ± 0.0^{b}	***
4-vinylguaiacol	2197	1316	A	nd	nd	0.9 ± 0.5	-

Table 3-7: Additional aroma compounds detected in commercial rice wines using SPE (continued).

volatile compounds	†LRI		‡ID	approximate concentration (mg/l)			§Sig
	WAX-DA	ZB-5MSi		RW1	RW2	PRW1	
Others							
dimethyl sulfoxide	1595	-	B	0.22 ± 0.0	0.18 ± 0.1	0.17 ± 0.1	ns
γ-butyrolactone	1653	916	A	0.2 ± 0.1	0.32 ± 0.1	0.16 ± 0.0	ns
γ-nonalactone	2034	1365	A	nd	nd	0.24 ± 0.0	-

Values with the same letter superscripts within each row are not significantly different (p = 0.05).

Data are presented as mean ± standard error, n=3. nd = not detected.

†Linear retention index calculated from a linear equation between each pair of straight chain alkanes (C_7–C_{40}).

‡ID, mass spectrum and LRI agree with those of authentic compound; A agreement on both column and B agreement on just one column.

§Sig = Probability, as obtained from ANOVA, that there is a difference between means; ns, no significant difference between means ($P > 0.05$), *Significant at the 5% level ($0.01 < p \leq 0.05$), **Significant at the 1% level ($0.001 < p \leq 0.01$) and ***Significant at the 0.1% level ($p \leq 0.001$).

3.3.2.3 GC-Olfactometry

It is important to identify which of the volatile aroma compounds are likely to be aroma active. According to table 3.8 and 3.10, twenty six aromas were presented in RW1, RW2 and PRW1. Moreover, the confirmation of their corresponding aroma compounds detected by GC-MS was showed in table 3.9 and 3.11. Ethyl propanoate, ethyl 2-methylpropanoate, ethyl butanoate, ethyl 2-methylbutanoate, ethyl 3-methylbutanoate, 2-methylbutyl acetate, 3-methylbutyl acetate, ethyl 3-hydroxybutanoate, ethyl hexanoate, ethyl octanoate and 3-methylbutyl lactate, were found to be the most important aroma compounds which impart fruity and sweet notes. Theses esters are in agreement with Chen et al. (2013b), except 2-methylbutyl acetate, ethyl 3-hydroxybutanoate and 3-methylbutyl lactate. The polished rice wine (RW1 and RW2) showed higher cheesy note, whereas PWR1 showed a higher fruity, floral, green and earthy note which were derived from esters, aldehydes and pyrazines.

Notably, PRW1 showed smoky-spicy note which were derived from guaiacol and 4-vinylguaiacol. These aroma compounds are likely to be formed from the degradation of vanillic acid and ferulic acid, which are liberated from the degradation of lignin in plants (Priefert et al., 2001, Sunao et al., 2016). The study of aroma in pigmented rice wine is not widely published. However, Chen et al. (2013b) showed that volatile phenols, including guaiacol, 4-ethylguaiacol, 4-methylphenol, 4-ethylphenol and 4-vinylguaiacol impart to smoky notes in Chinese rice wine which was brewed with polished rice and wheat Qu. They also showed that these volatile phenols are derived from the wheat Qu, and their concentrations were lower in those rice wines without wheat Qu. This is in agreement with Park et al. (2013) who showed that the smoky note is not found in the Korean rice wines, which was brewed using polished rice.

The analysis of GC-O also showed that the aroma intensity in rice wines reported from each assessor was quite variable (table 3.8 and 3.10). This is in agreement with Schranz et al. (2017) who showed that olfactory perception can be influenced by the aroma receptors of assessors. The variation of aroma receptors in humans is caused by the single nucleotide polymorphisms (SNPs). Human subjects with SNPs in the DNA sequence of aroma receptors might have highly sensitive aroma perception (Schranz et al., 2017). Although the concentration of each aroma in commercial rice wine samples was variable, the corresponding aroma attribute detected by assessors did not vary. Moreover, the nasal mucosa contains metabolic enzymes and aroma-binding proteins and these will vary between individuals. They can convert or decompose the aroma compounds to their derivatives before docking to the receptor proteins of the cilia (Nagashima and Touhara, 2010) thus reducing the concentration perceived and the intensity scores.

Table 3-8: Aroma description and intensity of the volatile compounds in commercial rice wines detected by GC–O.

aromas	responsible compound	aroma intensity from individual assessor												‡aroma intensity (mean)		
		RW1				RW2				PRW1						
		§A1		A2		A1		A2		A1		A2		RW1	RW2	PRW1
		†R_1	R_2	R_1	R_2	R_1	R_2	R_1	R_2	R_1	R_2	R_1	R_2			
strecker/cocoa	3-methylbutanal	7	7	nd	nd	5	4	3	3	3	3	nd	nd	4	4	2
pineapple	ethyl propanoate	5	4	3	3	nd	nd	nd	nd	nd	nd	nd	nd	4	nd	nd
strecker	3-methylbutanol	3	3	5	3	8	6	5	nd	7	5	5	3	4	5	5
fruity	ethyl 2-methylpropanoate	5	3	5	5	6	4	3	3	6	7	nd	nd	4	4	3
fruity	ethyl butanoate	7	7	7	7	nd	nd	5	7	4	5	5	3	7	3	4
sweet	ethyl lactate	5	5	nd	nd	nd	nd	nd	nd	5	6	nd	nd	3	nd	3
fruity	ethyl 2-methylbutanoate	nd	nd	nd	nd	nd	nd	nd	nd	4	4	nd	nd	nd	nd	2
strawberry	ethyl 3-methylbutanoate	nd	nd	nd	nd	nd	nd	nd	nd	5	6	5	5	nd	nd	5
meaty	*2-methyl 3-furanthiol	nd	nd	nd	nd	nd	nd	nd	nd	nd	3	5	3	nd	nd	3
fruity	3-methylbutyl acetate	3	4	5	nd	3	nd	3	nd	nd	nd	nd	nd	3	2	nd
fruity	2-methylbutyl acetate	7	8	5	5	4	4	3	3	nd	nd	nd	nd	6	4	nd
fruity	ethyl 3-hydroxybutanoate	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	3	3	nd	nd	2
pineapple	ethyl hexanoate	8	7	7	7	6	6	5	5	6	7	5	5	7	6	6
fruity	ethyl octanoate	nd	nd	3	3	nd	nd	nd	nd	nd	nd	3	3	2	nd	2
floral	2-phenylethanal	4	4	nd	nd	nd	nd	nd	nd	5	7	5	5	2	nd	6
floor cleaning	octanol	nd	nd	nd	nd	5	4	nd	nd	4	4	3	3	nd	2	4
earthy	*2-isopropyl-3-methoxypyrazine	4	4	nd	nd	5	4	nd	nd	6	6	3	3	2	2	5

Table 3-8: Aroma description and intensity of the volatile compounds in commercial rice wines detected by GC–O (continued).

aromas	responsible compound	aroma intensity from individual assessor												‡aroma intensity (mean)		
		RW1				RW2				PRW1						
		§A1		A2		A1		A2		A1		A2		RW1	RW2	PRW1
		†R_1	R_2	R_1	R_2	R_1	R_2	R_1	R_2	R_1	R_2	R_1	R_2			
smoky-spicy	guaiacol	nd	nd	nd	nd	nd	nd	nd	nd	6	6	7	7	nd	nd	7
rose	phenylethyl alcohol	5	4	3	3	nd	nd	7	7	4	3	7	7	4	4	7
green	*2-isobutyl-3-methoxypyrazine	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	3	3	nd	nd	2

‡The value for intensity is the mean of intensities observed from two assessors and two replicate analyses for each sample.

*Compounds with no peak in the GC-MS. These were based on finding the correct aroma at the correct LRI on one or two columns.

§A1 and A2 were the aroma intensity from rice wines detected by assessor 1 and assessor 2, respectively.

†R_1 and R_2 were a replication of the analysis of aroma intensity by GC-O from each assessor.

nd = not detected.

Table 3-9: Confirmation of aromas from volatile compounds in rice wines detected by GC-O with the corresponding aroma compounds from GC-MS.

aromas	responsible compound	†ZB-5MSi			†WAX-DA		
		GC-O	GC-MS	AC	GC-O	GC-MS	AC
strecker/cocoa	3-methylbutanal	657	654	657	908	-	928
pineapple	ethyl propanoate	722	714	712	957	-	961
strecker	3-methylbutanol	734	737	732	1215	1218	1207
fruity	ethyl 2-methylpropanoate	765	762	752	966	973	976
fruity	ethyl butanoate	811	802	801	1040	1046	1050
sweet	ethyl lactate	816	817	812	-	-	-
fruity	ethyl 2-methylbutanoate	856	852	850	1051	1062	1062
strawberry	ethyl 3-methylbutanoate	859	855	851	1069	1078	1082
meaty	*2-methyl 3-furanthiol	880	-	877	1322	-	1307
fruity	3-methylbutyl acetate	879	878	876	-	-	-
fruity	2-methylbutyl acetate	886	880	881	1120	1129	1130
fruity	ethyl 3-hydroxybutanoate	941	935	935	1535	1530	1540
pineapple	ethyl hexanoate	1005	1001	998	1237	1240	1237
fruity	ethyl octanoate	1200	1196	1194	1450	1444	1438
floral	2-phenylethanal	1056	1054	1058	1656	1664	1678
floor cleaning	octanol	1071	1071	1073	-	-	-
earthy	*2-isopropyl-3-methoxypyrazine	1085	-	1095	-	-	-
smoky-spicy	guaiacol	1097	1098	1092	1872	1875	1862

Table 3-9: Confirmation of aromas from volatile compounds in rice wines detected by GC-O with the corresponding aroma compounds from GC-MS (continued).

aromas	responsible compound	†ZB-5MSi			†WAX-DA		
		GC-O	GC-MS	§AC	GC-O	GC-MS	§AC
rose	phenylethyl alcohol	1122	1124	1119	1921	1925	1909
green	*2-isobutyl-3-methoxypyrazine	1185	-	1181	1513	-	1533

†LRI on both of ZB-5MSi and WAX-DA column, calculated from a linear equation between each pair of straight chain alkanes C_5–C_{25}.

§AC is LRI from authentic compounds.

*Compounds with no peak in the GC-MS. These were based on finding the correct aroma at the correct LRI on one or two columns.

Table 3-10: Additional aroma description and intensity of the semi-volatile compounds in commercial rice wines detected by GC–O.

aromas	responsible compound	aroma intensity from individual assessor												‡aroma intensity (mean)		
		RW1				RW2				PRW1						
		§A1		A2		A1		A2		A1		A2		RW1	RW2	PRW1
		†R_1	R_2	R_1	R_2	R_1	R_2	R_1	R_2	R_1	R_2	R_1	R_2			
cheese	3-methylbutanoic acid	nd	nd	3	5	nd	nd	3	3	nd	nd	nd	nd	2	2	nd
cheese	2-methylbutanoic acid	6	7	3	nd	nd	7	5	5	5	6	3	3	4	4	4
sweet	3-methylbutyl lactate	nd	nd	nd	nd	nd	nd	nd	nd	4	5	5	5	nd	nd	5
smoky/clove	4-vinylguaiacol	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	3	4	nd	nd	2

‡The value for intensity is the mean of intensities observed from two assessors and two replicate analyses for each sample.

*Compounds with no peak in the GC-MS. These were based on finding the correct aroma at the correct LRI on one or two columns.

§A1 and A2 were the aroma intensity from rice wines detected by assessor 1 and assessor 2, respectively.

†R_1 and R_2 were a replication of the analysis of aroma intensity by GC-O from each assessor.

nd = not detected.

Table 3-11: Confirmation of additional aromas from semi-volatile compounds in rice wines detected by GC-O with the corresponding aroma compounds from GC-MS.

aromas	responsible compound	†ZB-5MSi			†WAX-DA		
		GC-O	GC-MS	§AC	GC-O	GC-MS	§AC
cheese	3-methylbutanoic acid	830	839	839	1674	1671	1687
cheese	2-methylbutanoic acid	858	847	845	1689	-	1691
sweet	3-methylbutyl lactate	1072	1072	1084	-	-	-
smoky/clove	4-vinylguaiacol	1334	1316	1324	-	-	-

†LRI on both of ZB-5MSi and WAX-DA column, calculated from a linear equation between each pair of straight chain alkanes C_5–C_{25}.

§AC is LRI from authentic compounds.

3.4 Conclusion

This study has identified taste and aroma compounds in the commercial rice wines which were brewed from the polished rice and pigmented rice. All rice wines showed sweet taste and metallic note, which are imparted from glucose and DKPs (cyclo(leu-pro) and cyclo(pro-pro)). However, a sour taste from lactic acid and acetic acid was showed as the characteristic taste in the pigmented rice wine. PRW2 showed the highest centration of glucose, fructose and acetic acid, which is greatly different from the ordinary rice wine. Moreover, the unnatural colour from an alternative source was found in this sample. Thus, PRW2 might not be a genuine rice wine.

Several esters and alcohols were found to be the most abundant aroma compound, which derives fruity note and floral note in commercial rice wines. However, the pigmented rice wine (PRW1) showed a higher fruity, green and earthy note, compared to the polished rice wines. In addition, the smoky-spicy notes were found to be the characteristic aroma in PRW1. They are derived from guaiacol and 4-vinylguaiacol. According to the reviews in chapter 2 section 2.4.5, their possible precursors are vanillic acid and ferulic acid which are found in the bran of pigmented rice. Thus, the identification of the characteristic aroma compounds and their exact precursors were studied in chapter 5.

3.5 Publication

1) Yotmanee, S., Oruna-Concha, M.J. and Parker, J.K. (2015). The determination of flavour profiles in pigmented rice wine. The 3rd Nursten symposium, Northumbria University, Newcastle, UK, 6-7 July 2015.

   (*Oral presentation*)

2) Yotmanee, S., Oruna-Concha, M.J. and Parker, J.K. (2015). The determination of flavour profiles in pigmented rice wine. In: Bioflavour 2015, DECHEMA-Haus, Frankfurt am Main, Germany, 9-11 September 2015.

   (*Poster presentation*)

CHAPTER 4: THE OPTIMIZATION OF PIGMENTED RICE BREWING PROCESS

Abstract

The brewing of pigmented rice wine was investigated in this chapter. The brewing process was carried out using different rice cooking methods (steaming and pressure cooking) and brewing temperature (25 °C and 30 °). In order to find the selected brewing condition, sugars, ethanol and organic acids were investigated throughout the brewing process. Steaming was found to be the better cooking method. The saccharification conditions selected for subsequent experiments were 30 °C for 2 days due to a higher concentration of total sugars. Moreover, the selected fermentation procedure was 30 °C and 9 days due to a higher concentration of ethanol and a lower concentration of acetic acid. The challenge of this study is to find the suitable conditions for the parallel fermentation of pigmented rice wine in the presence of *Aspergillus oryzae* for starch saccharification, and *Saccharomyces cerevisiae* for alcoholic fermentation.

Keywords: saccharification, alcoholic fermentation, pigmented rice wine, brewing process

4.1 Introduction

The traditional rice brewing process mainly consists of steaming rice and then inoculation with fungi and yeast. While *Aspergillus oryzae* has been reported as the important fungi that produces α-amylase, glucoamylase and protease for the degradation of starch (Jiao et al., 2017), *Saccharomyces cerevisiae* is widely known as the important yeast for alcohol production, and has high stress tolerance during fermentation (Boulton and Quain, 2008).

Jha et al. (2017) showed that the optimum condition for the brewing of black rice, using *Aspergillus oryzae* was 43 °C for 64 h. This is in agreement with Thippeswamy et al. (2006) who showed the optimum temperature for α-amylase production from *Aspergillus oryzae* was found to be 50 °C. Although the increase in temperature resulted in increased enzyme activity, the excess temperature can terminate the activity of enzyme. In addition, Sundarram and Murthy (2014) showed that the saccharification at a high temperature caused the loss of moisture from the substrate, thus reducing the growth rate and enzyme activity of fungi.

During alcoholic fermentation, the temperature also influences the growth rate and biomass of yeast (Charoenchai et al., 1998). These results are related to the production of ethanol in wine and beer (Du et al., 2011, Charoenchai et al., 1998, Olaniran et al., 2011). According to Torija et al. (2003), the growth of yeast cells slowly increased at 15-20 °C, compared to the fermentation at 25-30 °C. This is consistent with Trott and Morano (2003) who showed that *Saccharomyces cerevisiae* produced high levels of ethanol at 25-30 °C. However, the fermentation temperature at 35 °C caused a decrease in the growth of the yeast and an increase in the mortality. This is similar to that of Casey and Ingledew (1986)

who showed that as the temperature increased, the production of ethanol decreased while the levels of glycerol and acetic acid increased.

Uscanga et al. (2003) also showed that *Saccharomyces cerevisiae* can produce more ethanol under small amounts of oxygen, whereas this process is terminated under excess oxygen content. This is in agreement with Wellala et al. (2006) who showed that the brewing of rice wine is mostly done under semi-aerobic conditions. However, the oxygen might decrease the ethanol production during the fermentation of rice wine. According to Du Toit et al. (2017), the acetaldehyde dehydrogenase from acetic acid bacteria produce acetic acid from the oxidation of ethanol.

The method used to cook the rice has an influence on the brewing of rice wine. Steaming is widely used for the cooking of rice during the manufacture of rice wine. This traditional process starts with rice soaking, followed by steaming. The moisture which is absorbed by the rice grain accelerates the starch gelatinization at high temperature (Xu et al., 2016). However, there are some disadvantages, including half-cooking and retrogradation of starch in rice which are frequently observed during the large scale steaming, thus affecting the subsequent fermentation (Xu et al., 2016). Therefore, a new method for rice cooking, including extrusion, liquefying, and roasting is used instead of the traditional steaming process (Chen and Xu, 2012, Li et al., 2013, Xu et al., 2015b).

During the artisanal brewing of rice wine, pressure cooking may be used because it is a domestic process found in many households. Rashmi and Urooj (2003) showed that rice which was cooked by pressure cooking had a high degree of gelatinization, which promotes the growth rate of brewing cultures and ethanol production. This is in agreement with Rani et al. (1994) who showed that the gelatinised starch was significantly more hydrolysed, compared to the corresponding native starch. One likely explanation is that gelatinisation

cause the disrupted of H-bond in linear structure of starch (crystallinity), thus making it more accessible to enzymes for the degradation.

The brewing process of pigmented rice wine is less documented and therefore the choice of rice cooking method and range of temperature needs to be considered. The challenge for this study is to find the suitable conditions for the initial saccharification process which is favourable for parallel fermentation that limited to 25 °C or 30 °C with the presence of characteristic cultures (*A. oryzae* ATCC 22787 and *S. cerevisiae* NCYC 478) and the pigmented glutinous rice. Thus, the aims of this study were to investigate the influence of (i) the rice cooking procedure (steaming and pressure cooking) on the saccharification process during the production of pigmented rice wine and (ii) the effect of the brewing temperature on the production of ethanol, sugars, organic acids and phenolic acids in pigmented rice wine.

4.2 Materials and methods

4.2.1 Materials

Glutinous pigmented rice (Double Elephant, Thailand) was purchased from a local supplier at Reading, UK. Brewing microorganisms were *Aspergillus oryzae* ATCC 22787 and *Saccharomyces cerevisiae* NCYC 478 obtained from LGC Standards (Teddington, UK) and The National Collection of Yeast Cultures (Norwich, UK), respectively.

4.2.2 Chemicals

Ethanol, 99.5% glucose, 99% fructose, 99% maltose, 95% maltotriose, 99% malic acid, 98% lactic acid, 99% citric acid, 99% sodium succinate and 99% sodium tartrate were purchased from Sigma-Aldrich (Dorset, UK), whereas >96% cyanidin-3-glucoside was purchased from Extrasynthese (Genay, France). Analytical grade sulfuric acid and HPLC grade methanol were purchased from Fisher Scientific (Loughborough, UK), whereas formic acid was purchased from BDH (Poole, UK).

4.2.3 Brewing process

4.2.3.1 Saccharification

Pigmented rice was steamed for 60 min at 100 ºC, or pressure cooked at 80 kPa for 60 min, and inoculated with *Aspergillus oryzae* ($3x10^6$ spores/ml), followed by incubation at 25 ºC or 30 ºC for 8 days. Sugars and organic acids were analysed every 24 h. The optimum saccharification process was determined by the conditions (time and temperature) that produced the highest concentration of glucose.

4.2.3.2 Alcoholic fermentation

The optimum saccharification process was applied to the cooked pigmented rice, which was subsequently inoculated with *Saccharomyces cerevisiae* ($6x10^6$ cells/ml) and left to ferment for 10 days at either 25 ºC or 30 ºC. Samples were collected every day to determine the concentration of sugars, organic acids, ethanol, phenolic acids and

anthocyanins. The optimum fermentation conditions were selected on the basic of high ethanol content and reduced levels of acetic acid. Samples were pasteurised at 70 ^{o}C for 10 min.

4.2.4 Sugars, ethanol and organic acids content

Sugars, ethanol and organic acids was analysed by HPLC from Agilent (Waldbronn, Germany). The method was adapted from Zeppa et al. (2001), and the details are shown in chapter 3, section 3.2.3.1. The standard curves for sugars, ethanol and organic acids were prepared in the range of 10-100 mg/l, 10-1,000 mg/l and 0.01-1 ml/100 ml, respectively, R^2>0.99.

4.2.5 Statistical analysis

IBM SPSS Statistics for Windows, Version 22.0 software (IBM Corp., Armonk, NY, USA) was used for the statistical analysis of experimental data. The difference between the mean values was considered at 95% confidence interval, using the analysis of variance (ANOVA) with the post hoc Duncan test.

4.3 Results and discussions

4.3.1 Effect of the rice cooking method and incubation temperature on the saccharification of pigmented rice

Sugars including maltotriose, maltose and glucose were monitored throughout the saccharification of steamed pigmented rice at two different temperatures, 25 °C and 30 °C. According to figure 4.1, the concentrations of sugars were low on day 1 regardless of the temperature however the concentration of maltotriose and maltose significantly increased by day 2 as the rice starch was degraded to maltotriose and maltose by the fungi. From day 2, an increase in glucose was observed as both maltotriose and maltose were converted to glucose. The highest concentration of glucose was observed at day 6. After that, all sugars decreased as their rate of formation was less than their rate of consumption by *Aspergillus oryzae*. A higher concentration of sugars was observed at 30 ^{o}C on day 2, compared to that at 25 ^{o}C ($p < 0.05$).

According to figure 4.2, the formation of sugars from pressure cooked pigmented rice followed a similar pattern to that of steamed pigmented rice, but their concentration were much less. The concentrations of sugars were low on day 1, regardless of temperature and then increased from day 2, especially at 25 ^{o}C. After that, the highest concentration of sugars was observed on day 6, and then they started to decrease on day 7. A higher concentration of sugars, except glucose was observed in the saccharified pressure cooked pigmented rice at 25 ^{o}C, compared to that at 30 °C ($p < 0.05$).

