University of Reading

The Investigation of 2-Acetyl-1-pyrroline Formation in Fragrant and Non-fragrant Rice during Cooking

Thesis submitted for degree of Doctor of Philosophy

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March 2019

Declaration

I confirm that this is my own work and the use of all material from other sources has been properly and fully acknowledged.

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March 2019

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Abstract

2-Acetyl-1-pyrroline (2-AP) is a popcorn-like aroma compound with low odour detection threshold. This compound is regarded as the key contributor of fragrant rice aroma. 2-AP can be detected in fragrant rice, whereas the content of this compound in non-fragrant rice is too low to be detected.

Levels of 2-AP in raw fragrant rice can be affected by abiotic stress and growth conditions, such as climate and soil conditions. Whereas it was originally thought that this compound could not be enhanced post-harvest, including cooking. One previous study in our research group demonstrated that 2-AP could be increased in fragrant rice after baking at 180 °C for 20 min. Hence, the objective of this project was to evaluate the mechanism of 2-AP formation in rice during cooking.

2-AP content was evaluated in fragrant and non-fragrant rice prepared by different cooking methods. The results of sensory evaluation on boiled milled rice confirmed that 2-AP and its popcorn-like aroma is the most important factor to differentiate fragrant and non-fragrant rice. Popcorn-like aroma was also noticed in boiled non-fragrant rice and the results from GC-olfactometry confirmed the presence of 2-AP in raw non-fragrant rice.

Levels of 2-AP increased on baking milled rice (without water addition) at 180 °C. As the high level of lipids and polyphenols in rice bran might affect 2-AP formation during baking, 2-AP was not observed in rice bran after baking.

When milled rice was baked at a range of temperatures, formation of 2-AP in fragrant and non-fragrant rice showed different trends; maximum 2-AP formation was observed in fragrant rice at around 100 °C, whereas 2-AP formation in non-fragrant rice only took place when the baking temperature was higher than 140 °C. Addition of intermediate compounds confirmed that the 2-AP formation at 180 °C occurred through Maillard reaction; the addition of external 1-pyrroline and methylglyoxal was shown to reduce 2-AP generation yield at 100 °C. These results suggested that formation of 2-AP in both fragrant and non-fragrant rice is driven by Maillard reaction at 180 °C baking. However, 2-AP generation in fragrant rice during baking at 100 °C may follow another reaction pathway and this pathway is sensitive to 1-pyrroline and methylglyoxal. Further research should focus on the effect of biosynthesis on 2-AP generation at 100 °C baking.

Acknowledgement

I would like to express my deepest gratitude to my supervisors, Dr J. Stephen Elmore and Prof. Lisa Methven. I would never finish my PhD and this thesis without their guidance, supervision, encouragement and patience.

I would like to thank Dr Jane Parker and Dr Dimitris Balagiannis who offered me support and advice on my whole PhD research, and thank Andy Dodson, who offered me the training and the technical support on GC-MS analysis.

Also, I would like to thank Giract, the Bursary for the 1st year PhD students in Flavour Research. I am extremely grateful to them for honouring me a scholarship that supported me to start my first year PhD research.

I would like to express my sincere acknowledgement to Prof. Don Mottram and British Society of Flavourists, who offered me to attend the 15th Flavourist Course, where I gained valuable knowledge on flavour chemistry and comprehensive understanding on this field.

I would like to thank Dr Dody. D. Handoko. As my PhD project is based on his research, he is the person who led me into fragrant rice research field. Also, I would like to thank the Indonesia Centre of Rice Research, the institute that Dr Handoko works for, to provide me Sintanur and Ciherang rice samples.

Last but not least, I would like to express my special gratitude to my parents, my entire family and my girlfriend Sherrie, for their loving, supporting, understanding, and encouraging on my PhD and entire life.

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List of Abbreviations

2-AP	2-acetyl-1-pyrroline
A-SDE	atmospheric pressure simultaneous distillation–extraction
ATHP	6-acetyl-1,2,3,4-tetrahydropyridine
BADH2	betaine aldehyde hydrogenase
CaM	Ca^{2+}-dependent calmodulin
CAR	Carboxen™
CI	chemical ionisation
DCM	dichloromethane
DHAP	dihydroxyacetone phosphate
DMT	dimethyltitanocene
DVB	divinylbenzene
EI	electron ionisation
ESI	electrospray ionization
FEMA	flavour and extract manufacturers association
fgr	recessive gene fragment
FID	flame ionisation detector
G3P	glyceraldehyde-3-phosphate
GABA	γ-aminobutyric acid
GABald	γ -aminobutyraldehyde
GAD	glutamic acid decarboxylase
GC	gas chromatography
GC-O	gas chromatography-olfactometry
GSA	glutamic-γ-semialdehyde
H_2O	water
HCl	hydrochloric acid
HPLC	high-performance liquid chromatography
HS-SPME	headspace solid-phase microextraction
ICRR	Indonesia Centre for Rice Research

LDA	lithium diisopropylamide
LOQ	limit of quantification
$MgSO_4$	magnesium sulfate
MS	mass spectrometry
NaOH	sodium hydroxide
OAT	ornithine aminotransferase
P5C	1-pyrroline-5-carboxylate
P5CDH	1-pyrroline-5-carboxylate dehydrogenase
P5CR	1-pyrroline-5-carboxylate reductase
P5CS	1-pyrroline-5-carboxylate synthetase
PCI	positive chemical ionisation
PDMS	polydimethylsiloxane
PEG	polyethylene glycol
PGA	immobilised penicillin G acylase
PRODH	proline dehydrogenase
QDA	quantitative descriptive analysis
Q-TOF	quadrupole–time-of-flight
SAFE	solvent-assisted flavour evaporation
SDE	simultaneous distillation–extraction
SIDA	stable isotope dilution assays
SIM	selected ion monitoring
SPE	solid-phase extraction
SPME	solid-phase microextraction
TFA	trifluoroacetic acid
THF	tetrahydrofuran
TMP	2,4,6-trimethylpyridine
V-SDE	vacuum simultaneous distillation–extraction
$ZnBr_2$	zinc bromide
$ZnCl_2$	zinc chloride
ZnI_2	zinc iodide

Chapter 1. Introduction

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Rice can be categorised into two types depending on its aroma: fragrant rice and non-fragrant rice. Although non-fragrant long-grain and medium-grain rice constitute the majority of world trade (79%), the price of fragrant rice is higher than non-fragrant rice on the world market. According to the 2017 Rice Market Monitor report, the price of fragrant rice was more than double that of high quality non-fragrant rice (FAO, 2017).

The aroma of fragrant rice has been researched over the past thirty years because of its high economic value. This aroma was first evaluated analytically in the 1980s. It was described as 'pandan-like' by Orientals or frequent rice consumers and it was described as 'popcorn-like' by non-Orientals or infrequent rice consumers because the different cultures and familiarity with samples of the assessors influenced the descriptors used (Paule & Powers, 1989). 2-Acetyl-1-pyrroline (2-AP) was considered as the most important contributor to this odour (Buttery, Ling, Juliano, & Turnbaugh, 1983). This volatile compound can contribute a popcorn-like aroma with a low detection threshold (0.1 nL/L in water and 0.02 ng/L in air) (Buttery et al., 1983; Schieberle, 1991). It was first identified in boiled fragrant rice (Buttery, Ling, & Juliano, 1982), and was found in many cooked cereal products, some vegetables (especially pandan), and some animal products (Adams & De Kimpe, 2006; Wakte et al., 2016).

A 2-AP generation pathway in model systems *via* Maillard reaction has been proposed; it is regarded as the 2-AP generation route in cooked food products. Proline is a precursor of 2-AP and provides the nitrogen source in the 2-AP molecule, while the acetyl group in 2-AP is provided by reducing sugars (Schieberle, 1988; Rewicki et al., 1993). It has been shown that Strecker degradation of proline with α-dicarbonyl compounds can give several intermediates, including 1-

pyrroline, and 2-AP can also be generated from the reaction between 1-pyrroline and methylglyoxal (Schieberle, 1995; Hofmann & Schieberle, 1998a).

2-AP is not only generated *via* Maillard reaction, it can also be formed in fragrant rice during rice cultivation. Because of the presence of a non-functional BADH2 enzyme in fragrant rice, γ-aminobutyraldehyde (GABald) is cyclised to 1-pyrroline and then acetylated to form 2-AP. In contrast, functional BADH2 catalyses GABald to GABA, preventing the formation of 2-AP from GABald in non-fragrant rice (Bradbury, Gillies, Brusheet, Waters & Henry, 2008). It was also found that the presence of 1-pyrroline-5-carboxylate (P5C) from the metabolism of glutamic acid, proline and ornithine is positively correlated with 2-AP accumulation in rice (Huang, Teng, Chang, Chuang, Ho and Wu, 2008).

2-AP levels in fragrant rice can be influenced by various factors during cultivation. Environmental stress, especially drought, can enhance proline accumulation and hence lead to higher 2-AP concentration (Yoshihashi, Nguyen, & Kabaki, 2004). Introduction of salinity, manganese or silicon into soil during cultivation was found to increase 2-AP content in fragrant rice (Poonlaphdecha et al., 2012; Li et al., 2016; Mo et al., 2017). However, 2-AP is regarded as an aroma compound which cannot be enhanced in rice during postharvest processing and cooking (Yoshihashi, 2002).

Handoko (2014) reported that 2-AP concentration increased in fragrant rice after baking without water at 180 °C. Moreover, his study also indicated that popcorn-like attributes were found in both baked fragrant and non-fragrant rice. Hence, 2-AP may also be generated in non-fragrant rice during baking. His research implied that 2-AP level may not only be increased during rice cultivation, but also can be enhanced by heat processing.

Following on from the research of Handoko (2014), the hypothesis of this study is that 2-AP can be generated not only in fragrant rice, but also in non-fragrant rice during baking at high temperature. Hence, this project aims to investigate the effect of cooking temperature on 2-AP

generation in both fragrant and non-fragrant rice, and tries to find out the key intermediates which can limit 2-AP formation during baking.

Objectives:

Develop techniques to extract and quantify 2-AP in rice.

Confirm the importance of popcorn-like aroma and 2-AP on sensory quality of fragrant rice.

Confirm the concentration of 2-AP in fragrant and non-fragrant rice by multiple detection techniques.

Confirm the increase of 2-AP in rice (fragrant *vs* non-fragrant; milled rice *vs* rice bran) after 180 °C baking without water addition and determine the change of free amino acids and reducing sugars during 180 °C baking.

Develop optimal baking conditions to enhance 2-AP concentrations in both fragrant and non-fragrant rice.

Explore 2-AP formation pathways during rice baking by adding additional intermediates.

Contents of this thesis:

Chapter 2 is a literature review based on Wei et al. (2017), which evaluated 2-acetyl-1-pyrroline from several different aspects, focusing on its importance in fragrant rice.

Chapter 3 is a method development chapter to select and optimise 2-AP extraction and determination techniques.

Chapter 4 emphasises the importance of popcorn-like aroma and 2-AP in the differentiation of fragrant and non-fragrant rice using sensory analysis. A key finding of this

chapter was that trace levels of 2-AP were present and contributed popcorn-like aroma in non-fragrant rice.

In **Chapter 5** 2-AP was measured in fragrant and non-fragrant milled rice and rice bran before and after baking at 180 °C. In addition, free amino acids and reducing sugars were also measured to explore the relationships between precursors and 2-AP formation. This chapter also examined the relationship between baking temperature, baking time and 2-AP formation, and found the optimal temperatures in fragrant and non-fragrant rice to form maximum concentrations of 2-AP.

In **Chapter 6** the intermediates 1-pyrroline, methylglyoxal and GABA were added into rice and 2-AP levels were measured after baking, to explore if 2-AP concentrations could be enhanced in fragrant and non-fragrant rice. 2-AP was enhanced in fragrant rice and GABA levels were enhanced in both fragrant and non-fragrant rice after 100 °C baking; addition of 1-pyrroline or methylglyoxal reduced these enhancements.

Chapter 7 is a general discussion of this thesis. The whole thesis is discussed from the importance of 2-AP in rice aroma, development of 2-AP extraction and analysis techniques and 2-AP formation mechanisms under different baking temperatures.

Chapter 2. Evaluation of 2-acetyl-1-pyrroline in foods, with an emphasis on rice flavour

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This chapter is based on the following paper: Wei, X., Handoko, D. D., Pather, L., Methven, L., & Elmore, J. S. (2017). Evaluation of 2-acetyl-1-pyrroline in foods, with an emphasis on rice flavour. *Food Chemistry*, 232, 531-544.

Abstract

The popcorn-like aroma compound 2-acetyl-1-pyrroline (2-AP) is a key contributor to the desirable aroma of fragrant rice and is also important in the aroma of other foods, such as pandan leaf and popcorn. It can be formed enzymatically in the rice grain as it grows and is also formed, as part of the Maillard reaction, when rice is heated. This review examines the formation of 2-AP in rice and other foods, particularly its formation during cooking, focusing on the importance of the Maillard reaction between reducing sugar breakdown products and 1-pyrroline derived from the amino acids proline and ornithine. The synthesis of 2-AP is discussed alongside the attempts that have been made to stabilise this relatively unstable compound. The analysis of 2-AP by instrumental techniques, particularly gas chromatography-mass spectrometry and gas chromatography-olfactometry, alongside the use of sensory studies, is also discussed.

Keywords: 2-acetyl-1-pyrroline, 2-AP, flavour, rice, pandan, popcorn, Maillard reaction, biosynthesis, analysis

2.1. Introduction

The IUPAC name of 2-acetyl-1-pyrroline (2-AP) is 1-(3,4-dihydro-2H-pyrrol-5-yl)ethanone, its CAS number is 85213-22-5 and its FEMA (Flavor and Extract Manufacturers Association) number is 4249. 2-AP was first identified in rice by Buttery, Ling, and Juliano (1982), and is regarded as the most important aroma compound in rice, especially fragrant rice (Buttery, Ling, Juliano, & Turnbaugh, 1983). In that study, 0.05 mg/kg 2-AP was described as popcorn-like and its odour threshold in water was measured as 0.1 nL/L, while its odour threshold in air was reported by Schieberle (1991) as 0.02 ng/L; this very low threshold makes it an important contributor to a food's aroma when present. As well as rice, it is also a key flavour compound in many cereal products, as well as some vegetable and animal products (Adams & De Kimpe, 2006; Wakte, Zanan, Hinge, Khandagale, Nadaf, & Henry, 2016).

Bioformation of 2-acetyl-1-pyrroline in both plants and microorganisms has been studied and several types of bacteria are able to form this compound (see *Section 2.2.3*). 2-Acetyl-1-pyrroline has also been shown to form in the Maillard reaction; it can be formed from the reaction between proline and reducing sugars/sugar degradation products upon heating (Schieberle, 1989).

Although there is a high commercial interest in 2-AP because of its desirable sensory attributes, the instability of this compound is a significant problem for its commercial application. Pure 2-AP will turn red and degrade within 10 minutes at room temperature (Fang & Cadwallader, 2014), and there is significant short-term reduction of 2-AP concentration in food products, such as popcorn (Schieberle, 1995) and raw fragrant rice (Widjaja, Craske, & Wootton, 1996a).

The occurrence of 2-acetyl-1-pyrroline in food products, its bioformation and thermal formation, synthesis, stabilisation, analysis and sensory evaluation will be reviewed in this paper, with particular emphasis on the role of 2-AP in fragrant rice aroma.

2.2. Food Sources of 2-Acetyl-1-pyrroline

2.2.1. Rice

Non-fragrant rice (long- and medium-grain *indicas* and short-grain *japonicas*), mainly grown in USA, Vietnam, Thailand and Australia, constitutes around 80% of the world rice trade (Singh, Singh, & Khush, 2000). Major producers of fragrant rice are India, Pakistan and Thailand. Most of the fragrant rice exported from India and Pakistan is basmati, while fragrant jasmine rice is a major export of Thailand (Singh et al., 2000). In 2010, Thailand was the biggest exporter of fragrant rice: 2.65 million tonnes of jasmine rice were exported, followed by India (1.80 million tonnes basmati) and Pakistan (1.05 million tonnes basmati) (Slayton & Muniroth, 2015).

The price of fragrant rice is more than double that of high quality non-fragrant rice (FAO, 2017). The commercial value of fragrant rice is higher than that of non-fragrant rice, partly because fragrant rice varieties are relatively low yielding. Fragrant rice is less resistant to disease and insect pests and is prone to high shedding, leading to losses in yield (Berner & Hoff, 1986; Golam et al., 2011). It has been shown that higher quality grains with stronger aromas are generated in crops grown in drought and saline conditions (Yoshihashi, Nguyen, & Kabaki, 2004). These adverse conditions do not favour high yields.

2-AP is the key discriminator between fragrant and non-fragrant rice and many studies have focused on the concentration of 2-AP in different rice cultivars. 2-AP concentrations in different fragrant cultivars vary substantially (**Table 2.1**). For example, 2-AP was present in milled Fowler Gourmet Aromatic rice (a US-grown aromatic rice) at 999 µg/kg, while, in a set of five basmati samples, levels of 2-AP from 19 µg/kg to 342 µg/kg were measured (Bergman, Delgado, Bryant, Grimm, Cadwallader, & Webb, 2000).

Table 2.1: 2-AP concentrations in fragrant and non-fragrant rice

rice variety	2-AP concentration (µg/kg)			
	milled	**brown**	**Cooking state**	**Extraction technique**
Fragrant				
Basmati	60[a]	170[a]	Boiling during extraction	SDE
		610[b]	Boiling during extraction	SDE
	87.4[d]		Boiling during extraction	SDE
	588[g]		Boiling during extraction	SDE
	68-342[h]	119[h]	uncooked	Solvent extraction (DCM)
Khao Dawk Mali 105 (jasmine)	70[a]	200[a]	Boiling during extraction	SDE
	156.1[d]		Boiling during extraction	SDE
	532[i]		uncooked	Solvent extraction (ethanol)
	810[h]	550[h]	uncooked	Solvent extraction (DCM)
Malagkit Sungsong	90[a]	200[a]	Boiling during extraction	SDE
		760[b]	Boiling during extraction	SDE
Milagross	70[a]		Boiling during extraction	SDE
Seratus Malam	60[a]		Boiling during extraction	SDE
Azucena	40[a]	160[a]	Boiling during extraction	SDE
Hieri	40[a]	100[a]	Boiling during extraction	SDE
Ir841-76-1	70[a]	200[a]	Boiling during extraction	SDE
		560[b]	Boiling during extraction	SDE
Della	76.2[d]		Boiling during extraction	SDE
Goolarah	691[e]		Boiling during extraction	SDE
Yrf9	670[e]		Boiling during extraction	SDE
		344[f]	Boiling during extraction	SDE
B5-3	2746[g]		Boiling during extraction	SDE
Amber Aromatic (Lundberg)		345[h]	uncooked	Solvent extraction (DCM)
Aromatic (Fowler Gourmet)	999[h]		uncooked	Solvent extraction (DCM)
Black Thai (Bulk)		259[h]	uncooked	Solvent extraction (DCM)
Jasmati (Rice Tec)	526[h]		uncooked	Solvent extraction (DCM)
Kasmati (Rice Tec)	496[h]		uncooked	Solvent extraction (DCM)
Texmati (Rice Tec)	266[h]		uncooked	Solvent extraction (DCM)
Aychade	575–638[j]		uncooked	SPME
Fidji	45–475[j]		uncooked	SPME
Giano	28–336[j]		uncooked	SPME
Kala Bhat	920[k]		uncooked	SPME
Kali Kumud	732[k]		uncooked	SPME
Amritbhog	787[k]		uncooked	SPME
Non-fragrant				
Calrose	<6[a]		Boiling during extraction	SDE
California long-grain	0.6[c]		Boiled before extraction	Dynamic headspace extraction (Tenax)
Pelde	15[e]		Boiling during extraction	SDE
Texas long-grain	<8[a]		Boiling during extraction	SDE
	6[b]		Boiling during extraction	SDE
Ariette	10.6[j]		uncooked	SPME
Ruille	24.7[j]		uncooked	SPME
Sonsali	72[k]		uncooked	SPME
Kolamb	125[k]		uncooked	SPME

Data are from the following references: [a]Buttery et al., 1983; [b]Buttery et al., 1986; [c]Buttery et al., 1988; [d]Tanchotikul and Hsieh, 1991; [e]Widjaja et al., 1996a; [f]Widjaja et al., 1996b; [g]Tava and Bocchi, 1999; [h]Bergman et al., 2000; [i]Yoshihashi et al., 2004; [j]Maraval et al., 2010; [k]Mathure et al., 2014.

Milled rice (commonly referred to as white rice) is obtained from the milling of brown rice to remove the outer bran layer. Whole rice grains are dehulled; then the dehulled (brown) rice is milled twice. Generally, 20–22% of the rice grain is hull, and another 8–10% is bran and embryo; therefore, the yield of milled rice is around 70% (Singh et al., 2000). As can be seen in **Table 2.1**, in most cases more 2-AP is present in brown rice compared to milled rice.

Caution should be applied when comparing data acquired by different authors. In some cases, 2-AP was measured in uncooked rice (e.g., Hopfer, Jodari, Negre-Zakharov, Wylie, & Ebeler, 2016), while in other cases the rice was cooked before analysis (e.g., Buttery et al., 1988) and even during analysis (e.g., Buttery et al., 1983, Widjaja et al., 1996a; Widjaja, Craske, & Wootton, 1996b). The effect of sample preparation on 2-AP content in rice is covered in more detail in Part 7 of this review.

Soil and climate conditions during cropping can also influence 2-AP concentration in rice cultivars. During cultivation, a dry climate or sandy soil with low moisture retention can induce the fragrant rice cultivar Khao Dawk Mali 105 to produce more 2-AP (518−532 μg/kg), compared with moisture-retentive soils (218−388 μg/kg) (Yoshihashi et al., 2004). It appears that moisture during cultivation could be one of the most important factors affecting 2-AP formation when rice grows.

Due to the instability of 2-AP, drying and storage of rice can also influence the 2-AP content of the final product (Wongpornchai et al., 2004). The unstable nature of 2-AP will be discussed in detail in Part 6 of this review.

2.2.2. Pandan

2-AP is an important component of pandan leaf; the aroma of 2-AP is often described as pandan-like. Pandan plays an important role in Southeast Asian cookery. The leaf of this plant is often boiled with rice to enhance flavour. When boiled with non-fragrant rice, it can provide the

popcorn-like flavour associated with boiled fragrant rice, allowing cheap non-fragrant cultivars to possess similar aroma to higher value fragrant rice cultivars (Peter, 2006). The treatment of pandan leaf can affect 2-AP content. The fresh or slightly withered leaf is normally torn into strips, tied in a bunch and then boiled together with rice. The pandan leaves are removed from the rice after cooking.