Maltotriose was observed as the most abundant sugar in saccharified pigmented rice. Maltotriose is produced from the degradation of starch by α-amylase from *Aspergillus oryzae*, and then broken down to maltose and glucose by glucoamylase (Santoyo et al.,

2003, Ghosh et al., 2015). This explains the observation that a low concentration of glucose and a high concentration of maltotriose were investigated on the first day saccharification process.

During the saccharification, the concentrations of sugars in steamed pigmented rice were higher than in the pressure cooked rice, regardless of the temperature. This result may be explained by the fact that the grain absorbed more water during the steaming, and its outer layer or the bran was broken. The starch granules in the steamed rice were likely to be more swollen and gelatinised. Under these conditions, the fungi grow well as they would have access to nutrients from the swollen substrate, rice starch.

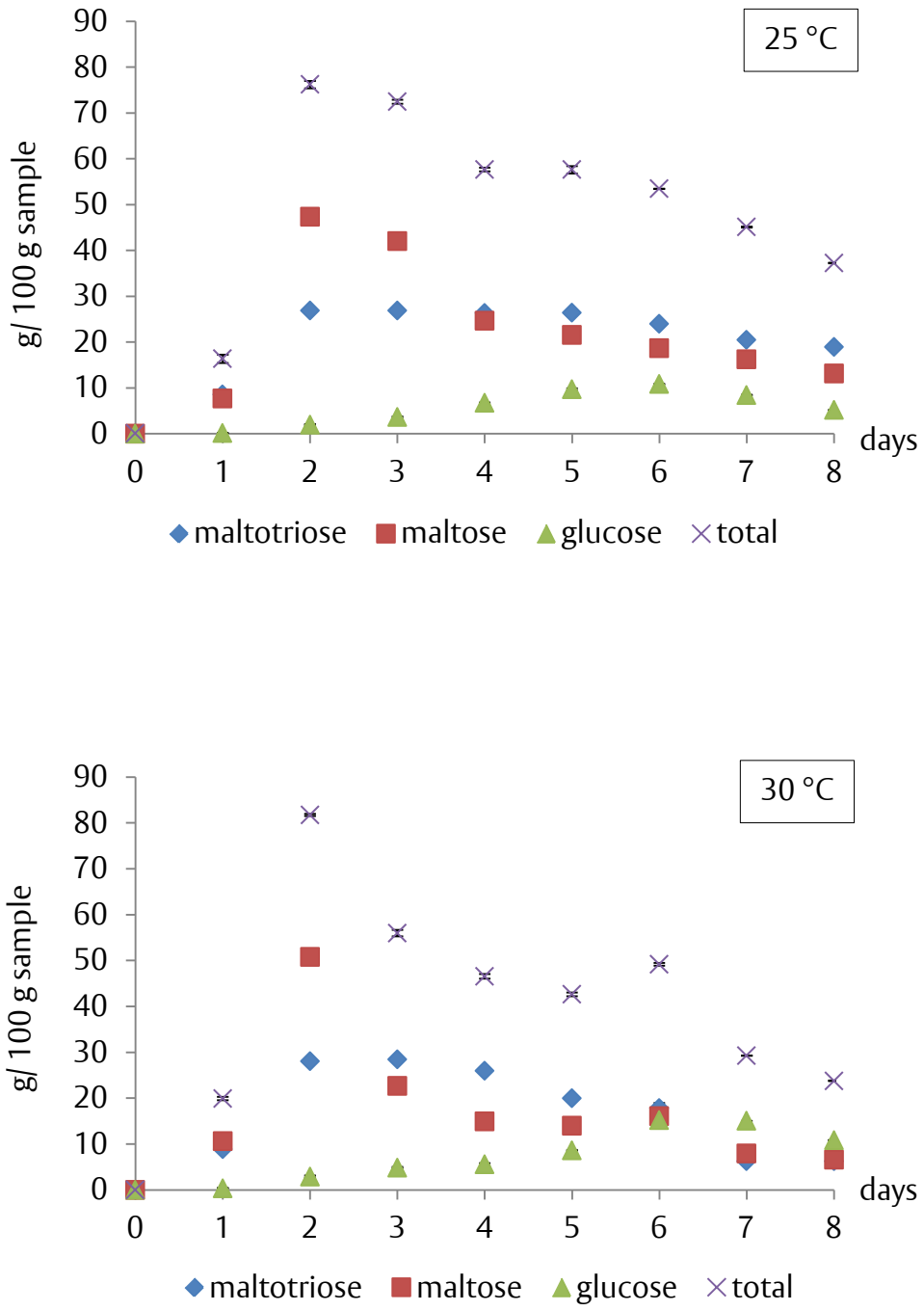

Figure 4-1: The concentration of glucose, maltose and maltotriose from the saccharification of steamed pigmented rice using *Aspergillus oryzae*. The saccharification temperatures were 25 ºC and 30 ºC and the saccharification time was 8 days, n=3.

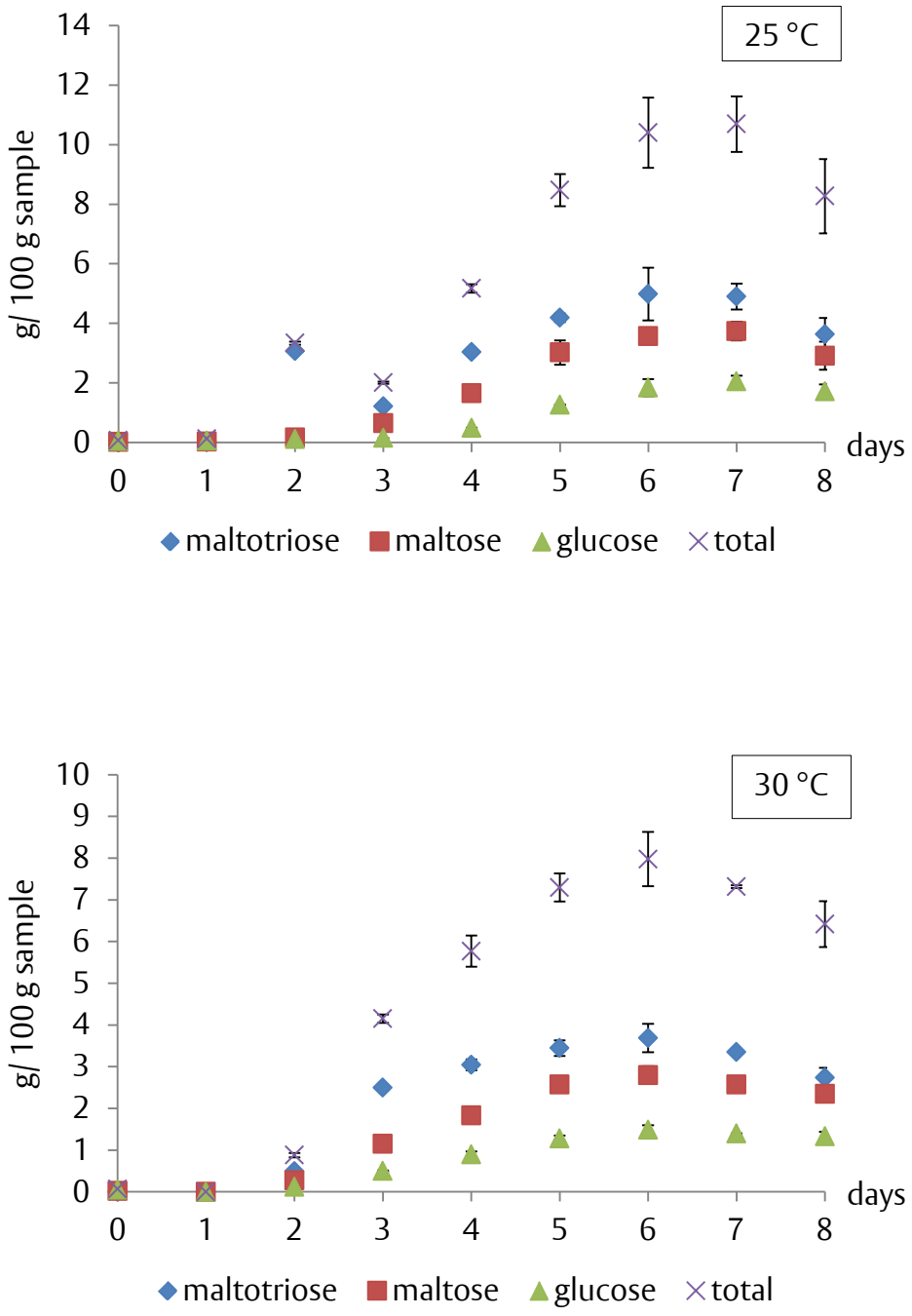

Figure 4-2: The concentration of glucose, maltose and maltotriose from the saccharification of pressure cooked pigmented rice using *Aspergillus oryzae*. The saccharification temperatures were 25 °C and 30 °C and the saccharification time was 8 days, n=3.

On the contrary, a lower concentration of sugars was found in saccharified pressure cooked pigmented rice. This finding suggests that the outer layer of the pigmented rice was not broken by the pressure cooking. Thus, starch granules were less swollen. Moreover, a higher temperature and shear from steam pressure cooking increase the degradation of amylopectin in starch (Byars, 2003). This results in the long term retrogradation by the recrystallization of de-branched amylopectin during cooling. This is consistent with Dundar and Gocmen (2013) who showed that starch which is autoclaved at higher temperature results in the increase of retrograded starch. Moreover, the recrystallized structure has been seen to be resistant to enzymatic hydrolysis (Ashwar et al., 2016).

For the saccharification of steamed pigmented rice, 30 °C was found to be better than 25 °C, however the reverse was found for the pressure cooked pigmented rice. This can be explained that pressure cooked pigmented rice which was saccharified at 25 °C possibly had a higher moisture, compared that to 30 °C. Therefore, the recrystallization of rice starch was less during cooling. This is consistent with Biliaderis et al. (1986) who showed that retrogradation of starch is decreased by an increase in moisture content, because moisture is a plasticizer in starchy foods which decrease the rearrangement of crystalline regions in starch granule during cooling.

In summary, the steaming was found to be the better method for rice cooking. The selected saccharification conditions were found at 30 ºC for 2 days due to the highest concentration of total sugars. These conditions were used for the next experiment.

4.3.2 Effect of temperature on the alcoholic fermentation of pigmented rice

Following saccharification, the rice was inoculated with *Saccharomyces cerevisiae* for alcoholic fermentation. During this step, and regardless of temperature, the levels of maltose and glucose decreased, whereas an increase in ethanol was observed, particularly on day 9 (figure 4.3). The concentration of ethanol formed during from both alcoholic fermentations was not significantly different. This result is in disagreement with Trott and Morano (2003) who showed that more ethanol is produced by yeast at 30 ºC.

The ethanol content found in the present study was around 9% (v/v), slightly lower that 12% ethanol reported by Singkong (2015) in black rice wine. However, Casey and Ingledew (1986) showed that ethanol in the range of 5-20% (v/v) is produced by *Saccharomyces cerevisiae*. The formation of ethanol was described by Dalawai et al. (2017) who showed that glucose is converted to pyruvate via the glycolytic pathway, and then undergoes decarboxylation to form acetaldehyde, which is converted to ethanol under anaerobic conditions.

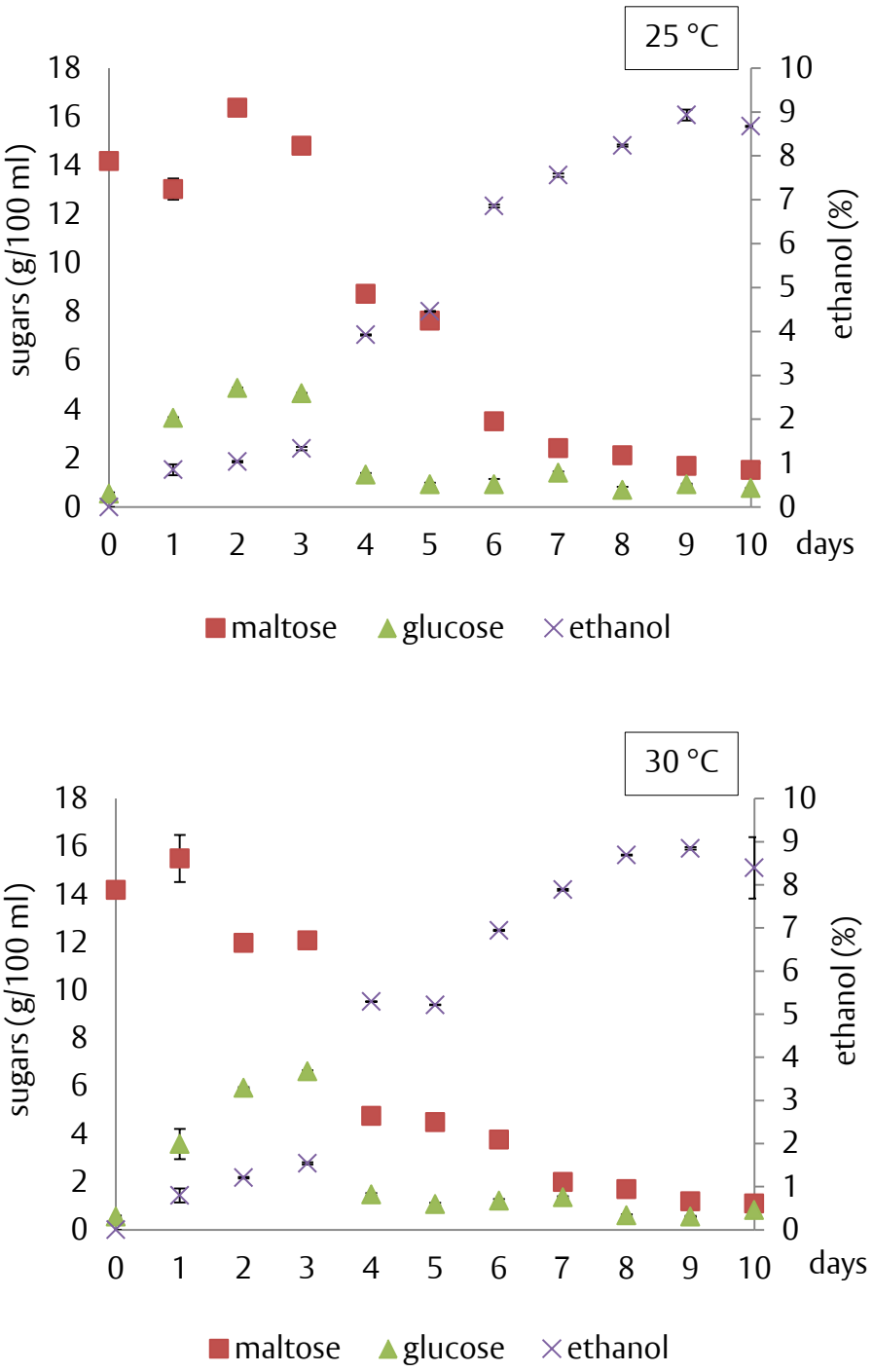

Figure 4-3: The concentration of maltose, glucose and ethanol from the fermentation of steamed pigmented rice using *Saccharomyces cerevisiae*. The fermentation temperatures were 25 °C and 30 °C and the fermentation time was 10 days, n=3.

Thus, glucose continuously decreased during alcohol production. Singkong (2015) also showed that sugars were reduced during 10 days of fermentation, because they were converted to ethanol by *Saccharomyces cerevisiae*. Moreover, Ma and Liu (2010) and Singkong (2015) showed that protein in the yeast cells can be denatured by ethanol at high concentration. This physical damage causes the dysfunction of yeast cell wall, thus affecting the termination of alcohol production.

The concentration of organic acids, including malic acid, lactic acid, succinic acid, and acetic acids increased throughout 10 days during the fermentation (figure 4.4). They were formed by yeast metabolism which used glucose as a substrate (Liu et al., 2014a). This is similar to wrok reported by Singkong (2015) who showed that the pH of black rice wine decreased during fermentation because organic acids and CO_2 were produced in parallel with ethanol production. In addition, a high concentration of acetic acid was found from fermentation at 25 ^{o}C.

Succinic acid was the most abundant organic acid in the pigmented rice wine. This is consistent with Yu et al. (2015) who also showed that succinic acid was the most abundant organic acid in rice wine. However, Liu et al. (2014a), Liu et al. (2014b) and Das et al. (2014) showed that lactic acid was the most abundant organic acid in Chinese rice wine and Indian rice beer. These differences could be linked to the use of different brewing strains. Moreover, Lee et al. (2012a) showed that high concentrations of acetic acid, tartaric acid and malic acid were found in static cultures fermentation (non-shaking condition) rather than agitated cultures fermentation (shaking condition).

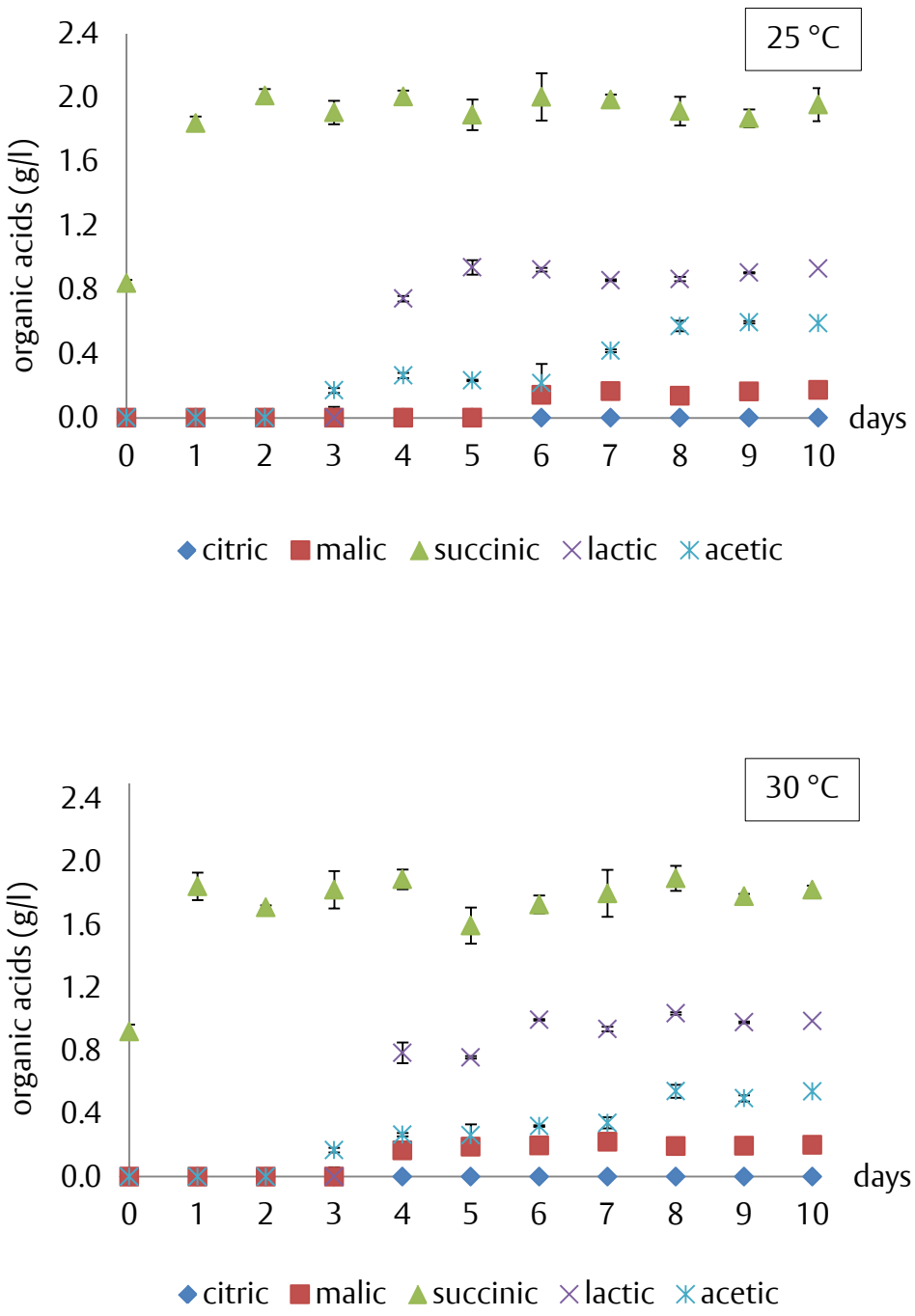

Figure 4-4: The concentration of citric acid, malic acid, succinic acid, lactic acid and acetic acid from the fermentation of steamed pigmented rice using *Saccharomyces cerevisiae*. The fermentation temperatures were 25 °C and 30 °C and the fermentation time was 10 days, n=3.

The effect of brewing temperature on the concentration of organic acids during the brewing of pigmented rice was also investigated. Organic acids, especially succinic acid and acetic acid were found at a higher concentration in the fermentation at 25 °C. A possible explanation for this might be that pigmented rice was brewed under semi-anaerobic condition, which allowed small amounts of oxygen into the fermentation system. This amount of oxygen might be slowly utilised, as the growth rate of brewing strains was slower at 25 °C. Therefore, the remaining of oxygen during the fermentation resulted in more ethanol being oxidise to acetic acid by *Acetobacter* under the respiratory metabolism (Bartowsky and Henschke, 2008). These results are not consistent with Rina et al. (2016) and Shang et al. (2016) who had previously reported a reduction in the concentration of acetic acid during fermentation at low temperature (20 °C), but an increase at higher temperature. However, Adachi et al. (1997) indicated that a low concentration of acetic acid can be found as a result of fermentation at high temperature, as acetic acid will be evaporated. Although acetic acid is the most abundant volatile acid in wine, its excessive concentration (> 0.9 g/l) affects negatively the quality of wine because it can contribute a sour taste (Shang et al., 2016). The brewing of pigmented rice at 30 ºC for 9 days showed a slightly higher ethanol, whereas the acetic acid was reduced. However, only acetic acid was different between samples ($p < 0.05$). Therefore, this condition was selected as the standard brewing method for the pigmented rice wine in this presented study.

4.4 Conclusions

Steaming was found as a suitable cooking for the pigmented rice because the steaming increased the moisture content and gelatinisation of starch, which increased the growth of *Aspergillus oryzae*. Thus, a higher concentration of sugars was produced from steamed pigmented rice, compared to pressure cooked pigmented rice. Moreover, the saccharification at 30 °C resulted in a greater increase of glucose concentration.

The concentration of sugars decreased during the parallel fermentation step because they were converted to ethanol by *Saccharomyces cerevisiae*. The concentration of ethanol at 30 ºC slightly differed from that at 25 ºC, whereas a high concentration of succinic acid and acetic acid was found in the rice wine which produced at 25 ºC.

In summary, for all future brewing experiments, steaming was confirmed as the preferred method for cooking the rice. Saccharification at 30 ºC for 2 days and parallel fermentation at 30 ºC for 9 days were the selected conditions for the brewing of steamed pigmented rice, due to a higher concentration of ethanol and a lower organic acids production. However, the ultimate aim of this study is to identify the characteristic taste and aroma compounds and their precursors in pigmented rice wine. In order to achieve the aim, this brewing process was used to produce the lab-scale pigmented rice wine in chapter 5.

4.5 Publication

1) Yotmanee, S., Oruna-Concha, M.J. and Parker, J.K. (2018). Influence of the brewing process and degree of milling on the taste characteristics of pigmented rice wine. In: Flavour Science: Proceedings of the 15th Weurman Flavour Research Symposium, Graz University of Technology, Austria, 18-22 September 2017, pp 155-158. (*Partly published*)

CHAPTER 5: INFLUENCE OF BRAN FROM PIGMENTED RICE ON FLAVOUR FORMATION IN PIGMENTED RICE WINE

Abstract

Pigmented rice wines were brewed using unpolished, 30% polished, 50% polished and 65% polished pigmented rice, and then analysed for the characteristic taste and aroma compounds. This study showed that the bran promoted the formation of phenolic acids, glutamic acid and γ-glutamyl peptides (γ-glu-gly, γ-glu-his and γ-glu-tyr). Moreover, a higher concentration of acetic acid, succinic acid, glutamic acid, gallic acid and protocatechuic acid was found in 0% RW, compared to other samples and their concentrations were above reported thresholds. Esters, alcohols, and organic acids were found to be the predominant aroma compounds in rice wines however guaiacol, 4-vinylguaicol and vanillin were significantly and substantially higher in 0% RW, compared to others. The GC-Olfactometry showed that 0% RW had smoky-spicy note as a characteristic aroma, and it was derived from guaiacol. This is consistent with the aroma profiling analysis which showed that a higher intensity of smoky-spicy note was found in 0% RW, compared to others. This study summarised that guaiacol was found as a characteristic aroma compound in 0% RW, and it was likely to have been formed from the bran of pigmented rice during brewing. However, the confirmation of its precursor in the pigmented rice bran was further studied.

Keywords: pigmented rice wine, guaiacol, 4-vinylguaiacol, smoky-note, characteristic aroma

5.1 Introduction

According to the analysis of taste and aroma compounds in commercial polished rice wines and pigmented rice wines (chapter 3), the smoky-spicy note was observed as a characteristic aroma in pigmented rice wine. However, the effect of bran from pigmented rice on the formation of characteristic taste and aroma compounds should be investigated.

The brewing process of unpolished black rice presents some challenge. Takeshita et al. (2015) showed that the fermentation of cooked black rice was slow on the first day because its grain was difficult to hydrolyse by enzymes, comparing to the corresponding polished rice. However, the concentration of ethanol (11-13.8%) was not different between samples. Moreover, Chay et al. (2017) also showed that yield, ethanol, total proteins, total sugars and reducing sugars from polished rice wine were higher than that in pigmented rice wine, regardless of brewing process. This attributed to the greater fermentation efficiency of the white rice.

The effect of the bran from aromatic red rice (*Oryza sativa* var. *Indica*, *Tapol*) on the aroma compounds in polished rice wine was found by Ueki et al. (1991). They showed that polished rice wine which was brewed by adding the bran of aromatic red rice contained a higher concentration of higher alcohols (2.5 times) and esters (3.5-5.0 times), compared that to polished rice wine which was brewed without the bran. Sukhonthara et al. (2009) showed that organic acids, including 3-methylbutanoic acid, hexanoic acid, heptanoic acid and octanoic acid were the most abundant aroma compounds in the bran of pigmented rice, especially back rice. These are likely to form the corresponding esters during brewing.

However, much less is known about the taste and aroma compounds in pigmented rice wine. Previous studies have shown that pigmented rice wine had characteristic aromas, particularly smoky-spicy notes. We know from chapter 3 that guaiacol and 4-vinylguaiacol are the compounds must likely to contribute to those notes. Since these aromas are not detected in commercial polished rice wines, they are likely to have arisen from the bran, or precursors in the bran. The aim of this study was to investigate the source of these smoky-spicy notes.

5.2 Materials and methods

5.2.1 Materials

Black glutinous rice (Double Elephant, Thailand) was purchased from a local supplier at Reading, UK. The brewing strains, including *Aspergillus oryzae* ATCC 22787 and *Saccharomyces cerevisiae* NCYC 478 were purchased from LGC Standards (Teddington, Middlesex, UK) and The National Collection of Yeast Cultures (Colney, Norwich, UK), respectively. The polishing machine was purchased from Twinbird (Tsubame, Niigata. Japan). The black glutinous rice was polished to remove the bran with various degree of polishing to produce 0% (unpolished grain), 30%, 50% and 65% (bran was fully removed). The degree of polishing was calculated by the following equation. The polished rice is show in figure 5.1.

$$DOM = \left(1 - \frac{weight\ of\ polished\ rice}{weight\ of\ brown\ rice}\right) \times 100$$

unpolished grain (0%) | partially polished grain (30%) | partially polished grain (50%) | fully polished grain (65%)

Figure 5-1: Black glutinous rice subject to various degree of polishing (from 0% unpolished to 65% polished)

5.2.2 Chemicals

The chemicals in this study were methyl acetate, diethyl ether, >98% 4-coumaric acid, 98% epicatechin, >97% vanillic acid, >98% sinapic acid, >97% protocatechuic acid, >99% ferulic acid, >95% syringic acid, >98% caffeic acid, 98% catechin, 97% gallic acid, 99% 4-hydroxybenzoic acid, 99.5% glucose, >99% fructose, 99% maltose, 95% maltotriose, >99% malic acid, 98% lactic acid, 99% citric acid, 99% sodium succinate, 99% sodium tartrate, 99% 1,2-dichlorobenzene, saturated alkane standard C_5-C_{30} and C_7-C_{40}, >99% guaiacol and 98% 4-vinylguaiacol were purchased from Sigma-Aldrich (Dorset, UK). Cyanidin-3-glucoside (>96%) chloride was purchased from Extrasynthese (Genay, France). Analytical grade sulfuric acid, HPLC grade methanol, >99% acetic acid, Optima™ 0.1% formic acid and Pierce® acetonitrile purchased from Fisher Scientific (Loughborough, UK). Analytical grade formic acid was purchased from BDH (Poole, UK). EZfaast™ amino acid test kit was purchased from Phenomenex (CA, USA). The standard of DKPs were >99% cyclo(proline-valine), >99% cyclo(alanine-valine), >99% cyclo(isoleucine-proline), >99% cyclo(leucine-proline), >98% cyclo(proline-proline), >99% cyclo(alanine-proline) and standard of γ-glutamyl peptides including >99% γ-glutamyl tyrosine (γ-glu-tyr), >99% γ-glutamyl phenylalanine (γ-glu-phe),

>99% γ-glutamyl histidine (γ-glu-his), >99% γ-glutamyl methionine (γ-glu-met), >99% γ-glutamyl glutamic acid (γ-glu-glu), >99% γ-glutamyl leucine (γ-glu-leu), >99% γ-glutamyl valine (γ-glu-val), >99% γ-glutamyl alanine (γ-glu-ala) and >99% γ-glutamyl glycine (γ-glu-gly) were purchased from Bachem (Bubendorf, Switzerland).

5.2.3 Brewing process of pigmented rice wines

Black glutinous rice which had been subjected to various degree of polishing from 0% to 65% (120 g) was cleaned using water, and soaked in water for overnight. The rice was steamed at 100 °C for 60 min, and then left to cool. It was inoculated with *Aspergillus oryzae* ($3x10^6$ spores/ml), and then incubated at 30 °C for 2 days under aerobic conditions to promote saccharification. For fermentation, *Saccharomyces cerevisiae* ($6x10^6$ cells/ml) was added to the rice mash, and saccharification and fermentation continued in parallel for a further 9 days at 30 °C under anaerobic conditions. The rice cake was then removed from the rice wine using Heraeus Multifuge $3SR^+$ centrifuge from Thermo Scientific (Paisley, UK) and Piramoon Fiberlite 6x250 LE rotor from Marshall Scientific (Hampton, UK) at 7,300 g for 15 min at 15 °C. Rice wine samples were pasteurised at 70 °C for 10 min, and cooled in a water bath to ambient temperature. The rice wines were stored in the freezer at -20 °C until further analysis.

0% RW 30% RW 50% RW 65% RW

Figure 5-2: Rice wines produced from pigmented rice subject to a different degree of polishing from 0% (unpolished grain) to 65% (fully polished grain)

5.2.4 Chemical analysis of raw pigmented rice

5.2.4.1 Free amino acids

To prepare the rice flour, pigmented rice (unpolished grain and 65% polished grain) was milled using the cryogenic tissue grinder from Stratech scientific (Cambridge, UK). The rice bran or rice flour samples (2 g) were mixed with 10 ml of 0.01M hydrochloric acid, and then shaken for 30 min using the Multi Reax shaker from Heidolph (Schwabach, Germany). The samples were left for 15 min, and then centrifuged to remove the supernatant at 7,300 g for 15 min, using a Howe laborzentrifugen, series 3K10 and 19776-H rotor from Sigma (Osterode am Harz, Germany). The pellet was re-extracted again under the same conditions, and then both supernatants were combined. Amino acids from the extracts were derivatised using the EZfaast™ test kit from Phenomenex (CA, USA). The samples were analysed for amino acids by gas chromatography-mass spectrometry (GC-MS) from Agilent (CA, USA) as reported by Elmore et al. (2005). To quantify the amino acids, the calibration curves of basic amino acids were carried out in the range of 50-200 µM, $R^2 > 0.9$.

5.2.4.2 Acid soluble and insoluble lignin

This analysis was adapted by Sluiter et al. (2010). The rice flour (unpolished and 65% polished grain) or pigmented rice bran (300 mg) were placed into autoclavable bottles, and then 72% sulfuric acid (3 ml) was added. The slurry was stirred and then incubated in a water bath at 30 °C for 60 min. During the incubation, the samples were stirred every 5 min. After that, 84 ml of water was added into the slurry, which then autoclaved at 121 °C for 60 min. The residues were separated from the supernatant using vacuum filtration, and then placed to pre-weighed crucibles. The samples were dried at 105 °C to constant weight using a hot air oven from Gallenkamp (Loughborough, UK), and then burned at 575 °C overnight using the muffle furnace from Carbolite (Bamford, UK). The crucibles were cooled in the desiccator, and then weighed. The supernatant was measured for acid soluble lignin at 320 nm using a spectrophotometer, series CE1021 from Cecil instrument (Cambridge, UK). To quantify the acid soluble and non-soluble lignin, the following equations were used.

$$\%\ acid\ insoluble\ lignin\ (AIL) = \frac{(weight_{dried\ residue} - weight_{ash}) - weight_{protein}}{ODW_{sample}} \times 100 \qquad (a)$$

$$\%\ acid\ soluble\ lignin\ (ASL) = \frac{UV_{abs} \times volume_{filtrate} \times dilution}{\varepsilon \times ODW_{sample} \times pathlenght} \times 100 \qquad (b)$$

Where UV_{abs} = the mean of the absorbance from the sample at 320 nm

volume = volume of filtrate, 87 ml

$dilution = \frac{volume_{sample} \times volume_{diluting\ solvent}}{volume_{sample}}$

ε= absorbability of biomass at the specific wavelength, 30

ODW_{sample} = weight of sample in milligram

pathlength = 1 cm

Weight $_{protein}$ = Amount of protein present in the acid insoluble residue. The measurement is only necessary for biomass containing high amounts of protein.

5.2.4.3 Free phenolic acids and anthocyanins

The pigmented rice flour (unpolished and 65% polished grain) and pigmented rice bran were defatted, to prevent oxidation, by solvent extraction method (Jung et al., 2007). Briefly, 5 g of sample was mixed with 20 ml of hexane, and then stirred for 1 h. Then, the solvent was removed from the sample using Whatman™ qualitative filter paper grade 1 from Whatman (Buckinghamshire, UK). The process was repeated for a second time to ensure the removal of all lipid material from the samples. Defatted samples were dried in a desiccator at room temperature overnight. To extract the phenolic acids and anthocyanins, 0.1% formic acid in 70% methanol (10 ml) was added into defatted sample (0.2 g), and then shaken for 45 min using the Multi Reax shaker from Heidolph (Schwabach, Germany). The supernatant was removed from the samples, using a Howe laborzentrifugen series 3K10 and 19776-H rotor from Sigma (Osterode am Harz, Germany) at 7,300 g for 15 min. The pellet was re-extracted to ensure the complete extraction of phenolic acids and anthocyanins. The supernatants were combined, and then concentrated at 40 °C by using a rotary evaporator, series R110 from Büchi (Flawil, Switzerland). Dried samples were re-dissolved with 0.1% formic acid in 70% methanol (2 ml), and then filtrated with 0.22 µm Minisart® syringe filter from Sartorius (Goettingen, Germany). The samples were analysed for phenolic acids and anthocyanins using high performance liquid chromatography (HPLC) from Agilent (CA, USA) as described by Seal (2016). Details of this method was shown in chapter 3, section 3.2.3.3. All phenolic acids and anthocyanins were quantified using the

external standard method. Quantification was based on peak area. Calibration curves of the standards were carried out by diluting stock standards in 0.1% formic acid in 70% methanol to yield 2-6 mg/l (phenolic acids) or 1-200 mg/l (anthocyanins), $R^2 > 0.99$.

5.2.5 Analysis of taste compounds in pigmented rice wines

5.2.5.1 Sugars, ethanol and organic acids

Rice wines were filtered using a 0.22 µm Minisart® syringe filter from Sartorius (Goettingen, Germany), and then analysed for sugars, ethanol and organic acids using HPLC from Agilent (CA, USA). The analytical conditions were adapted from Zeppa et al. (2001), and the information of this procedure was shown in chapter 3, section 3.2.3.1. Sugars, ethanol and organic acids were quantified using the external standard method. Quantification was based on peak area. Calibration curves of the standards were carried out by diluting stock standards to yield 0.01-5 g/l (sugars), 0.1-1 % (ethanol) and 0.1-1 g/l mg/l (organic acids), $R^2 > 0.99$.

5.2.5.2 Free amino acids

The pre-filtered samples were derivatised using the EZfaast™ kit from Phenomenex (CA, USA) as described by Elmore et al. (2005). The amino acids in the derivatised samples were analysed, using GC-MS from Agilent (CA, USA). All basic amino acids were quantified using the external standard method. Quantification was based on peak area. Calibration curves of the standards were carried out by diluting stock standards to yield 50-300 µM, $R^2 > 0.99$.

5.2.5.3 Free phenolic acids and anthocyanins

The pre-filtered rice wines were analysed for phenolic acids and anthocyanins using HPLC from Agilent (CA, USA) as described by Seal (2016). This procedure was shown in chapter 3, section 3.2.3.3. The phenol acids and anthocyanins were quantified using the external standard method. Quantification was based on peak area. Calibration curves of the standards were carried out by diluting stock standards to yield 0.5-50 mg/l (phenolic acids) or 0.4-100 mg/l (anthocyanins), $R^2 > 0.99$.

5.2.5.4 Diketopiperazines (DKPs)

Pigmented rice wines were extracted for DKPs, using Strata-X 33 µm polymeric reversed phase giga tube from Phenomenex (CA, USA) as described by Oruna-Concha et al. (2015). Prior the extraction, 50 µl of internal standard (1,2-dichlorobenzene, 100 mg/l) was added into 15 ml pigmented rice wine to obtain the final concentration of internal standard in sample was 0.33 mg/l. The extract was analysed for DKPs using GC-MS from Agilent (CA, USA), and the analytical condition was shown in chapter 3, section 3.2.3.4. DKPs were quantified using the external standard method. Quantification was based on peak area. Calibration curves of the standards were carried out by diluting stock standards to yield 0.1-5 mg/l, $R^2 > 0.98$.

5.2.5.5 γ-glutamyl peptides

To prepare the samples, rice wine (500 µl) containing 0.2 mg/l of γ-glu-met (internal standard) was filtered through an Amicon® Ultra, MWCO 3kDa filter from Sigma-Aldrich

(Steinheim, Germany) at 12,045 g for 10 minutes, using Eppendorf centrifuge (Ontario, Canada). The filtered samples were analysed for γ-glutamyl peptides by liquid chromatography-tandem mass spectrometry (LC-MS/MS) from Agilent (CA, USA) as described by Toelstede et al. (2009), and the procedure was shown in chapter 3, section 3.2.3.5. All γ-glutamyl peptides in samples were quantified using the external standard method. Quantification was based on peak area. Calibration curves of the standards were carried out by diluting stock standards to yield 0.01-5 mg/l, $R^2 > 0.93$.

5.2.6 Analysis of aroma compounds in pigmented rice wine

5.2.6.1 Volatile compounds

The volatile compounds in pigmented rice wines were extracted using solid phase microextraction (SPME) as described by Chen and Xu (2010). The samples were analysed for volatile compounds using GC-MS from Agilent (CA, USA), and the procedure was shown in chapter 3, section 3.2.4.1. The quantification of guaiacol and 4-vinylguaiacol was carried out using the external standard. Quantification was based on peak area. Calibration curves of the standards were carried out in the range of 0.01-5 mg/l for guaiacol and 0.002-2 mg/l for 4-vinylguaiacol, $R^2 > 0.99$.

5.2.6.2 Semi-volatile compounds

The semi-volatile compounds in pigmented rice wines were extracted using solid phase extraction (SPE) as shown by Lignou et al. (2013). The extract wad analysed for semi-

volatile compounds using GC-MS from Agilent (CA, USA), and the procedure was shown in chapter 3, section 3.2.4.2.

5.2.6.3 GC-Olfactometry

Pigmented rice wines which produced from unpolished grain and 65% polished grain were analysed for aroma description using GC-O. This method was adapted from Lignou et al. (2013), and it was shown in chapter 3, section 3.2.4.3. Four assessors (two males and two females) were used for the detection and verbal description of aroma active compounds in samples. The assessors had at least 2 years of experience in recognising odorants by GC-O.

5.2.7 Sensory evaluation for the pigmented rice wine

A professional panel of eight trained assessors (one male and 7 females), each with a minimum of six months' experience, was used to develop vocabularies for aroma profiling of pigmented rice wines. Unpolished pigmented rice wine (0% RW) and 65% polished pigmented rice wine (65% RW) were labelled with a random symbol, and presented to each assessor. To develop vocabularies for the aroma profiling, assessors were asked to sniff all samples to produce a list of descriptive terms for aromas in the samples. Following this initial session, reference materials were provided (table 5.1). These terms were discussed by the panel of assessors as a group, assisted by a panel leader, to agree on a final profile consisting of 7 aroma terms. The quantitative sensory assessment took place in individual sensory booths under red light and room temperature controlled to 23 °C. 5 ml of unpolished pigmented rice wine (0% RW), 30% polished pigmented rice wine (30% RW) and

65% polished pigmented rice wine (65% RW) presented in an amber bottles which were covered with aluminium foil, coded with random 3 digit codes, and then cooled at 4 °C. Samples were presented to the assessors in a balanced and randomised order, and assessors were asked to sniff the samples and score them on aroma attributes. The intensity of each attribute was recorded on an unstructured line scale (scaled 0–100), and data were collected using Compusense® version 5 software from Compusense (ON, Canada). A duplicate assessment was carried out in a separate session.

Table 5-1: Reference materials provided to help assessors to standardise attribute descriptors.

descriptor	reference material	brand
soy sauce	light soy sauce	Blue dragon
acid	balsamic vinegar	M&S
sweet	dried jujube	-
smoky-spicy	aromatic hoisin dip	ASDA
creamy-cheesy	Yorkshire blue cheese	M&S
beefy	beef yeast extract	Bovril
mushroom	-	-

5.2.8 Statistical analysis

IBM SPSS Statistics for Windows, Version 22.0 software (IBM Corp., Armonk, NY, USA) was used for the statistical analysis of experimental data. The statistical significant difference of the mean value was considered significant at $p<0.05$ by using the analysis of variance (ANOVA). All ANOVA were conducted using a 95% confidence interval and post hoc Duncan test was used for multiple pairwise comparisons. The Pearson's correlation was

used to identify the relation between two factors, if required. To analyse the data from the sensory evaluation, the Senpaq software version 4.2 (Qi Statistics, Reading, UK) was applied.

5.3 Results and discussions

5.3.1 The chemical composition of pigmented rice and polished pigmented rice

5.3.1.1 Free amino acids

Amino acids tended to be higher in the unpolished grain, compared to polished grain. The amino acid composition of the bran, unpolished and 65% polished pigmented rice is shown in the table 5.2. Alanine, glycine, asparagine and glutamic acid were found to be the predominant amino acid in the bran and unpolished pigmented rice. This is consistent with Shen et al. (2015) who showed that alanine, aspartic acid, threonine, glutamic acid and serine were found to be the predominant amino acid in black rice. Moreover, Liu et al. (2017) showed that aspartic acid, valine, leucine, arginine and proline were observed as the most abundant amino acid in unpolished non-pigmented rice.

Amino acids isoleucine, threonine, methionine, phenylalanine, glutamine, lysine, histidine, tyrosine and tryptophan were not present in 65% polished pigmented rice, and all other amino acids decreased as the degree of polishing increased ($p < 0.05$). This is consistent with Liu et al. (2017) who showed that an increase in polishing (from 0% to 14.8%) resulted in the decrease of amino acids, regardless of the rice variety. Saikusa et al. (1994) also studied the amino acids in the fraction of rice which was prepared by polishing at 5-10, 10-14, 14-18, 18-23, 23-27 and 27-100 %. They found that the highest concentration of amino acids was observed in the first fraction (5-10% polishing), and then

it was decreased by the abrasive process of polishing. Moreover, the decrease of amino acids was observed towards the centre of the kernel. Thus, confirming that the amino acids are more concentrated in the bran of the rice grain.

Table 5-2: Free amino acid composition in the bran, unpolished and 65% polished pigmented rice.

compounds	concentration in each fraction of pigmented rice (mg/ 100 g)			§Sig
	Bran	unpolished grain	65% polished grain	
alanine	45 ± 1.0^{c}	26 ± 0.7^{b}	12 ± 0.6^{a}	***
glycine	15 ± 0.1^{c}	11 ± 0.3^{b}	5.9 ± 0.3^{a}	***
valine	9.9 ± 0.1^{c}	5.7 ± 0.1^{b}	4.2 ± 0.2^{a}	***
leucine	11 ± 0.2^{c}	5.4 ± 0.1^{b}	4 ± 0.3^{a}	***
isoleucine	6.9 ± 0.1	4.9 ± 0.1	nd	ns
threonine	10.3 ± 0.1	6.3 ± 0.1	nd	ns
serine	23 ± 0.4^{c}	8 ± 0.3^{b}	4.4 ± 0.2^{a}	***
proline	8.6 ± 0.1^{c}	6 ± 0.1^{b}	4.5 ± 0.1^{a}	***
asparagine	36 ± 0.9^{c}	20.2 ± 0.5^{b}	11.9 ± 0.2^{a}	***
aspartic acid	44 ± 2.0^{c}	17 ± 0.6^{b}	10.2 ± 0.2^{a}	***
methionine	3 ± 0.1	4 ± 1.3	nd	ns
glutamic acid	106 ± 7^{c}	28 ± 3^{b}	11.3 ± 2^{a}	***
phenylalanine	10.9 ± 0.1	6.7 ± 0.1	nd	ns
glutamine	12.6 ± 0.6^{b}	10.3 ± 0.2^{a}	nd	*
lysine	28.9 ± 0.3^{b}	13.6 ± 0.2^{a}	nd	*
histidine	14.8 ± 0.7^{b}	13 ± 0.1^{a}	nd	*
tyrosine	14.6 ± 0.6^{b}	12 ± 0.1^{a}	nd	*
tryptophan	17.6 ± 0.2^{b}	13.5 ± 0.1^{a}	nd	*

Values with the same letter superscripts within each row are not significantly different ($p = 0.05$).