The concentration of 2-AP in pandan leaves ranges from 40 ± 10 to 450 ± 10 μg/kg (Yahya, Lu, Santos, Fryer, & Bakalis, 2010). Dried and ground pandan leaves were extracted in this study. However, these treatments disrupted the papillae structure in epidermal cells on the surface of the pandan leaves. 2-AP is contained in the papillae; therefore, a proportion of 2-AP is lost during drying and grinding.

2.2.3. Cereal products

2-Acetyl-1-pyrroline has also been detected in cooked cereal-based products. Wheat bread crusts contain around 75 μg/kg 2-AP compared to 1–4 μg/kg in sourdough processed rye bread (Schieberle & Grosch, 1987). Popcorn-like aroma compound 2-AP is, unsurprisingly, present in popcorn. However, in popcorn 6-acetyl-1,2,3,4-tetrahydropyridine and 2-propionyl-1-pyrroline also contribute roasty and popcorn-like flavour. The alkyl side-chains of those compounds are short; only one or two carbon atoms length. In contrast, 2-butanoyl-1-pyrroline and 2-hexanoyl-1-pyrroline, compounds with similar structure but with longer alkyl side-chains, do not possess roasty or popcorn-like aroma (Schieberle, 1991).

2-AP was also identified in a cereal coffee brew at 8 μg/L and contributed intense popcorn-like odour attributes when analysed by gas chromatography-olfactometry (Majcher, Klensporf-Pawlik, Dziadas, & Jeleń, 2013). The cereal coffee was a roasted mixture of 40% barley, 25% rye, 25% chicory, and 10% sugar beet.

2.2.4. Other foods

2-AP has also been detected in non-cereal-based food. A high concentration of 2-AP of up to 750 µg/kg was found on the surface of Mediterranean dried sausages, while values at the core were up to 100 µg/kg. *Penicillium nalgiovense,* the dominant mould species present, was shown to synthesise 2-AP during sausage processing (Stahnke, 2000). Using gas chromatography-olfactometry (GC-O), Blank, Devaud, Fay, Cerny, Steiner, and Zurbriggen (2001) identified 2-AP as a key contributor to the aromas of both Parma ham and Italian-type salami. They described the compound as it eluted from the GC column as having a 'roasty' aroma in the Parma ham and a 'roasty, popcorn' aroma in the salami.

2-AP was also isolated in Manuka honey at concentrations of 80–450 µg/kg. It was formed from methylglyoxal, which is responsible for the antibacterial activity in Manuka honey. Reaction of methylglyoxal with proline through the Strecker reaction can form 2-AP (Ruckriemen, Schwarzenbolz, Adam, & Henle, 2015). In addition, 2-AP was also isolated from two kinds of cooked edible fungus: huitlacoche and oyster mushroom (Lizarraga-Guerra, Guth, & Lopez, 1997), but the compound was mistakenly identified as 2-acetyl-2-pyrroline (Adams & De Kimpe, 2006). The importance of 2-AP in mushroom (*Agaricus bisporus*) aroma increased significantly as a result of pan-frying (Grosshauser & Schieberle, 2013), its concentration rising from 0.4 to 5.3 µg/kg. Similarly, 2-AP was also detected in both raw and roasted hazelnuts; a significant increase of 2-AP concentration was observed, from trace levels (< 3 µg/kg) to 85 µg/kg, when hazelnuts were roasted (Kiefl, Pollner, & Schieberle, 2013).

2-AP may not always make a positive contribution to food aroma. An undesirable 'mousy' flavour in wetted raw pearl millet grits was attributed to 2-AP. Although 2-AP concentration was not quantified in this study, it was implied that there was a higher concentration of 2-AP in millet than in rice, which was reflected in the difference in their odour quality (Seitz, Wright, Waniska, & Rooney, 1993).

2-AP has been identified and quantified in many food products. **Table 2.2** shows those foods other than rice where 2-AP has been quantified. Even at a very low concentration, such as 3 μg/kg in milk chocolate (Liu et al., 2015), this compound can still be considered a key odorant. In a recent review, a comprehensive list of food sources of 2-AP was provided, which included fruit and vegetables, fungi, cooked meat and fish, dairy and egg products (Wakte et al., 2016).

Table 2.2: 2-AP concentrations in foods other than rice

food sample	**2-AP concentration (μg/kg)**
wheat bread crusts	75[a]
Mediterranean dried sausages	750[b]
bread flowers (*Vallaris glabra* Ktze)	3.36 (fresh) / 26.1 (dry)[c]
palm wine	11.4[d]
roasted Criollo cocoa beans	4.2[e]
pandan leaves	40–450[f]
pan-fried mushrooms	4.2–7.0[g]
roasted in-shell peanuts	1920[h]
roasted hazelnuts	85[i]
cereal coffee brew	8[j]
squid broth	97.3[k]
dark chocolate	21[l]
milk chocolate	3[l]
cocoa liquor	11[l]
Manuka honey	80–450[m]
raw liquorice	9.41[n]
roasted almonds	12 (dry roasted) / 30 (oil roasted)[o]

Data are from the following references: [a]Schieberle and Grosch, 1987; [b]Stahnke, 2000; [c]Wongpornchai et al., 2003; [d]Lasekan et al., 2007; [e]Frauendorfer and Schieberle, 2008; [f]Yahya et al., 2010; [g]Grosshauser & Schieberle, 2013; [h]Kaneko et al., 2013; [i]Kiefl et al., 2013; [j]Majcher et al., 2013; [k]Carrascon et al., 2014; [l]Liu et al., 2015; [m]Ruckriemen et al., 2015; [n]Wagber et al., 2016; [o]Erten et al., 2017.

2.2.5. 2-Acetyl-1-pyrroline as a flavouring

Several patents have suggested that 2-AP could be applied as a food flavouring and they are summarised in **Table 2.3**. 2-AP was included in GRAS 22 (Smith et al., 2005) and the average and maximum levels for its addition to various food products have been summarised (Adams & De Kimpe, 2006). However, due to the high cost and low yield of 2-acetyl-1-pyrroline synthesis

(discussed in Section 2.5) and its stability issues (introduced in Section 2.6), 2-AP has not been widely applied as a flavouring in the food industry.

Table 2.3: Patents referring to the use of 2-AP as a food flavouring

Patent Number	Inventors	Date	Patent title	Patent Description
US4522838A	Ronald G. Buttery; Louisa C. Ling, Bienvenido O. Juliano	1985	2-Acetyl-1-pyrroline and its use for flavoring foods	2-acetyl-1-pyrroline was described as scented rice aroma; pure or substantially pure 2-AP was synthesised and a stable salt of 2-AP was prepared as a flavouring to give scented rice aroma in food products.
JP2002034549A	Kimisuke Asano; Nobuyuki Hirai; Sadao Kawakita; Naotaka Kurose; Masahiro Nagatomo; Keiji Ogawa; Katsuhiro Sakai; Kojiro Takahashi; Shoji Tarumi	2000	Distilled liquor product and method for producing the same	The invention gives fragrant rice flavour to distilled liquor containing 0.2–200 µg/kg of 2-AP.
US6274183B1	Travis Richard	2001	Rice composition for coating foods	A food coating was invented by grinding a fragrant rice containing at least 40 µg/kg of 2-AP, to give popcorn-like flavour to food products and improve crispness.
JP2012075350A	Hide Kaneko; Kenji Kumazawa; Hiroyuki Nishimura; Akiko Otani	2010	Flavour improving agent of soy milk or food and drink containing soy milk	A flavour improving agent was invented containing 0.001 ng/kg to 10 µg/kg of 2-AP to improve the flavour of soy milk or food and drink containing soy milk
JP6309181B1	巧弥 大橋; 誠一 瀧下; 太一 西塚	2016	Beer-like alcoholic beverages	10–200 µg/kg of 4-vinylguaiacol and 1 µg/kg of 2-AP were added into a reduced-sugar and reduced- alcohol beverage, to give balanced beer-like flavour.

2.3. Biological Formation of 2-Acetyl-1-pyrroline

2.3.1. Fragrant rice

It was originally thought that 2-AP was only produced during the cooking of rice *via* the Maillard reaction (Buttery et al., 1982). However, further research has shown that 2-AP is produced by the rice plant and is detected in the majority of plant tissues (Sakthivel, Sundaram, Rani, Balachandran, & Neeraja, 2009; Sood & Siddiq, 1978; Yoshihashi, Huong, & Inatomi, 2002).

It is now generally accepted that although some 2-AP in rice is produced during cooking, 2-AP is predominantly biosynthesised in rice.

Yoshihashi (2002) reported that 2-AP could not be formed during the cooking of fragrant rice (when heated with or without water at 90 °C for 8, 10, 12, 14 min, the concentration of 2-AP showed a slight decrease), nor in postharvest processes like drying and storage. It can only be formed in the aerial parts of plants during growing in paddy fields. In a later paper by the same author, excised callus (cells covering a plant wound) and seedlings were floated on labelled amino acid (200 mg/kg ^{15}N-glycine, ^{15}N-L-proline or 1-^{13}C-L-proline; pH 5.5) solutions. After incubation at 27 °C in darkness for 8 hours, increasing 2-AP concentrations were detected. Results showed clearly that the labelled derivative was only found in seeding and callus incubated with ^{15}N-L-proline. This result indicated that one of the precursors in 2-AP biosynthesis could be proline but not glycine and that the nitrogen source of 2-AP is proline. On the other hand, because no labelled derivative was found in the 1-^{13}C-L-proline sample, the acetyl group in 2-AP could not be provided by proline (Yoshihashi, Huong, & Inatomi, 2002).

It appears that moisture during cultivation could be one of the most important factors affecting 2-AP formation when rice grows. 2-AP concentrations in fragrant rice Khao Dawk Mali 105 from the Tung Kula Rong Hai region in north-east Thailand, where there is a drought-prone climate with sandy soil, were much higher than in the same rice grown in other areas of Thailand. Rice samples planted in clay soil can retain moisture during growth, resulting in lower 2-AP concentrations than those grown in sandy soil (Yoshihashi et al., 2004). Numerous studies have shown that proline accumulation occurs in higher plants due to different environmental stresses, such as drought, high salinity, high light and UV irradiation, heavy metals, oxidative stress and in response to biotic stresses (Szabados & Savouré, 2010). For example, Rhodes, Handa, and Bressan (1986) showed that proline will accumulate in water-deficient plant cells through the glutamate pathway. In this study, tomato cells adapted to water stress induced by polyethylene glycol (PEG);

a tenfold increase of proline synthesis was observed in the water-stressed cells. This research and that of Yoshihashi et al. (2004) suggest that more 2-AP will be synthesised when rice is grown in a dry climate, due to increased accumulation of its precursor proline. Inducing salinity at the vegetative phase (28 days) during fragrant rice cultivation in a greenhouse can enhance proline and 2-AP accumulation in rice leaves and finally result in a significant increase of 2-AP in rice grain. In contrast, although huge amounts of 2-AP can also accumulate in fragrant rice grain when salinity is induced at the later reproductive phase (14 days), the grain yield is reduced due to the salt stress. In addition, reduction of plant height, weight of leaves and weight of rice grain were observed in salinity induced samples (Poonlaphdecha et al., 2012). Manganese fertilisation was found to not only enhance 2-AP content and relative enzyme activities, but also improve grain quality including protein and amylose contents in two fragrant rice varieties, Meixiangzhan and Nongxiang 18 (Li et al., 2016). In addition, silicon application during cultivation was also reported to improve proline accumulation and 2-AP formation in the above-mentioned two fragrant rice varieties (Mo et al., 2017).

In addition, a cool climate and early harvest could increase 2-AP concentration in fragrant rice varieties. Between 1992 and 1994, three brown fragrant rice cultivars from Japan (Hieri, Miyakaori and Sari Queen) were harvested once a year and their 2-AP concentrations were analysed. It was found that 2-AP was higher in rice crops exposed to low temperature (day 25 °C/night 20 °C) than high (day 35 °C/night 30 °C) or moderate temperature (day 30 °C/night 25 °C). In addition, from results across three years, 2-AP concentrations of samples harvested early were higher than those in samples harvested at normal time (Itani, Tamaki, Hayata, Fushimi, & Hashizume, 2004).

In a recent study, a partial least squares model was built, based on planting and harvesting conditions, that could predict 2-AP concentrations in Thai Jasmine Pathumthani 1 rice (Funsueb, Krongchai, Mahatheeranont, & Kittiwachana, 2016). The status of the rice plants was recorded

during cultivation and after harvest, including number of tillers (grain-bearing branches), plant height, root length, number of grains per plant and grain weight. Nitrogen and sodium concentrations, rice yield, shoot dry weight and number of tillers per plant all had significant influences on 2-AP concentration.

There are two mechanisms proposed for the accumulation of 2-AP in mature grains. In the first 2-AP is synthesised in leaves and stem sheaths and transported to the mature grains, while, in the second, proline translocates from leaves into grains and 2-AP synthesis occurs in grains. Hinge, Patil, and Nadaf (2016) showed maximum 2-AP concentrations in mature grains, with less proline in the grain at that time than at other developmental stages. These results suggested that the first mechanism was more likely in the fragrant rice cultivars they were studying (Ambemohar-157 and Basmati-370).

The gene *badh2* encodes an enzyme, betaine aldehyde hydrogenase (BADH2) (Bradbury, Fitzgerald, Henry, Jin, & Waters, 2005), which catalyses the oxidation of γ-aminobutyraldehyde to γ-aminobutyric acid (GABA). γ-Aminobutyraldehyde is a high affinity substrate for the BADH2 enzyme (Oishi & Ebina, 2005; Trossat, Rathinasabapathi, & Hanson, 1997). In solution, γ-aminobutyraldehyde exists in equilibrium with its cyclic form, 1-pyrroline (Struve & Christophersen 2003), a 2-AP precursor. Hence the oxidation of γ-aminobutyraldehyde reduces the potential for 2-AP synthesis (Kovach, Calingacion, Fitzgerald, & McCouch, 2009). Bradbury et al. (2005) identified a mutated version of the *badh2* gene as being responsible for determining fragrance in rice, which has since been confirmed (Arikit et al., 2011; Fitzgerald, Waters, Brools, & Henry, 2010; Kovach et al., 2009; Siddiq, Vemireddy, & Nagaraju, 2012). The mutated *badh2* gene incurs a deletion of eight base pairs in exon 7, leading to early gene termination and production of a truncated non-functional BADH2 enzyme (Bradbury et al., 2005). Non-fragrant rice cultivars contain the *badh2* gene and hence a functional BADH2 enzyme; whereas fragrant cultivars have the mutated *badh2* gene and so produce a non-functional enzyme (Bradbury, Gillies,

Brusheet, Waters, & Henry, 2008). This non-functional enzyme will not be able to oxidise γ-aminobutyraldehyde, leading to a build-up of 1-pyrroline and hence increased 2-AP synthesis. Therefore, 2-AP formation *via* non-functional BADH2 in fragrant rice was proposed to originate from conversion between proline and ornithine. γ-Aminobutyraldehyde is catabolised from proline *via* putrescine oxidation. Non-functional BADH2 in fragrant rice then leads to 1-pyrroline formation. Acetylation in 1-pyrroline either *via* enzyme catalysis or methylglyoxal involvement finally leads to 2-AP accumulation in fragrant rice. Functional BADH2 in non-fragrant rice catalyses the conversion of γ-aminobutyraldehyde to GABA, which is then involved in other pathways (**Figure 2.1**) (Bradbury et al., 2008).

Recent studies have shown that there are various other mutations in the *badh2* gene that may also lead to increased 2-AP production, such as a deletion of seven base pairs in exon 2 (Amarawathi, Singh, Singh, Singh, Mohaoatra, & Sharma, 2008; He & Park, 2015). Similar biosynthetic pathways for the formation of 2-AP have been reported in soybeans (Arikit et al., 2011) and sorghum (Zanan, Khandagale, Hinge, Elangovan, Henry, & Nadaf 2016).

Huang, Teng, Chang, Chuang, Ho and Wu (2008) reported that 1-pyrroline-5-carboxylate (P5C) level positively correlated to 2-AP concentration in fragrant rice varieties and proposed that 2-AP can be synthesised *via* P5C from amino acids without non-functional BADH2 involvement. Using ^{15}N-labelled amino acids suggested that both glutamic acid and proline are nitrogen sources of 2-AP synthesis (Huang et al., 2008; Yoshihashi, Huong, & Inatomi, 2002); the accumulation of proline could correlate with 2-AP synthesis (Poonlaphdecha et al., 2012). 1-Pyrroline-5-carboxylate (P5C) is synthesised from glutamic acid and ornithine with the catalysation of 1-pyrroline-5-carboxylate synthetase (P5CS) and ornithine aminotransferase (OAT) in fragrant rice (Huang et al., 2008). P5C can also be reduced to proline by P5C reductase (P5CR) (Huang et al., 2008), in contrast, conversion from proline to P5C *via* proline dehydrogenase (PRODH) was also reported (Kishor et al., 2005; Bradbury et al., 2008). Therefore, enzyme catalysis of proline,

ornithine and glutamic acid can finally result to high level of P5C accumulation in fragrant rice. It was suggested that the 1-pyrroline-5-carboxylate undergoes a reaction with methylglyoxal, giving rise to 2-AP, either directly or *via* degradation to 1-pyrroline (**Figure 2.1**) (Huang, et al., 2008).

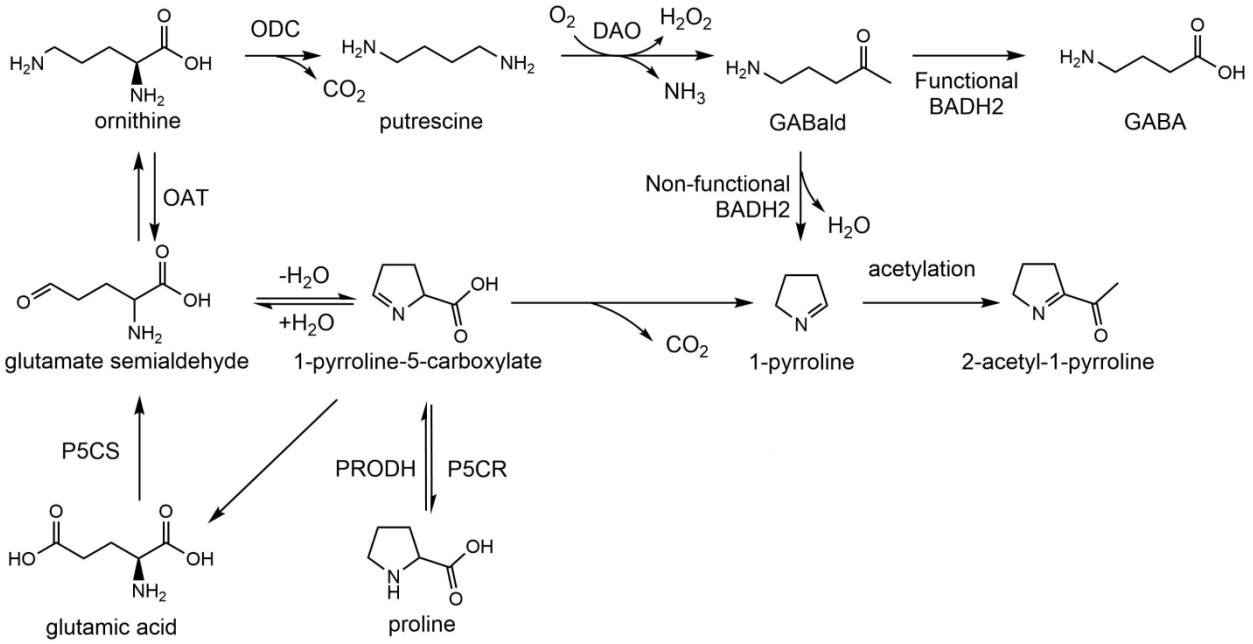

Figure 2.1: A comparison of the BADH2-dependent 2-AP biosynthetic pathway (Bradbury et al., 2008) and the BADH2-independent 2-AP biosynthetic pathway (Sakthivel et al., 2009; Huang et al., 2008, Shelp et al., 2012, Li et al., 2016). Transformation of ornithine, glutamic acid and proline through 1-pyrroline-5-carboxylate and glutamate semialdehyde were summarised from Kavi Kishor et al. (2005). DAO: diamine oxidase, OAT: ornithine aminotransferase, ODC: ornithine decarboxylase, P5CR: 1-pyrroline-5-carboxylate reductase, P5CS: 1-pyrroline-5-carboxylate synthetase, PRODH: proline dehydrogenase.

Although very different (**Figure 2.1**), both pathways could require 1-pyrroline in order to produce 2-AP. 1-Pyrroline has been shown to be a limiting substrate of the biosynthesis of 2-AP in a recent study, where both fragrant and non-fragrant rice callus were incubated with 1-pyrroline. In both cases, a significant increase in 2-AP production was observed, proving 1-pyrroline to be a key intermediate of 2-AP biosynthesis (Poonlaphdecha et al., 2016).

2.3.2. Formation of 2-acetyl-1-pyrroline by microorganisms

Microorganisms could also play an important role in 2-AP formation. During cocoa bean fermentation, yeasts, lactic acid, acetic acid, and various spore-forming bacteria, such as *Bacillus cereus*, are involved in the flavour-forming reactions. Some *Bacillus cereus* strains produce popcorn-like notes and 2-AP was produced by several of these strains incubated on standard plate count agar at 35 °C; 30–75 µg/kg 2-AP was produced after 2 days. A series of ^{13}C and ^{15}N experiments showed that 2-AP could be formed from glucose as carbon source, and glutamic acid and proline as nitrogen sources, through *Bacillus cereus* metabolism (Romanczyk, McClelland, Post, & Aitken, 1995).

In Mediterranean dried sausages, which have a popcorn-like odour and are very different from Northern European sausages, 2-AP is also regarded as the key aroma compound. The main difference between Northern European sausages and Mediterranean dried sausages is a coverage of mould on the latter. 2-AP concentration on the surface of Mediterranean dried sausages is much higher than at the core. Therefore, it was suggested that the mould on the surface of Mediterranean dried sausages is able to produce 2-AP. *Penicillium nalgiovense* was isolated from the sausage surface and it was the dominating mould species. When incubated with and without various supplements, it was found that *Penicillium nalgiovense* could only produce popcorn odour when the sausage was present (Stahnke, 2000).

2-AP, together with other *N*-heterocyclic compounds 2-ethyltetrahydropyridine and 6-acetyl-1,2,3,4-tetrahydropyridine, could cause mousy off-flavour in wine (Herderich, Costello, Grbin, & Henschke, 1995; Strauss & Heresztyn, 1984), through the action of lactic acid bacteria (LAB). *Lactobacillus hilgardii* DSM 20176 was incubated with a defined *N*-heterocycle assay medium, which included D-fructose, ethanol, L-lysine, L-ornithine and mineral salts. It was found that L-ornithine stimulated 2-AP formation and repressed 6-acetyl-1,2,3,4-tetrahydropyridine formation, while L-lysine had the opposite effect (Costello & Henschke, 2002). It had previously

been suggested that D-fructose and ethanol could provide the acetyl side-chain for 2-AP and 6-acetyl-1,2,3,4-tetrahydropyridine (Strauss & Heresztyn, 1984). A possible mechanism of fermentable carbohydrate and amino acid, forming 2-AP and 6-acetyl-1,2,3,4-tetrahydropyridine through LAB fermentation was proposed by Costello and Henschke (2002). This pathway is shown in **Figure 2.2**. L-Lysine could form the intermediate 1-piperideine *via* cadaverine pathways, with the enzymes L-lysine decarboxylase and cadaverine aminotransferase involved (Fothergill & Guest, 1977).