Data are presented as mean ± standard error, n=3. nd = not detected.

§Sig = Probability, as obtained from ANOVA, that there is a difference between means; ns, no significant difference between means ($P > 0.05$), *Significant at the 5% level ($0.01 < p \leq 0.05$) and ***Significant at the 0.1% level ($p \leq 0.001$).

5.3.1.2 Acid soluble and acid insoluble lignin

Lignin is a dietary fibre which is found in plant cell walls (DeVries et al., 1999). It consists of a highly branched three dimensional phenolic structure, including 4-coumaril, coniferyl and sinapyl alcohols (Brebu and Vasile, 2010). The content of lignin in the bran, unpolished and 65% polished pigmented rice is shown in the table 5.3. The concentration of acid soluble and acid insoluble lignin in the bran was 1.25 and 2 fold higher than unpolished pigmented rice, respectively. Moreover, the content of lignin in unpolished pigmented rice is in agreement with Fardet et al. (2008) who showed that lignin in wheat was found in the range of 0.6-1.3 g/ 100 g sample. The bran removal caused the decrease of lignin, especially in 65% polished grain because lignin is mostly found in the bran or cell walls of rice grain. This is consistent with Knudsen (2014) who showed that a higher content of lignin is found in the outer part of the kernel, compared that to endosperm.

Table 5-3: Lignin composition of the bran, unpolished and 65% polished pigmented rice.

compound	concentration (%)			§Sig
	Bran	unpolished grain	65% polished grain	
acid soluble lignin	1.5 ± 0.1^{c}	1.1 ± 0.1^{b}	0.74 ± 0.0^{a}	***
acid insoluble lignin	3.1 ± 0.3^{c}	1.8 ± 0.4^{b}	0.18 ± 0.1^{a}	***

Values with the same letter superscripts within each row are not significantly different ($p = 0.05$).

Data are presented as mean ± standard error, n = 3.

§Sig = Probability, as obtained from ANOVA, that there is a difference between means;
***Significant at the 0.1% level ($p \leq 0.001$).

5.3.1.3 Free phenolic acids and anthocyanins

The bran, unpolished and 65% polished pigmented rice were analysed for free phenolic acid and anthocyanins. According to the results in table 5.4, protocatechuic acid, catechin, vanillic acid, synringic acid, 4-coumaric acid and ferulic acid were found in unpolished pigmented rice. This is consistent with Shao et al. (2017) who showed that protocatechuic acid, 2,5-dihydroxybenzoic acid, ferulic acid, 4-coumaric acid, vanillic acid, 4-hydroxybenzoic acid, sinapic acid and isoferulic acid were found in unpolished pigmented rice. However, a higher concentrations of those compounds were observed from our study, compared that to Shao et al. (2017).

One anthocyanin, cyaniding-3-glucoside was found in unpolished pigmented rice. Anthocyanins have been shown as contributing the red brown or dark purple colour of the kernel (Yodmanee et al., 2011). This is consistent with Sompong et al. (2011) who showed that cyanindin-3-glucoside was found in black rice samples from Thailand, however their concentrations (19.3-137.4 mg/ 100 g sample) were lower than our study. Moreover, Ichikawa et al. (2001) also showed that cyanindin-3-glucoside represents around 94% of the total anthocyanins composition in pigmented rice and Yawadio et al. (2007) showed that this compound was found in Japanese black rice at 86% of the total anthocyanins.

The concentration of both phenolic acids and anthocyanin decreased as the degree of polishing increased, especially in the 65% polished pigmented rice ($p < 0.05$). Kong and Lee (2010) and Paiva et al. (2014) showed that free phenolic acids and anthocyanins are mostly distributed in the bran layer and therefore are affected by physical process such as polishing. This is consistent with our results which showed that the highest concentration of phenolic acids and anthocyanins were found in the bran of pigmented rice ($p < 0.05$).

Table 5-4: Phenolic acids and anthocyanins identified in the bran, unpolished and 65% polished pigmented rice.

compounds	concentration (mg/ 100 g sample)			§Sig
	bran	0% DM	65% DM	
protocatechuic acid	11.4 ± 0.6^{c}	7.5 ± 0.4^{b}	2.2 ± 0.9^{a}	***
catechin	4.1 ± 0.1^{b}	2.7 ± 0.4^{a}	nd	*
vanillic acid	2.9 ± 0.3^{c}	1.6 ± 0.1^{b}	0.45 ± 0.0^{a}	***
synringic acid	3.5 ± 0.5^{b}	2.4 ± 0.1^{a}	nd	*
4-coumaric acid	0.43 ± 0.0^{b}	0.32 ± 0.0^{a}	nd	**
ferulic acid	0.88 ± 0.1^{b}	0.52 ± 0.0^{a}	nd	*
cyanidin-3-glucosude	174 ± 10^{b}	94 ± 7^{a}	nd	***

Values with the same letter superscripts within each row are not significantly different ($p = 0.05$).

Data are presented as mean ± standard error, n=3. nd = not detected.

§Sig = Probability, as obtained from ANOVA, that there is a difference between means; *Significant at the 5% level ($0.01 < p \leq 0.05$), **Significant at the 1% level ($0.001 < p \leq 0.01$) and ***Significant at the 0.1% level ($p \leq 0.001$).

The concentration of phenolic acids corresponded to the concentration of lignin (table 5.3) because lignin is formed by enzymatic dehydrogenation of cinnamyl, coiferyl and sinapyl alcohol. Thus, the corresponding phenolic acids such as 4-coumaric acid, ferulic acid, 4-hydroxybenzoic acid and protocatechuic acid are from the degradation of lignin (chapter 2, section 2.2.6). Dick et al. (2011) also showed that anthocyanins in plants are formed from phenylalanine via the anthocyanin biosynthetic pathway.

5.3.2 The effect of degree of polishing on the formation of taste compounds in pigmented rice wine

5.3.2.1 Sugars, ethanol and organic acids

The sugar and organic acid compositions as well as ethanol and pH values for the pigmented rice wines with different degree of polishing are presented in table 5.5. The concentration of maltose and glucose in lab-scale brewed pigmented rice wines were lower than that in commercial rice wines (chapter 3), whereas fructose was not found in in lab-scale brewed pigmented rice wines. Maltose was only found in 0% RW, whereas glucose was found in all lab-scales brewed pigmented rice wines with concentrations lower than the reported thresholds. These fermentable sugars were likely generated from the degradation of rice starch using *Aspergillus oryzae* via the saccharification (Dung et al., 2006, Saranraj and Stella, 2013). Moreover, an increase in polishing resulted in an increase in glucose content in rice wines ($p<0.05$). This can be explained by the fact that polishing increases the concentration of carbohydrate in rice grain (Payakapol et al., 2011). This is consistent with Eun et al. (2007) who showed that the concentration of glucose in Jinyangju rice wine increased as the polishing of glutinous rice increased. However, Lee et al. (2012b) and Park et al. (2015) showed that there was no correlation between degree of polishing and total sugars content in rice wine.

The concentration of ethanol in rice wines was not significantly different between samples, although the concentration of glucose was found at the highest concentration ($p < 0.05$) in 65% RW. This is in agreement with Park et al. (2015) who showed that the concentration of ethanol was not significantly different between alcoholic beverages which were produced from barley with different degree of polishing. This corresponded to the concentration of total sugars which were not different between samples.

Glucose is the substrate for ethanol formation via the EMP pathway (Bai et al., 2008). According to this, a higher concentration of ethanol was found in 65% RW, compared to the other samples. However, no significant differences were observed between rice wines. This might be explained by the fact that *Saccharomyces cerevisiae* produces ethanol in the range of 8-12% v/v. Moreover, the concentration of ethanol which is above 15% can terminate the fermentation because the yeast cannot survive at those conditions (Basso et al., 2011).

Five organic acids including citric acid, malic acid, succinic acid and acetic acid were found in 0% RW. Among these compounds, only succinic acid in 0% RW was higher than the threshold value. The concentration of organic acids, except acetic acid in our study were consistent with that reported by Chun et al. (2012). The polishing process changed and reduced the concentration of organic acids in rice wines as only citric acid, malic acid and succinic acid were detected in 0% RW with the concentration ranging from 0.33-2.03 g/l. This is in agreement with Park et al. (2015) who showed that the concentration of total organic acids in wines which were brewed from Huinchalssal-bori barley was decreased by the increase of degree of polishing. Chun et al. (2012) showed that citric acid and succinic acid decreased as the polishing process increased. Moreover, they also showed acetic acid was not found in 30-40% polished rice wine, compared that to 10-20% polished rice wine. This might be explained by the fact that not only glucose was used as the precursor for organic acid formation, but also fatty acids in the rice bran might have been used too. Free fatty acids are converted to the acetyl CoA via fatty acid oxidation by *Saccharomyces cerevisiae* (Van Roermund et al., 2003), and they go through the TCA cycle to form citric acid, malic acid and succinic acid (Rezaei et al., 2015). According to the redox pathway in *Saccharomyces cerevisiae*, acetyl CoA is also converted to acetaldehyde using acetaldehyde dehydrogenase. It is possibly converted to ethanol using alcohol dehydrogenase, and acetic

acid using non-acetylating acetaldehyde dehydrogenase (Henningsen et al., 2015). Thus, an increase in the bran content resulted in an increase in the concentration of citric acid, malic acid, succinic acid and acetic acid.

Table 5-5: Sugars, organic acids, ethanol and pH values in rice wines brewed from pigmented rice with different degree of polishing and their reported threshold values.

compounds	†TC (g/l)	concentration				§Sig
		0% RW	30% RW	50% RW	65% RW	
ethanol (% v/v)	-	12 ± 0.1	12.2 ± 0.3	11.6 ± 0.3	11.8 ± 0.7	ns
pH	-	4.8 ± 0.0^{d}	4.2 ± 0.0^{c}	3.9 ± 0.0^{b}	3.7 ± 0.1^{a}	***
sugars (g/l)						
maltose	-	1.3 ± 0.4	nd	nd	nd	-
glucose	3.2	0.8 ± 0.1^{a}	1.2 ± 0.1^{b}	1.2 ± 0.1^{b}	1.8 ± 0.1^{c}	***
organic acid (g/l)						
citric acid	0.5	0.33 ± 0.0	nd	nd	nd	-
malic acid	0.5	0.44 ± 0.1	nd	nd	nd	-
succinic acid	0.1	2.03 ± 0.2	nd	nd	nd	-
lactic acid	1.4	0.73 ± 0.2	0.9 ± 0.1	1.04 ± 0.2	0.76 ± 0.0	ns
acetic acid	0.1	0.65 ± 0.1^{c}	0.39 ± 0.1^{ab}	0.36 ± 0.1^{a}	0.48 ± 0.0^{b}	***

Values with the same letter superscripts within each row are not significantly different (p = 0.05).

Data are presented as mean ± standard error, n=3. nd = not detected.

†Taste threshold concentrations, (TC) of sugars (sweet) and organic acids (sour) were obtained from Hufnagel and Hofmann (2008b).

§Sig = Probability, as obtained from ANOVA, that there is a difference between means; ns, no significant difference between means ($P > 0.05$) and ***Significant at the 0.1% level ($p \leq 0.001$).

5.3.2.2 Free amino acids

The free amino acid compositions of pigmented rice wine samples are presented in table 5.6. Higher concentrations of all amino acids were found in lab-scale brewed rice wines, compared to commercial rice wine (chapter 3). Moreover, glycine, proline, leucine, asparagine, glutamic acid and glutamine were found to be the predominant compounds in all rice wine samples. This amino acid profile in pigmented rice wine was similar to those reported in rice beer by Das et al. (2014) who found arginine, serine, aspartic acid, glutamic acid, glycine, tyrosine, proline, valine, phenylalanine, isoleucine, leucine, histidine and lysine in those samples. Kang et al. (2014) also showed that alanine, proline, tyrosine, valine, methionine, leucine, phenylalanine and lysine were found in Korean rice wine samples. Moreover, the concentration of glutamic acid in rice wines from our study was above the reported threshold, thus contributing to the umami taste.

Amino acids, especially glycine, proline, methionine, leucine, aspartic acid and glutamic acid in rice wines decreased as the degree of polishing increased. Moreover, the concentration of total amino acids was also decreased by the increase in the rice polishing. This is consistent with Chun et al. (2012) and Kang et al. (2014). The rice bran is the source of amino acids however they are decreased by the progressive polishing process (Liu et al., 2017).

Shen et al. (2010) and Kang et al. (2014) showed that amino acids in Chinese rice wine mostly originate from the hydrolysis reaction of proteins in rice and wheat Qu, using protease and carboxypeptidase from brewing strains. The formation of amino acids is important for rice wine because they are the source of nitrogen which is used for the growth of the brewing strains. However, the concentration of some amino acids in rice wines from our study was not decreased by the increase of polishing. This can be explained

by the fact that the amino acids might be formed by the autolysis of yeast during the alcoholic fermentation (Feuillat and Charpentier, 1982). Autolysis normally takes place at the end of the stationary phase of growth, and usually correlates to the mortality of yeast cell. This is an irreversible process caused by an intracellular yeast enzyme. During the autolysis, nucleotides, amino acids, peptides and proteins are liberated from the yeast cells because their cell walls are degraded by intracellular enzymes (Alexandre and Guilloux-Benatier, 2006). Thus, the amino acids in rice wines were not only liberated from the bran of pigmented rice, they could have also formed from the yeast cells.

Table 5-6: Free amino acids concentration in rice wines brewed from pigmented rice with different degree of polishing and their reported threshold values.

compounds	†TC (mM)	taste	concentration (mM)				§Sig
			0% RW	30% RW	50% RW	65% RW	
alanine	12	sweet	7.1 ± 0.1^{a}	8.7 ± 0.4^{c}	7.9 ± 0.2^{b}	8.3 ± 0.2^{bc}	***
glycine	25	sweet	5.9 ± 0.1^{b}	6.2 ± 0.2^{b}	5.1 ± 0.3^{a}	5.2 ± 0.2^{a}	***
threonine	35	sweet	1.9 ± 0.1^{b}	1.9 ± 0.1^{b}	1.5 ± 0.1^{a}	1.8 ± 0.1^{b}	**
serine	25	sweet	3.7 ± 0.2^{ab}	4.2 ± 0.3^{b}	3.4 ± 0.4^{a}	4.1 ± 0.3^{b}	*
proline	25	sweet	4.5 ± 0.1^{d}	3.8 ± 0.2^{c}	3.1 ± 0.2^{a}	3.4 ± 0.1^{b}	***
methionine	5	sweet	0.8 ± 0.0^{b}	0.9 ± 0.0^{b}	0.6 ± 0.0^{a}	0.7 ± 0.0^{a}	***
valine	20	bitter	3.4 ± 0.1^{d}	2.9 ± 0.2^{c}	2.3 ± 0.3^{a}	2.6 ± 0.1^{b}	***
leucine	11	bitter	4.1 ± 0.1^{b}	4.5 ± 0.3^{b}	3.3 ± 0.3^{a}	3.6 ± 0.2^{a}	***
isoleucine	10	bitter	1.7 ± 0.1^{c}	1.8 ± 0.1^{c}	1.2 ± 0.1^{a}	1.5 ± 0.1^{b}	***
phenylalanine	45	bitter	2.8 ± 0.1^{b}	3.2 ± 0.2^{c}	2.4 ± 0.2^{a}	2.6 ± 0.1^{ab}	***
lysine	80	bitter	2.2 ± 0.2^{a}	2.9 ± 0.5^{b}	2.3 ± 0.1^{ab}	2.6 ± 0.4^{ab}	*
histidine	45	bitter	1.6 ± 0.1^{a}	1.9 ± 0.2^{b}	1.4 ± 0.2^{a}	1.6 ± 0.1^{a}	**
tyrosine	4	bitter	2.9 ± 0.1^{c}	1.9 ± 0.2^{a}	2.1 ± 0.2^{a}	2.5 ± 0.2^{b}	***
aspartic acid	20	umami	6.9 ± 0.3^{c}	5.3 ± 0.4^{b}	3.7 ± 0.5^{a}	4.1 ± 0.3^{a}	***
glutamic acid	1.2	umami	7.9 ± 0.2^{c}	5.5 ± 0.4^{b}	4.2 ± 0.2^{a}	4.2 ± 0.1^{a}	***
asparagine	50	umami	0.7 ± 0.0^{a}	1.2 ± 0.0^{b}	1.3 ± 0.1^{b}	1.6 ± 0.1^{c}	***

Table 5-6: Free amino acids concentration in rice wines brewed from pigmented rice with different degree of polishing and their reported threshold values (continued).

compounds	†TC (mM)	taste	concentration (mM)				§Sig
			0% RW	30% RW	50% RW	65% RW	
glutamine	50	umami	4.6 ± 0.3^{a}	6.2 ± 0.3^{c}	4.5 ± 0.3^{a}	5.2 ± 0.3^{b}	***
ornithine	-	-	0.22 ± 0.0^{a}	0.56 ± 0.1^{b}	0.27 ± 0.0^{a}	0.3 ± 0.0^{a}	***
tryptophan	-	-	0.64 ± 0.1^{b}	0.66 ± 0.1^{b}	0.43 ± 0.0^{a}	0.41 ± 0.0^{a}	***
cysteine	-	-	0.54 ± 0.1^{a}	0.77 ± 0.1^{b}	0.46 ± 0.0^{a}	0.47 ± 0.1^{a}	**
total amino acids	-	-	64 ± 1.2^{b}	65 ± 4^{b}	51 ± 3^{a}	57 ± 3^{a}	***

Values with the same letter superscripts within each row are not significantly different ($p = 0.05$).

Data are presented as mean ± standard error, $n = 3$.

†Taste threshold concentrations, (TC) were obtained from Hufnagel and Hofmann (2008b).

§Sig = Probability, as obtained from ANOVA, that there is a difference between means; *Significant at the 5% level ($0.01 < p \leq 0.05$), **Significant at the 1% level ($0.001 < p \leq 0.01$) and ***Significant at the 0.1% level ($p \leq 0.001$).

5.3.2.3 Free phenolic acids and anthocyanins

The phenolic acids and anthocyanin identified in rice wines are shown in table 5.7. Gallic acid and protocatechuic acid were found to be the predominant phenolic acid in all rice wine samples, followed by 4-hydroxybenzoic acid, vanillic acid, 4-coumaric acid and ferulic acid. This is in agreement with Wang et al. (2014) who showed that gallic acid, protocatechuic acid, 4-hydroxybenzoic acid, vanillic acid, ferulic acid and cyanidin-3-glucoside were detected in unpolished black rice wines, however their concentrations were lower than that in 0% RW from our study. Moreover, the concentrations of gallic acid, protocatechuic acid, 4-hydroxybenzoic acid, vanillic acid, 4-coumaric acid and gerulic acid in commercial pigmented rice were also lower than that in lab-scale brewed pigmented rice wine. The phenolic acids and anthocyanins in rice wine samples can be correlated with the corresponding compounds in raw pigmented rice (table 5.4).

The concentration of gallic acid in 0% RW, 30% RW and 65% RW, and the concentration of protocatechuic acid in 0% RW were higher than reported thresholds. Thus, these compounds might contribute to the astringent mouthfeel in the rice wines, especially in 0% RW. Furthermore, 0% RW had a rosé colour which may have been as a results of the cyanidin-3-glucoside being released from the bran of pigmented rice (Yodmanee et al., 2011, Samyor et al., 2017).

An increase in degree of polishing resulted in the decrease in all phenolic acids and anthocyanins in rice wines. This is in agreement with their higher concentrations were found in pigmented rice grain, compared that to polished rice grain (table 5.4).

Table 5-7: Phenolic acids and anthocyanins identified in rice wines brewed from pigmented rice with different degree of polishing and their reported threshold values.

compounds	†TC (mg/l)	taste/mouthfeel	concentration (mg/l)				§Sig
			0% RW	30% RW	50% RW	65% RW	
gallic acid	50	astringent	74 ± 3.0^{c}	55 ± 3.4^{b}	48 ± 5.0^{a}	55 ± 3.0^{b}	***
protocatechuic acid	32	astringent	58 ± 2.0^{b}	2.4 ± 0.2^{a}	3.6 ± 0.3^{a}	2.4 ± 0.3^{a}	***
4-hydroxybenzoic acid	92	astringent	22 ± 4.5^{c}	14 ± 0.2^{b}	14 ± 0.5^{b}	9.4 ± 0.2^{a}	***
vanillic acid	53	astringent	3.7 ± 0.1^{c}	2.2 ± 0.3^{a}	2.1 ± 0.3^{a}	2.6 ± 0.4^{a}	***
4-coumaric acid	23	astringent	2.5 ± 0.1^{b}	0.5 ± 0.0^{a}	0.5 ± 0.0^{a}	0.5 ± 0.0^{a}	***
ferulic acid	13	astringent	13 ± 0.1^{c}	8.5 ± 0.8^{b}	6.3 ± 0.3^{a}	5.6 ± 0.3^{a}	***
sinapic acid	-	-	1.8 ± 0.2	nd	nd	nd	-
cyanidin-3-glucoside	-	-	7.8 ± 0.5	nd	nd	nd	-

Values with the same letter superscripts within each row are not significantly different ($p = 0.05$).

Data are presented as mean ± standard error, n = 3. nd = not detected.

†Taste threshold concentrations (TC) were obtained from Hufnagel and Hofmann (2008b).

§Sig = Probability, as obtained from ANOVA, that there is a difference between means; ***Significant at the 0.1% level ($p \leq 0.001$).

5.3.2.4 Diketopiperazines (DKPs)

Four proline-based DKPs, namely cyclo(pro-val), cyclo(pro-ile), cyclo(pro-leu) and cyclo(pro-pro) were found in the pigmented rice wines (table 5.8). However, their concentrations were lower than the reported thresholds. These DKPs were previously found to be odour active in fermented food products such as cheese, red wine and yeast extract (Borthwick and Da Costa, 2017). Moreover, they were also detected in commercial rice wines with the concentration in the range of 0.03-12.8 mg/l (chapter 3).

DKPs are possibly formed from the condensation of an equimolar amounts of amino acids under heating process (Mishra et al., 2017). However, Borthwick and Da Costa (2017) showed that they are likely to be formed from the heating of the acyclic tripeptides under the acidic conditions. This is consistent with our study which showed that the DKPs were likely to be formed form the corresponding amino acids under the acidic condition, and the Pearson's correlation showed a correlation coefficient from -0.76 to -0.9. Therefore, a higher concentration of DKPs was found in 65% RW, compared to other rice wines.