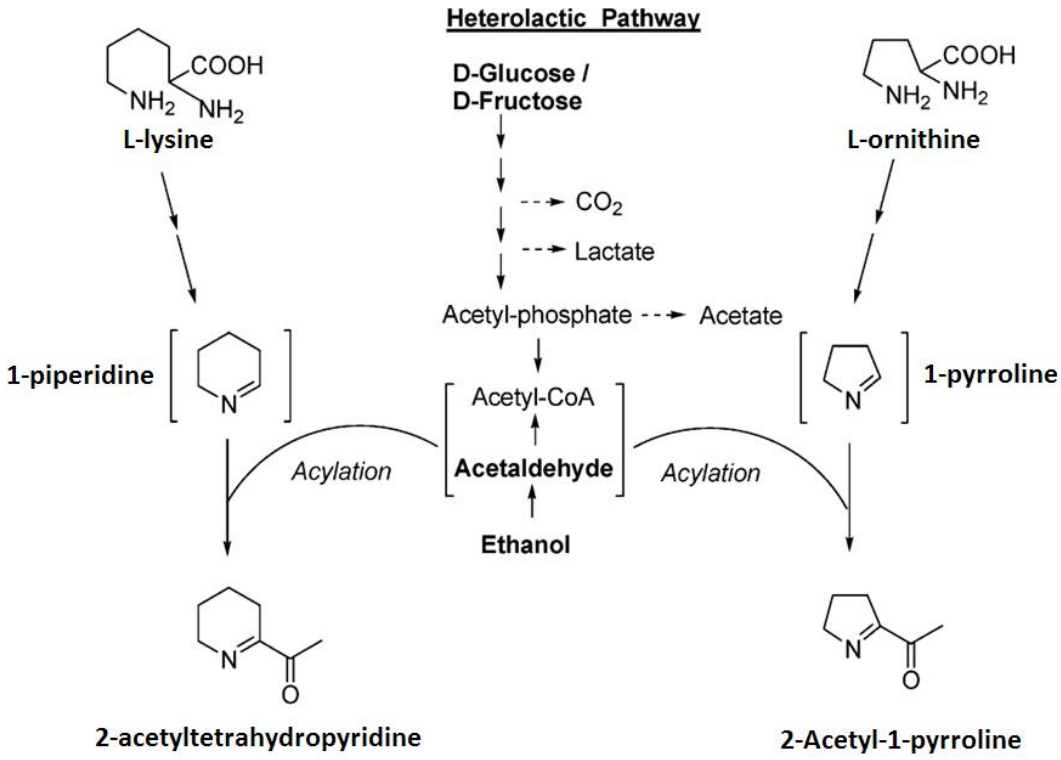

Figure 2.2: Mechanism of 2-AP formation through the heterolactic pathway (Costello & Henschke, 2002)

Pathways from putrescine to succinate *via* 1-pyrroline in *P. fluorescens* and *E. coli* (Jacoby & Fredericks, 1959; Kim, 1964) have been reported. Putrescine is the decarboxylation product of ornithine (Fothergill & Guest, 1977); hence, 1-pyrroline could be formed through the putrescine pathway from L-ornithine. Due to the presence of carbohydrates, such as ethanol and glucose/fructose, acetyl-CoA accumulated through the heterolactic pathway and reacted with

intermediates 1-pyrroline and 1-piperideine to form 2-AP and 6-acetyl-1,2,3,4-tetrahydropyridine, respectively.

Adams and De Kimpe (2007) reproduced the work of Romanczyk et al. (1995) and suggested *B. cereus* formed 2-AP by enzymatic acetylation of 1-pyrroline. 1-Pyrroline was formed from the degradation of ornithine and proline, as proposed by Costello and Henschke (2002; see above and **Figure 2.2**).

2.4. Formation of 2-Acetyl-1-pyrroline through the Maillard Reaction

2-AP is not only present in raw food, like rice and pandan leaf, but is also formed in many cooked products. Therefore, the Maillard reaction is also an important route to 2-acetyl-1-pyrroline. Schieberle (1988) tested model systems containing proline, lysine, and alanine, and indicated that, when heated with reducing sugars, only proline could form 2-AP. Two ^{13}C-labelling experiments were then designed: in the first experiment, 1-^{13}C-proline reacted with unlabelled glucose, and in the second experiment, unlabelled proline reacted with U-^{13}C-glucose (all six carbon atoms labelled with ^{13}C). Both experiments were carried out at 170 °C for 30 min. In both experiments labelled carbon was only found in the acetyl group of 2-AP and much more ^{13}C was detected in the second experiment, which indicated that glucose could provide the acetyl group in 2-AP formation (Schieberle, 1989).

Rewicki et al. (1993) reacted unlabelled proline with 1-^{13}C-glucose, and noted the formation of a 1:1 mixture of unlabelled and labelled 2-AP. A hypothetical mechanism based on the labelling experiment is shown in **Figure 2.3a**. Two isomers of 1-deoxy-2,3-glucosone form in a ratio of 1:1 from the labelled sugar. They are converted to the dihydro form of diacetylformoin, which reacts with 1-pyrroline, to form 2-acetylpyrrolidine, which then oxidises to 1:1 labelled and non-labelled 2-AP (Rewicki et al., 1993). The 1:1 ^{13}C label was due to the 1:1 ratio of the reducing

sugar fragments. The ^{13}C from labelled proline did not exist in the final 2-AP product; supporting the theory that 2-AP is formed by acylation of 1-pyrroline by a two-carbon sugar fragment.

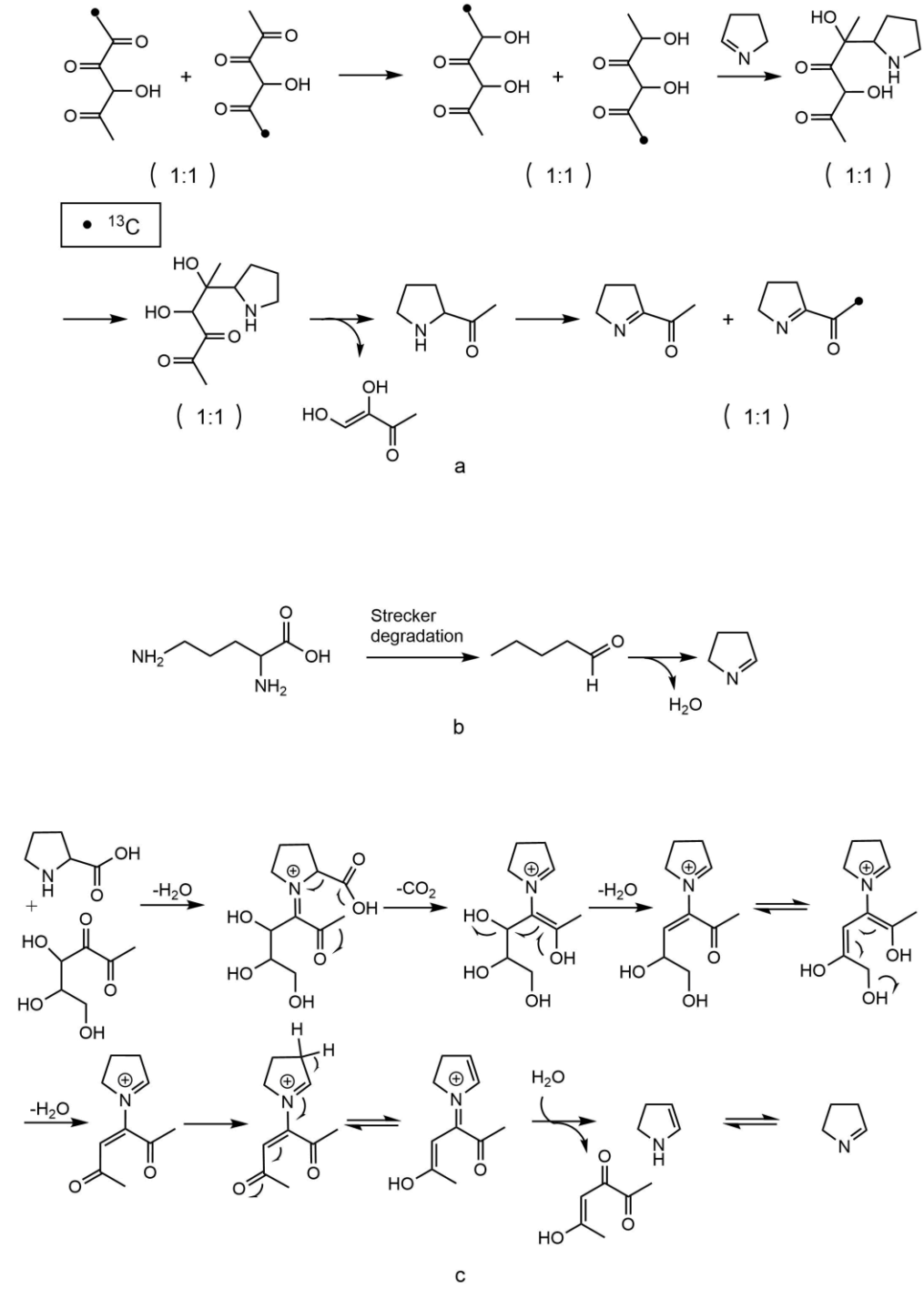

Figure 2.3: 2-AP Maillard Reaction formation pathways: (a) hypothetical formation of ^{13}C-labelled and unlabelled 2-AP from 1-pyrroline and ^{13}C-glucose, based on labelling studies (Rewicki et al., 1993); (b) hypothetical formation of 1-pyrroline from ornithine (Schieberle, 1990); (c) hypothetical formation of 1-pyrroline from proline and 1-deoxyosone (Schieberle, 1995).

Strecker degradation of proline can generate 1-pyrroline by α-dicarbonyl compounds catalysation, with intermediates such as methylglyoxal (2-oxopropanal) and deoxyosone compounds (Adams & De Kimpe, 2006). Schieberle (1990) showed that both proline and ornithine could react with methylglyoxal to form 2-AP, and there was a higher yield with ornithine than with proline. **Figure 2.3b** shows the hypothetical formation of 1-pyrroline *via* Strecker degradation of ornithine; both ornithine and citrulline, another amino acid, can generate γ-aminobutyraldehyde and finally result in 1-pyrroline. High levels of proline are present in plants, especially cereals, but ornithine can rarely be detected in plants. Schieberle (1995) also indicated that 155 mg/kg of free proline can be detected in freeze dried maize but ornithine was not detectable (<5 mg/kg). Hence, further 2-AP thermal generation researches more focused on the formation from proline.

Hofmann and Schieberle (1998a) indicated that the reaction pathway proposed by Hodge, Mills and Fisher (1972) between proline and methylglyoxal can not only result in 6-acetyl-1,2,3,4-tetrahydropyridine (ATHP), but also form the important intermediates 1-pyrroline and 1-hydroxy-2-propanone. Schieberle (1995) hypothesised a mechanism of 1-pyrroline formation from proline and 1-deoxyglucosone through Strecker degradation (**Figure 2.3c**). This reaction starts with the formation of an iminium ion. After decarboxylation and water elimination, 1-pyrroline can be generated from hydrolysis of the iminium ion. Hofmann and Schieberle (1998a) also proposed that proline and 1-deoxyglucosone can generate 1-pyrroline, glyceraldehyde and 1-hydroxy-2-propanone at the first stage and methylglyoxal is yielded through loss of water from glyceraldehyde.

As the important intermediate, 1-pyrroline can generate 2-AP with methylglyoxal and generate 6-acetyl-1,2,3,4-tetrahydropyridine with 1-hydroxy-2-propanone. Hofmann and Schieberle (1998b) emphasised the importance of 2-acetylpyrrolidine in 2-AP generation. 1-Pyrroline and methylglyoxal formed 2-acetylpyrrolidine *via* a number of steps, and then 2-acetylpyrrolidine was readily oxidised to 2-AP (Hofmann & Schieberle, 1998a). It was found that

in a 1-pyrroline and methylglyoxal model system, a higher ratio of methylglyoxal:1-pyrroline could result in a higher 2-AP yield (Hofmann & Schieberle, 1998a).

Therefore, 2-AP formation in a proline/reducing sugar model system through the Maillard reaction can be concluded from the above research (**Figure 2.4**). Proline can react with sugar degradation products, either methylglyoxal (Hodge, Mills, & Fisher, 1972; Schieberle, 1990; Hofmann & Schieberle, 1998a) or 1-deoxyglucosone (Schieberle, 1995; Hofmann & Schieberle, 1998a), to form several intermediates, including 1-pyrroline, which can generate 2-AP with methylglyoxal *via* 2-acetylpyrrolidine oxidation and generate ATHP with 1-hydroxy-2-propanone. The ratio of precursors in the proline/methylglyoxal system can significantly influence the yield of products; more 2-oxopropanal than proline in this reaction can lead to more 2-AP formation, whereas more proline than methylglyoxal can result in more ATHP (Hofmann & Schieberle, 1998a).

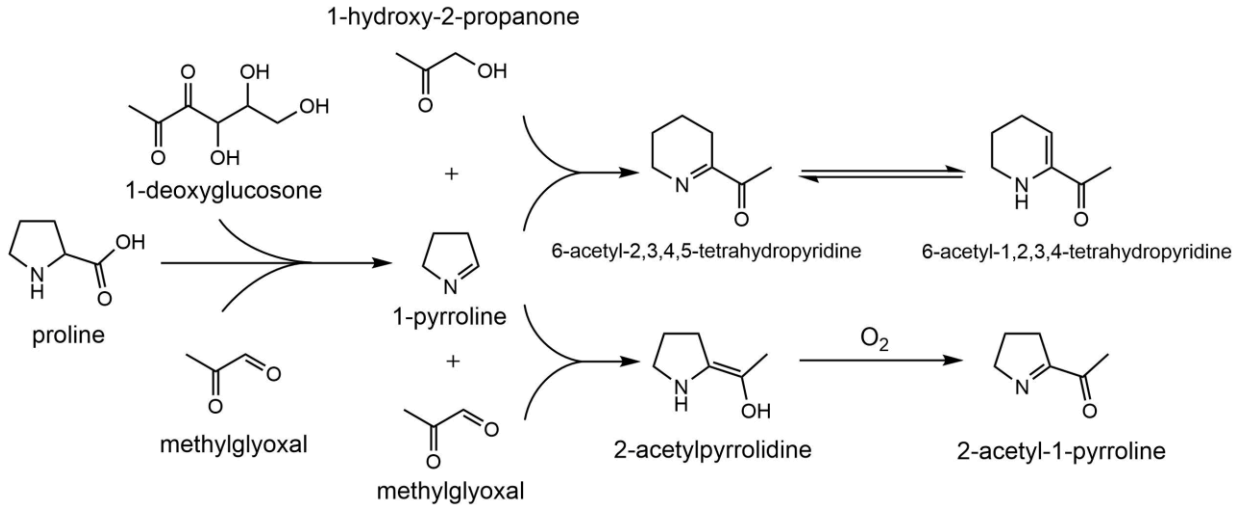

Figure 2.4: Formation of 2-AP and ATHP from proline and α-dicarbonyl compounds (2-oxopropanal or 1-deoxyglucosone) via 1-pyrroline (Hofmann & Schieberle, 1998a).

The involvement of water can lead to higher 2-AP yield. Hofmann and Schieberle (1998a) proposed that hydrated methylglyoxal, which was formed in aqueous conditions, could be the reason for higher 2-AP yield. This hydrated compound attacked carbon-2 of 1-pyrroline to form

the *N*-analogous reductone structure; after further oxidation and hydration, 2-acetylpyrrolidine was formed by carbon dioxide loss and then this compound was oxidised to 2-AP (**Figure 2.5a**). When methylglyoxal and 1-pyrroline reacted without water involvement, the pathway is shown in **Figure 2.5b**. They proposed that this reaction started from tautomeric 2-pyrroline attacking carbon-1 in non-hydrated methylglyoxal. After hydration and pyrroline ring opining, a carbon atom is lost with formamide generation. Then further reaction can lead to 2-acetylpyrrolidine and finally oxidation to 2-AP. The authors also indicated that those hypotheses needed to be further verified by several carbon labelling experiments (Hofmann & Schieberle, 1998a). The above hypothetical reaction pathways were proposed according to the observation of reactions in model systems; the reaction conditions and observations during these experiments are summarised in **Table 2.4**.

Phosphate ion could significantly increase the yield of 2-AP, through increased formation of methylglyoxal *via* 1,3-dihydroxyacetone phosphate (Schieberle, 1989). If malonate buffer replaced phosphate buffer, there was a one-third reduction of 2-AP formation (Schieberle, 1995). Blank, Devaud, Matthey-Doret, and Robert (2003) examined the effect of pH and heating time on the formation of various Maillard-derived compounds in two phosphate-buffered model systems: one an equimolar mixture of proline and glucose, the other the Amadori compound fructose-proline. 2-AP yield was similar in both systems across all treatments and was shown to increase with increasing pH and heating time, when samples were refluxed for 1, 2 and 4 hours at pH 6, 7 and 8.

From the above hypothesised mechanisms of 2-AP thermal formation, it is agreed that 2-AP is formed through an acylation of 1-pyrroline. Certain amino acids, i.e., proline, ornithine and citrulline, reacting with 2-oxpropanal from reducing sugar fragmentation, are the most important intermediates of 2-AP formation during the Maillard reaction (Adams & De Kimpe, 2006).

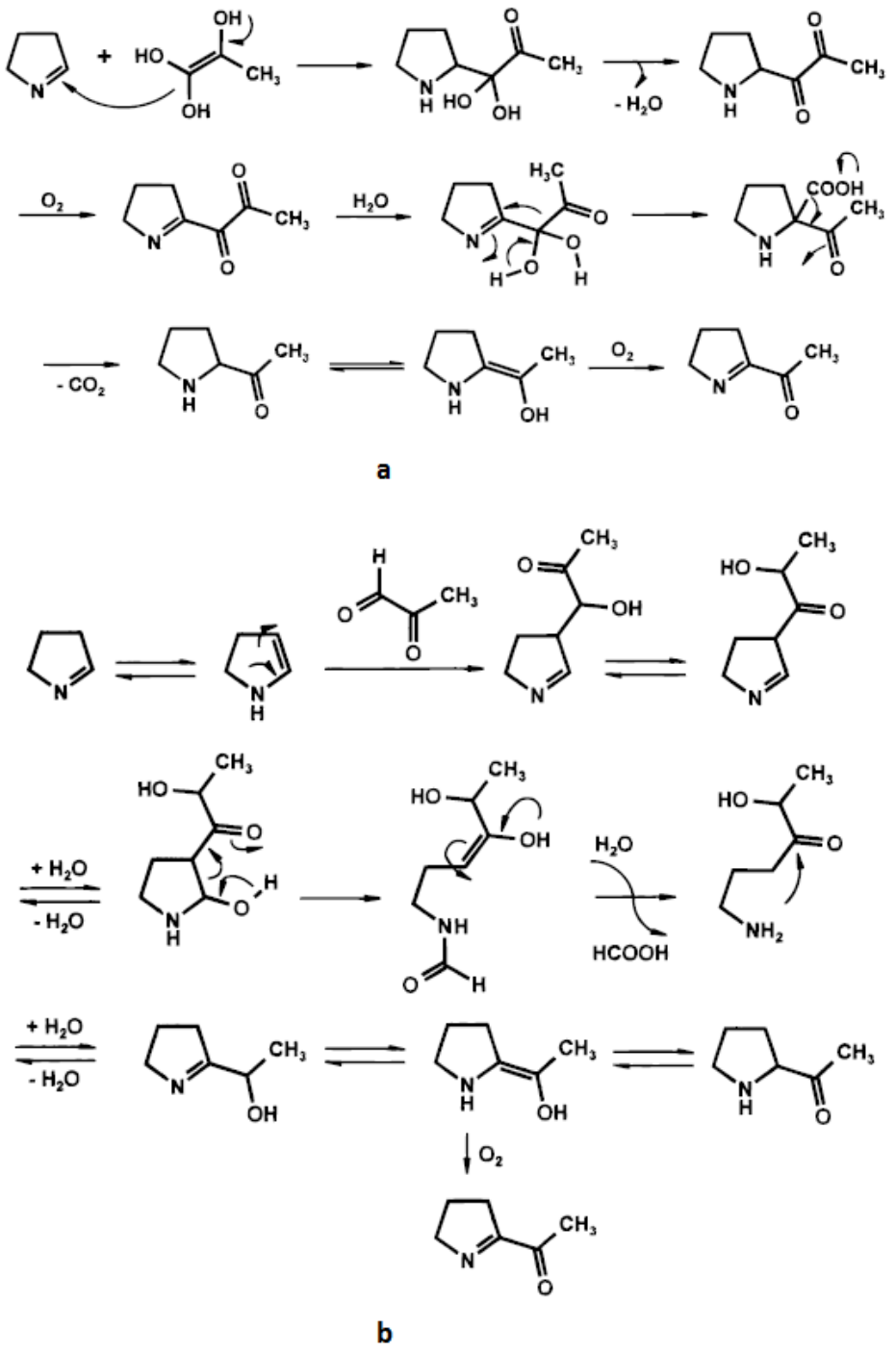

Figure 2.5: 2-AP Maillard Reaction formation pathways from 1-pyrroline: (a) 1-pyrroline reacts with methylgloxal hydrate; (b) 1-pyrroline reacts with methylgloxal without water involvement (from Hofmann & Schieberle, 1998a).

Table 2.4: Summary of hypothetical 2-AP Maillard reaction pathways

reaction pathway	experimental conditions	experimental observations	hypothesis based on experiment	authors	year
Figure 2.3a	Proline and labelled glucose heated in model system. Reaction conditions were absent.	1:1 labelled and unlabelled 2-AP were detected from the reaction of proline and labelled glucose	Two isomers of 1-deoxy-2,3-glucosone gave labelled carbon in two different positions, and caused the detection of 1:1 labelled and unlabelled 2-AP 2-AP is formed by acylation of 1-pyrroline by a two-carbon sugar fragment	Rewicki et al.	1993
Figure 2.3b	Amino acids and methylglyoxal boiled in water for 30 min at 100 °C during SDE	Only proline and ornithine among 11 amino acids reacted with methylglyoxal to form 2-AP	formation of 1-pyrroline from ornithine through GABald	Schieberle	1990
Figure 2.3c	————	De Kimpe et al. (1994) cannot generate ATHP from the key intermediate N-acetonyl-4-aminobutanal from Hodge et al. (1972) pathway	pathway modified from Hodge et al., 1972 without N-acetonyl-4-aminobutanal as intermediate	Schieberle	1995
Figure 2.4	1-Pyrroline and 1-hydro-2-propanone reacted for 30 min at 100 °C in phosphate buffer	ATHP can be generated from the reaction of 1-pyrroline and methylglyoxal	Reaction of proline and 1-deoxyglucosone can generate the key intermediates 1-hydroxy-2-propanone, 1-pyrroline and methylglyoxal and finally lead to the generation of 2-AP and ATHP	Hofmann and Schieberle	1998
	1-Pyrroline and methylglyoxal reacted for 30 min at 100 °C in phosphate buffer	2-AP can be generated from the reaction of 1-pyrroline and methylglyoxal			
	Proline and methylglyoxal reacted for 30 min at 100 °C in phosphate buffer	2-AP and ATHP were detected in the reaction of proline and methylglyoxal	Reaction of proline and methylglyoxal can generate the key intermediates 1-hydroxy-2-propanone and 1-pyrroline and finally lead to the generation of 2-AP and ATHP		
	Proline and glucose reacted for 30 min at 100 °C in phosphate buffer and for 10 min at 160 °C in silica gel	2-AP and ATHP were detected in the reaction of proline and glucose			
Figure 2.5	1-Pyrroline and methylglyoxal reacted for 30 min at 100 °C in phosphate buffer or for 5 min at 180 °C in silica gel in the presence of phosphate buffer	More 2-AP can be generated in hydrated conditions than in dry conditions from the reaction of 1-pyrroline and methylglyoxal	Higher yield of 2-AP due to the hydration of methylglyoxal	Hofmann and Schieberle	1998

2.5. Synthesis of 2-Acetyl-1-pyrroline

The first synthesis of 2-AP was reported by Buttery et al. (1983) and is shown in **Figure 2.6a**. This method is based on an earlier synthesis of a six-membered ring compound 6-acetyl-1,2,3,4-tetrahydropyridine (Büchi & Wüest, 1971). However, the yield of 2-AP from this reaction was only around 10%.

The first large-scale method for 2-AP synthesis was developed in 1993. Methyl prolinate is oxidised to 2-(methoxycarbonyl)-1-pyrroline, which then reacts with methylmagnesium iodide in a Grignard reaction. However, this is not a completed reaction with a 45–83% yield and 8–39% starting material in the final product (De Kimpe, Stevens, & Keppens, 1993). Methyllithium in ether also converted 2-(methoxycarbonyl)-1-pyrroline into a mixture of 2-acetyl-1-pyrroline (47%) and a side-product, 2-(1-hydroxy-1-methylethyl)-1-pyrroline (32%) (**Figure 2.6b**). To prevent formation of this side-product, which was also formed in the methylmagnesium iodide reaction, a cyanide functional group can replace the ester (**Figure 2.6c**). In this modified method, the reaction started with oxidation of pyrrolidine to tripyrroline. The tripyrroline was hydrocyanated into 2-cyanopyrrolidine, which can form 2-cyano-1-pyrroline through the Grignard reaction. 2-Cyano-1-pyrroline can form 2-AP with a yield of 60% when treated with methylmagnesium iodide (De Kimpe et al., 1993).

Over subsequent years, other synthesis methods focused on the stabilisation of 2-AP during the reaction, by using protected carbonyl groups, e.g., Duby and Huynh-Ba (1993), or amino groups, e.g., De Kimpe and Keppens (1996). De Kimpe and Keppens (1996) used diacetyl as a starting material to generate an α-diimine, which then reacted with a stabase derivative to form the 1-pyrroline ring structure (**Figure 2.6d**). Another synthesis method applied the high substrate selectivity of immobilised penicillin G acylase (PGA) as the catalyst in the last reaction step (Favino, Fronza, Fuganti, Fuganti, Grasselli, & Mele, 1996). 1-Aminohex-4-yne reacted with phenylacetyl chloride, and then ozone oxidised the product to form 1-[*N*-(phenylacetyl)amino]-

4,5-dioxohexane, which when treated with PGA could form 2-AP spontaneously, as shown in **Figure 2.6e**. An 80% yield of 2-AP could be achieved using this method.

A four-step synthesis was reported by Hofmann and Schieberle (1998a), starting from *N*-*tert*-butoxycarbonyl-protected proline, while 2-pyrrolidinone was selected as a raw material for 2-AP synthesis by Harrison and Dake (2005). Another 'popcorn' compound, 6-acetyl-1,2,3,4-tetrahydropyridine, was a by-product of this latter method. A three-step synthesis was reported by Fuganti, Gatti, and Serra (2007), starting from the reaction of *N*-Boc-pyrrolidinone with ethylmagnesium bromide. The yield of 2-AP was only 20–30% but with 98% purity. Maraval et al. (2010) formed *N*,5-diacetylpyrrolidin-2-one from L-glutamic acid and acetic anhydride with 78% yield. Sodium carbonate was used for deacetylation to form 5-acetylpyrrolidin-2-one; then lithium aluminium hydride was used for reduction to form 2-(1-hydroxyethyl)-pyrrolidine, which was oxidised by silver carbonate to 2-AP. The overall yield of 5-acetylpyrrolidin-2-one formation was 37% but the yield of 2-AP was not reported.

A recent publication reported the synthesis of three major popcorn-like Maillard aroma compounds, 2-acetyl-1-pyrroline, 6-acetyl-1,2,3,4-tetrahydropyridine and 2-acetyl-5,6-dihydro-4*H*-1,3-thiazine (Deblander, Van Aeken, Adams, De Kimpe, & Abbaspour Tehrani, 2015). The authors noted that existing synthetic procedures for these compounds suffered a number of problems, including extensiveness of some reaction pathways, and the use of costly and/or harmful reagents. The 2-AP synthesis they proposed started from *N*-Boc-prolinate to give a final yield of 2 AP of 28%, *via* a four-step reaction (**Figure 2.6f**). The authors considered this method to be relatively straightforward, as the starting materials were readily available, only one general procedure was involved and the vinyl ether intermediate prepared was a stable precursor, which could be readily converted to 2-AP.

Although numerous procedures have been published for the synthesis of 2-AP, these methods all require an experienced organic chemist to make them work. Synthesis of 2-AP is

difficult, due to the unstable nature of this compound, which degrades very rapidly upon standing (see Part 6). This is reflected in the high price of commercial 2-AP and the small number of 2-AP suppliers.

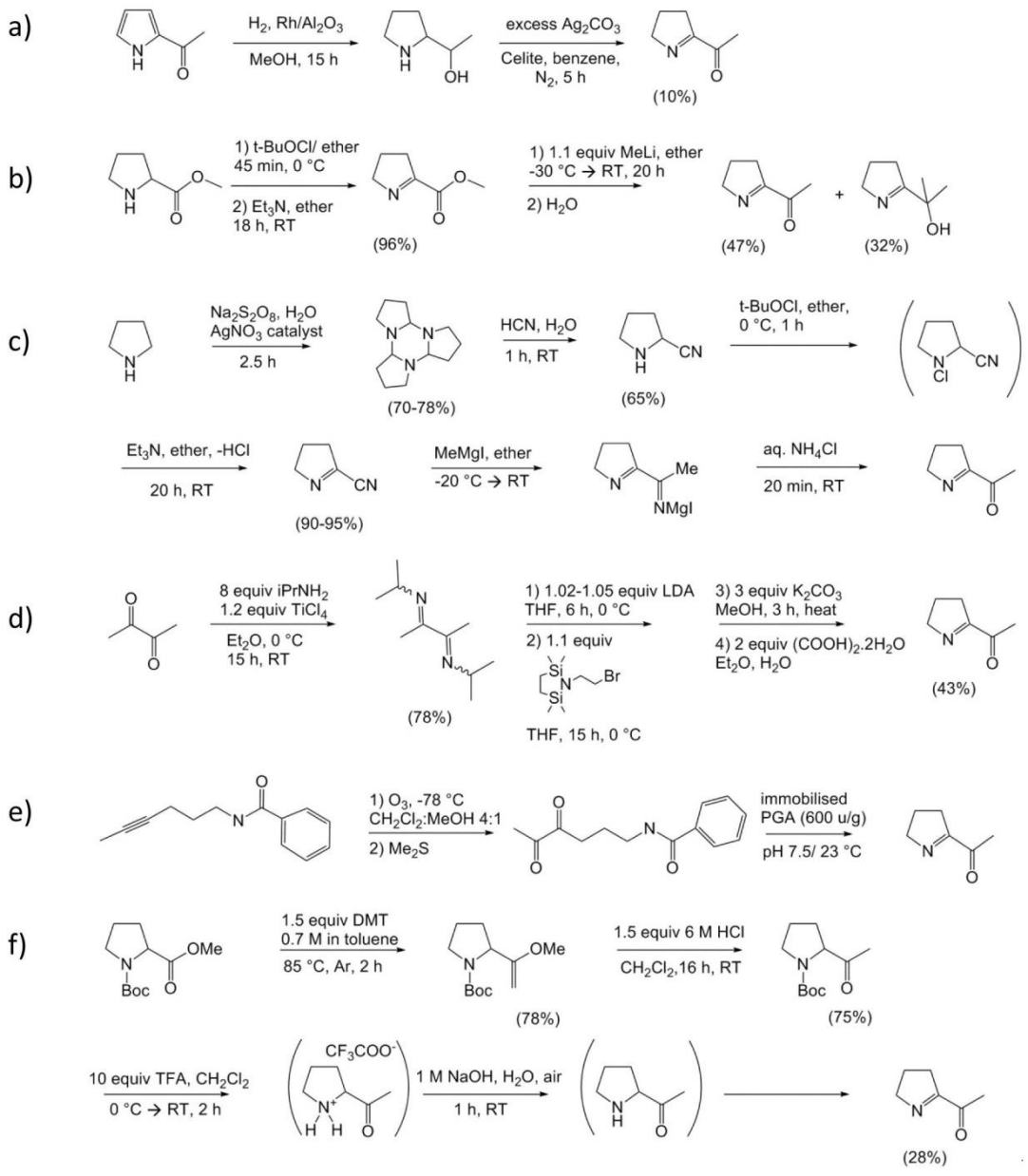

Figure 2.6: 2-AP synthesis strategies: (a) Buttery et al., 1983; (b) De Kimpe et al., 1993; (c) De Kimpe et al., 1993; (d) De Kimpe & Keppens, 1996; (e) Favino et al., 1996; (f) Deblander et al. (2015). LDA: lithium diisopropylamide; THF: tetrahydrofuran; PGA: immobilised penicillin G acylase; DMT: dimethyltitanocene; TFA: trifluoroacetic acid.

2.6. Stability and Stabilisation of 2-Acetyl-1-pyrroline

Stability is very important for a flavour compound in food products. Unfortunately, 2-acetyl-1-pyrroline has limited stability. Pure 2-AP will turn red and degrade within 10 min at room temperature (Fang & Cadwallader, 2014). This instability of 2-AP was noticed when it was first identified by Buttery et al. (1982), and this instability was assumed to be due to polymerisation. Loss of 2-AP in stored foods could be due to complexation, decomposition, diffusion to the environment and generation of other compounds (Adams & De Kimpe, 2006).

An experiment was designed to investigate the effect of storage on 2-AP levels in rice (Widjaja et al., 1996a). In this experiment, using the fragrant cultivar YRF9, paddy (rice with husk and rice bran), brown (rice without husk, but with rice bran) and white (rice without husk and rice bran) rice samples were stored under two conditions: atmospheric pressure and reduced pressure, at 84% RH and 30 °C. After three months' storage, 2-AP level was reduced by 40–50% in all cases. Another study aimed to compare the effect of different drying methods and storage time on 2-AP reduction in fragrant rice (Wongpornchai et al., 2004). Six different drying methods (sun drying, 30 °C modified air, 40 °C modified air, 40 °C air, 50 °C air and 70 °C air) were applied to fresh paddy rice, to reduce moisture content from 28% to below 14%, and then the rice was stored in gunny sacks at 20–35 °C. 2-AP concentration in rice stored for 10 months was only 25% of that in freshly dried rice and it was shown in a concentration–storage time curve that a significant decrease occurred at the beginning of storage. The sun-dried sample retained less 2-AP than the other drying methods; this could be due to the longer drying time. Sun drying took 54 hours in this study, while the average time for the other drying methods was 10 hours. Although the authors did not provide details of the modified air used, drying with this kind of air maintained 2-AP in rice better than normal hot air drying, while lower air temperatures also resulted in less 2-AP loss (Wongpornchai et al., 2004).

In addition to rice, 2-AP decreases in other food products during storage. Hot air popped popcorn was sealed in commercial polyethylene food bags and stored in the dark at room temperature. After two days, the 2-AP level reduced by 20%, and after seven days storage, it reduced by 75% (Schieberle, 1995).

Therefore, it is important to develop a stabilisation method to defer 2-AP breakdown. Encapsulation is a popular technique to protect unstable volatile compounds for commercial processing, and several studies have applied this technique. Encapsulation of 2-AP by β-cyclodextrin (Duby & Huynh-Ba, 1996) showed some success. When stored at room temperature (20 °C), 99% 2-AP decomposed after 110 days' storage, when the 2-AP load of the β-cyclodextrin was 1%. However, if the storage temperature decreased, encapsulation performed better, with 10% losses at 4 °C and no losses at –20 °C. If the loading of cyclodextrin was increased to 10%, the stability of the 2-AP was reduced.

Apintanapong and Noomhorm (2003) extracted 2-AP from pandan leaves and examined its stability at 30 mg/kg in acidic and basic solution at room temperature. They also microencapsulated 2-AP in various maltodextrin and gum acacia mixtures. In basic solution, 2-AP was reduced by 63% after 7 days, and in acidic solution by 30% after 35 days. When 2-AP was microencapsulated with 70:30 gum acacia:maltodextrin, only 28% of the encapsulated 2-AP was lost after 72 days at room temperature. Gum acacia and/or starch mixed materials were used in a patented form by Srinivas, Sulochanamma, Raghavan, and Gurudutt (2006), to form a stable 2-AP powder using spray drying, but the stability of this powder was not reported.

Fang and Cadwallader (2014) recently reported a novel stabilisation method, using zinc ions to solve 2-AP powder storage problems. Anhydrous 2-AP and ZnI_2 were added into diethyl ether to form a yellowish precipitate, which was the desired 2-AP-ZnI_2 complex. Excess ZnI_2 and other impurities were removed through dissolving the complex in anhydrous diethyl ether. The complex compound was obtained as a powder after drying through nitrogen evaporation. Other 2-

AP-zinc halide complexes could be obtained in the same way. When stored at 25 °C, there was only 6% loss of 2-AP from a 2-AP-ZnI_2 complex (2-AP content = 14.4%) after 3 months, and 3% reduction of 2-AP after 3 months when a 12.5% 2-AP content complex was stored at –20 °C. It was found that compared with ZnI_2(2-acetyl-1-pyrroline)$_n$, which had a yield of 62%, complexes of $ZnBr_2$(2-acetyl-1-pyrroline)$_n$ and $ZnCl_2$(2-acetyl-1-pyrroline)$_n$ had better yields of 96% and 86%, respectively. A $ZnCl_2$-2-AP complex would be the preferred food agent because $ZnCl_2$ has been approved for food use (CFR – Code of Federal Regulations Title 21; April 1st, 2016).

This method can also be applied to similar volatile compounds, such as 2-propionyl-1-pyrroline, 6-acetyl-1,2,3,4-tetrahydropyridine, 2-acetyl-2-thiazoline, 2-acetylthiazole, 2-acetylpyrazine and 2-acetylpyridine. Although this is an effective technique for 2-AP stabilisation compared with others, this high yield was only confirmed in a dry powdered complex. It may be reduced by moisture, temperature and other conditions when applied in foods. Therefore, it may be necessary to combine this technique with an encapsulation technique to protect the 2-AP-zinc halide complex in a changeable food environment (Fang & Cadwallader, 2014).

2.7. Extraction and Instrumental Analysis of 2-Acetyl-1-pyrroline

2.7.1. Solvent-based extraction techniques

Simultaneous distillation-extraction (SDE) was used as the extraction method when 2-AP was first discovered by Buttery et al. (1982). SDE was widely used in the 1980s and 1990s for volatile compound extraction (Chi, Yeung, & MacLeod, 1981). The sample is heated in water to produce steam and the steam transfers volatile material to a boiling non-polar solvent, which condenses to give an aroma extract (Likens & Nickerson, 1964). However, this vigorous heating process may cause volatile compound formation or breakdown. Buttery et al. (1983) reported that the boiling conditions used in this extraction may decompose 2-AP in rice and cause a lower concentration than in raw samples.

In Buttery's study, 500 g rice were extracted with 6 L water in a Likens-Nickerson type extraction equipment (Likens & Nickerson, 1964); diethyl ether was used as solvent and the isolation process was carried out at atmospheric pressure for 2 hours. After concentration, the solvent extract was dissolved in hexane and then extracted with 3 N hydrochloric acid and then ether. The ether extract was then concentrated to a small amount for analysis. For subsequent quantitative measurements (Buttery, Ling, & Mon, 1986), an internal standard (5 mL of 30 mg/kg 2,4,6-trimethylpyridine (collidine) solution) was added to the rice before extraction. Buttery's group published a number of papers on 2-AP in fragrant rice. They detected 2-AP in 10 different varieties of cooked rice (both milled and brown) using SDE and found that the concentrations of 2-AP in brown rice were much higher than in white rice (Buttery et al., 1983).

Likens-Nickerson extraction was continuously developed and used in the following decade for 2-AP extraction in rice, pandan leaf and other food samples. Addition of magnesium sulfate (MgSO4) during rice SDE inhibited starch gelatinisation, water absorption, swelling of rice and foaming of the mixture during distillation (Widjaja et al., 1996a & b). Dichloromethane (DCM) has also been used as the extraction solvent in SDE of 2-AP (Nadaf, Krishnan, & Wakte, 2006).

Rice was boiled before extraction in some studies and in others the rice was boiled during SDE. For example, when fresh and stored brown and white fragrant YRF9 rice were compared, samples were boiled during the SDE process (Widjaja et al., 1996a & b). When white Italian Line B5-3 and basmati, two fragrant rice species, were compared, they were also boiled during SDE. A 4-fold higher 2-AP concentration was found in the Italian variety than in basmati (Tava & Bocchi, 1999). Several different varieties of brown fragrant rice (Malagkit Sungsong, 370 basmati, Khashkani and an unknown *indica* cultivar) were boiled for 25 min in tap water before SDE analysis. 2-AP was found in all four species but the concentration in the *indica* was much lower than in the others (Jezussek, Juliano, & Schieberle, 2002). 2-AP was also detected in five boiled fragrant rice cultivars; four of them were white rice (Hyangmibyeo 1, Hyangmibyeo 2, Royal,

Golden Elephant) and one a Korean black rice called Goemjeongssal. Boiled non-fragrant rice Jeongilpum also contained 2-AP. Those six cultivars were boiled 30 min with distilled water (Yang, Shewfelt, Lee, & Kays, 2008). Three white fragrant cultivars (Aychade, Fidji, and Giano) and one white non-fragrant cultivar (Ruille) were boiled for 20 min. 2-AP was found in all four cultivars, but the concentration in Ruille was too low to quantify; it was lower than 2 μg/kg while the concentrations in the other fragrant cultivars were 150–300 μg/kg (Maraval et al., 2008).

When using SDE at atmospheric pressure, sample and water mixture are boiled. Therefore, this kind of extraction technique cannot be used to study uncooked foods. Buttery et al., when first identifying 2-AP, used simultaneous distillation/extraction under vacuum (V-SDE) to study rice aroma. However, compared to SDE at atmospheric pressure (A-SDE), V-SDE showed a low efficiency of extraction (Buttery et al., 1983). Levels of 2-AP in rice extracted by A-SDE were 10 times higher than in rice that was cooked and then extracted with V-SDE. The authors suggested that most of the 2-AP may be lost during cooking. Therefore, compared with A-SDE, where rice is cooked during the isolation process, less 2-AP is present in the already cooked sample in V-SDE. In addition, 2-AP may be generated during cooking, which can also cause the significant difference in concentrations obtained between V-SDE and A-SDE.

Another solvent-based extraction technique, solvent-assisted flavour evaporation (SAFE), first introduced in 1999 (Engel, Bahr, & Schieberle, 1999), is a useful technique for 2-AP extraction. The volatile compounds in a solvent extract, usually in diethyl ether or dichloromethane, are removed from non-volatile material using high-vacuum distillation. The procedure takes place at around 30 °C, keeping sample decomposition to a minimum. When using 1:1 diethyl ether:dichloromethane as the solvent, 2-AP was isolated from cereal coffee brew (Majcher et al., 2013) and this compound was also isolated from hazelnut when using diethyl ether as solvent (Kiefl et al., 2013).

Solid-phase extraction (SPE) can also be applied for 2-AP extraction. Several commercial SPE cartridges (Strata™ X from Phenomenex, LiChrolut® EN from Merck Millipore and Isolute® ENV+ from Biotage) have been successfully used for volatile compound extraction (Du & Qian, 2008; Metafa & Economou, 2013), particularly for isolation of relatively polar aroma compounds, such as 2-AP. An advantage of SPE is that no heating is applied when using this technique, which is the same as SAFE, but SPE is much easier to perform than SAFE. 2-AP was generated during high-temperature cooking of fragrant rice (180 °C for 20 min in an open system), and extracted by SPE, followed by GC-MS (Handoko, 2014). This result suggests that there may be a component of fragrant rice that is formed enzymatically, which can be converted to 2-AP by the application of higher temperatures. Although there was no 2-AP detected in Ciherang rice (a non-fragrant rice) heated under the same conditions, a sensory panel perceived popcorn-like odour (Handoko, 2014), suggesting that compounds besides 2-AP could generate popcorn-like odour in rice heated at 180 °C.

2.7.2. Headspace techniques

Dynamic headspace extraction using an adsorbent polymer such as Tenax can also be used in 2-AP extraction, Buttery, Turnbaugh, and Ling (1988) used this technique to analyse the volatile compounds in cooked rice. Seventeen volatile compounds including 2-AP were identified through this method. Around 0.6 µg/kg 2-AP was found in white Californian long-grain rice (a kind of non-fragrant rice) boiled in water for 20 min before Tenax trapping (Buttery et al., 1988). Around 30 odorants were identified in cooked rice using the same technique (Yang et al., 2008).

Headspace solid-phase microextraction (HS-SPME) is now the most widely used extraction method for 2-AP. As with all extraction techniques, increased extraction time or higher temperatures could result in better release of 2-AP from the sample. However, the reported instability of 2-AP may cause its loss during isolation at higher temperatures, while at lower

temperatures enzymatic changes may occur during this extraction. These conflicting reactions make 2-AP quantification difficult.