The degree of rice polishing did not have an effect on the formation of DKPs. Moreover, an increase in the concentration of amino acids did not result in an increase in DKPs content. This could be due to amino acids being in excess in all lab-scale brewed rice wines, which were used for the formation of the corresponding DKPs.

Table 5-8: Diketopiperazines (DKPs) identified in rice wines brewed from pigmented rice with different degree of polishing and their reported threshold values.

compounds	†TC (mg/l)		concentration (mg/l)				§Sig
	metallic	bitter	0% RW	30% RW	50% RW	65% RW	
cyclo(pro-val)	62	251	1.1 ± 0.2^{a}	1.9 ± 0.1^{b}	1.8 ± 0.3^{b}	2.1 ± 0.4^{b}	**
cyclo(pro-ile)	25	101	5.1 ± 0.6^{a}	14.2 ± 1.2^{b}	16.3 ± 2.3^{b}	17.2 ± 2.7^{b}	***
cyclo(pro-leu)	25	250	8.9 ± 1.3	9.7 ± 1.1	9.3 ± 1.8	9.8 ± 1.6	ns
cyclo(pro-pro)	147	501	2.9 ± 0.2^{a}	4.1 ± 0.1^{b}	4.3 ± 0.5^{b}	4.9 ± 0.4^{c}	***

Values with the same letter superscripts within each row are not significantly different ($p = 0.05$).

Data are presented as mean ± standard error, n=3.

†Taste threshold concentrations, (TC) were obtained from Stark and Hofmann (2005).

§Sig = Probability, as obtained from ANOVA, that there is a difference between means; ns, no significant difference between means ($P > 0.05$), **Significant at the 1% level ($0.001 < p \leq 0.01$) and ***Significant at the 0.1% level ($p \leq 0.001$).

5.3.2.5 γ-glutamyl peptides

Five γ-glutamyl peptides were identified in lab-scale brewed rice wines (table 5.9). γ-glu-gly and γ-glu-his were observed as the most abundant compound, and their concentrations were in the range of 2.53-3.29 and 1.21-1.62 mg/l, respectively. Moreover, γ-glu-leu, γ-glu-phe and γ-glu-tyr were found to be the less abundant compounds with concentrations in the range of 0.04-0.07, 0.04-0.08 and 0.05-0.1 mg/l, respectively. Lab-scale brewed rice wine samples showed a lower concentration of those γ-glutamyl peptides, compared to commercial rice wines. Moreover, γ-glu-val and γ-glu-glu were found as an additional compound in commercial rice wines (chapter 3).

According to Toelstede et al. (2009) and Zhao et al. (2016), γ-glutamyl peptides impart kokumi taste, responsible to the long lasting mouthfeel in fermented products. Although, the concentration of these γ-glutamyl peptides in rice wines was lower than the individual threshold, it can be assumed that the combination of all γ-glutamyl peptides might contribute to the overall kokumi taste.

The concentrations of γ-glu-gly and γ-glu-his in the rice wines decreased as the polishing process increased. This suggested that the bran of pigmented rice contained the corresponding precursors which are used for the formation of γ-glutamyl peptides. This is consistent with Zhao et al. (2016) and Hillmann et al. (2016) who showed that γ-glutamyl peptides are formed, using free amino acids from the fermented materials and γ-glutamyl transferase from fungi, including *Aspergillus*, *Bacillu*s and *Lactobacillus* and yeast *Saccharomyces cerevisiae*.

Table 5-9: γ-glutamyl peptides identified in rice wines brewed from pigmented rice with different degree of polishing and their reported threshold values.

compounds	†TC (mg/l)	concentration (mg/l)				§Sig
		0% RW	30% RW	50% RW	65% RW	
γ-glu-gly	3.6	3.29 ± 0.0^{d}	3.09 ± 0.0^{c}	2.7 ± 0.1^{b}	2.53 ± 0.0^{a}	***
γ-glu-leu	1.3	0.05 ± 0.0^{b}	0.06 ± 0.0^{b}	0.04 ± 0.0^{a}	0.07 ± 0.0^{c}	***
γ-glu-his	2.8	1.62 ± 0.1^{c}	1.42 ± 0.0^{b}	1.21 ± 0.1^{a}	1.21 ± 0.2^{a}	**
γ-glu-phe	0.8	0.07 ± 0.0^{b}	0.07 ± 0.0^{b}	0.04 ± 0.0^{a}	0.08 ± 0.0^{c}	***
γ-glu-tyr	1.5	0.1 ± 0.0^{d}	0.07 ± 0.0^{c}	0.05 ± 0.0^{a}	0.06 ± 0.0^{b}	***

Values with the same letter superscripts within each row are not significantly different ($p = 0.05$).

Data are presented as mean ± standard error, n=3.

†Taste threshold concentrations, (TC) for γ-glu-gly, γ-glu-leu and γ-glu-his were obtained from Zhao et al. (2016); for γ-glu-phe and γ-glu-tyr were obtained from Shibata et al. (2017).

§Sig = Probability, as obtained from ANOVA, that there is a difference between means; **Significant at the 1% level ($0.001 < p \leq 0.01$) and ***Significant at the 0.1% level ($p \leq 0.001$).

5.3.3 The effect of degree of polishing on the formation of aroma compounds in pigmented rice wine

5.3.3.1 Volatile compounds

Aroma volatile compounds identified in lab-scale brewed pigmented rice wines are presented in table 5.10. Esters and alcohols were found to be the predominant volatile compounds in all rice wines. The esters identified in lab-scale brewed rice wines are in agreement with those in commercial rice wine samples which reported by Yotmanee et al. (2015). However, ethyl dodecanoate, ethyl tetradecanoate and ethyl pentadecanoate were found as additional lipid-derived esters in lab-scale brewed rice wines. Ethyl acetate and 3-methylbutyl acetate were found to be the most abundant esters in rice wines, and their higher concentrations were observed in 0% RW, compared that to other rice wines. Notably, 2-methylbutyl acetate, ethyl 2-methylbutanoate and ethyl 2-hydroxyhexanoate were observed as the additional esters in pigmented rice wines, and those esters were not detected in rice wine by Niu et al. (2017) and Son et al. (2018).

Higher alcohols were also found to be the predominant volatile compounds in rice wines. Their formation involved the use of amino acids (leucine, valine, isoleucine and phenylalanine) under anaerobic conditions or chemical reduction of the corresponding aldehydes via the metabolism of yeast during the brewing (Fan et al., 2011). In this study, higher alcohols identified in rice wines were 2-methylpropanol, 3-methylbutanol, 2-methylbutanol and 2-phenylethyl alcohol. This is in agreement with Xu et al. (2015a) who showed that 2-methylpropanol and 3-methylbutanol were found in Chinese rice wines, and Son et al. (2018) showed that 3-methylbutanol, 2-methylbutanol and 2-phenylethyl alcohol were found to be the predominant higher alcohols in rice wines. Moreover, 65% RW

contained a higher concentration of these higher alcohols, compared to 0% RW and commercial rice wines in chapter 3.

A higher concentration of guaiacol and 4-vinylguaiacol was observed in lab-scale brewed unpolished pigmented rice wines. This is consistent with Yotmanee et al. (2015) who showed that a higher concentration of those volatile phenols was found in commercial pigmented rice wine. Chen et al. (2013a) also showed that guaiacol and 4-vinylguaiacol were found in Chinese rice wines which were produced using wheat Qu. The concentration of guaiacol and 4-vinylguaiacol in lab-scale brewed rice wine samples was decreased by the increase of polishing. This showed that the bran of pigmented rice had the precursors for the formation of guaiacol and 4-vinylguaiacol. This is in agreement with Witthuhn et al. (2012) who showed that guaiacol is formed from vanillic acid which is derived from the degradation of lignin in grain, and Coghe et al. (2004) also showed that 4-vinylguaiacol is formed from ferulic acid which is liberated from lignin.

In summary, most of the volatile aroma compounds were not significantly different between 0% RW and 65% RW. This can be explained that the bran of pigmented rice did not have an effect on their formations. Volatile phenol compounds, including phenol, guaiacol and 4-vinylguaiacol) were found in 0% RW using SPME technique. They were significantly and substantially higher in 0% RW, compared that to 65% RW. This can be explained that the bran of pigmented rice promoted the formation of guaiacol and 4-vinylguaiacol during brewing process. Moreover, their formations were shown by Ito et al. (2016) and presented in chapter 2, figure 2.12.

Table 5-10: Aroma volatile compounds identified in rice wines brewed using pigmented rice with varying degree of polishing.

volatile compounds	†LRI		‡ID	§approximated concentration				¦Sig
	ZB-5MSi	WAX-DA		0% RW	30% RW	50% RW	65% RW	
acetate esters								
ethyl acetate	623	900	A	161 ± 16^{b}	88 ± 4^{a}	90 ± 18^{a}	104 ± 17^{a}	***
propyl acetate	715	-	B	0.71 ±0.0^{a}	0.75 ± 0.1^{a}	0.78 ± 0.2^{a}	1 ± 0.1^{b}	*
2-methylpropyl acetate	774	1002	A	4.1 ± 0.2	3.6 ± 0.7	3.9 ± 1.2	5.4 ± 1.3	ns
3-methylbutyl acetate	877	1130	A	75 ± 13^{b}	47 ± 10^{a}	52± 12^{a}	62 ± 5^{ab}	*
2-methylbutyl acetate	879	-	B	12 ± 0.6^{b}	8.8 ± 1.7^{a}	8.7 ± 2.2^{a}	10 ± 1.7^{ab}	*
ethyl 2-phenylacetate	1251	1784	A	1.3 ± 0.1^{a}	5.3 ± 0.2^{b}	5.3 ± 0.8^{b}	5.6 ± 0.9^{b}	***
2-phenylethyl acetate	1264	1835	A	5.3 ± 0.7^{a}	6.4 ± 0.6^{ab}	6.6 ± 0.9^{ab}	7.6 ± 1.6^{b}	*
ethyl esters								
ethyl propanoate	713	-	B	0.3 ± 0.0^{a}	0.39 ± 0.0^{b}	0.38 ± 0.1^{b}	0.33 ± 0^{ab}	*
ethyl 2-methylpropanoate	760	-	B	0.63 ± 0.0^{a}	0.98 ± 0.1^{b}	0.76 ± 0.2^{a}	0.77 ± 0.1^{a}	*
ethyl butanoate	800	1050	A	2.4 ± 0.1^{b}	1.7 ± 0.2^{a}	1.7 ± 0.3^{a}	1.9 ± 0.3^{a}	*
ethyl 2-methylbutanoate	851	-	B	0.24 ± 0.0^{a}	0.35 ± 0.1^{b}	0.31 ± 0.1^{ab}	0.37 ± 0.0^{b}	*
ethyl 3-methylbutanoate	854	-	B	0.23 ± 0.0^{a}	0.46 ± 0.1^{b}	0.47 ± 0.1^{b}	0.6 ± 0.0^{c}	***
ethyl pentanoate	899	-	B	0.33 ± 0.0	0.4 ± 0.1	nd	nd	ns
ethyl hexanoate	998	1237	A	12 ± 0.4^{b}	9 ± 0.8^{a}	9 ± 1.5^{a}	10 ± 1^{ab}	*
ethyl heptanoate	1096	1351	A	0.36 ± 0.1^{a}	0.98 ± 0.2^{b}	1 ± 0.2^{b}	1.1 ± 0.2^{b}	**
ethyl octanoate	1195	1438	A	23 ± 2.1	25 ± 3	25 ± 2	27 ± 5	ns

Table 5-10: Aroma volatile compounds identified in rice wines brewed using pigmented rice with varying degree of polishing (continued).

volatile compounds	[†]LRI		[‡]ID	[§]approximated concentration				[¦]Sig
	ZB-5MSi	WAX-DA		0% RW	30% RW	50% RW	65% RW	
ethyl nonanoate	1293	1541	A	0.32 ± 0.3	1.4 ± 1.2	1.4 ± 0.3	1.3 ± 0.1	ns
ethyl decanoate	1392	1643	A	2.7 ± 2.5	4.8 ± 1.2	4.2 ± 0.3	4.4 ± 0.8	ns
ethyl dodecanoate	1596	1847	A	0.26 ± 0.2	0.67 ± 0.6	0.52 ± 0.5	0.85 ± 0.8	ns
ethyl tetradecanoate	-	2067	B	3.9 ± 0.1	4 ± 3.1	6.3 ± 2.4	4.5 ± 0.1	ns
ethyl pentadecanoate	-	2151	B	0.22 ± 0.0	0.5 ± 0.2	0.58 ± 0.2	0.42 ± 0.0	ns
alcohols								
propanol	568	1032	A	5.4 ± 0.6^{a}	13 ± 1^{b}	11 ± 1^{b}	13 ± 2^{b}	***
2-methylpropanol	633	1097	A	54 ± 2^{a}	51 ± 4^{a}	54 ± 4^{a}	71 ± 12^{b}	*
butanol	664	1132	A	0.27 ± 0.0^{a}	1.1 ± 0.1^{b}	0.81 ± 0.2^{b}	0.78 ± 0.1^{b}	***
3-methylbutanol	732	1207	A	27 ± 2^{a}	31 ± 3^{ab}	34 ± 4^{b}	31 ± 3^{ab}	*
2-methylbutanol	739	-	B	20 ±1	23 ± 2	22 ± 4	21 ± 3	ns
pentanol	-	1249	B	0.26 ± 0.0	0.24 ± 0.1	0.17 ± 0.1	0.14 ± 0.0	ns
hexanol	876	-	B	0.45 ± 0.1^{b}	0.39 ± 0.0^{ab}	0.39 ± 0.1^{ab}	0.31 ± 0.1^{a}	*
2-ethylhexanol	1029	-	B	0.16 ± 0.0^{b}	0.08 ± 0.0^{a}	0.07 ± 0.0^{a}	nd	***
octanol	1070	1542	A	0.21 ± 0.1^{ab}	0.32 ± 0.0^{c}	0.27 ± 0.1^{b}	0.18 ± 0.0^{a}	*
2-phenylethyl alcohol	1123	1909	A	32 ± 2^{a}	60 ± 5^{c}	54 ± 5^{c}	44 ± 6^{b}	***

Table 5-10: Aroma volatile compounds identified in rice wines brewed using pigmented rice with varying degree of polishing (continued).

volatile compounds	†LRI		‡ID	§approximated concentration				¦Sig
	ZB-5MSi	WAX-DA		0% RW	30% RW	50% RW	65% RW	
acids								
acetic acid	-	1486	B	4.6 ± 0.3^{b}	1 ± 0.4^{a}	1.1 ± 0.1^{a}	1.3 ± 0.3^{a}	**
2-methylbutanoic acid	846	-	B	0.67 ± 0.2^{a}	0.98 ± 0.1^{ab}	0.75 ± 0.2^{a}	1.1 ± 0.2^{b}	*
volatile phenols								
phenol	980	1997	A	0.2 ± 0.1	nd	nd	nd	-
guaiacol	1096	1856	A	2.2 ± 0.2^{b}	0.08 ± 0.0^{a}	0.03 ± 0.0^{a}	nd	***
4-vinylguaiacol	1326	2211	A	0.75 ± 0.2^{c}	0.41 ± 0.1^{b}	0.21 ± 0.0^{a}	0.04 ± 0.0^{a}	***
aldehydes								
3-methylbutanal	658	-	B	0.04 ± 0.0	0.04 ± 0.0	0.03 ± 0.0	0.04 ± 0.0	ns
methional	913	-	B	0.02 ± 0.0^{b}	0.01 ± 0.0^{a}	nd	nd	***
benzaldehyde	968	1539	A	0.16 ± 0.0^{a}	1.2 ± 0.2^{b}	1.7 ± 0.3^{c}	1.3 ± 0.2^{b}	***
phenylacetaldehyde	1052	-	B	0.15 ± 0.1^{a}	0.35 ± 0.1^{bc}	0.36 ± 0.1^{c}	0.25 ± 0.0^{ab}	**
2(E)-decenal	-	1652	B	1.2 ± 0.0	1.2 ± 0.3	1.1 ± 0.2	0.96 ± 0.2	ns
2-undecenal	-	1773	B	1.1 ± 0.0^{a}	1.6 ± 0.3^{c}	1.4 ± 0.1^{bc}	1.2 ± 0.1^{ab}	**
others								
2-heptanone	891	-	A	0.18 ± 0.0^{b}	0.13 ± 0.0^{a}	0.13 ± 0.0^{a}	0.13 ± 0.0^{a}	**
butyrolactone	916	-	A	1.1 ± 0.1^{a}	2.7 ± 0.5^{b}	2.9 ± 0.7^{b}	2.4 ± 0.3^{b}	**
1-octen-3-one	983	1444	A	0.07 ± 0.0^{a}	0.22 ± 0.0^{b}	0.26 ± 0.1^{b}	0.21 ± 0.0^{b}	***

Table 5-10: Aroma volatile compounds identified in rice wines brewed using pigmented rice with varying degree of polishing (continued).

volatile compounds	[†]LRI		[‡]ID	[§]approximated concentration				[¦]Sig
	ZB-5MSi	WAX-DA		0% RW	30% RW	50% RW	65% RW	
2-nonanone	1093	-	A	0.23 ± 0.1^{b}	0.15 ± 0.0^{a}	0.14 ± 0.0^{a}	0.15 ± 0.0^{a}	*
γ-nonalactone	1373	-	A	0.28 ± 0.1	0.26 ± 0.0	0.26 ± 0.0	0.19 ± 0.0	ns

Values with the same letter superscripts within each row are not significantly different ($p = 0.05$).

Data are presented as mean ± standard error, n=3. nd = not detected.

[†]Linear retention index calculated from a linear equation between each pair of straight chain alkanes (C_5–C_{30}).

[‡]ID, mass spectrum and LRI agree with those of authentic compound; A agreement in both column and B agreement on one column.

[§]Estimated quantities (µg/l) in the headspace from 5 g sample, calculated by comparison with internal standard, the experiment was carried out using ZB-5MSi column.

If the compounds were not detected on ZB-5MSi column, they were semi-quantified using WAX-DA column.

The guaiacol and 4-vinylguaiacol were quantified in mg/l, using their external calibration curves.

[§]Sig = Probability, as obtained from ANOVA, that there is a difference between means; ns, no significant difference between means ($P > 0.05$), *Significant at the 5% level ($0.01 < p \leq 0.05$), **Significant at the 1% level ($0.001 < p \leq 0.01$) and ***Significant at the 0.1% level ($p \leq 0.001$).

5.3.3.2 Semi-volatile compounds

The semi-volatile aroma compounds identified in the pigmented rice wines are shown in table 5.11. Ethyl 2-oxopropanoate, ethyl 2-phenylethanoate and 3-methylbutyl dodecanoate were detected as the additional esters using this extraction technique. This is in agreement with Yotmanee et al. (2015), however ethyl 2-oxopropanoate and ethyl 2-phenylethanoate were not detected in the commercial rice wines. Zea et al. (2001) and Moreno et al. (2005) showed that ethyl 2-oxopropanoate (ethyl pyruvate) was found in sherry wines. Moreover, Duarte et al. (2010) showed that this aroma compound was formed during fermentation of raspberry wines, using *Saccharomyces cerevisiae* and *Saccharomyces bayanus*. The increase in degree of polishing resulted in an increase in the concentration of ethyl 2-oxopropanoate, ethyl lactate and ethyl 2-phenylethanoate, however the concentration of ethyl 3-hydroxybutanoate decreased.

Several alcohols were found in lab-scale brewed rice wine samples. This is consistent with Yotmanee et al. (2015) who showed that those alcohols were found in commercial rice wines. However, 3-(methylthio)propanol was found as an additional compound in lab-scale brewed rice wines. Among them, 4-hydroxybenzeneethanol was observed as the predominant alcohols in those rice wines, and its concentration in 0% RW was higher than other samples. However, this was not significantly different between samples. Overall, most of the alcohols were not affected by the polishing.

A large number of organic acids were found using SPE, as this extraction technique is more suitable for the polar compounds, compared with SPME (table 5.11). Among them, 3-(methylthio)propanoic acid was detected as the additional compound in rice wines from this study, and this organic acid was not found in commercial rice wine by Yotmanee et al. (2015). Overall, no significant differences were found for most of the organic acids between

rice wine samples. However, the concentration of propanoic acid, 2-methylpropanoic acid, butanoic acid, 3-methylbutanoic acid and benzeneacetic acid slightly increased, whereas the concentration of 3-(methylthio)propanoic acid lightly decreased by the increase in degree of polishing. Moreover, 2-methylpropanoic acid, 3-methylbutanoic acid and 3-(methylthio)propanoic acid were formed from valine, isoleucine and methionine in rice wines via the Ehrlich pathway. This was explained by Ravasio et al. (2014) and presented in chapter 2, figure 2.9.