One large study used manual SPME to analyse 91 different uncooked cultivars, including 77 non-basmati fragrant cultivars, 9 basmati fragrant cultivars and 5 non-fragrant cultivars. A 1-cm divinylbenzene/Carboxen/polydimethylsiloxane (DVB/CAR/PDMS) fibre was used in this experiment. Samples were extracted for 15 min at 80 °C after a 30-min equilibration period. 2-AP was detected in some non-fragrant cultivars but its average concentration was around 10-fold higher in basmati fragrant cultivars and around 20-fold higher in selected non-basmati fragrant cultivars (Mathure, Wakte, Jawali, & Nadaf, 2011; Mathure, Jawali, Thengane, & Nadaf, 2014). Bryant and McClung (2011) used an automated SPME system to compare seven uncooked fragrant and two uncooked non-fragrant rice samples extracted with a 1-cm DVB/CAR/PDMS SPME fibre at 80 °C for 18 min after a 5-min equilibration period. 2-AP was only found in the fragrant rice samples.

A recent study measured 2-AP in 48 fragrant rice samples using manual SPME, followed by GC-MS/MS. The ion transition from *m/z* 111 to *m/z* 82 was used to quantify 2-AP. Optimised conditions were 10 minutes extraction at 40 °C after a 5-min incubation period. The technique was sensitive enough both to quantify 2-AP below its odour threshold concentration (Buttery et al., 1983) and to obtain successful 2-AP measurements using a single grain of rice (Hopfer et al., 2016). Although DVB/CAR/PDMS fibres performed better than DVB/PDMS fibres, the latter was preferred, because carry-over (the presence of volatile material from the fibre in a subsequent blank analysis) observed when using the DVB/CAR/PDMS fibre was not observed with the DVB/PDMS fibre. A limit of quantification of 103 ng per kg was reported for 2-AP in this work.

2.7.3. Gas chromatography-mass spectrometry

Gas chromatography-mass spectrometry (GC-MS) is the most common technique used for volatile compound analysis. Column choice in GC-MS analysis of 2-AP is very important. A polar phase (e.g., Carbowax) is normally chosen; 2-AP is a relatively polar compound and its peak shape is more symmetrical and sharper on a polar column. The use of base-deactivated phases is recommended for basic compounds that may possess poor peak shape under normal GC conditions (De Zeeuw, Stricek, & Stidsen, 2011). A base-deactivated column may also be useful for quantifying 1-pyrroline (Poonlaphdecha et al., 2016). Because of the instability of 2-AP, Buttery et al. (1986) recommended a relatively low injector temperature of 150–170 °C to minimise its decomposition. Base-deactivated injection port liners may also have a protective role (De Zeeuw et al., 2011).

When quantifying 2-AP using GC-MS, separation of 2-AP from other compounds is a common challenge. This interference problem was first reported by Paule and Powers (1989) when using a packed column coated with 10% Carbowax 20M on Chromosorb® W; 1-hexanol eluted very close to 2-AP and could interfere in its quantification. The mass spectrum of 6-methyl-5-hepten-2-one contains all the major ions present in the mass spectrum of 2-AP (*m/z* 43, 41, 111, 83, 68, 69); these two compounds often co-elute in fragrant rice extracts run on polar columns, affecting the quantification of 2-AP, especially when the concentration of 2-AP is similar to or lower than that of 6-methyl-5-hepten-2-one. A long isothermal stage of 65 °C for 70 min at the start of the GC run was reported by Tanchotikul and Hsieh (1991) when performing sample analysis by GC-MS using a 60-m length Supelcowax® 10 (Supelco, Bellefonte, PA) column. A similar method was reported by Seitz et al. (1993), to obtain better separation of 2-AP and 6-methyl-5-hepten-2-one. They used a shorter, 30 m Supelcowax 10 column in this analysis at an initial temperature of 60 °C for 15 min.

Although electron ionisation (EI) is the usual ionisation mode used for GC-MS, chemical ionisation (CI) is an option. CI is a softer ionisation technique, which can reduce interference during MS analysis compared with EI, and hence could result in increased signal-to-noise ratio for compounds of interest. In a study on bread flavour by Schieberle and Grosch (1987), 2-AP was analysed in CI mode using isobutane as the reagent gas. Compared with EI mode in GC-MS analysis, Maraval et al. (2010) reported that positive ion CI mode could be better for 2-AP quantification in rice, especially when MS-MS was applied for analysis. The EI mass spectrum of 2-AP possesses few defining peaks: a characteristic ion at m/z 83 and a less intense molecular ion at m/z 111. However, in PCI mode, using acetonitrile as the reagent gas, only an intense pseudomolecular ion at *m/z* 112 was observed. Under MS-MS conditions, *m/z* 112 ion yielded a fragment ion at *m/z* 70 and this transformation was used for 2-AP quantification, with a low limit of quantification of 0.4 µg/kg.

2.7.4. Quantification of 2-acetyl-1-pyrroline

When Buttery et al. (1983) first quantified 2-AP in rice they measured peak areas by flame ionisation detector and performed an approximate quantification, in order to determine the relative amounts of 2-AP in the 16 types of rice that they analysed. In subsequent work they used collidine (2,4,6-trimethylpyridine) as an internal standard, adding it in solution to the rice/water mixture prior to extraction. Collidine was chosen because it has similar physicochemical properties to 2-AP (basic, similar water solubility, similar volatility), is stable, has a GC retention time similar to 2-AP on a wax column and is commercially available (Buttery et al., 1986). Known amounts of 2-AP were added to the rice prior to extraction alongside a fixed amount of collidine, in order to provide a calibration curve for quantification. Collidine was subsequently used as the internal standard in a number of papers where 2-AP was quantified in rice (Tanchotikul & Hsieh, 1991; Widjaja, et al. 1996a & b; Tava & Bocchi, 1999; Bergman et al., 2000).

Stable isotope dilution assays (SIDA) are now widely used in flavour science. An isotopomer of the compound of interest is added to the sample under study, in order to permit accurate quantification of the compound of interest. The quantification of 2-AP by SIDA was carried out for the first time by Schieberle and Grosch (1987). They prepared a 2-AP analogue, which was partially deuterated in the heterocyclic ring, giving a product with a range of molecular masses from 113 to 116. They then used the deuterated isotopomer to quantify 2-AP in wheat and rye bread.

SIDA was used to measure 2-AP in rice for the first time by Yoshihashi et al. (2004). Instead of deuteration, a ^{13}C atom was introduced in the methyl position of the acetyl side-chain giving an isotopomer with a mass of 112. Naturally-occurring 2-AP has an M+1 ion with a mass of 112, which has 7% of the intensity of its molecular ion. It is not clear if this was considered by the authors in their calculations. This issue was highlighted by Maraval et al. (2010), who used SPME with deuterated 2-AP to quantify 2-AP in rice. Unlike Schieberle and Grosch (1987), the deuteration was defined. Deuterium-hydrogen exchange can occur in aqueous solution and to reduce the chances of this happening the authors replaced both hydrogen atoms at the 5-position of the heterocyclic ring with deuterium. In order to provide a calibration curve for quantification, ground rice from a non-fragrant rice cultivar were spiked with nine different amounts of 2-AP in solution.

The key reason for performing SIDA is that several steps of enrichment of the compounds can be performed without losses in accuracy, provided that the initial ratio between the compound and its labelled analogue remains unchanged during the entire procedure (Schieberle & Grosch, 1987). As the compound of interest and its isotopomer should have the same physicochemical properties, SIDA provides a degree of confidence that is lacking when other internal standards are used. The incompletely deuterated 2-AP synthesised by Schieberle and Grosch is now available

commercially from aromaLAB GmbH (Planegg, Germany) and has been used to quantify 2-AP in rice (Hopfer et al., 2016).

2.8. Sensory Evaluation of 2-Acetyl-1-pyrroline

2-AP is described as a popcorn-like odour compound and it has a very low odour threshold. When Buttery et al. (1983) first identified this compound, they ranked the amount of popcorn-like odour in different rice varieties. Malagkit Sungsong, a kind of Philippine fragrant rice, had the greatest popcorn aroma and Texas long-grain was determined as the rice with the least popcorn aroma. The most famous fragrant rice, basmati, was ranked in the middle of this list. When the Malagkit Sungsong was compared with Calrose (a non-fragrant rice), it was easy to distinguish them. However, when a 2-AP solution was added to the Calrose rice, they became much more difficult to tell apart. It was clear that the popcorn aroma of 2-AP is a key component of rice flavour.

Lexicons of aroma and flavour of rice are being continuously developed by researchers (Goodwin et al., 1996; Piggott, Morrison, & Clyne, 1991; Yau & Liu, 1999). When comparing these studies, some descriptors are similar, but some are different; it is difficult to estimate which research has an intact lexicon and which needs more development. The choice of descriptors depends on the culture and familiarity with the sample of the panellists in each study (Paule & Powers, 1989). A study aiming to build an intact lexicon tested 36 different varieties of rice, which were mainly jasmine and basmati rice samples from different regions, but also included many other fragrant and non-fragrant rice species (Limpawattana & Shewfelt, 2010). Twenty-four attributes were listed by 8 trained panellists, of which 6 did not vary across the 36 varieties. The 18 attributes finally used in this study were ‘popcorn’, ‘starchy’, ‘woody’, ‘cooked-grain’, ‘grain’, ‘sulfury’, ‘corn’, ‘nutty’, ‘floral’, ‘dairy’, ‘hay-like’, ‘barny’, ‘buttery’, ‘green’, ‘rancid’, ‘waxy’, and ‘earthy’. A standard and intensity of standard for each attribute was also defined. Of the 18 significant attributes, ‘popcorn’, which was mainly attributed to 2-AP, was positively correlated with ‘buttery’ and ‘corn’ and negatively correlated with ‘earthy’ and ‘smoky’.

In an earlier study from Limpawattana's group, sensory profiling was conducted on 13 varieties of rice by using trained panels, and aroma-active compounds were analysed by GC-olfactometry (GC-O) and GC-MS (Limpawattana, Yang, Kays, & Shewfelt, 2008). In this study, a predictive model was built for correlation analysis of attributes and volatile compounds. Unexpectedly, 'popcorn' in this model was negatively correlated with guaiacol and (*E*,*E*)-2,4-decadienal, while 2-AP was not present in this model as a 'popcorn' descriptor. Guaiacol and (*E*,*E*)-2,4-decadienal contributed smoky and fatty attributes, respectively. The authors suggested that the thermal process of reference standard preparation may have influenced the 'popcorn' descriptor analysis. In addition, these authors suggested that the contribution of 2-AP to popcorn-like odour was always overemphasised relative to many other compounds that also contribute to this aroma in fragrant rice.

Three types of fragrant rice (jasmine, basmati, and Jasmati) were studied in a recent paper, to compare their main aroma active compounds using GC-O and GC-MS (Mahattanatawee & Rouseff, 2014). Hexanal, octanal, 2-AP, (*E*,*E*)-2,4-nonadienal, (*E*)-2-nonenal, 4-vinyl-2-methoxyphenol and indole were identified as the aroma-active compounds common to all three species. Across all three types of rice, 30 compounds were identified as aroma-active compounds and were described by 8 attributes. Jasmati contained 35% less 'roasty/nutty' total aroma intensity than jasmine and basmati, while 'medicine' flavour was not detected in jasmine rice. Jasmine contained 35% more 'sweet fruity/floral' total intensity than basmati and 79% more than Jasmati.

2.9. Conclusions

2-AP contributes important aroma in many foods, like pandan leaf, mushroom and especially fragrant rice. Amino acids, in particular proline and ornithine, have been identified, alongside reducing sugars, as precursors of 2-AP in both biosynthesis and Maillard reaction. The presence of a non-functional betaine aldehyde dehydrogenase (non-functional BADH2) allows the formation of 2-AP in fragrant rice and several bacteria like *Bacillus cereus* and *Penicillium nalgiovense* may also form 2-AP.

It appears that 1-pyrroline is a key intermediate in both biosynthesis and thermal formation of 2-AP, and this intermediate could form 2-AP through an acylation reaction. Methylglyoxal and 2-acetylpyrrolidine are other intermediates hypothesised to form 2-AP during the Maillard reaction and the presence of phosphate ion could increase yields of 2-AP. 2-AP formation mechanisms, particularly in rice, still need to be researched. The work of Poonlaphdecha et al. (2016) showed the importance of 1-pyrroline in 2-AP formation, and future work with this intermediate may provide useful information.

Synthesis of 2-AP is still difficult but its stabilisation in a zinc halide complex has increased its applicability. New synthesis strategies and stabilisation techniques could reduce the cost of 2-AP, which may increase its use in the food industry, adding desirable popcorn-like aroma to rice products, such as rice cakes. Another possibility is the addition of 2-AP intermediates, such as 1-pyrroline, to rice, which can then readily form 2-AP during processing, providing a desirable fragrance to rice products.

Chapter 3. Methods development of 2-acetyl-1-pyrroline extraction and quantification

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Abstract

2-Acetyl-1-pyrroline is a volatile compound with low detection threshold and low stability. This compound is present in some cereal products at low concentration and contributes key popcorn-like aroma. Therefore, the extraction and determination of 2-AP in these food products is important. In order to investigate 2-AP concentrations in fragrant and non-fragrant rice, 2-AP extraction and determination techniques were developed and modified in this chapter. Solid-phase extraction (SPME) and headspace solid-phase microextraction (HS-SPME) were optimised for 2-AP extraction. For SPE, Isolute ENV+ sorbent was selected to extract 2-AP from rice matrix, and dichloromethane was used as eluting solvent. For SPME, 2-AP was extracted at a low incubation temperature (40 °C) and the extraction was conducted for 1 hour. The limit of quantification of 2-AP reference compound was 5 µg/kg for both extraction techniques. The GC-MS analysis program was optimised for each extraction technique.

Keywords: 2-acetyl-1-pyrroline, fragrant rice, non-fragrant rice, GC-MS, SPME, SPE

3.1. Introduction

2-Acetyl-1-pyrroline (2-AP) is a volatile compound and it can contribute the popcorn-like odour in rice. The detection threshold of this compound is very low: 0.02 ng/L in air (Schieberle, 1991) and 0.1 ng/L in water (Buttery et al., 1982). Because of this low detection threshold, 2-AP is an important aroma contributor for rice especially fragrant rice.

Steam Distillation Extraction (SDE) was used to extract 2-AP from boiled fragrant rice when this compound was first identified as a popcorn-like aroma contributor in rice (Buttery et al., 1982). In the following decade, SDE was widely used for 2-AP extraction from boiled rice and other cooked food products such as popcorn (Schieberle, 1991) and bread crust (Schieberle and Grosch, 1987). Although vacuum-SDE can be operated at a lower heating temperature, Buttery et al. (1982) reported that vacuum-SDE only had a 1/10 yield compared with atmospheric-SDE in 2-AP extraction from rice. Hence, SDE is not suitable for 2-AP extraction from raw rice or other raw food materials.

Headspace extraction techniques, especially headspace solid-phase microextraction (HS-SPME), has been used in 2-AP extraction from raw rice in recent years. Bryant and McClung (2011) used an automated SPME system to compare 2-AP levels in seven uncooked fragrant and two uncooked non-fragrant rice samples. A manual SPME method was used for 2-AP quantification in 91 Indian rice varieties; 2-AP was not only detected in fragrant rice, but also found in some non-fragrant rice samples. However, the concentration in non-fragrant rice was 10 times lower than in Basmati varieties (Mathure, Jawali, Thengane, & Nadaf, 2014). Hopfer et al. (2016) optmised a manual SPME method for 2-AP extraction from raw rice, 2-AP levels in 48 fragrant rice varieties were measured using this method. They reported that this method is sentive enough to measure 2-AP at concentations lower than its odour threshold (Hopfer et al., 2016).

Several papers have reported the usage of solid-phase extraction (SPE) as a means of extracting and, hence, evaluating polar volatile compounds in low-fat foods. Pet'ka, Leitner and

Parameswaran (2012) reported that furaneol and mesifuran in musk strawberry were quantified using SPE with a LiChrolut EN cartridge. LiChrolut EN was also used in the extraction of sotolon from sweet Chinese rice wine (Chen, Wang & Xu, 2013). LiChrolut EN and Strata-X cartridges led to good recovery of aroma compounds in melons and strawberries when eluted using ethyl acetate and methyl acetate (Lignou, Parker, Oruna-Concha & Mottram, 2013; Lignou, Parker, Baxter & Mottram, 2014). An earlier study on wine compared 7 different SPE cartridges (Oasis HLB, Oasis MAX, Isolute ENV+, Isolute 101, Resprep C18, LiChrolut C18 and XAD-2) eluted with dichloromethane (DCM), and found that Isolute ENV+ was the most suitable for polar aroma compounds (Metafa & Economou, 2013). However, Strata-X stationary phase was not compared in that study. Another study examining the extraction of furaneol from fruit juice compared different organic solvents used with three different cartridges: LiChrolut EN, ODS C18 and Oasis HLB cartridges were eluted with methanol, ethyl ether, DCM, ethyl acetate, isopropanol, and acetone. The most suitable solvent to elute furaneol from all three cartridges was methanol (Du & Qian, 2008).

In this chapter, SPME and SPE, two popular volatile compound extraction techniques were compared, and optimised SPME and SPE methods were developed for 2-AP extraction from raw and cooked rice.

3.2. Materials and methods

3.2.1. Plant materials and Chemicals

A milled fragrant rice cultivar, Sintanur and two milled non-fragrant rice cultivars, Ciherang and long-grain rice, were used in the method development. Both milled Sintanur and Ciherang samples were provided by Indonesian Centre for Rice Research (ICRR), Sukamandi, Indonesia. ASDA brand long-grain rice was obtained from the local supermarket. Samples were stored at 4 °C before analysis.

HPLC-grade water, methanol (⩾99.8%) and dichloromethane (DCM, ⩾99.8%) were purchased from Fisher Scientific (Loughborough, UK); 2,4,6-trimethylpyridine (TMP, ⩾99%) was purchased from Sigma-Aldrich (St Louis, MO). 2-AP (30000 mg/kg in dichloromethane) and deuterated 2-AP (2-AP-d_2, 30000 mg/kg in dichloromethane) were purchased from AromaLAB (Planegg, Germany).

3.2.2. Rice sample preparation

Rice samples were ground to rice flour using a De'Longhi grinder (KG40; De'Longhi). Rice flour (10 g ± 0.01 g) was put into 20-mL glass ampoules (Wheaton, Millville, NJ) for baking without water at 180 °C for 20 min in GC oven. Whole rice grain (1 g ± 0.01 g) with 1.5 mL HPLC grade water was boiled at 100 °C for 20 min in GC oven in a 20-mL SPME glass vials and capped with metal screw-caps with PTFE-faced silicone septa.

3.2.3. 2-Acetyl-1-pyrroline extraction from rice samples

3.2.3.1. Solid-phase extraction

Accurately weighed (10.00 ± 0.01 g) rice flour was put into a 50-mL centrifuge tube and 35 mL HPLC-grade water were added. Then the tube was shaken for 20 min at 1700 rpm (Multi Reax; Heidolph, Schwabach, Germany), before centrifuging at 7000 rpm (5095 *g*) and 15 °C for

15 min (Sigma 3K10 laboratory centrifuge; Sigma, Osterode, Germany). A 20-mL aliquot of the supernatant was collected for solid-phase extraction (SPE). The Isolute ENV+ cartridge (200 mg/6 mL; Biotage, Uppsala, Sweden) was firstly conditioned with 10 mL methanol, then with 10 mL HPLC-grade water. Then 20 mL rice supernatant were loaded onto the cartridge. After sample loading, the cartridge was washed with 10 mL HPLC-grade water. The washed cartridge was dried under vacuum for 30 min. Finally, compounds were eluted with 2 mL DCM and collected in a 2-mL autosampler vial (Supelco, Bellefonte, PA) with metal crimp cap. 2,4,6-Trimethylpyridine (TMP) has been widely used as the internal standard in 2-AP quantification with different extraction techniques because the physicochemical properties of TMP are similar to 2-AP, including solubility, volatility and GC retention time on wax columns (Buttery et al., 1986, Wei et al., 2017). TMP was added together with rice samples at the beginning of simultaneous distillation/extraction (Tanchotikul & Hsieh, 1991; Widjaja, et al. 1996a & b; Tava & Bocchi, 1999), and was added with rice samples before solid-phase microextraction (Bryant & McClung, 2011). TMP was also selected as the internal standard for 2-AP quantification in this study. However, this compound was poorly retained by the Isolute ENV+ sorbent; therefore, 5 μL of 50 mg/kg 2,4,6-trimethylpyridine (TMP) solution in DCM were added to the collected sample before the sample was concentrated with a nitrogen stream to around 100 μL. Finally, the concentrated sample was transferred to a 200-μL glass insert (Thermo Scientific; Langerwehe, Germany), which was then sealed in a 2-mL autosampler vial with metal cap for GC-MS analysis.

3.2.3.2. Headspace solid phase-microextraction

Deuterated 2-AP (2-AP-*d2*) was used as an internal standard in SPME. Two hundred microlitres of prepared 2-AP-*d2* solution (approximately 200 mg/kg in dichloromethane) were evaporated by nitrogen gas to remove solvent, and then 300 mL of HPLC grade water were added to dissolve the 2-AP-d_2. Accurately weighed rice samples (1.000 ± 0.001 g) were put in 20-mL solid-phase microextraction (SPME) glass vials and capped with metal screw-caps with PTFE-

faced silicone septa; 1.5 mL of internal standard solution were added to the vial before analysis. SPME was conducted with an SPME autosampler (GC Sampler 120; Agilent, Santa Clara, CA). Rice samples were pre-incubated with magnetic shaking for 10 min at 40 °C, and then extracted with a 1-cm Supelco divinylbenzene/Carboxen™/polydimethylsiloxane (DVB/CAR/PDMS) SPME fibre for 1 hour. The SPME fibre was desorbed in a GC injector at 250 °C for 20 min during GC-MS analysis.

3.2.4. GC-MS analysis

A Zebron™ ZB-Wax column (30 m length × 0.25 mm i.d. × 0.25 μm film thickness; Phenomenex, Torrance, CA) was used for analysis of the SPE extract and a Zebron ZB-Wax column (30 m × 0.25 mm and 1 μm film thickness; Phenomenex, Torrance, CA) was used after SPME. One microlitre of the SPE extract was injected into the GC-MS system after SPE, and the SPME fibre was desorbed in a GC injector for 20 min after SPME; the injector temperature was 250 °C with splitless mode, the splitter was opened at 1 min in both cases. The carrier gas was helium at a constant column flow rate of 1 mL/min. The GC temperature program was developed in both extractions and discussed in *Section 3.3.1.* Electronic ionisation (EI) mode was applied; ionisation energy was 70eV, and the electron multiplier was set at 2000 V. Full scan mode was used for analysis from *m/z* 10 to 200. A 50 mg/L standard of 2-AP in dichloromethane (aromaLAB GmbH, Planegg, Germany) was run as a reference compound under the same conditions.