Table 5-11: Semi-volatile aroma compounds identified in rice wines brewed using pigmented rice with varying degree of polishing.

volatile compounds	†LRI		‡ID	§approximated concentration (µg/l)				¦Sig
	WAX-DA	ZB-5MSi		0% RW	30% RW	50% RW	65% RW	
ethyl esters								
ethyl 2-oxopropanoate	1342	-	B	0.4 ± 0.1^{a}	1.2 ± 0.1^{b}	1.4 ± 0.1^{b}	2 ± 0.3^{c}	***
ethyl lactate	1348	-	B	6.2 ± 0.7^{a}	9.9 ± 0.8^{b}	12.3 ± 0.2^{b}	16 ± 2.4^{c}	***
ethyl 3-hydroxybutanoate	1524	929	A	5.8 ± 1.4^{c}	3.3 ± 0.3^{b}	3.6 ± 1^{b}	1.1 ± 0.6^{a}	**
ethyl 2-hydroxyhexanoate	1544	-	B	0.02 ± 0.0^{a}	0.08 ± 0.0^{b}	0.1 ± 0.0^{b}	nd	***
ethyl succinate	1666	1179	A	0.17 ± 0.0^{ab}	0.25 ± 0.0^{b}	0.18 ± 0.0^{ab}	0.11 ± 0.0^{a}	*
ethyl 2-phenylethanoate	1797	1251	A	0.12 ± 0.0^{a}	0.53 ± 0.0^{b}	0.53 ± 0.1^{b}	0.51 ± 0.1^{ab}	*
3-methylbutyl dodecanoate	2066	-	B	0.2 ± 0.2	0.05 ± 0.0	0.04 ± 0.0	nd	ns
alcohols								
3-ethoxypropanol	1379	-	B	0.23 ± 0.1^{a}	0.34 ± 0.0^{b}	0.4 ± 0.0^{b}	0.56 ± 0.1^{c}	*
3-(methylthio)propanol	1715	980	A	0.39 ± 0.2	0.29 ± 0.0	0.36 ± 0.1	0.2 ± 0.0	ns
benzyl alcohol	1871	-	B	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	ns
homovanillyl alcohol	2830	-	B	0.01 ± 0.0	nd	nd	nd	-
4-hydroxybenzeneethanol	3012	1427	A	26 ± 8	19 ± 3	19 ± 3	18 ± 3	ns
acids								
acetic acid	1448	-	B	5.9 ± 2.5	5.4 ± 1.9	6.2 ± 1.9	8.6 ± 4.1	ns
propanoic acid	1548	-	B	0.05 ± 0.0^{a}	0.14 ± 0.0^{ab}	0.14 ± 0.0^{ab}	0.16 ± 0.0^{b}	**
2-methylpropanoic acid	1568	753	A	8 ± 1^{a}	16 ± 3^{b}	14 ± 1^{b}	16 ± 2^{b}	***

Table 5-11: Semi-volatile aroma compounds identified in rice wines brewed using pigmented rice with varying degree of polishing (continued).

volatile compounds	†LRI		‡ID	§approximated concentration (µg/l)				¦Sig
	WAX-DA	ZB-5MSi		0% RW	30% RW	50% RW	65% RW	
butanoic acid	1647	775	A	0.94 ± 0.1	1.1 ± 0.1	1.1 ± 0.1	1.3 ± 0.3	ns
3-methylbutanoic acid	1687	839	A	3.2 ± 0.7^{a}	5.1 ± 0.3^{b}	5.2 ± 0.6^{b}	5.8 ± 0.7^{b}	***
hexanoic acid	1857	-	B	0.7 ± 0.2	0.6 ± 0.1	0.7 ± 0.1	0.64 ± 0.2	ns
octanoic acid	2083	-	B	0.66 ± 0.2	0.66 ± 0.1	0.59 ± 0.1	0.62 ± 0.2	ns
nonanoic acid	2173	-	B	0.05 ± 0.0	0.04 ± 0.0	0.04 ± 0.0	0.05 ± 0.0	ns
decanoic acid	2258	-	B	0.32 ± 0.2	0.15 ± 0.0	0.12 ± 0.0	0.31 ± 0.3	ns
3-(methylthio)propanoic acid	2298	-	B	0.1 ± 0.0^{b}	0.06 ± 0.0^{a}	0.04 ± 0.0^{a}	nd	***
dodecanoic acid	2469	-	B	0.5 ± 0.6^{b}	0.11 ± 0.0^{a}	nd	nd	*
benzeneacetic acid	2561	-	B	6.9 ± 0.4	9.3 ± 3.4	9.6 ± 3.2	13 ± 4	ns
hexadecanoic acid	2912	990	A	0.58 ± 0.2	0.5 ± 0.2	0.6 ± 0.1	0.5 ± 0.1	ns
octadecanoic acid	3104	-	B	0.29 ± 0.0^{ab}	0.23 ± 0.1^{ab}	0.33 ± 0.0^{b}	nd	***
4-hydroxybenzeneacetic acid	3587	1557	A	2.4 ± 1.4	4.7 ± 2.6	3.5 ± 1	4.4 ± 2.7	ns

Table 5-11: Semi-volatile aroma compounds identified in rice wines brewed using pigmented rice with varying degree of polishing (continued).

volatile compounds	[†]LRI		[‡]ID	[§]approximated concentration (µg/l)				[¦]Sig
	WAX-DA	ZB-5MSi		0% RW	30% RW	50% RW	65% RW	
aldehydes								
vanillin	2556	-	B	0.15 ± 0.0^{b}	0.14 ± 0.0^{b}	0.1 ± 0.0^{ab}	0.08 ± 0.0^{a}	***
4-hydroxybenzaldehyde	2949	-	B	0.06 ± 0.0^{a}	0.44 ± 0.1^{a}	1.5 ± 0.3^{b}	2.5 ± 0.7^{c}	***

Values with the same letter superscripts within each row are not significantly different ($p = 0.05$).

Data are presented as mean ± standard error, n=3. nd = not detected.

[†]Linear retention index calculated from a linear equation between each pair of straight chain alkanes (C_7–C_{40}).

[‡]ID, mass spectrum and LRI agree with those of authentic compound; A agreement in both column and B agreement on one column.

[§]Estimated quantities (µg/l) in 15 ml sample, calculated by comparison with internal standard, the experiment was carried out using WAX-DA column.

If the compounds were not detected on WAX-DA column, they were semi-quantified using ZB-5MSi column.

[¦]Sig = Probability, as obtained from ANOVA, that there is a difference between means; ns, no significant difference between means ($P > 0.05$), *Significant at the 5% level ($0.01 < p \leq 0.05$), **Significant at the 1% level ($0.001 < p \leq 0.01$) and ***Significant at the 0.1% level ($p \leq 0.001$).

5.3.3.3 GC-Olfactometry

The GC-O analysis of the SPME extract yielded a total 23 odours descriptions (table 5.12, 5.13 and 5.14). These included aromas like cocoa, fruity, peach, cheesy, beefy, potato, sweet, plastic, floral, mushroom, earthy, green, bell pepper and smoky-spicy. The intensity of each aroma in pigmented rice wines varied significantly between assessors. This is consistent with Schranz et al. (2017) who showed that the olfactory perception of assessors can be influenced by their aroma receptors.

The aromas in lab-scale brewed rice wines detected by GC-O from this experiment is in agreement with Yotmanee et al. (2015). Moreover, Chen et al. (2013b), Chen et al. (2013a) and Niu et al. (2017) also showed that rice wine which is produced using wheat Qu mostly contributed fruity, floral, sweet, cheese, earthy, green and smoky-spicy aroma. However, the smoky-spicy aroma was not detected in the Japanese rice wine which was brewed without wheat Qu (Isogai et al., 2005, Yoshizaki et al., 2010). Thus, indicating that the wheat might be containing the lignin which contain the precursor of volatile phenols in Chinese rice wines.

Notably, green, earthy and bell pepper aroma from 2,3-diethyl-5-methylpyrazine and 2-(1-methylpropyl)-3-methoxypyrazine, and roasted aroma from methyl 2-methyl-3-furyl disulfide were reported for the first time in rice wines, compared to Chen et al. (2013b) and Yotmanee et al. (2015). Although, these aromas were found in both of 0% RW and 65% RW, the higher intensity was found in 0% RW. Moreover, Champagne (2008) showed that 2-isobutyl-3-methoxypyrazine and 2-methyl-3-furanthiol were naturally found in unpolished rice. They can contribute to bell pepper and meaty note in cooked unpolished rice. Thus, these green and meaty aromas might not be derived from the pigmented rice grain during the brewing process.

Overall, fruity, sweet, floral, earthy and green notes were found in both of 0% RW and 65% RW. They were likely derived from esters, alcohols and pyrazines. However, smoky-spicy notes from guaiacol were only found to be the characteristic aroma in 0% RW. Thus, the bran of pigmented rice wine had an effect on the formation of smoky-spicy notes in 0% RW.

Table 5-12: Aroma description and intensity of the volatile compounds (individual score from assessors) in unpolished pigmented rice wines (0% RW) detected by using GC–O.

aromas	responsible compound	aroma intensity from individual assessor							
		0% RW							
		§A1		A2		A3		A4	
		†R_1	R_2	R_1	R_2	R_1	R_2	R_1	R_2
cocoa	3-methylbutanal	4	2	4	nd	nd	nd	nd	nd
strecker	3-methylbutanol	5	3	4	3	5	2	5	3
fruity/ peach	ethyl 2-methylpropanoate	5	5	5	7	5	2	5	5
fruity	ethyl butanaote	3	6	6	4	5	7	5	3
cheese	3-methylbutanoic acid	3	6	6	6	nd	5	3	5
fruity/strawberry	ethyl 2-methylbutanoate	4	5	6	7	3	4	6	5
fruity	ethyl 3-methylbutanoate	3	4	6	7	5	3	6	6
meat	*2-methyl-3-furanthiol	3	5	4	5	7	8	6	6
sweet/fruity	3-methylbutyl acetate	3	5	7	7	nd	3	nd	nd
potato	methional	3	4	5	6	2	3	6	5
sweet/plastic	benzaldehyde	2	2	4	3	3	nd	nd	nd
mushroom	1-octen-3-one	2	2	3	3	nd	nd	nd	nd
fruity	ethyl hexanoate	2	5	5	4	2	5	5	5
floral	phenylacetaldehyde	4	6	3	7	nd	nd	nd	nd
earthy	unknown	6	7	4	6	7	5	5	5

Table 5-12: Aroma description and intensity of the volatile compounds (individual score from assessors) in unpolished pigmented rice wines (0% RW) detected by using GC–O (continued).

aromas	responsible compound	aroma intensity from individual assessor							
		0% RW							
		§A1		A2		A3		A4	
		†R_1	R_2	R_1	R_2	R_1	R_2	R_1	R_2
green/earthy	*2-isopropyl-3-methoxypyrazine	6	nd	6	7	7	7	6	7
smoky-spicy	guaiacol	7	8	8	10	7	7	8	8
rose	2-phenylethyl alcohol	6	4	nd	nd	4	3	nd	nd
earthy/smoky	*2,3-diethyl-5-methylpyrazine	6	5	5	6	3	nd	5	nd
green	*2-(1-methylpropyl)-3-methoxypyrazine	3	5	3	5	4	4	3	nd
bell pepper	*2-isobutyl-3-methoxypyrazine	6	6	5	5	nd	nd	nd	nd
roasted	*methyl 2-methyl-3-furyl disulfide	nd	3	3	nd	nd	2	nd	nd
sweet/fruity	ethyl octanoate	3	3	3	2	nd	nd	nd	nd
rose/honey	ethyl 2-phenylacetate	3	4	3	2	nd	nd	nd	nd

*Compounds with no peak in the GC-MS. These were based on finding the correct aroma at the correct LRI on one or two columns.

§A1, A2, A3 and A4 were the aroma intensity from rice wines detected by assessor 1, assessor 2, assessor 3 and assessor 4, respectively.

†R_1 and R_2 were a replication of the analysis of aroma intensity by GC-O from each assessor.

nd = not detected.

Table 5-13: Aroma description and intensity of the volatile compounds (individual score from assessors) in 65% polished pigmented rice wines (65% RW) detected by using GC–O.

aromas	responsible compound	‡aroma intensity from individual assessor							
		65% RW							
		§A1		A2		A3		A4	
		†R_1	R_2	R_1	R_2	R_1	R_2	R_1	R_2
cocoa	3-methylbutanal	2	2	4	3	nd	nd	nd	nd
strecker	3-methylbutanol	3	3	5	4	3	3	5	4
fruity/ peach	ethyl 2-methylpropanoate	5	4	6	6	2	2	5	5
fruity	ethyl butanaote	5	5	4	5	2	nd	5	5
cheese	3-methylbutanoic acid	6	4	6	6	1	6	6	6
fruity/strawberry	ethyl 2-methylbutanoate	5	6	6	5	2	4	6	7
fruity	ethyl 3-methylbutanoate	5	6	4	6	2	3	6	6
meat	*2-methyl-3-furanthiol	6	5	5	5	2	5	5	5
sweet/fruity	3-methylbutyl acetate	3	5	7	7	nd	nd	nd	nd
potato	methional	3	6	5	3	1	2	5	4
sweet/plastic	benzaldehyde	2	2	4	3	1	1	2	2
mushroom	1-octen-3-one	4	3	6	2	1	nd	2	3
fruity	ethyl hexanoate	4	4	4	3	3	3	3	2
floral	phenylacetaldehyde	4	4	2	5	nd	nd	nd	nd
earthy	unknown	6	7	3	6	2	3	6	nd

Table 5-13: Aroma description and intensity of the volatile compounds (individual score from assessors) in 65% polished pigmented rice wines (65% RW) detected by using GC–O (continued).

aromas	responsible compound	‡aroma intensity from individual assessor							
		65% RW							
		§A1		A2		A3		A4	
		†R_1	R_2	R_1	R_2	R_1	R_2	R_1	R_2
green/earthy	*2-isopropyl-3-methoxypyrazine	6	7	7	6	5	6	6	6
smoky-spicy	guaiacol	nd	nd	nd	nd	nd	nd	nd	nd
rose	2-phenylethyl alcohol	4	5	nd	nd	4	3	nd	nd
earthy/smoky	*2,3-diethyl-5-methylpyrazine	6	4	2	5	3	nd	3	nd
green	*2-(1-methylpropyl)-3-methoxypyrazine	3	4	3	4	3	2	nd	nd
bell pepper	*2-isobutyl-3-methoxypyrazine	5	5	4	5	nd	nd	nd	nd
roasted	*methyl 2-methyl-3-furyl disulfide	nd	nd	nd	nd	nd	nd	nd	nd
sweet/fruity	ethyl octanoate	2	3	3	4	nd	nd	nd	nd
rose/honey	ethyl 2-phenylacetate	5	5	3	4	nd	1	nd	nd

*Compounds with no peak in the GC-MS. These were based on finding the correct aroma at the correct LRI on one or two columns.

§A1, A2, A3 and A4 were aroma intensity in rice wines detected by assessor 1, assessor 2, assessor 3 and assessor 4, respectively.

†R_1 and R_2 were replication (duplicate) of the analysis of aroma intensity by GC-O from each assessor.

nd = not detected.

Table 5-14: Intensity of volatile compounds (mean) in 65% polished (65% RW) and unpolished (0% RW) pigmented rice wines, and their corresponding volatile aroma compounds.

aromas	responsible compound	†LRI ZB-5MSi			†LRI WAX-DA			¦aroma intensity (mean)	
		SPME	SPE	§AC	SPME	SPE	§AC	0% RW	65% RW
cocoa	3-methylbutanal	654	-	657	915	905	928	3	3
strecker	3-methylbutanol	733	723	732	1216	1221	1204	4	4
fruity/ peach	ethyl 2-methylpropanoate	754	743	752	965	-	976	5	4
fruity	ethyl butanoate	797	-	801	1037	1041	1050	5	4
cheese	3-methylbutanoic acid	829	831	839	1673	1661	1687	5	5
fruity/strawberry	ethyl 2-methylbutanoate	845	830	840	1052	-	1042	5	5
fruity	ethyl 3-methylbutanoate	850	-	851	1069	-	1082	5	5
meat	*2-methyl-3-furanthiol	869	852	863	1324	1321	1307	6	5
sweet/fruity	3-methylbutyl acetate	873	-	878	1143	-	1128	6	6
potato	methional	905	912	912	-	-	-	4	4
sweet/plastic	benzaldehyde	961	-	969	-	-	-	3	2
mushroom	1-octen-3-one	976	-	978	1302	1315	1302	3	3
fruity	ethyl hexanoate	994	-	998	1232	1239	1237	4	3
floral	phenylacetaldehyde	1050	1055	1058	-	-	-	5	4
earthy	unknown	1084	-	-	-	-	-	6	5
green/earthy	*2-isopropyl-3-methoxypyrazine	1090	1080		1449	-	1439	7	6
smoky-spicy	guaiacol	1092	1097	1095	1869	1875	1862	8	nd

Table 5-14: Intensity of volatile compounds (mean) in 65% polished (65% RW) and unpolished (0% RW) pigmented rice wines, and their corresponding volatile aroma compounds (continued).

aromas	responsible compound	†LRI ZB-5MSi			†LRI WAX-DA			¦aroma intensity (mean)	
		SPME	SPE	§AC	SPME	SPE	§AC	0% RW	65% RW
rose	2-phenylethyl alcohol	1124	1110	1119	1936	1947	1925	4	4
earthy/smoky	*2,3-diethyl-5-methylpyrazine	1155	1163	1151	1478	1467	1488	5	4
green	*2-(1-methylpropyl)-3-methoxypyrazine	1170	-	1175	1498	-	1509	4	3
bell pepper	*2-isobutyl-3-methoxypyrazine	1181	1182	1181	1526	1522	1533	6	5
roasted	*methyl 2-methyl-3-furyl disulfide	1184	-	1178	-	-	-	3	nd
sweet/fruity	ethyl octanoate	1192	-	1194	-	-	-	3	3
rose/honey	ethyl 2-phenylacetate	1249	-	1251	1837	-	1818	3	4

†LRI from GC-O using ZB-5MSi and WAX-DA column, calculated from a linear equation between each pair of straight chain alkanes C_5–C_{25}.

¦Aroma intensity (mean) in samples was analysed using ZB-5MSi. Aroma compounds were extracted using SPME.

§AC is LRI from authentic compounds.

*Compounds with no peak in the GC-MS. These were based on finding the correct aroma at the correct LRI on one or two columns.

nd = not detected.

5.3.4 Sensory analysis of pigmented rice wine

Attributes including yeasty note (soy sauce), acid (balsamic vinegar), sweet aroma (dried jujube), smoky-spicy note (hoisin sauce), cheese (creamy blue cheese), mushroom and beefy note were used to describe the aroma of the pigmented rice wines by the panel. The highest scores were obtained for yeasty and cheesy notes, regardless of the presence of the bran.

According to table 5.15, a higher intensity of smoky-spicy note (hoisin sauce) was shown 0% RW, compared to 30% RW and 65% RW. However, the cheesy note was significantly higher in 65% RW, compared to other samples. This is in agreement with Yotmanee et al. (2015) who showed that smoky-spicy note was found in commercial pigmented rice wine. The increase in degree of polishing resulted in the decrease in smoky-spicy note (hoisin sauce) (Pearson's correlation coefficient, $r = -0.87$).

Table 5-15: Aroma profiling in rice wines brewed using pigmented rice with varying degree of polishing (0%, 30% and 65%).

aroma attribute	rice wine samples			§Sig
	0% RW	30% RW	65% RW	
yeasty note (soy sauce)	40.9 ± 3.7	44.4 ± 1.1	42.4 ± 0.8	ns
acid (balsamic vinegar)	27.8 ± 1.5	25.3 ± 1.6	26.6 ± 2.1	ns
sweet aroma (dried jujube)	23.9 ± 1.7^{b}	21.4 ± 0.3^{ab}	19.3 ± 2.7^{a}	*
smoky-spicy note (hoisin sauce)	22.9 ± 1.8^{b}	19.4 ± 0.8^{a}	16.8 ± 2.1^{a}	**
cheese (creamy blue cheese)	19.4 ± 3.4^{a}	28.2 ± 3.1^{b}	32.5 ± 0.5^{b}	**
mushroom	1.1 ± 0.7	2.6 ± 1.3	3.1 ± 1.2	ns
beefy note	22.9 ± 0.3^{b}	19.9 ± 1.7^{a}	19.7 ± 1.2^{a}	*

Values with the same letter superscripts within each row are not significantly different ($p = 0.05$).

Data are presented as mean ± standard error.

§Sig = Probability, as obtained from ANOVA, that there is a difference between means; ns, no significant difference between means ($P > 0.05$), *Significant at the 5% level ($0.01 < p \leq 0.05$) and **Significant at the 1% level ($0.001 < p \leq 0.01$).

The PC plot shows that the first two dimensions accounted for 63.3 and 13.8% of the variance respectively. PC1 separated the 0% RW from all samples, characterised by aroma attributes which included smoky-spicy (hoisin sauce), sweet aroma (dried jujube), beefy note and acid (balsamic vinegar). Contrary to 0% RW, the polished samples (30% RW and 65% RW) were characterised by cheesy note, mushroom and yeasty aroma (figure 5.3).

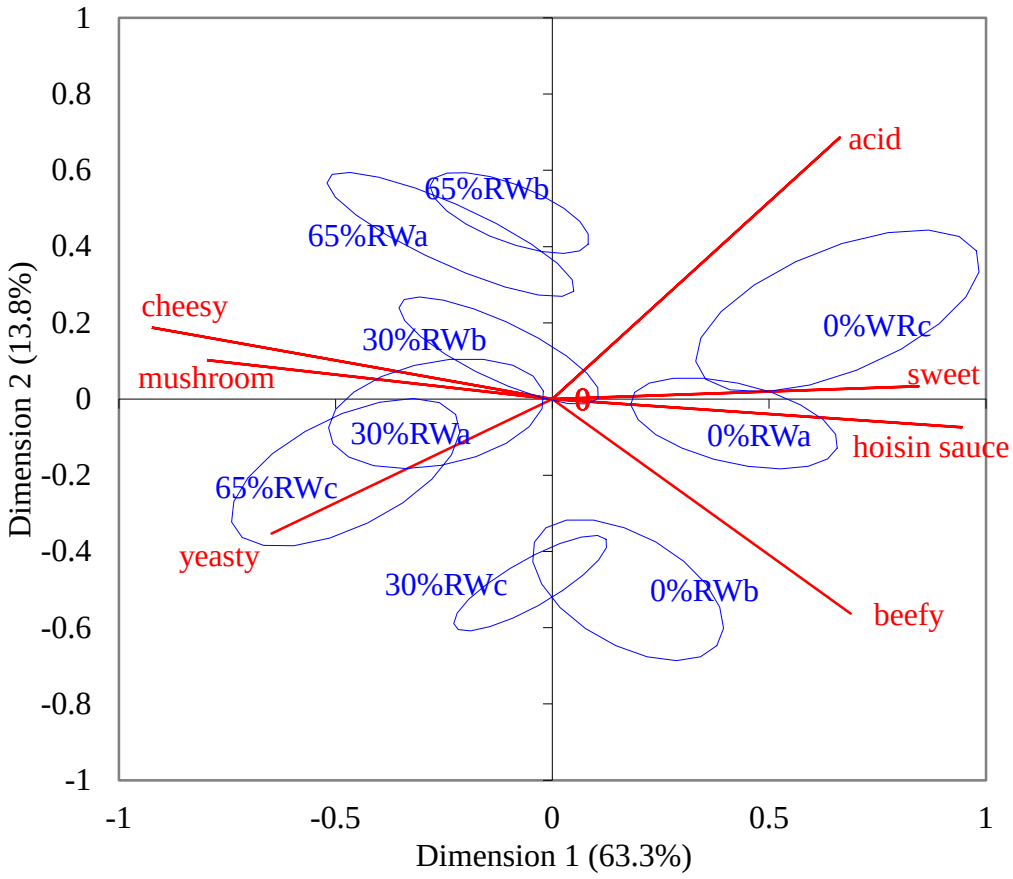

Figure 5-3: Principal component analysis (PCA) biplots of sensory descriptive trait scores for aromas and pigmented rice wine samples (0% RW (unpolished pigmented rice wine), 30% RW (30% polished pigmented rice wine and 65% RW (fully polished pigmented rice wine)). PC1 vs. PC2 accounts for 77.1% of the explained variation.

The sensory characteristics of rice wines have been previously reported by Jung et al. (2014) who showed that Korean rice wines produced by using polished rice were described with aroma attributes which included alcohol, sour, sweet, fruity, roasted cereal, yeasty and mouldy notes. Yang et al. (2017b) also showed that aroma attributes such as alcohol, fruity and cereal-like aromas were found in Chinese rice wines which were produced by polished glutinous rice. Contrary to the polished rice wines, the sensory characteristics of pigmented rice wines are less known. However, our study has shown that rice wine which is produced from unpolished pigmented rice has the smoky-spicy notes (hoisin sauce). This can be correlated with the results from the analysis of aroma profile and GC-Olfactometry, which showed that the smoky-spicy note identified as guaiacol was only found in pigmented rice wine. To confirm this, the effect of volatile phenols (guaiacol and 4-vinylguaiacol) on the smoky-spicy notes in pigmented rice wine was further studied using the standard spiking technique.

5.4 Conclusions

Rice wines produced using pigmented rice with varying degree of polishing were analysed for characteristic taste and aroma compounds as well as their precursors. The concentration of sugars (glucose and maltose), organic acids (citric acid, malic acid, succinic acid, lactic acid and acetic acid), free amino acids and DKPs did not change as the pigmented rice was polished. However, phenolic acids and γ-glutamyl peptides (γ-glu-gly, γ-glu-his and γ-glu-tyr) were formed in greater amount in 0% RW, compared to other rice wines.

The analysis of aroma compounds showed that esters, alcohols and fatty acids were found to be the predominant aroma compound in rice wines. Most of the aroma compounds were unaffected by the presence of the pigmented rice bran. However, phenol, guaiacol, 4-vinylguaiacol and vanillin were significantly and substantially higher in 0% RW, compared to others. This can be explained by the fact that those compounds were formed from their precursors in the bran of pigmented rice. The analysis of GC-Olfactometry showed that fruity, sweet, floral, green and earthy note were detected in 0% RW and 65% RW, whereas smoky-spicy aroma which was derived from guaiacol was only found in 0% RW. However, Yotmanee et al. (2015) showed both of guaiacol and 4-vinylguaiacol were detected in commercial rice wine samples by GC-O. This can be explained that a lower concentration of 4-vinylguaiacol was found in 0% RW, Thus, it was not detected by GC-O. The sensory analysis showed that a higher intensity of sweet and smoky-spicy note was found in 0% RW, whereas a higher intensity of mushroom and cheesy notes was found in 65% RW. This is in agreement with the analysis of GC-O which showed that the smoky-spicy note was found in 0% RW.

In summary, the bran of pigmented rice increases the formation of guaiacol which contributed characteristic aroma, smoky-spicy note in pigmented rice wine during brewing. This aroma compound is likely to be formed from vanillic acid or ferulic acid which is derived from lignin in the bran of pigmented rice. To confirm this result, the effect of those phenolic acids on the formation of guaiacol in pigmented rice wine was further studied in next chapter.

5.5 Publication

1) Yotmanee, S., Oruna-Concha, M.J. and Parker, J.K. (2018). Influence of the brewing process and degree of milling on the taste characteristics of pigmented rice wine. In: Flavour Science: Proceedings of the 15th Weurman Flavour Research Symposium, Graz University of Technology, Austria, 18-22 September 2017, pp 155-158.
   (Partly published)
2) Yotmanee, S., Oruna-Concha, M.J. and Parker, J.K. (2018). The impact of volatile phenols on the aroma, and the identification of their precursors in pigmented rice wine. The 6th Nursten symposium, University of Nottingham, UK, 27-28 September 2018.
   (Oral presentation)

CHAPTER 6: CONFIRMATION OF THE PRECURSORS FOR THE CHARACTERISTIC AROMA COMPOUNDS, SMOKY-SPICY NOTE IN PIGMENTED RICE WINE

Abstract

Pigmented rice wine contained smoky-spicy note which was derived from guaiacol or 4-vinylguaiacol. These aroma compounds were formed from their corresponding precursor in the bran of pigmented rice. In order to identify these, 65% polished pigmented rice wines were spiked with vanillic acid and ferulic acid at different concentrations in the range of 0-20 mg/100 g rice, and then analysed for the corresponding aroma compounds. This study showed that vanillic acid and ferulic acid were decarboxylated to form guaiacol and 4-vinylguaiacol, respectively. These phenolic acid compounds were likely to have been liberated from the bran of pigmented rice during brewing. Moreover, guaiacol was formed from unpolished pigmented rice during steaming. The sensory aroma profiling analysis also confirmed that guaiacol was a key aroma compound, responsible for the smoky-spicy note in unpolished pigmented rice wine.

Keywords: guaiacol, 4-vinylguaiacol, smoky-spicy aroma, pigmented rice wine

6.1 Introduction

Aroma compounds in pigmented rice wine were studied by Yotmanee et al. (2015) and Yotmanee et al. (2018). They showed that guaiacol and 4-vinylguaiacol were the characteristic aromas which were responsible for smoky-spicy notes in commercial pigmented rice wine. This is in agreement with the results in chapter 5 which showed that those aroma compounds were also identified as a characteristic aroma in lab-scale brewed pigmented rice wine. However, the analysis of GC-Olfactometry showed that only guaiacol contributed a smoky-spicy note in lab-scale brewed unpolished pigmented rice wine, whereas 4-vinylguaiacol was not detected due to its low concentration. Moreover, the bran of pigmented rice contained precursors which are likely to be converted to guaiacol and 4-vinylguaiacol during brewing.

Ito et al. (2016) showed that ferulic acid is converted to 4-vinylguaiacol and guaiacol, whereas vanillic acid is converted to guaiacol via decarboxylation during brewing. These aroma compounds were generated using brewing microorganisms such as *Saccharomyces cerevisiae* and *Aspergillus oryzae*. However, Mo and Xu (2010) showed that 4-vinylguaiacol in Chinese rice wine was likely to be formed from wheat Qu (a starter containing *Aspergillus oryzae*) rather than from yeast. Moreover, Witthuhn et al. (2012) showed that ferulic acid is possibly metabolised to formed vanillic acid by *Paecilomyces variotii* (Rahouti et al., 1989), *Rhodotorula rubra* (Huang et al. (1993) and *Sporotrichum thermophile* (Topakas et al., 2003), and then further converted to guaiacol. However, Yang et al. (2007) and Yang et al. (2010) showed that guaiacol was found to be the characteristic aroma compound in unpolished black rice.

Guaiacol and 4-vinylguaiacol were found to be the characteristic aroma compounds, responsible for smoky-spicy notes in pigmented rice wines however their formation in the brewing process of pigmented rice wine has never been studied. Therefore, the aim of this study was (i) to identify and confirm their precursors which are derived from the bran of pigmented rice and (ii) confirm the aroma compound which contributes to smoky-spicy notes in lab-scale brewed pigmented rice.

6.2 Materials and methods

6.2.1 Materials

Black glutinous rice, *Aspergillus oryzae* ATCC 22787 and *Saccharomyces cerevisiae* NCYC 478 were used for this study and obtained from the same suppliers as in Chapter 5

Black glutinous rice was polished to obtain 65% polished grain, using a polishing machine from Twinbird (Niigata. Japan). The degree of polishing was calculated by the equation that was shown in chapter 5, section 5.2.1 which is shown as following equation.

$$DOM = \left(1 - \frac{weight\ of\ polished\ rice}{weight\ of\ brown\ rice}\right) \times 100$$

6.2.2 Chemicals

The chemicals in this study, including >98% 4-coumaric acid, 98% epicatechin, >97% vanillic acid, >98% sinapic acid, >97% protocatechuic acid, >99% ferulic acid, >95% syringic acid, >98% caffeic acid, 98% catechin, 97% gallic acid, 99% 4-hydroxybenzoic acid, 1,2-dichlorobenzene, saturated alkane standard C_5-C_{30} and C_7-C_{40}, >99% guaiacol and 98% 4-vinylguaiacol were purchased from Sigma-Aldrich (Dorset, UK). Cyanidin-3-glucoside (>96) was purchased from Extrasynthese (Genay, France). HPLC grade methanol and formic acid were purchased from BDH (Dorset, UK).

6.2.3 Brewing process

In the laboratory, 65% polished pigmented (60 g) was steamed and then spiked with vanillic acid and ferulic acid at two concentrations (10 and 20 mg/100 g of sample) which were higher than naturally present in the sample to access their contribution. The samples were brewed using the brewing process which was shown by Yotmanee et al. (2018). The samples were centrifuged using a Howe laborzentrifugen, series 3K10 and 19776-H rotor from Sigma (Osterode am Harz, Germany) at 7,300 g for 15 min at room temperature. The clarified samples were stored at -20 °C for further analysis.

6.2.4 Analysis of precursors for guaiacol and 4-vinylguaiacol in pigmented rice wines

Volatile compounds were extracted from the rice wines using solid phase microextraction (SPME) technique as adapted from Chen and Xu (2010). The compounds of interest were analysed by gas chromatography-mass spectrometry (GC-MS) from Agilent

(CA, USA) coupled to a Zebron™ ZB-5MSi column (30 m × 250 µm internal diameter, 1 µm film thickness) from Phenomenex (CA, USA). The analysis procedure was shown in chapter 3. The quantification of guaiacol and 4-vinylguaiacol was carried out using the external standard. Quantification was based on peak area. Calibration curves of the standards were carried out in the range of 0.002-20 mg/l for guaiacol and 0.001-10 for 4-vinylguaiacol, $R^2 >$ 0.99.

6.2.5 Aroma profiling of rice wines by sensory analysis

To identify the aroma compounds which were responsible for smoky-spicy notes in pigmented rice wine, 65% polished rice wines were spiked with guaiacol and 4-vinylguaiacol at their natural concentrations in unpolished pigmented rice wine (table 6.1), and the aroma profile was assessed by the trained professional panel (one male and 7 females) as shown in chapter 5, section 5.2.7.

Table 6-1: Rice wine samples prepared for aroma profiling analysis.

sample	standard spiking (mg/l)	
	guaiacol	4-vinylguaiacol
65% polished pigmented rice wine (65% RW)	×	×
65% RW + guaiacol (65% RW G^+)	2	×
65% RW + 4-vinylguaiacol (65% RW PVG^+)	×	0.8
65% RW + guaiacol and 4-vinylguaiacol (65% RW GP^+)	2	0.8
0% polished rice pigmented rice wine (0% RW)	×	×

G^+ = guaiacol; PVG^+ = 4-vinylguaiacol; GP^+ = guaiacol and 4-vinylguaiacol.

6.2.6 Analysis of precursors for guaiacol and 4-vinylguaiacol

6.2.6.1 Analysis of guaiacol and 4-vinylguaiacol in steamed pigmented rice

Sample (1 g) was mixed with 15 ml of HPLC water, and spiked with 20 µl of internal standard (1,2-dicholrobenzene, 1 mg/l). The sample was incubated at 40 °C for 30 min for the aroma extraction using SPME, and then analysed for compounds of interest by GC-MS from Agilent (CA, USA) which coupled with a Zebron™ ZB-5MSi column (30 m × 250 µm internal diameter, 1 µm film thickness) from Phenomenex (CA, USA). This procedure was described in chapter 3. The quantification of guaiacol and 4-vinylguaiacol was carried out using the external standard. Quantification was based on peak area. Calibration curves of the standards were carried out in the range of 0-4 mg/l, $R^2 > 0.99$.

6.2.6.2 Analysis of phenolic acids during brewing process

To investigate the formation of phenolic acids during brewing, pigmented rice wines were analysed for those compounds throughout the brewing process by high performance liquid chromatography (HPLC) from Agilent (Waldbronn, Germany). The analytical method was adapted from Seal (2016) and it was shown in chapter 3. Quantification was based on peak area. Calibration curves of the standards were carried out by diluting stock standards to yield 0.5-100 mg/l for phenolic acids or 0.5-1.5 g/l for anthocyanins, $R^2 > 0.99$.

6.2.7 Statistic analysis

IBM SPSS Statistics for Windows, Version 22.0 software (IBM Corp., Armonk, NY, USA) was used for the statistical analysis of experimental data. The statistical significant difference of the mean value was considered significant at $p<0.05$ by using the analysis of variance (ANOVA). All ANOVA were conducted using a 95% confidence interval and post hoc Duncan test was used for multiple pairwise comparisons. The Pearson's correlation was used to identify the relation between two factors, if required. To analyse the data from the sensory evaluation, the Senpaq software version 4.2 (Qi Statistics, Reading, UK) was applied.

6.3 Results and discussions

6.3.1 Analysis of precursors for guaiacol and 4-vinylguaiacol in pigmented rice wines

To identify and confirm the precursors from which guaiacol and 4-vinylguaiacol are generated in pigmented rice wine during the brewing process, the 65% polished rice grain (control) was spiked with ferulic acid and vanillic acid, and then brewed using the brewing method from Yotmanee et al. (2018). The rice wine samples were analysed for compounds of interest which were guaiacol and 4-vinylguaiacol. According to results in table 6.2, guaiacol was formed in 65% RW spiked with vanillic acid, and it was observed that the concentration increased with the increase of vanillic acid. Similar effect was observed for the formation of 4-vinylguaiacol when 65% RW was spiked with ferulic acid. These are consistent with Ito et al. (2016) who showed that guaiacol and 4-vinylguaiacol were decarboxylated to form vanillic acid and ferulic acid during brewing, respectively.

Table 6-2: Concentration of guaiacol and 4-vinylguaiacol in 65% RW in the presence of ferulic acid and vanillic acid standards.

compounds	concentration (mg/l)								§Sig
	spiked with ferulic acid (mg/ 100 g sample)				spiked with vanillic acid (mg/ 100 g sample)				
	control (0)	natural (0.5)	10	20	control (0)	natural (1.5)	10	20	
guaiacol	nd	nd	nd	nd	nd	nd	0.01 ± 0.0^{a}	0.08 ± 0.0^{b}	***
4-vinylguaiacol	0.27 ± 0.1^{a}	0.6 ± 0.1^{a}	0.59 ± 0.1^{a}	41 ± 6^{b}	0.27 ± 0.1^{a}	0.04 ± 0.0^{a}	0.26 ± 0.1^{a}	0.5 ± 0.5^{a}	***

Values with the same letter superscripts within each row are not significantly different ($p = 0.05$).

Data are presented as mean ± standard error, n = 3. nd = not detected.

§Sig = Probability, as obtained from ANOVA, that there is a difference between means; ***Significant at the 0.1% level ($p \leq 0.001$).

6.3.2 Aroma profiling of rice wines spiked with standard of guaiacol and 4-vinylguaiacol

In order to investigate the effect of guaiacol and 4-vinylguaiacol on the aroma profile of pigmented rice wine, 65% polished rice wine (control) was spiked with guaicol and 4-vinylguaiacol at the concentrations that were naturally present in unpolished pigmented rice wine, and then scored for the aroma profiling (table 6-3).

The aroma attributes, including were yeasty, acidic, sweet, dried jujube beefy/meaty and alcoholic aroma were not significantly different between samples (table 6.3). The addition of guaiacol resulted in an increase in smoky-spicy (hoisin sauce) aroma in 65% RW, and this was not significantly different to 0% RW. However, the smoky-spicy aroma in 65% RW was slightly increased by the addition of 4-vinylguaiacol. The addition of both guaiacol and 4-vinylguaiacol resulted in an increase in smoky-spicy aroma in 65% RW. These results confirmed that guaiacol was the key aroma compound which contributed smoky-spicy note in unpolished pigmented rice wine.

The aroma intensity in rice wine samples was quite variable between assessors. As discussed above, assessors have different aroma receptors which influence their sensitivity towards certain compounds. In particular, they have shown this a wide range of sensitivities for phenol compounds (Schranz et al., 2017). Moreover, assessors have different metabolite enzymes in the nasal cavities which have an impact on different bioconversions of aroma compounds before docking with aroma receptors. Therefore, the intensity and attribute of aromas were variable between assessors (Nagashima and Touhara, 2010).

Table 6-3: Aroma profiling in 65% polished rice wines spiked with guaiacol, 4-vinylguaiacol and both of guaiacol and 4-vinylguaiacol.

aroma attribute	rice wine samples					§Sig
	0% RW	65% RW	65% RW GP^+	65% RW PVG^+	65% RW G^+	
yeasty aroma (soy sauce)	39.7	41.7	35.8	38	34.9	ns
acidic aroma (balsamic vinegar)	18.6	22.8	21.5	19.7	22.8	ns
overall sweet aroma	29.5	23.1	27.1	27.8	25.7	ns
sweet aroma (dried jujube)	12.5	9	12.1	12.2	10.6	ns
beefy/meaty aroma	25.1	19.6	19.9	21	20.5	ns
smoky-spicy aroma (hoisin sauce)	22.3^a	13.6^b	19.9^a	18.1^{ab}	23.2^a	**
cheesy/mushroom aroma	22.9	32.6	33.8	28.7	27.4	ns
alcoholic aroma	15.7	20.8	18.2	18.2	19.7	ns

Values with the same letter superscripts within each row are not significantly different ($p = 0.05$).

Data are presented as mean.

G^+ = guaiacol; PVG^+ = 4-vinylguaiacol; GP^+ = guaiacol and 4-vinylguaiacol.

§Sig = Probability, as obtained from ANOVA, that there is a difference between means; ns, no significant difference between means ($P > 0.05$) and **Significant at the 1% level ($0.001 < p \leq 0.01$).

6.3.3 Analysis of precursors for guaiacol and 4-vinylguaiacol

6.3.3.1 Analysis of guaiacol and 4-vinylguaiacol in steamed pigmented rice

In order to find the source of guaiacol and 4-vinylguaiacol, steamed pigmented rice was analysed for these key compounds. Only guaiacol was found in the steamed unpolished pigmented rice (table 6.4). This is in agreement with Yang et al. (2007) who studied the formation of these aroma compounds in cooked black rice which was mixed with polished rice at different ratios, 100:0, 50:50, 20:80, 5:95 and 0:100 %. They showed that guaiacol was found at higher concentration in 100% cooked black rice, whereas its concentration decreased as the polished rice ratio was increased. Moreover, Choi et al. (2018) showed that guaiacol was the characteristic aroma compound in cooked black rice and that was removed by polishing. Furthermore, Brebu and Vasile (2010) also showed that the thermal degradation of lignin in plants was the cause of the formation of guaiacol. Thus, guaiacol in the pigmented rice wine originates from the degradation of lignin in the bran of pigmented rice during the steaming process.

Table 6-4: Concentration of guaiacol and 4-vinylguaiacol in steamed pigmented rice.

compounds	concentration (mg/g sample)	
	steamed unpolished grain (0%)	steamed polished grain (65%)
guaiacol	0.23 ± 0.04	nd
4-vinylguaiacol	nd	nd

Data are presented as mean ± standard error, n=3.

nd = not detected.

6.3.3.2 Analysis of phenolic acids during brewing process

The concentration of all phenolic acids and anthocyanins were found at a lower concentration on day 0, regardless of the brewing temperature (figure 6.1). After that, their concentrations increased by the degradation of the corresponding precursors. This is consistent with de Gonzalo et al. (2016) who also showed that lignin is degraded to the monomers, including 4-hydroxyphenyl group (4-hydroxybenzoic acid and 4-coumaric acid), guaiacyl group (ferulic acid, vanillic acid and protocatechuic acid) and syringyl group (sinapic acid and gallic acid) using the enzyme from the microorganisms. Shin et al. (2019) showed that the derivatives of hydroxycinnamic acids (ferulic acid, 4-coumaric acid and caffeic acid) are likely to be present in rice bran.

At the end of the saccharification process, the concentration of phenolic acids and cyanidin-3-glucoside had decreased. They either bonded to insoluble polysaccharides (Shin et al., 2019) or metabolised to form other compounds, for example protocatechuic acid is converted to catechol, 4-coumaric is converted to 4-vinylphenol (Filannino et al., 2015), caffeic acid is converted to 4-vinylcatechol and ferulic acid is converted to 4-vinylguaiacol (Belda et al., 2017). Moreover, Zhang et al. (2001) showed that the sugar groups in anthocyanins can be removed by anthocyanase from fungi, leading to the anthocyanin decolourisation.

Gallic acid and protocatechuic acid were found to be the most abundant compounds from saccharified pigmented rice. This is in agreement with Hiemori et al. (2009) who showed that protocatechuic acid is formed from the degradation of cyanidin-3-glucoside which is the most abundant anthocyanin in rice, and then degraded to gallic acid. The results also showed that the concentration of phenolic acids and cyanidin-3-

glucoside from the saccharification at 25 °C were different to that at 30 °C, especially from day 2 onwards.

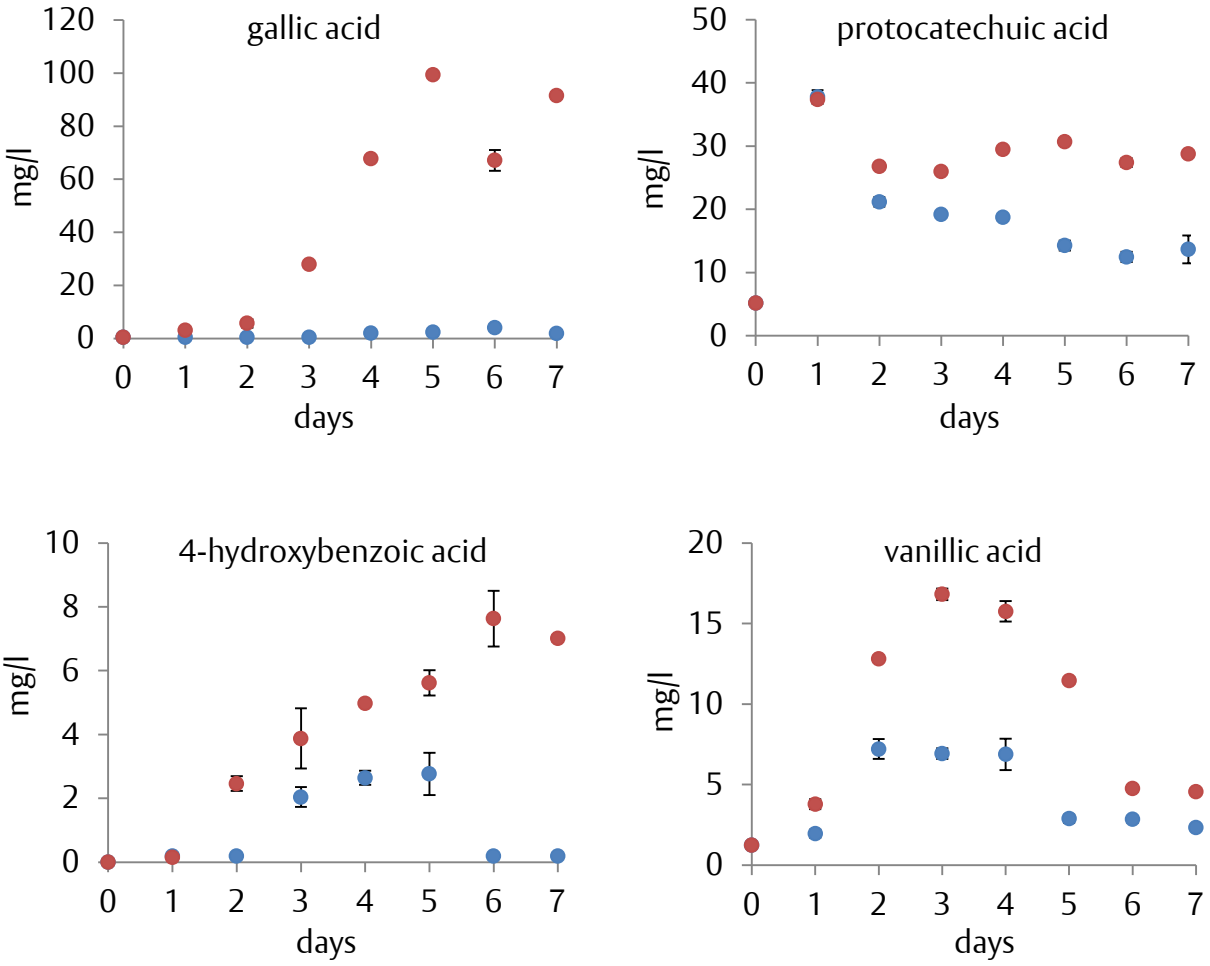

Figure 6-1: Concentration of phenolic acids and anthocyanins from the saccharification of steamed pigmented rice using *Aspergillus oryzae* at 25 °C (blue) and 30 °C (red) for 7 days, n=3.