3.3. Results and discussions

3.3.1. Optimisation of GC-MS analysis

3.3.1.1. Separation of 2-AP

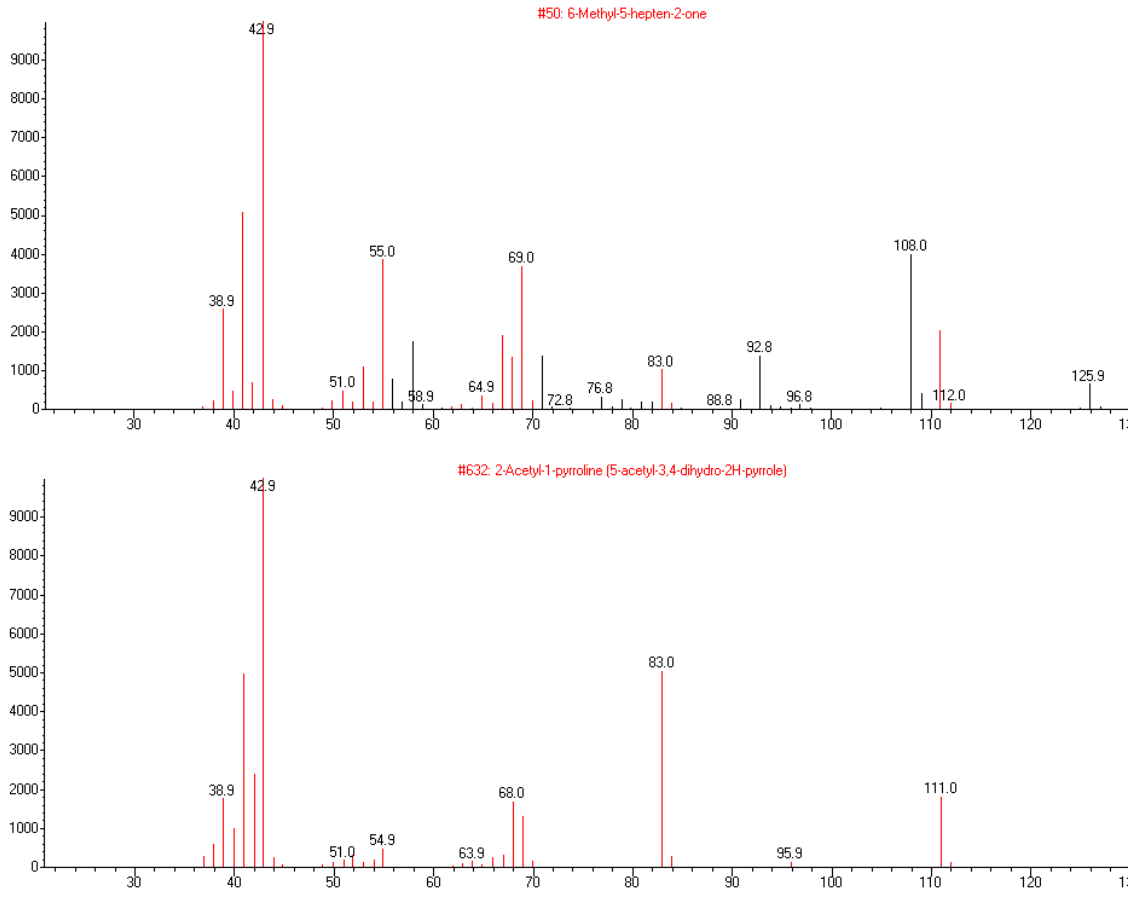

Figure 3.1: Mass spectrum of 6-methyl-5-hepten-2-one and 2-AP. The ions highlighted with red colour represent the ions common to both 6-methyl-5-hepten-2-one and 2-AP.

Co-elution of 2-AP and 6-methyl-5-hepten-2-one was noticed when SPE extract was analysed by GC-MS, especially when 2-AP concentration was much lower than 6-methyl-5-hepten-2-one. Mass spectra of 6-methyl-5-hepten-2-one and 2-AP are shown in **Figure 3.1**. It can be seen that the ion fragments in 2-AP can be found in 6-methyl-5-hepten-2-one, including the key ions *m/z* 68, *m/z* 83 and *m/z* 111 which are normally used for 2-AP identification and quantification. This makes 2-AP difficult to quantify when it co-elutes with 6-methyl-5-hepten-2-one. The co-elution of these two compounds has been reported in other studies. Both Tanchotikul and Hsieh

(1991), and Seitz et al. (1993) reported the co-elution of 2-AP and 6-methyl-5-hepten-2-one when wetted ground pearl millet extract was analysed by Supelcowax 10 column (30 m or 60 m).

A GC oven temperature program was developed to separate 2-AP from 6-methyl-5-hepten-2-one. Both Tanchotikul and Hsieh (1991), and Seitz et al. (1993) reported a GC program that started with holding at an initial oven temperature for longer time than usual (65 °C for 75 min and 60 °C for 15 min, respectively). It was previously reported that 2-AP was not present in non-fragrant rice after baking at 180 °C for 20 min (Handoko, 2015). Extracted ion chromatograms (*m/z* 68, 83, 111 and 108) of 180 °C and 20 min baked Ciherang (non-fragrant rice) SPE extract analysed by GC-MS using a common isothermal program (40 °C hold 2 min, increased to 200 °C at the rate of 4 °C/min, increased to 250 °C at the rate of 15 °C/min and held for 15 min) is shown in **Figure 3.2a**, 2-AP cannot be identified in this chromatogram. However, when the methods reported by Tanchotikul and Hsieh (1991) and Seitz et al. (1993) were applied in the analysis, 2-AP was isolated from the peak of 6-methyl-5-hepten-2-one.

Figure 3.2b shows the extracted ion chromatograms with 60 °C initial temperature held for 20 min at the beginning of analysis. A broader gap between ethylpyrazine and 6-methyl-5-hepten-2-one can be seen with this method compared with the temperature program in **Figure 3.2a**. The ion peaks of *m/z* 68, 83 and 111 were found in this gap but the peak of *m/z* 108 was not found; these peaks were identified as 2-AP. However, the tail of the 2-AP ion peaks still cannot be well separated from 6-methyl-5-hepten-2-one with 20 min holding at 60 °C. Therefore, the holding duration was extended to 25 min, 30 min and 35 min in **Figure 3.2c**, **Figure 3.2**d and **Figure 3.2e**, respectively. **Figure 3.2e** showed the peaks of *m/z* 68, 83 and 111 with individual shape in between ethylpyrazine and 6-methyl-5-hepten-2-one and resolved from other compounds. Hence, the isothermal program used in **Figure 3.2e** (the initial oven temperature was 60 °C, held for 35 min, and then increased to 250 °C at 5 °C/min and finally held for 10 min) was applied in the further GC analysis for rice SPE extracts.

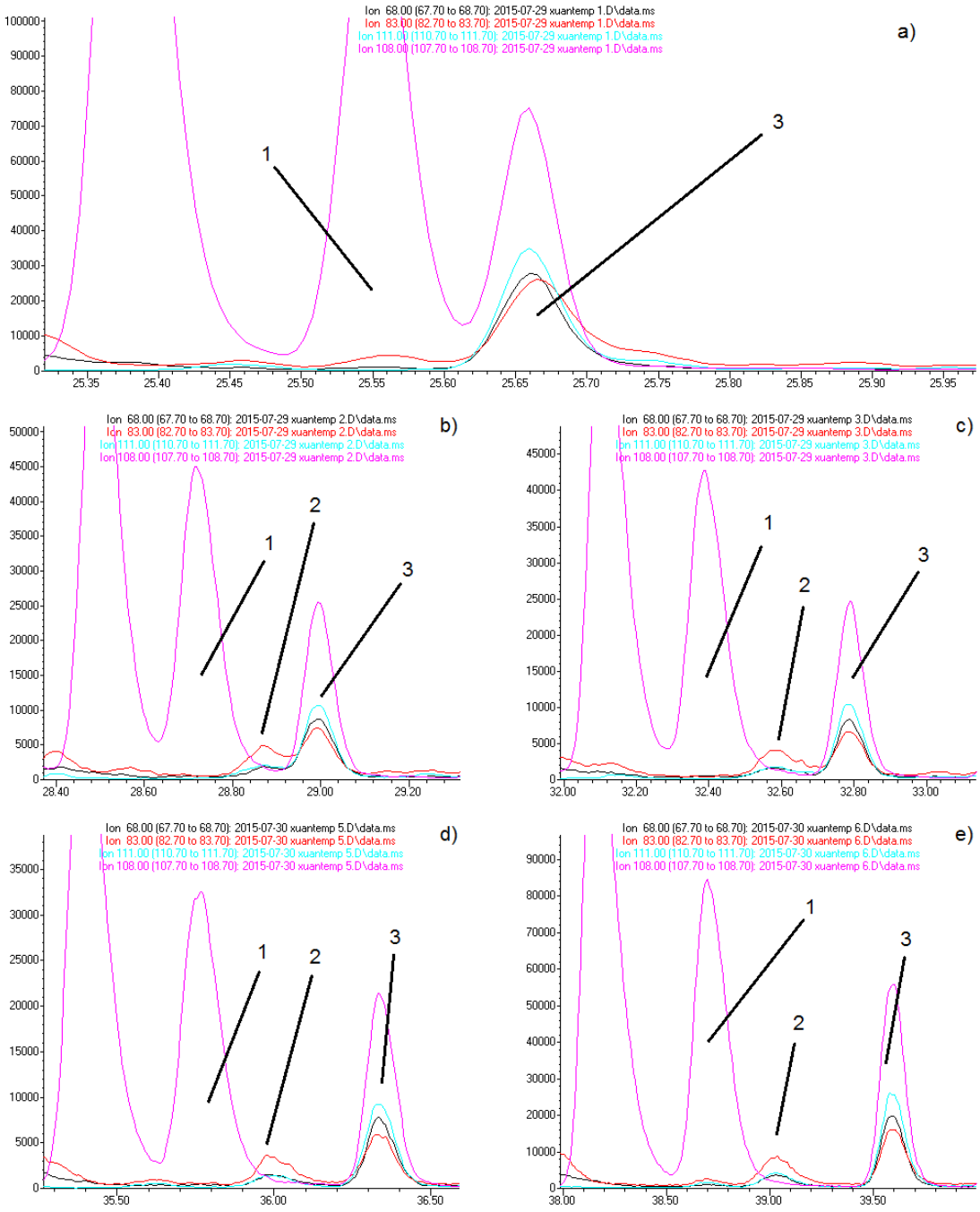

Figure 3.2: The selective ion chromatograms of 2-AP separation from 6-methyl-5-hepten-2-one in 180 °C and 20 min baked Ciherang rice with longer holding of lower temperature at the beginning of GC analysis, Zebron ZB-Wax column (30 m × 0.25 mm and 1 µm film thickness; Phenomenex, Torrance, CA) was used in the analysis. a) 40 °C held 2 min, increase to 200 °C at the rate of 4 °C/min, increased to 250 °C at the rate of 15 °C/min and held for 15min. b) 60 °C held 20 min, increased to 250 °C at 5 °C/min and held for 10 min. c) 60 °C held 25 min, increased to 250 °C at 5 °C/min and held for 10 min. d) 60 °C held 30 min, increased to 250 °C at 5 °C/min and held for 10 min. e) 60 °C held 35 min, increased to 250 °C at 5 °C/min and held for 10 min. Different ion chromatograms are represented as different colours, *m/z* 68 is represented as black, *m/z* 83 is represented as red, *m/z* 111 is represented as light blue, and *m/z* 108 is represented as purple. '1' represents ethylpyrazine, '2' represents 2-AP, '3' represents 6-methyl-5-hepten-2-one.

When this modified isothermal program (35 min holding at 60 °C) was applied in the analysis by SPME with the same type column (wax column but thicker film), the co-elution of 2-AP and 6-methyl-5-hepten-2-one was observed. However, this co-elution was not observed when using the common temperature program (40 °C hold 2 min, increase to 200 °C at the rate of 4 °C/min, increased to 250 °C at the rate of 15 °C/min and hold for 15min) in SPME extraction. The column with 0.25 µm stationary film is commonly used for GC-MS analysis, whereas, a column with thicker film is suitable for more volatile compounds, such as the analysis by SPME, and this thicker film gave a better separation of 2-AP and 6-methyl-5-hepten-2-one in the chromatogram (Zeeuw, 2015). Therefore, the common temperature program was used in SPME analysis and the modified temperature program was used in SPE analysis.

3.3.1.2. Determination of limit of quantification (LOQ) for 2-AP and establishment of calibration curve

Determination of LOQ and establishment of calibration curve were conducted individually for the rice samples extracted by SPE and SPME because of the different extraction yield in these two extraction techniques. LOQ determination was conducted together with the establishment of a calibration curve for 2-AP in the rice matrix.

The calibration curve was established in boiled rice matrix and dry rice matrix (raw and dry baked), respectively. Non-fragrant long-grain rice was used as the matrix for calibration curves; a prepared 2-AP standard solution (5.5 mg/kg in dichloromethane) was used for this curve. Calibration standards were prepared to create a calibration curve for 2-AP as well as determine 2-AP LOQ in GC-MS. The 2-AP-d_2 and 2-AP aqueous solutions were prepared from a dichloromethane solution of 2-AP; dichloromethane was evaporated by N_2 gas and replaced by an equal amount of HPLC-grade water. For SPE, 500 µL 2-AP aqueous solutions (0.01 mg/kg, 0.05 mg/kg, 0.1 mg/kg, 0.5 mg/kg, 1 mg/kg, 2 mg/kg, 5 mg/kg) were added into 10 g raw long-grain rice flour, and then extracted by SPE (see method section). For SPME, 50 µL 2-AP aqueous

solutions (0.01 mg/kg, 0.05 mg/kg, 0.1 mg/kg, 0.5 mg/kg, 1 mg/kg, 2 mg/kg, 5 mg/kg) were added into 1 g raw long-grain rice flour or boiled rice grain, then extracted by SPME (see method section).

2-AP was detected and quantified in the rice with 0.1 mg/kg, 0.5 mg/kg, 1 mg/kg, 2 mg/kg, and 5 mg/kg 2-AP standards by GC-MS using both extraction techniques. Trace ions could be detected in 0.01 mg/kg and 0.05 mg/kg standards; however, they were not quantifiable. Seven concentrations of 2-AP standards (0.01 mg/kg, 0.05 mg/kg, 0.1 mg/kg, 0.5 mg/kg, 1 mg/kg, 2 mg/kg, 5 mg/kg) were equivalent to 0.5 µg/kg, 2.5 µg/kg, 5 µg/kg, 25 µg/kg, 50 µg/kg, 100 µg/kg and 250 µg/kg of 2-AP in rice. Therefore, the LOQ through both SPE and SPME in dry and boiled rice matrix was 5 µg/kg.

3.3.2. Optimisation of extraction techniques for 2-AP in rice

3.3.2.1. Solid phase-extraction

In an earlier unpublished study, when compared with the similar phases Strata-X (Phenomenex, Torrance, CA,) and LiChrolut EN (Millipore, Billerica, MA) cartridges, Isolute ENV$^+$ (Biotage, Uppsala, Sweden) was regarded as the solid phase with the best recovery for several commonly-analysed polar volatile compounds (diacetyl, furfural, propionic acid, 2-methoxyphenol (guaiacol), maltol, pantolactone, furaneol, sotolon, 5-(hydroxymethyl)furfural, vanillin and raspberry ketone). Drying for 30 min between the washing and eluting steps could remove moisture from the sorbent and retain most of the extract on the cartridge. Relatively non-polar solvent DCM could elute more of the extract, compared with methanol, ethanol, methyl acetate, ethyl acetate and acetone; 2 mL DCM were enough to elute compounds from 200 mg sorbent.

3.3.2.2. Solid-phase microextraction

HS-SPME is a solvent-free headspace extraction technique; volatile compounds in the headspace of the extraction vial are absorbed or adsorbed by the SPME fibre, depending on its type. Although extraction can be conducted at room temperature, heating during SPME can improve volatile compounds release into the headspace above the food matrix, particularly for relatively polar aroma compounds such as 2-AP (Grimm, Bergman, Delgado, & Bryant, 2001). Some studies suggested that 2-AP in rice should be extracted with high temperatures (80 °C to 120 °C), in order to get a better release of 2-AP and improve 2-AP sensitivity during analysis (Mathure, Jawali, Thengane, & Nadaf, 2014; Bryant & McClung, 2011). Grimm et al. (2001) indicated that a slight change of extraction temperature, time and moisture content can significantly affect the amount of compounds extracted from a food matrix. They found that double the amount of 2-AP was extracted from rice when the extraction temperature was increased from 60 °C to 80 °C, and hence they suggested to heat rice at 80 °C during SPME (Grimm et al., 2001). However, higher levels of 2-AP in our study were also observed when rice was heated before SPE (**Figure 3.3**). As SPE is a solvent-based extraction technique, 2-AP increase should be because of generation rather than release of 2-AP as a result of heating. Hopfer, Jodari, Negre-Zakharov, Wylie, and Ebeler (2016) also proposed the possibility of 2-AP generation at elevated temperatures when they extracted 2-AP from single rice kernels. Fig. 3 shows that 2-AP can be generated during heating even where the heating temperature was lower than normal cooking temperatures.

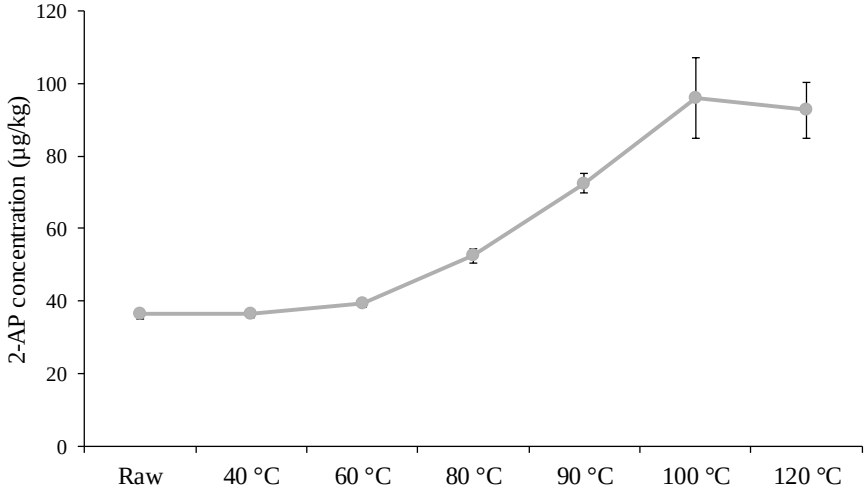

Figure 3.3. 2-AP concentration (µg/kg) in raw and baked (20 min) milled Sintanur (*n* = 3), 2-AP was extracted through SPE. Error bar represents standard deviations of triplicate analyses.

When SPME was applied in this project, heating during SPME needed to be controlled at a low temperature, in order to minimise 2-AP generation, yet still allow quantifiable levels of 2-AP to be released during extraction. Therefore, extraction in the current study followed a procedure modified from that of Hopfer et al. (2016); the heating temperature was set at 40 °C to minimise 2-AP generation during extraction, since no significant difference of 2-AP content was found between raw and 40 °C baked fragrant rice in the current study. In contrast, a longer extraction (1 hour) was used to trap sufficient volatile compounds for GC-MS analysis. SPME was conducted for 15 min by Hopfer et al (2016), since an increase of 2-AP was not obvious in single rice kernels when extraction time was longer than 15 min. However, more rice (1 g) was extracted in our study, and it was found that extracted 2-AP increased until 1 hour when SPME was conducted at 40 °C. Because it was confirmed by SPE that heating at 40 °C cannot generate 2-AP, a one-hour extraction by SPME can release more 2-AP from the rice flour matrix, peak areas of 2-AP under different extraction time at 40 °C is shown in **Figure 3.4**. Although SPME can be conducted at room temperature, chromatograms showed improved extraction of 2-AP after SPME at 40 °C relative to room temperature.

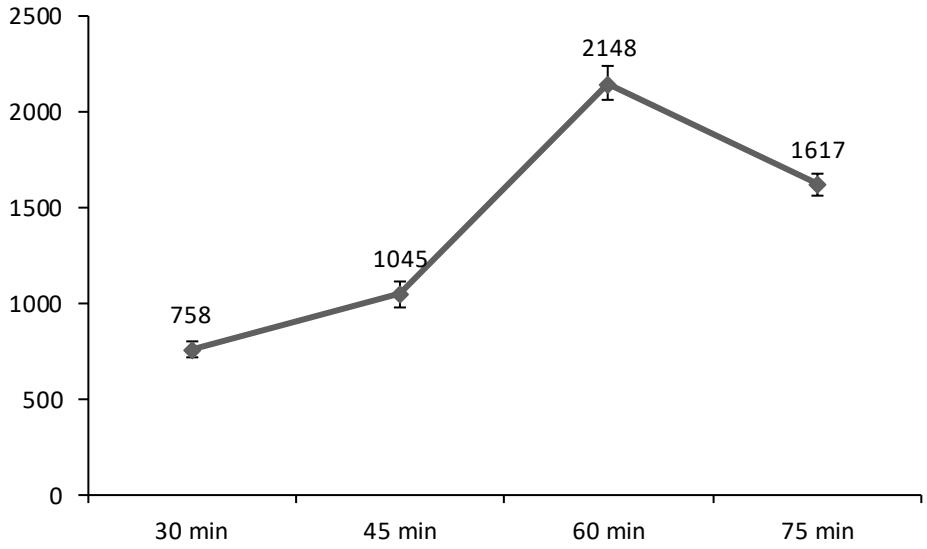

Figure 3.4: Peak areas*1000 of 2-AP extracted from raw Sintanur rice with SPME for different extraction time at 40 °C (*n* = 3). Error bar represents standard deviations of triplicate.

3.3.2.3. The effect of the chosen extraction techniques on 2-acetyl-1-pyrroline analysis

Both solid-phase extraction (SPE) and headspace solid-phase microextraction (SPME) were used in this project. SPE is a solvent-based extraction technique, where compounds can be extracted from water by SPE sorbent based on their polarity, and organic solvent is used to elute compounds of interest from the SPE sorbent. It was reported that up to 50 mL of sample can be extracted using 200 mg sorbent in SPE (Piñeiro, Palma, & Barroso, 2004); 20 mL supernatant from 10 g rice flour extracted with 35 mL HPLC water mixture was loaded onto the cartridge in our study.

During SPE, the supernatant of centrifuged rice/water mixture was loaded onto the SPE cartridge, compounds were trapped by the sorbent, and then the compounds of interest were eluted by organic solvent for analysis; polar non-volatile compounds were removed during washing before the eluting step. However, this process was difficult to apply on boiled rice. After boiling of rice, gelled rice starch increased viscosity of the supernatant, and the gelled starch resided in the SPE sorbent. Hence it could not be completely removed during washing. Therefore, some of the gelled starch was eluted into the extract and caused high viscosity. The high viscosity of the

extract made the extract difficult to inject into the GC system. Moreover, the high viscosity could also prevent volatile compounds release in the inlet liner. Therefore, in order to prevent the problems due to gelled starch, headspace SPME was used for boiled rice.

Compared to SPE, SPME is more often used for volatile compound extraction, since non-volatile compounds are not extracted with this technique. In contrast, SPE extracts compounds based on their polarity and can extract non-volatile material. SPME is more economical than SPE; a re-usable SPME fibre rather than disposable SPE cartridge is used as the sorbent during extraction. Also, SPME is a solvent-free technique and hence ecologically friendly. Moreover, the SPME autosampler has been widely used for extraction, and this sampler was used in our project. Human error is reduced on temperature and extraction time controlled with the SPME autosampler compared with manual extraction. Only 1 g of rice could be loaded into a 20-mL SPME vial when the sample was extracted with SPME autosampler, whereas more rice can be loaded into a bigger headspace vial when SPME is operated manually, and 20 mL supernatant from 10 g rice can be loaded during manual SPE. Theoretically, the large sample size in extraction may improve LOQ of compounds with low concentrations in food. However, the LOQ determination section showed that the lowest concentration of 2-AP can be quantified in rice matrix was 5 µg/kg when rice was extracted through both SPE and SPME, which indicated that the sensitivity of SPME is relatively acceptable compared to SPE.

In this project, SPME was used on boiled rice, raw and baked rice in experiments which did not specifically focus on the effect of temperature on 2-AP change. SPE was used for GC-O analysis, since a large amount of rice sample (10 g) could be loaded. With a higher sample loading during extraction, 2-AP can be more easily detected in GC-O, especially for non-fragrant rice, and the results in **Chapter 4** showed the presence of 2-AP in non-fragrant rice. SPE was also used in the research focusing on the effect of cooking temperature on 2-AP generation in fragrant and non-fragrant rice. Although heating at 40 °C did not lead to 2-AP generation, which will be discussed

in **Chapter 5**, heating should still be avoided during extraction; hence SPE rather than SPME was selected in this chapter.

3.4. Conclusion

To conclude, 2-AP extraction and analysis techniques were optimised in this chapter. The GC isothermal program was developed for SPE, in order to get better separation of 2-AP from other compounds. LOQ values of 2-AP in rice through both extraction techniques were determined. In this study, 5 µg/kg was the lowest 2-AP concentration that could be detected by GC-MS in both dry rice flour and boiled rice. SPE and SPME extraction conditions were optimised in this chapter. Since there was no difference in 2-AP detection sensitivity between the two extraction techniques, the reason for the studies was the main consideration when choosing extraction techniques subsequently. SPE was selected in the study which was focusing on the effect of cooking temperature on 2-AP concentration; otherwise, SPME was selected.

Chapter 4. Comparison of the Sensory Properties of Fragrant and Non-Fragrant Rice, Focusing on the Role of the Popcorn-like Aroma Compound 2-Acetyl-1-pyrroline

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This chapter was presented in 15th Weurman flavour research symposium as a poster presentation; it has been partly published as a book chapter: Wei, X., Methven, L., & Elmore, J. S. (2018). Comparison of the Sensory Properties of Fragrant and Non-Fragrant Rice, Focusing on the Role of the Popcorn-like Aroma Compound 2-Acetyl-1-pyrroline. *Food Science: Proceedings of the XV Weurman Flavour Research Symposium*. Siegmund, B., & Leitner, E. (eds). Verlag der Technischen Universität Graz. 427-432.

Abstract

2-Acetyl-1-pyrroline (2-AP) has been widely reported as a key contributor to the popcorn-like aroma of fragrant rice. To understand more about its contribution to the aroma of fragrant rice, sensory profiling was conducted to examine the sensory properties of six boiled rice samples, three fragrant and three non-fragrant. Intensities of popcorn odour, flavour and after-effect in fragrant rice were higher than in non-fragrant rice. However, panellists could not differentiate between fragrant rice varieties using these three attributes. 2-AP was extracted from the boiled rice samples by headspace solid-phase microextraction and quantified by gas chromatography-mass spectrometry (GC-MS). 2-AP was quantified in fragrant rice samples but could not be quantified in non-fragrant varieties. The differences in 2-AP content between different boiled fragrant rice samples may be too small to allow perceptual discrimination. In addition, popcorn-like notes were perceived in non-fragrant rice samples, despite levels of 2-AP being below the limit of quantification of GC-MS.

Keywords: 2-acetyl-1-pyrroline, Sensory evaluation, fragrant rice, GCMS, SPME, GC-olfactometry

4.1. Introduction

Rice (*Oryza sativa*) provides energy for 25% of the world's population (FAO, 2002). It can be categorised into two types depending on its aroma: fragrant rice and non-fragrant rice. Although non-fragrant long-grain and medium-grain rice constitute the majority of world trade (79%), the price of fragrant rice is much higher than non-fragrant rice on the world market. According to the 2017 Rice Market Monitor report, the price of fragrant rice was more than double that of high quality non-fragrant rice (FAO, 2017).

The aroma of fragrant rice was first evaluated analytically in the early 1980s and was described as 'popcorn-like'. Perceived popcorn odour intensities in several fragrant rice varieties were ranked, and 2-acetyl-1-pyrroline was considered as the most important contributor to this odour (Buttery, Ling, Juliano, & Turnbaugh, 1983). This volatile compound can contribute a popcorn-like aroma with a low detection threshold (0.1 nL/L in water). It was first identified in boiled fragrant rice (Buttery, Ling, & Juliano, 1982). This compound is not only present in fragrant rice, it can also be detected in many different raw food materials, such as hazelnuts (Kiefl, Pollner, & Schieberle, 2012), pandan leaf (Peter, 2006), and Manuka honey (Ruckriemen, Schwarzenbolz, Adam, & Henle, 2015). In addition, 2-AP can also be detected in some manufactured food products, such as popcorn (Schieberle, 1991), wheat bread crusts (Schieberle & Grosch, 1987), and on the surface of Mediterranean dried sausages (Stahnke, 2000), Parma ham and Italian-type salami (Blank et al., 2001), where it contributes key odour characteristics.

Sensory profiling, using techniques such as quantitative descriptive analysis (QDA), is an effective sensory evaluation technique that has already been used in rice research to describe and quantify product attributes. Lexicons of rice descriptors have been established in several studies, especially for fragrant rice (Goodwin et al., 1996; Piggott, Morrison, & Clyne, 1991; Yau & Liu, 1999). The selection of descriptors depends on culture and familiarity with samples among panellists (Paule & Powers, 1989). However, no rice lexicon has previously been reported using a

UK sensory panel. In addition, several studies have indicated that aroma contributed by 2-AP may be overemphasised in boiled fragrant rice. Yang, Shewfelt, Lee and Kays (2008) reported that popcorn-like note might not be the only important attribute in boiled fragrant rice. In addition, Limpawattana, Yang, Kays, and Shewfelt (2008) reported that there was no correlation between popcorn flavour and 2-AP in boiled rice.

In this study, different boiled rice varieties were evaluated using quantitative descriptive analysis (QDA). A lexicon was developed for both boiled fragrant and non-fragrant rice varieties using a UK-based panel. Differences in flavour and odour between fragrant and non-fragrant rice were evaluated. In addition, 2-AP in boiled fragrant and non-fragrant rice varieties was quantified using headspace solid-phase microextraction (HS-SPME) and gas chromatography-mass spectrometry (GC-MS). The primary aim of this study was to determine the strength of the relationship between perceived popcorn-like flavour and 2-AP content in boiled fragrant and non-fragrant rice.

4.2. Materials and Methods

4.2.1. Plant materials and chemicals

Six varieties of milled (white) rice were obtained, including three fragrant rice varieties (basmati and Thai Jasmine from ASDA supermarket (Reading, UK), Sintanur from Indonesian Centre for Rice Research) and three non-fragrant rice varieties (American long-grain from ASDA supermarket (Reading), Arirang from Korea Foods Company Limited (Reading), and Ciherang from Indonesian Centre for Rice Research). Still mineral water (1.5 L, Harrogate, UK) was used for sensory analysis and HPLC-grade water (Fisher, Loughborough, UK) was used for chemical analysis. 2-AP and deuterated 2-AP (2-AP-d_2) standards were used for 2-AP quantification (both 30,000 mg/kg in dichloromethane (DCM); aromaLAB GmbH, Planegg, Germany).

4.2.2. Quantitative descriptive analysis (QDA) in boiled rice

Milled rice (200 ± 1 g) was weighed and then boiled using 300 mL mineral water in a rice cooker (0.8 L capacity; Lloytron PLC, Leigh, UK). Rice samples were initially cooked with tap water. During vocabulary development, panellists provided 'tap water' or 'kettle-like' attributes from samples cooked in tap water. However, these attributes were absent in samples cooked in mineral water. Subsequently, Harrogate mineral water was used for rice boiling. Cooking proceeded for 20 min before the rice cooker automatically turned to warm mode. The samples were kept warm (>65 °C) in the rice cooker for 20 min before serving to panellists for evaluation.

Sensory profiling using a quantitative descriptive analysis (QDA) approach was conducted for six rice samples, using 11 trained UK panellists (each with between 6 months and 10 years' experience). A consensus vocabulary was developed for appearance, odour, taste, flavour, mouthfeel and after-effects. After-effects included all attributes within the modalities of taste, flavour and mouthfeel that remained after samples were swallowed. Attribute definitions and references are given in **Table 4.1**. A pre-heated (120 °C for 20 min in the oven) ceramic cup (50 mL) filled with boiled rice (20 g) covered by foil was served to panellists for developing odour attributes and another 20-g sample was then served in the same manner for developing all the other attributes. The scoring for each sample attribute was conducted in individual booths in duplicate on separate days; samples were labelled with three-digit codes and presented randomly in a balanced order. Data were collected *via* Compusense at-hand software (Compusense, Guelph, Canada) using unstructured line scales (0–100), except for the attribute "popcorn odour", where a structured scale was used with anchors at positions defined by the panel after sniffing various concentrations of the reference 2-AP.

Table 4.1: Vocabulary of boiled rice developed by sensory profiling using 11 trained UK panellists.

attributes	definition	reference	anchors
appearance			
brown lines	extent of brown lines on the surface of rice grains		nil to extreme
wet	moistness of rice grain		dry to wet
yellow	colour of rice grain		white to yellow
uniform	shape of rice grain		irregular to regular
separated grain	separation between rice grains after cooking		unseparated to separated
length	length of rice grain		short to long
thickness	thickness of rice grain		thin to thick
mouthfeel			
smooth	smoothness of the sample on chewing		nil to extreme
effort to chew	springiness of the sample on chewing		nil to extreme
drying	mouth drying		nil to extreme
cohesive	stickiness of rice grain		nil to extreme
watery	how moist the sample felt in the mouth		nil to extreme
odour			
popcorn	aroma of popcorn	four levels of 2-acetyl-1-pyrroline standard in sniff bottle (10, 100, 1000 and 5000 µg/kg)	nil to extreme, standards were given as three anchors at 12, 40, and 75 along the line scale
sweet	aroma of Demerara sugar		nil to extreme
porridge	aroma of cooked oat porridge	Quaker wholegrain rolled oats porridge (Quaker, UK)	nil to extreme
rice pudding	aroma of rice pudding	Ambrosia original tinned rice pudding (Ambrosia, UK)	nil to extreme
milky	aroma of uncooked milk	pasteurised Tesco skim milk (Tesco, UK)	nil to extreme
starchy water	aroma of starch water from boiled non-fragrant rice	cold starchy water collected from boiled non-fragrant rice	nil to extreme
eggy	aroma of boiled egg		nil to extreme

Table 4.1. continue

taste			
sweet	elicited by sucrose		nil to extreme
bitter	elicited by caffeine		nil to extreme
salty	elicited by sodium chloride		nil to extreme
savoury	brothy or meaty like		nil to extreme
metallic	metal like		nil to extreme
flavour			
popcorn	flavour of popcorn		nil to extreme
porridge	flavour of oat porridge	Quaker wholegrain rolled oats porridge (Quaker, UK)	nil to extreme
rice pudding	flavour of cooked oat porridge	Ambrosia original tinned rice pudding (Ambrosia, UK)	nil to extreme
milky	flavour of uncooked milk	pasteurised Tesco skim milk (Tesco, UK)	nil to extreme
starchy water	flavour of starch water from boiled non-fragrant rice	cold starchy water collected from boiled non-fragrant rice	nil to extreme
eggy	flavour of boiled egg		nil to extreme
after-effect			
popcorn	popcorn odour and flavour residual in mouth after swallowing		nil to extreme
salty	salty residual in mouth after swallowing		nil to extreme
sweet	sweet residual in mouth after swallowing		nil to extreme
bitter	bitter residual in mouth after swallowing		nil to extreme
drying	mouth drying after swallowing		nil to extreme
residue	particulates left in mouth after swallowing		nil to extreme
starchy water	starchy water flavour in mouth after swallowing		nil to extreme

References for ‘porridge’, ‘rice pudding’, ‘milky’ and ‘starchy water’ attributes were provided (**Table 4.1**). The panellists were trained in recognition and scaling of popcorn odour, using a series of dilutions of 2-AP standard. Five sniff strips (Sigma-Aldrich, St Louis, MO) were wetted with four concentrations of 2-AP in dichloromethane (10, 100, 1000 and 5000 µg/kg) and a blank dichloromethane solution. After all solvent was evaporated using a nitrogen stream, each strip was sealed in a 5-mL glass vial with screw lid. Each vial was only opened once and sniffed by one panellist. Blank and 5000 µg/kg standard references were first provided to each panellist for Nil and Extreme values on the 0–100 unstructured line scale. Then the panellist was asked to score the 10, 100 and 1000 µg/kg standard references on the same line scale. The average score for each 2-AP reference was added onto all 0–100 line scales used to measure ‘popcorn’ odour in the rice sample scoring session. Five concentrations of 2-AP standard (blank, 10, 100, 1000 and 5000 µg/kg) were also provided to panellists before sample profiling. Panellists were asked to sniff the 2-AP standards in a separate room prior to the profiling session.

4.2.3. 2-Acetyl-1-pyrroline quantification in boiled rice using solid-phase microextraction and gas chromatography-mass spectrometry

Rice samples (1.000 g ± 0.001 g) and 1.5 mL HPLC-grade water were put in 10-mL SPME glass vials with screw metal caps with PTFE-faced silicone septa. Vials were heated in a GC oven at 100 °C for 20 min and then cooled to room temperature. Finally, a 1.5-mL aliquot of 2-AP-d_2 aqueous solution was added into the vials (post rice heating and cooling). The 2-AP-d_2 aqueous solution was prepared from 2-AP-d_2 dichloromethane solution (100 µg/kg); dichloromethane was evaporated by N_2 gas and replaced by an equal amount of HPLC-grade water.

Headspace solid-phase microextraction (HS-SPME) followed by gas chromatography-mass spectrometry (GC-MS) has been widely used in the aroma compound analysis in rice, especially for 2-AP detection (Tulyathan, Srisupattarawanich & Suwanagul, 2008; Bryant & McClung, 2011; Mathure et al., 2014; Poonaphdecha et al., 2016). Believing that a higher

extraction temperature can improve volatile compounds release from the food matrix, several studies have extracted 2-AP from rice using a high extraction temperature (80 °C to 120 °C) (Grimm et al., 2001; Bryant & McClung, 2011; Mathure et al., 2014; Poonaphdecha et al., 2016). However, Hopfer et al. (2016) suggested the use of a lower extraction temperature; they indicated that 2-AP may be generated at a high extraction temperature. Hence, in order to minimise 2-AP changes during extraction, the HS-SPME method used in this paper was modified from that of Hopfer et al. (2016).

2-AP in boiled rice was extracted by an HS-SPME autosampler (GC Sampler 120; Agilent, Santa Clara, CA), attached to a 6890 gas chromatograph with 5975 mass spectrometer (Agilent). Rice sample was pre-incubated with agitation for 10 min at 40 °C, and then extracted with a 1-cm divinylbenzene/Carboxen™/polydimethylsiloxane (DVB/CAR/PDMS) SPME fibre (Supelco, Bellefonte, PA) for 1 hour at 40 °C with agitation.

After extraction, the SPME fibre was desorbed in the GC injector at 250 °C for 20 min, in splitless mode, onto the front of a Zebron ZB-Wax column (30 m × 0.25 mm; 1 µm film thickness; Phenomenex, Torrance, CA). The carrier gas was helium at a constant column flow rate of 0.9 mL/min. The initial GC oven temperature was 40 °C held for 2 min, then increased to 60 °C at the rate of 2 °C/min, at which point the rate was increased to 6 °C/min and held for 35 min after the oven temperature reached 250 °C. Electron ionisation (EI) was applied; ionisation energy was 70 eV, and the electron multiplier was set at 2824 V. The full scan mode was used for analysis from *m/z* 30 to 280. Selected ion monitoring was also applied; *m/z* 68, *m/z* 83 and *m/z* 111 were monitored for 2-AP; *m/z* 86 and *m/z* 114 were monitored for 2-AP-d_2. Dwell time of monitored ions was set at 100 ms/ion.

A matrix-matched calibration curve was established for accurate quantification of 2-AP. Boiled long-grain rice (non-fragrant rice) was used as the matrix for calibration curves because it gave the lowest response for 2-AP in chromatograms among all of the rice samples studied. A

prepared 2-AP standard solution (5.5 mg/kg in dichloromethane) was used for this curve. Long-grain rice (1 g) with 1.5 mL HPLC grade water was boiled in an SPME glass vial with lid in a GC oven at 100 °C for 20 min and then the vial was cooled to room temperature. Five calibration standards were prepared to create a calibration curve for 2-AP. For each calibration standard, 50 µL 2-AP aqueous solutions (0.1 mg/kg, 0.5 mg/kg, 1 mg/kg, 2 mg/kg, 5 mg/kg; prepared in the same manner as 2-AP-d_2 aqueous solution) with 1.5 mL 2-AP-d_2 aqueous solution (the same concentration as in the extracted rice samples) were then added into the boiled long-grain rice matrix and analysed by GC-MS. A blank sample was prepared from 1.5 mL 2-AP-d_2 aqueous solution (100 µg/kg) with no rice present in a 20-mL SPME vial, and it was run by GC-MS before calibration standards and rice samples.

4.2.4. Gas chromatography-olfactometry of raw fragrant and non-fragrant rice extracts prepared using solid-phase extraction

Raw milled Sintanur or Ciherang rice flour (10 g ± 0.01 g) was placed into a 50-mL centrifuge tube and 35 mL HPLC-grade water were added. The tube was shaken for 20 min at 1700 rpm (Multi Reax; Heidolph, Schwabach, Germany), and then it was centrifuged at 7000 rpm (40 g) and 15 °C for 15 min (Sigma 3K10 laboratory centrifuge; Sigma, Osterode, Germany). A 20-mL aliquot of the supernatant was collected for solid-phase extraction (SPE). The Isolute ENV+ cartridge (200 mg/6 mL; Biotage, Uppsala, Sweden) was firstly conditioned with 10 mL methanol, then with 10 mL HPLC-grade water. Then 20 mL rice supernatant were loaded onto the cartridge. After sample loading, the cartridge was washed with 10 mL HPLC-grade water. The washed cartridge was dried under vacuum for 30 min. Finally, compounds were eluted with 2 mL DCM. The DCM extract was then concentrated with a nitrogen stream to around 100 µL. This concentrated extract was transferred to a 200-µL glass insert (Thermo Scientific, Loughborough, UK) and then it was sealed in a 2-mL autosampler vial with metal crimp-cap prior to gas chromatography-olfactometry (GC-O) analysis.

A Zebron ZB-Wax column (30 m × 0.25 mm; 0.25 µm film thickness; Phenomenex, Torrance, CA) was used in this analysis. One microlitre of the extract was injected manually in 1:20 split mode into the injection port of a Hewlett Packard 5890 Series II gas chromatograph with olfactometer and flame ionisation detector (FID). The injection inlet temperature was 250 °C and the carrier gas was helium. The total flow was set at 103.9 mL/min and 6.204 psi. The initial GC oven temperature was 40 °C held for 2 min, then increased to 200 °C at the rate of 4 °C/min, at which point the rate was increased to 15 °C/min and held for 15 min after the oven temperature reached 250 °C. Four trained sniffers were asked to sniff both Sintanur and Ciherang samples in duplicate. A timer was started at the beginning of sample injection. The sniffers were asked to describe the odour they perceived, record the time point on the timer when they perceived the odour and rate the intensity of the odour from 0 (nil) to 10 (extreme). An alkane standard (C5–C18) was used to calculate linear retention index (LRI) value.

4.2.5. Statistical analysis

Sensory profiling data were collected by Compusense at-hand (version 8.8, Guelph, Canada) and analysed using Senpaq (v4.2; Qi Statistics 2008, Reading, UK). Two-way ANOVA was used with sample fitted as a fixed effect and panellists as a random effect; effects were tested against the sample by panellist interaction. Significant differences between samples were assessed by Fisher's LSD pairwise comparison, and significance level was set at $p \leq 0.05$. In order to compare fragrant and non-fragrant rice samples as two groups, Student's *t*-test was carried out using XLSTAT software (2012, Addinsoft, Paris, France).

4.3. Results and Discussion

4.3.1. Quantitative descriptive analysis (QDA) in boiled rice

4.3.1.1. 2-Acetyl-1-pyrroline reference standard training

The understanding of attributes could vary for panellists from different cultures and with different experiences. Paule and Powers (1989) reported that descriptions of fragrant rice aroma by different groups were different. Orientals or frequent rice consumers described this aroma as 'pandan-like'; however, non-Orientals or infrequent rice consumers described it as popcorn-like. The fragrance in fragrant rice in this study was initially described as 'popcorn-like', 'basmati-like' or 'Jasmine rice-like' by 11 trained UK based panellists. 'Popcorn-like' is the major descriptor for this aroma. Buttery et al (1982) firstly described the odour as 'popcorn-like' in fragrant rice and reported that it was contributed by 2-acetyl-1-pyrroline. The popcorn-like aroma in boiled rice was described as 'a dry, dusty, slightly toasted and slightly sweet aroma that can be specifically identified as popcorn' in the lexicon developed by Kansas State Expert Sensory Panel (Goodwin et al., 1996). Mahattanatawee and Rouseff (2014) described the fragrance in basmati, jasmine and Jasmati varieties as 'cooked jasmine rice-like' using GC-O analysis. In the present study this aroma was finally unified to 'popcorn-like' with unanimous consent of all panellists. 2-AP standard was provided to panellists, to compare it with the fragrant odour in boiled rice samples.

The training of popcorn odour started from a set of 0 (blank), 100, 1,000, 5,000 and 25,000 µg/kg 2-AP standards, which were prepared in the same manner as in *Section 3.2.3*. Panellists (*n* = 11) were asked to sniff five different concentrations of 2-AP standards and rate perceived intensity on an unstructured line scale (0–100). The average value of each reference was added onto the line scale, and this line scale was used for further training. Panellists were subsequently asked to sniff all six boiled rice samples with reference to the 2-AP standards and perceived popcorn odour intensities were added onto the line scale with the 2-AP standard anchors; the average values of all samples and standard references on the line scale are shown in **Figure 4.1**. It

can be seen that the highest intensity (48) was perceived for jasmine rice; this mean score was slightly higher than the perceived intensity of the 1,000 µg/kg 2-AP standard. This result suggested that lower concentrations of 2-AP standard may be necessary to provide a more valid calibration. In addition, the average score for the blank standard sample was 6 rather than 0 which may have been influenced by sniffing the other 2-AP standards in the same environment; this could cause the score of popcorn odour in non-fragrant rice samples to be lower than the blank reference.