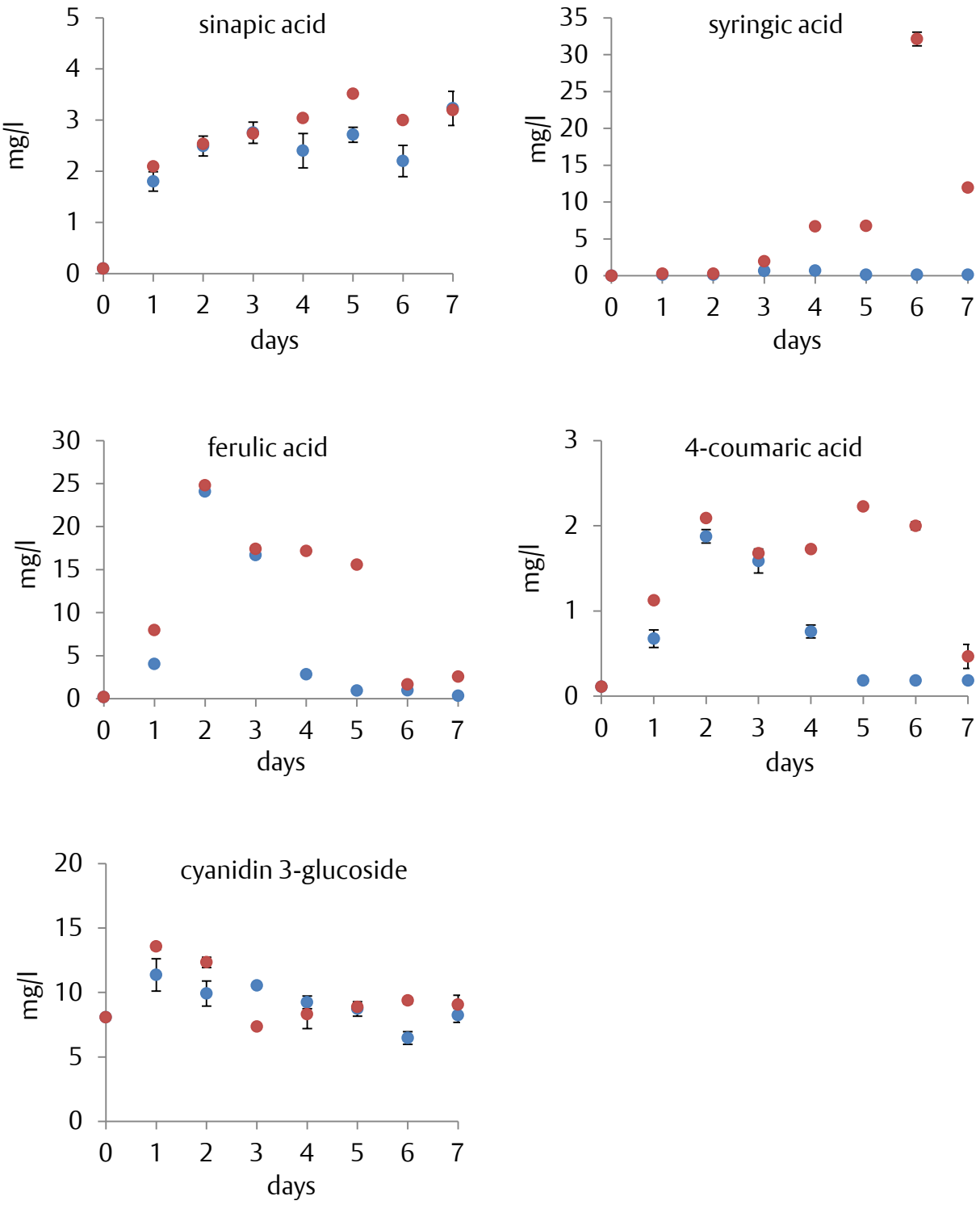

Figure 6-1: Concentration of phenolic acids and anthocyanins from the saccharification of steamed pigmented rice using *Aspergillus oryzae* at 25 °C (blue) and 30 °C (red) for 7 days, n=3 (continued).

To investigate the concentration of phenolic acids during fermentation, steamed pigmented rice was saccharified and then fermented using the selected brewing process from chapter 4. The rice wine samples were analysed for phenolic acids every 24 h. This study showed that the concentration of all phenolic acids and anthocyanins were detected at a lower concentration on day 0, regardless of the brewing temperature (figure 6.2). After that, their concentrations were increased by the degradation of the corresponding precursors. The concentration of phenolic acids and cyanidin-3-glucoside from the alcoholic fermentation at 25 °C were different from that at 30 °C ($p < 0.05$). Gallic acid and protocatechuic acid were found to be the predominant compounds in those rice wine samples. This corresponded to those in the saccharified pigmented rice. Hiemori et al. (2009) and Shin et al. (2019) showed that phenolic acids, including 4-hydroxyphenyl group, guaiacyl group and syringyl group are formed from the degradation of lignin. The concentration of all phenolic acids and cyanidin-3-glucoside were decreased during fermentation. This can be explained by the fact that ferulic acid, 4-coumaric acid and caffeic acid are converted to 4-vinyl derivatives, and then further reduced to 4-ethyl derivatives by microorganisms (Belda et al., 2017).

In summary, all phenolic acids and anthocyanins were increased by the metabolism of fungi and yeast during brewing process. However, the concentration of vanillic acid, syringic acid, ferulic acid and 4-coumaric acid was decreased during the brewing, especially the saccharification because they were converted to the volatile phenol compounds. Moreover, cyaniding-3-glucoside which was presented in flavylium cation (red colour) was converted to carbinol base (colourless) and chalcone (pale yellow colour) due to the pH of pigmented rice wines which were in the range of 4-6. This is consistent with Castaneda-Ovando et al. (2009).

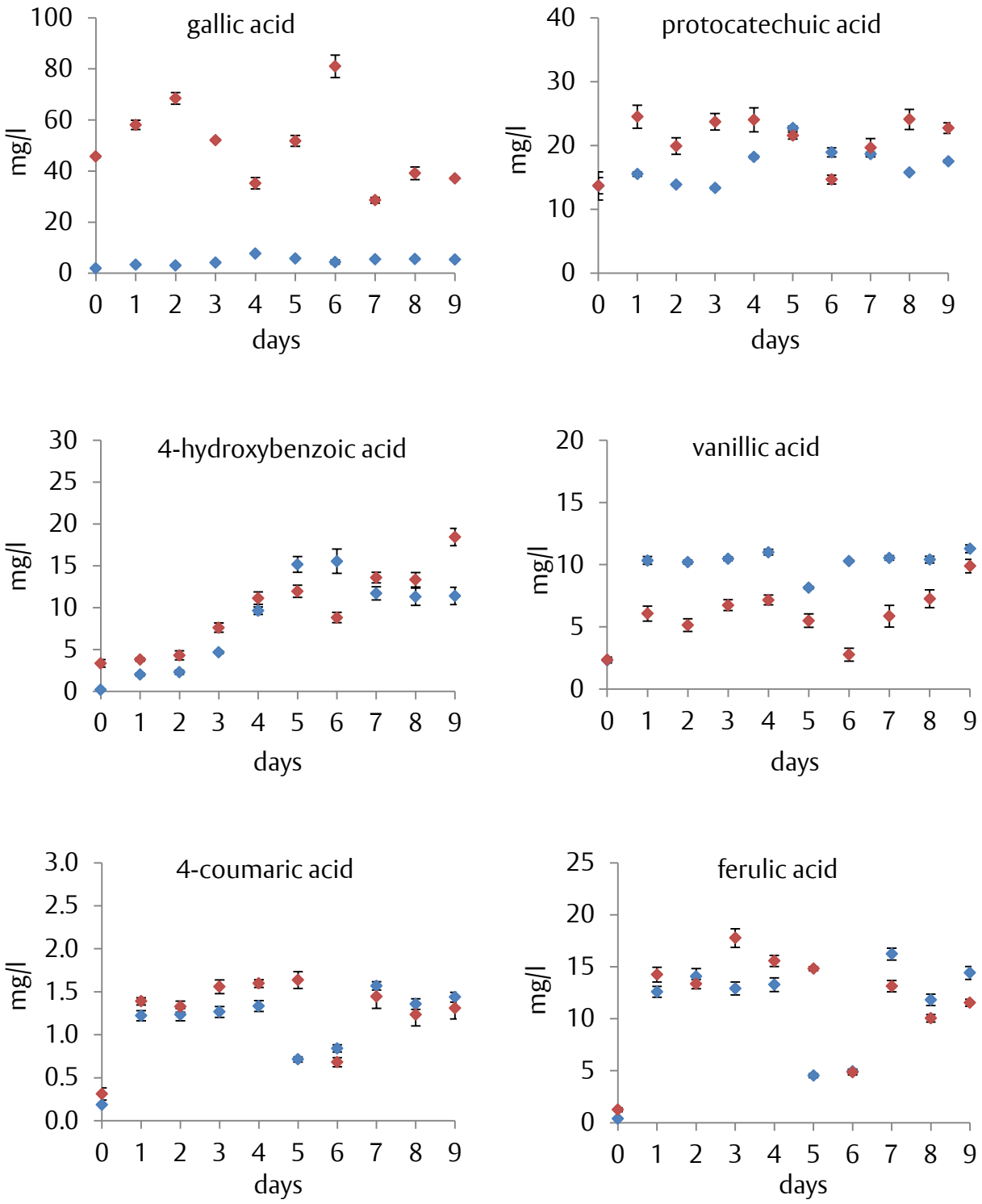

Figure 6-2: Concentration of phenolic acids and anthocyanins from the fermentation of steamed pigmented rice using *Saccharomyces cerevisiae* at 25 °C (blue) and 30 °C (red) for 9 days, n=3.

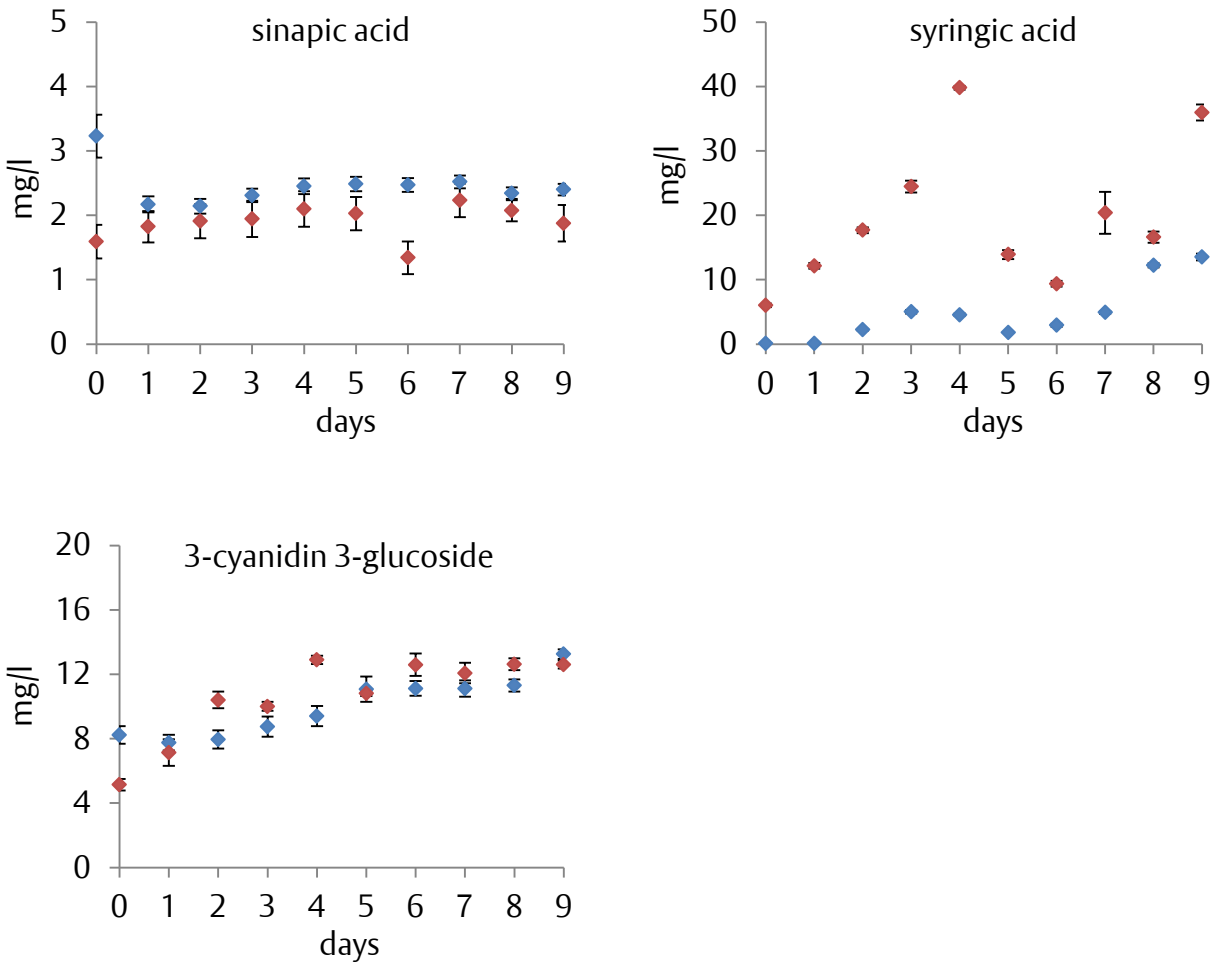

Figure 6-2: Concentration of phenolic acids and anthocyanins from the fermentation of steamed pigmented rice using *Saccharomyces cerevisiae* at 25 °C (blue) and 30 °C (red) for 9 days, n=3 (continued).

6.4 Conclusions

This study showed that phenolic acids were derived from the bran of pigmented rice during the brewing process. One of these phenolic acids, vanillic acid was decarboxylated to form guaiacol which was responsible for the smoky-spicy note in pigmented rice wine. Moreover, guaiacol was also found to be generated from the bran of unpolished pigmented rice during steaming process. The aroma profiling study confirmed that guaiacol was the key aroma compound responsible for smoky-spicy note in unpolished pigmented rice wine rather than 4-vinylguaiacol.

6.5 Publication

1) Yotmanee, S., Oruna-Concha, M.J. and Parker, J.K. (2018). The impact of volatile phenols on the aroma, and the identification of their precursors in pigmented rice wine. The 6th Nursten symposium, University of Nottingham, UK, 27-28 September 2018.
   (Oral presentation)

CHAPTER 7: GENERAL DISCUSSIONS

7.1 Discussion

Pigmented rice wine is produced using pigmented rice. Similar to Chinese rice wine, the brewing process of pigmented rice wine consists of fungi to saccharify rice starch to glucose, and brewing yeast to convert glucose to ethanol (Yang et al., 2017b, Chen et al., 2013b). Pigmented rice wine has been used by Chinese for drinking and medical purposes for a long time. However, the aroma and taste compounds in pigmented rice wine are less known. To characterise the taste and aroma compounds in pigmented rice wine, and identify their precursors, this study was conducted with the following interrelated aims:

I. Characterise and compare the characteristic taste and aroma compounds in commercial polished rice wine and commercial pigmented rice wine
II. Optimise the brewing process for the pigmented rice wine, using parallel fermentation
III. Directly compare and identify the characteristic taste and aroma compounds in rice wines which are brewed using pigmented rice polished to various degrees (0% for unpolished grain, 30%, 50% and 65% for bran fully removed)
IV. Confirm the corresponding precursors for the characteristic aroma in the pigmented rice wine, using standard spiking technique

To identify the characteristic taste and aroma in pigmented rice wine, commercial rice wines, including polished rice wines and pigmented rice wines, were analysed for taste compounds and aroma compounds (chapter 3). Most taste compounds were not likely to be formed from the bran of pigmented rice because their concentrations did not increase in the presence of the bran. Moreover, esters, alcohols and organic acids were the most abundant aroma compound in commercial rice wines. This is consistent with Chuenchomrat et al. (2008), Chen and Xu (2010) and Yang et al. (2017b). However, the analysis of GC-O showed that guaiacol and 4-vinylguaiacol which were responsible for the smoky-spicy notes which were found in pigmented rice wine. This is in agreement with Chen et al. (2013b) who showed that guaiacol and 4-vinylguaiacol contribute smoky note in Chinese rice wine which was brewed using wheat Qu. These aroma compounds are be formed from their precursors in the bran of pigmented rice. Thus, the influence of the bran from pigmented rice on the formation of guaiacol and 4-vinylguaiacol was further studied.

In chapter 4, a reproducible labscale process for brewing rice wine was developed. This brewing process was used for the production of pigmented rice wines which were analysed for the characteristic taste and aroma compounds (chapter 5). The challenge of this study was the application of pigmented rice cooking methods (steaming and pressure cooking) and the parallel fermentation which were suitable for the growth and activities of *Aspergillus oryza*e ATCC 22787 and *Saccharomyces cerevisiae* NCYC 478. The brewing process was monitored for sugars, ethanol and organic acids at 25 °C or 30 °C. The steaming process was selected for the pigmented rice cooking method. The selected saccharification process was observed at 30 °C for 2 days due to a higher concentration of sugars (figure 4-1). The selected alcoholic fermentation was selected at 30 °C for 9 days due to a lower concentration of acetic acid and higher concentration of ethanol (figure 4-3 and

4-4). According to this experiment, the brewing process for further pigmented rice wine producdtion was defined. However, the ultimate aim of this study was to observe the formation of guaiacol and 4-vinylguaiacol in pigmented rice wine. Thus, the selected brewing process was used to brew pigmented rice wines, which were analysed for characteristic aroma compounds, and their precursors.

In chapter 5, rice wines brewed using unpolished pigmented rice, 30% polished pigmented rice, 50% polished pigmented rice and 65% polished rice were analysed for taste and aroma compounds as well as the precursors for the characteristic aroma compounds. Unpolished pigmented rice wines had a higher sour (acetic acid and succinic acid), umami (glutamic acid) and astringent mouthfeel (gallic acid and protocatechuic acid), compared to others. Moreover, the bran of pigmented rice promoted the formation of glutamic acid, phenolic acids and γ-glutamyl peptides (γ-glu-gly, γ-glu-his and γ-glu-tyr). Esters, alcohols and organic acids were found to be the predominant aroma compound in rice wines. The bran promoted the formation of guaiacol and 4-vinylguaiacol, and they were significantly and substantially higher in unpolished pigmented rice wine (table 5-10). This is consistent with Yotmanee et al. (2015). However, the GC-Olfactometry showed that only guaiacol was found to be the characteristic aroma compound which was responsible for smoky-spicy note in unpolished pigmented rice wine (table 5-14). The aroma profiling analysis showed that a higher intensity of smoky-spicy aroma was observed in unpolished pigmented rice wine, compared to other samples. This showed that guaiacol which was responsible for smoky-spicy note was found to be the characteristic aroma compound in pigmented rice wine, and it was formed from the bran of pigmented rice thus the precursors for both guaiacol and 4-vinylguaiacol were further studied.

In chapter 6, the identification of the corresponding precursors of guaiacol and 4-vinylguaiacol was studied using standard spiking technique. The results showed that vanillic acid and ferulic acid were found to be precursor for guaiacol and 4-vinylguaiacol, respectively. These phenolic acid compounds were likely derived from the bran of pigmented rice during the brewing process. This is in agreement with Butsat and Siriamornpun (2010) and Zhang et al. (2010). Moreover, the aroma profiling analysis also showed that guaiacol likely contributes smoky-spicy note in unpolished pigmented rice wine rather than 4-vinylguaiacol.

7.2 Contribution to knowledge

According to the abundance literature regarding the taste and aroma compounds in pigmented rice wine, this study provides useful information for person who is interested in flavour and brewing sciences, due to the fact that this information can be used to understand the flavour in other alcoholic beverages. The contributions to scientific knowledge are summarized as follows:

I. The reliable methods and techniques for the analysis of volatile compounds (SPME/GC-MS), semi-volatile compounds (SPE/GC-MS) and GC-Olfactometry for pigmented rice wine were successfully developed.
II. The method of analysis for γ-glutamyl peptides in pigmented rice wine was successfully developed (without derivatisation). This method is more convenient when compared to that provided by Miyamura et al. (2015).

III. The selected condition of the brewing process for the pigmented rice wine was observed. This process used the parallel fermentation which shorts the time for the fermentation.

IV. The effect of the bran from pigmented rice on the taste and aroma compounds in pigmented rice wine was investigated. This showed that the bran promoted the formation of guaiacol, responsible for smoky-spicy note which was a characteristic aroma in pigmented rice wine.

7.3 Limitations of the research

There are some limitations that need to be considered in the present research:

I. The polished rice wines and pigmented rice wine were purchased for the analysis of taste and aroma compounds however commercial pigmented rice wine was limited and difficult to find. Ideally, three or four samples per each treatment would have been needed for the study.

II. The impact of the bran from pigmented rice on the taste in pigmented rice wine was studied however taste thresholds and taste profiling analysis were not studied due to local restrictions on producing pigmented rice wines. The samples in this study were precluded for health and safety reasons because (i) they were produced using brewing strains which had not officially been declared as food-grade, and (ii) as a result, they were brewed in a microbiology laboratory as the non-food grade brewing strains were not allowed to be used in the food processing plant in our department, thus resulted in samples not being tasted by the panel. Therefore, the taste thresholds from the published studies were used instead, and taste attributes were omitted from the sensory analysis.

7.4 Future studies

During this research, several interesting phenomena were observed regarding the brewing process for pigmented rice wine and the impact of the bran from pigmented rice on the characteristic taste and aroma compounds in pigmented rice wine. However, not all were explored fully due to the limitation with respect to the time frame and scope of the study. The following can be explored further to fulfil the gap of knowledge and provide the interesting results regarding the taste and aroma in pigmented rice wine.

I. It would be of interest to study the taste and aroma thresholds in pigmented rice wine. This will provide the precise taste and aroma thresholds for the pigmented rice wine, compared to the published thresholds that were used in this study which were done in different food models.

II. To identify the active aroma compounds in pigmented rice wine, the odour active value (OAV) should be studied in the future. Moreover, the accurate quantification of aroma compounds should be further studied as well. This will be used to calculate the OAV, as described in the following equation.

$$OVA = \frac{concentration\ of\ individual\ aroma\ compound}{the\ corresponding\ threshold}$$

III. To understand the consumer perceptions and acceptability of pigmented rice wine, consumer tests in both Asian and Caucasian should be carried out. This can be used to develop the quality of pigmented rice wine which is most acceptable to consumers of differing ethnic origin.

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