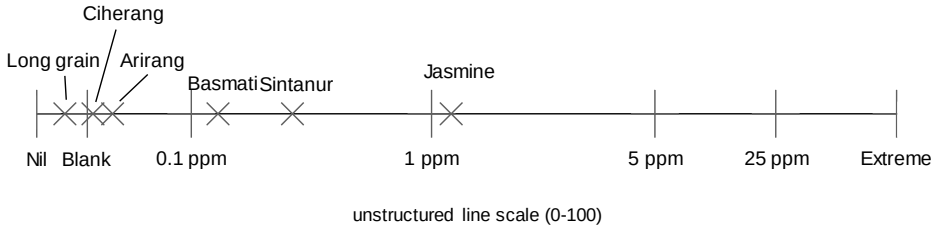

Figure 4.1: Unstructured line scale (0-100) with anchors of 2-AP standard references and average values of perceived popcorn odour intensity for the six boiled rice samples.

Considering this result, concentrations of 2-AP standard were subsequently reduced to blank (0), 10, 100, 1,000 and 5,000 µg/kg. Panellists were asked to rank these five 2-AP standards in triplicate to ensure that all the panellists could differentiate and rank 2-AP standards without difficulty. This ranking result suggested that 5- to 10-fold difference in 2-AP standards could be detected by a trained UK panel. The blank standard was subsequently labelled as Nil and the 5,000 µg/kg standard was labelled as Extreme; these two standards were scored as 0 and 100 on the unstructured line scale. The other three standards (10, 100, 1000 µg/kg) were labelled as '1', '2', '3' from low to high concentration and panellists (n = 11) were asked to sniff and rate these three references using the line scale relative to the Nil and Extreme references. Results are shown in **Figure 4.2a**. Mean scores were then used as anchors at 12, 40 and 75 on the 0–100 line scales for popcorn odour in the subsequent sample rating tests.

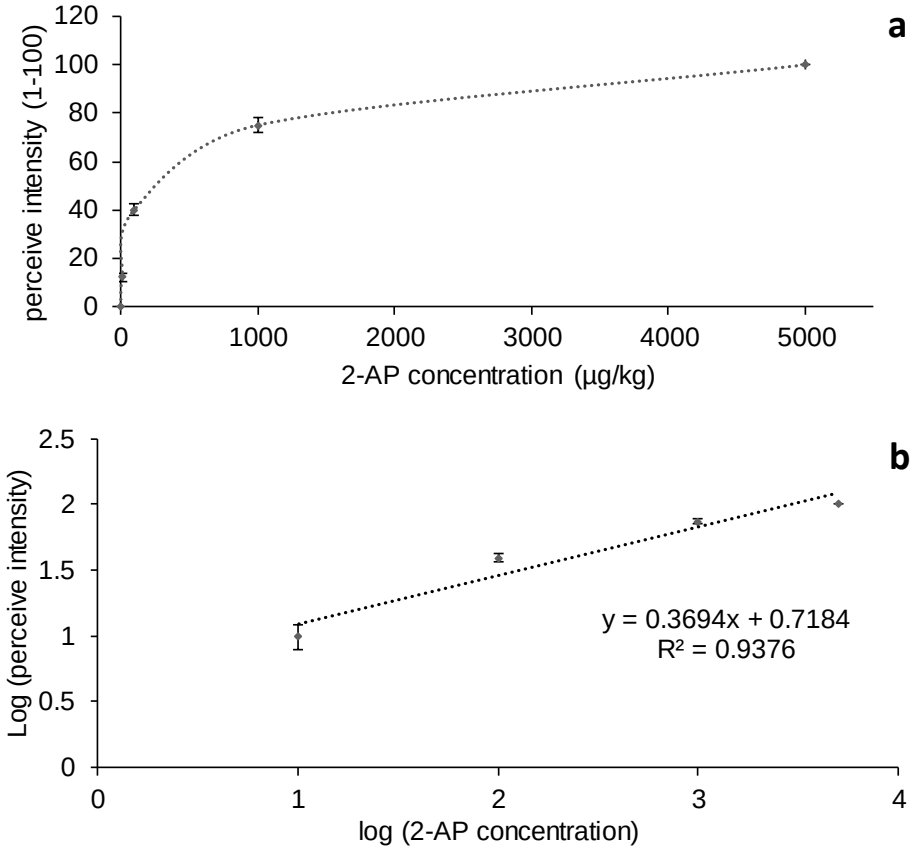

Figure 4.2: (a) Mean perceived intensity of odour of 2-AP standard references (0, 10, 100, 1000 and 5000 µg/kg) from 11 panellists; (b) log stimulus *vs* log response plot of perceived intensities of odour of 2-AP standard references (10, 100, 1000 and 5000 µg/kg) from 11 panellists. Error bars represent standard error of the mean.

According to Steven's law: "equal stimulus ratios result in equal sensation ratios rather than equal sensation difference", and his psychophysical power law was proposed as

$$R = kS^n$$

therefore

$$\log R = n \log S + \log k$$

where R is the response, k is a constant, S is the stimulus concentration, and n is the modality-dependent exponent (Stone, Bleibaum, & Thomas, 2012). The log–log plot between 2-AP concentration and perceived popcorn odour intensity follows Steven's law and is shown in

Figure 4.2b; *n* is 0.338, denoting a decelerating relationship, as expected for aroma perception. This result indicates that with increasing 2-AP concentration, the perceived popcorn-like odour intensity increases but to a less than proportional extent. Therefore, it may be more difficult for panellists to notice changes of 2-AP concentration at higher concentrations than at lower concentrations.

4.3.1.2. Boiled rice sensory attributes

Thirty-seven attributes (covering appearance, mouthfeel, odour, taste, flavour and after-effects) were quantified in the six boiled rice samples; however, significant differences between samples were only found in 8 attributes (**Table 4.2**). In physical modalities (appearance and mouthfeel), significant differences between samples were found for cohesive mouthfeel ($p < 0.0001$) and appearance attributes ($p < 0.0001$). The highest number of brown lines was observed in long-grain and Ciherang rice, and the lowest number of brown lines was found in jasmine rice. Brown lines cannot be observed on raw rice, they only appeared after rice boiling and they were only found on the surface of rice grain. Brown lines were not present in every rice grain and this attribute was evaluated by how many grains with brown lines could be observed in one sample portion (50 g). The brown lines may be due to crack formation during rice postharvest processing or storage, where perhaps incomplete drying or long-term storage may cause more brown lines to develop. However, to our knowledge, this has not been reported in the literature.

Arirang rice had the shortest rice grain and basmati rice had the longest rice grain after boiling, while basmati rice also gave the thinnest grains. The physical attributes in boiled rice, especially moisture content, stickiness and hardness are influenced by rice grain length and their starch content. Arirang rice had the highest 'wet' score and basmati had the lowest score. Visible moisture differences may be caused by different water absorption abilities of the different rice varieties. Water absorption of rice grain is dependent on surface area, amylose and protein contents

and gelatinisation temperature. Generally, long-grain varieties tend to absorb more water than short-grain varieties (Bett-Garber, Champagne, Ingram, & McClung, 2007). Therefore, as the same amount of water was added to all samples for boiling in this study, the shorter grain rice varieties (Arirang, Ciherang and Sintanur) appeared wetter than the three longer grain varieties.

The ratio of amylose to amylopectin in rice grain can significantly influence stickiness and hardness of boiled rice. Long-grain rice types (*indica*) usually contain more amylose and less amylopectin and can be harder and less sticky. In contrast, short-grain rice types (*japonica*) contain more amylopectin and less amylose, they are softer and stickier (Bao & Bergman, 2004; Li et al., 2016). The stickiness of boiled rice is caused by leached amylose and amylopectin interacting with each other and forming a coating on the surface of the grains (Bett-Garber et al., 2007). The differences in starch composition in the different rice varieties were expressed in their sensory attributes. High stickiness was expressed as lower grain separation appearance and higher cohesive mouthfeel scores. Effort to chew reflected the hardness of boiled rice grain. **Table 4.2** showed that basmati had highest grain separation and lowest cohesive mouthfeel. Arirang rice had the highest score for cohesive mouthfeel. However, no significant difference was found in this attribute between the six boiled rice varieties.

Table 4.2: Mean value and significance of sensory attributes for six boiled rice types. Where values in a row do not share the same letter, they are significantly different ($p < 0.05$, Fishers LSD)

Attributes	Mean value of perceived intensities (0-100)						Sample significance (*p*-value)
	Fragrant rice		Non-fragrant rice				
	jasmine	basmati	Sintanur	Arirang	long-grain	Ciherang	
Appearance							
wet	28.9^{ab}	5.86^{c}	30.3^{ab}	35.2^{a}	15.4^{bc}	28.9^{ab}	< 0.0001
yellow	14.8	16.2	20.7	24.3	13.7	14.6	0.203
brown lines	1.99^{c}	4.50^{abc}	2.93^{bc}	10.0^{ab}	10.6^{a}	11.4^{a}	< 0.0001
uniform	64.4^{a}	71.4^{a}	57.5^{ab}	56.3^{ab}	41.9^{b}	55.5^{ab}	< 0.0001
separated grain	47.8^{ab}	56.5^{a}	41.5^{ab}	31.5^{b}	31.4^{b}	36.9^{b}	< 0.0001
length	56.6^{ab}	71.9^{a}	38.1^{c}	37.7^{c}	46.1^{bc}	49.1^{bc}	< 0.0001
thickness	51.5^{a}	29.7^{b}	55.7^{a}	65.4^{a}	51.9^{a}	53.0^{a}	< 0.0001
Odour							
popcorn	49.1^{a}	43.2^{ab}	39.1^{ab}	32.2^{ab}	42.0^{ab}	24.9^{b}	0.028
sweet	39.3	32.1	28.0	31.1	34.8	28.3	0.297
porridge	31.8	26.6	25.5	35.6	30.2	30.7	0.647
rice pudding	16.8	9.90	11.9	14.5	13.7	10.1	0.609
milky	16.1	12.0	10.6	11.0	10.0	7.34	0.479
starchy water	21.3	22.0	22.6	16.0	19.2	27.8	0.442
eggy	9.32	10.1	7.42	2.72	6.30	10.8	0.572
Taste							
sweet	27.5	23.8	27.0	24.9	23.2	21.0	0.819
bitter	8.92	3.15	4.44	8.12	10.8	8.70	0.472
salty	7.88	4.79	6.91	8.88	6.94	8.70	0.843
savoury	19.0	23.3	19.3	22.3	15.6	18.7	0.875
metallic	4.20	3.29	5.19	4.15	6.57	4.49	0.909
Flavour							
popcorn	24.5	16.2	23.5	17.3	12.8	12.2	0.134
porridge	22.7	22.8	22.2	30.8	24.0	22.8	0.691
rice pudding	13.5	6.51	11.2	11.0	5.76	7.98	0.235
milky	12.8	4.15	9.27	7.85	7.66	5.94	0.254
starchy water	25.7	23.0	30.0	32.6	23.1	32.4	0.402
eggy	7.68	1.46	1.98	2.49	1.89	6.45	0.173
Mouthfeel							
smooth	50.7	48.9	52.7	46.0	38.6	44.5	0.316
effort to chew	38.3	45.7	36.6	43.8	47.0	41.3	0.489
drying	33.7	36.8	32.3	31.9	34.5	34.9	0.981
cohesive	44.1^{ab}	22.4^{c}	44.6^{ab}	54.8^{a}	28.1^{bc}	39.6^{abc}	< 0.0001
watery	12.2	4.55	10.5	12.3	8.09	11.0	0.376
After-effect							
popcorn	18.1	11.3	22.1	12.2	10.7	7.70	0.057
salty	7.84	11.2	7.98	11.4	7.85	4.48	0.765
sweet	22.6	18.8	21.6	20.1	17.8	20.5	0.912
bitter	5.77	3.49	3.35	6.99	8.43	7.18	0.664
drying	27.8	31.8	28.0	23.7	29.9	32.9	0.766
residue	27.8	16.8	28.4	26.0	22.8	24.6	0.664
starchy water	23.0	20.2	20.7	27.0	19.6	26.8	0.639

Of the 18 odour, taste and flavour attributes used to describe the boiled rice samples, only popcorn odour differed significantly between the samples (p = 0.028). Only 2 samples differed significantly for popcorn odour: the fragrant jasmine was significantly and substantially higher in popcorn odour than the non-fragrant Ciherang (difference of 24 in odour intensity rating score, p = 0.0013). The difference in popcorn flavour in mouth was not significant (p = 0.13), although the trend was the same (jasmine highest and Ciherang lowest) with a difference of 12 in flavour intensity rating score. Where popcorn was rated as an after-effect (flavour post-swallowing) the trend (p = 0.057) was for the fragrant Sintanur and jasmine varieties to be rated higher than the Ciherang.

When the six rice varieties were grouped into fragrant rice (jasmine, basmati and Sintanur) and non-fragrant rice (long-grain, Arirang and Ciherang), *t*-test results of all of the odour and flavour-related attributes showed significant differences between fragrant and non-fragrant rice types in popcorn odour (p = 0.016), popcorn flavour (p = 0.026) and popcorn after-effect (p = 0.019), as shown in **Figure 4.3**. However, no differences were observed in the other rice and cereal-related odour and flavour attributes. Yang et al. (2008) reported that the popcorn-like note may not be the only important characteristic in boiled rice and other key characteristics contributed by other volatile compounds could be found in boiled fragrant rice. The results of this study concur with Yang et al., in that there were other aroma and flavour attributes present in boiled rice. However, none of these additional odours or flavours (**Figure 4.3**) differentiates fragrant and non-fragrant rice types.

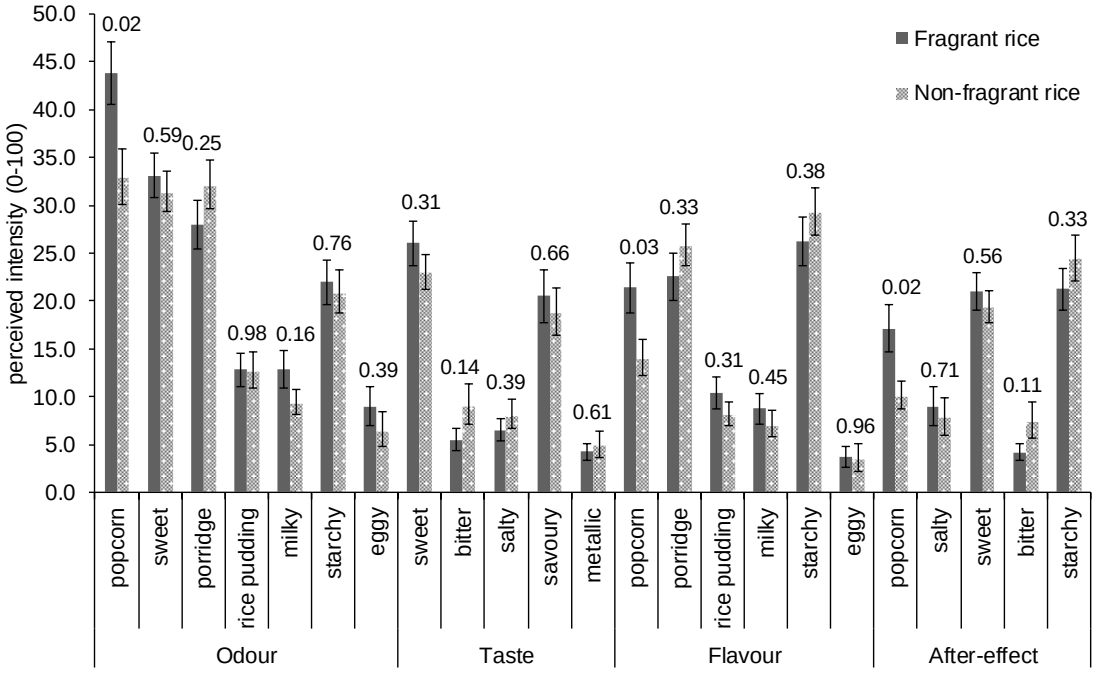

Figure 4.3: Perceived intensities of odour, taste and flavour related attributes between fragrant and non-fragrant rice types. P value labelled on the top of bars. Error bars represent standard error of the mean.

As discussed earlier, the differences in popcorn attributes between all different rice varieties was not obvious (**Figure 4.4**). The significant difference in perceived popcorn odour was driven by jasmine and Ciherang. However, panellists found it difficult to differentiate popcorn odour in the other four boiled rice samples (basmati, Sintanur, long-grain and Arirang). Although jasmine and Sintanur tended to show higher perceived popcorn flavour and after-effect than other samples, any differences between rice varieties were not significant (**Figure 4.4**). These results indicate that although the panellists could not differentiate individual boiled rice varieties based on popcorn odour, flavour or after-effect; fragrant and non-fragrant rices could be distinguished as two separate groups based on all three of these modalities.

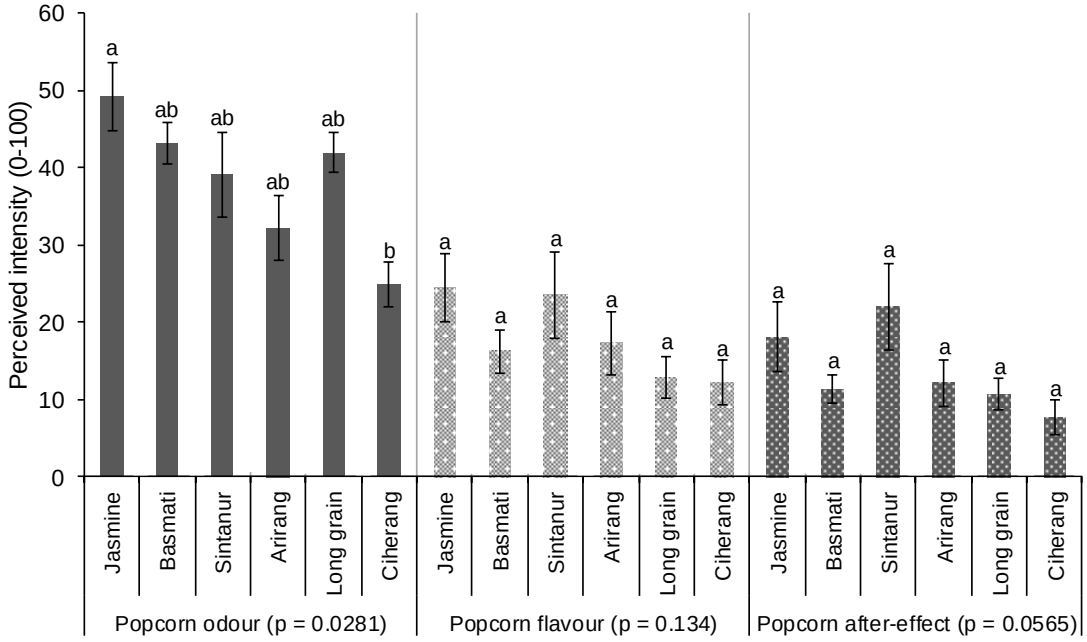

Figure 4.4: Perceived popcorn odour, popcorn flavour and popcorn after-effect among six rice samples. Scores for each attribute not sharing a common letter are significantly different ($p < 0.05$). Error bars represent standard error of the mean.

Where the difference in popcorn odour between varieties was significant and any differences between in-mouth popcorn flavour and popcorn as an aftertaste were not, this may have been due to the use of the four reference anchors (2-AP standards) for training the assessors (*Section 4.2.2*). This may have helped panellists to improve their discrimination of different boiled rice samples based on popcorn odour. However, the 2-AP standard training would have less effect in improving the discrimination of popcorn flavour and after-effect because the standards can only be sniffed; hence no standard levels of popcorn flavour and after-effect were provided to panellists. The lack of flavour and aftertaste standards may have resulted in higher variation between panellists in popcorn flavour and after-effect than in odour, and hence resulted in a reduced likelihood of discrimination.

Popcorn was used as a reference material for 'popcorn' attributes in previous studies (Limpawattana et al., 2008; Limpawattana & Shewfelt, 2010); it could have been used in the training of 'popcorn' odour, flavour and after-effect. However, other aromas present in popcorn,

such as ‘smoky’, may influence the understanding of ‘popcorn-like’ for panellists. Schieberle (1991) suggested that not only ‘popcorn-like’, but also ‘fatty’, ‘coffee-like’ and ‘spicy’ play important roles in the aroma of popcorn. In addition, intensities of ‘popcorn’ attributes cannot be controlled and adjusted in popcorn product during training.

4.3.2. Quantification of 2-acetyl-1-pyrroline in boiled rice

Boiled long-grain rice was used as a matrix for 2-AP calibration in this study. Although 2-AP cannot be quantified, trace of 2-AP ions (*m/z* 68, *m/z* 83, and *m/z* 111) could still be detected in long-grain rice samples. However, increasing viscosity or gelation of a food matrix can significantly decrease mass transfer and therefore influence flavour release (Silva, Castro, & Delgadillo, 2002). It was reported that release of aroma compounds is influenced by the amylose fraction in a gelation matrix; in contrast amylopectin is unlikely to form strong inclusion complexes with aroma compounds (Silva et al., 2002). It was discussed in *Section 3.3.2* that the starch composition was reflected in grain separation, cohesiveness and effort to chew, and long-grain rice scored average values for these attributes, indicating that long-grain rice contained an intermediate level of amylose amongst the six rice varieties. In addition, long-grain rice was the rice variety in which the smallest peak areas for 2-AP ions were detected among the three non-fragrant samples. Therefore, boiled long-grain rice was arguably the best choice as a matrix material for 2-AP calibration in this study.

Concentrations of 2-AP in the six boiled rice samples are shown in **Figure 4.5**. Significant differences in 2-AP concentrations were found between the three boiled fragrant rice samples ($p = 0.028$); jasmine rice contained most 2-AP (146 µg/kg), while the lowest 2-AP concentration in a boiled fragrant rice was in Sintanur (80 µg/kg). The most popular fragrant rice on the UK market, basmati, contained 113 µg/kg of 2-AP which would explain why it was ranked in the middle of the three boiled fragrant rice varieties in this study for perceived intensity of popcorn odour, even though the difference of intensities between the three was not significant. Although a significant

difference was found between the three fragrant rice samples in 2-AP concentration. There was only a two-fold difference between jasmine and Sintanur rice. The concentrations of 2-AP in the standard references used for popcorn odour training varied by 5 or 10-fold in difference. Popcorn odour intensity of blank (0), 10, 100, 1000 and 5000 µg/kg 2-AP reference standards were ranked, and results showed that the trained panellists could differentiate and rank these samples in order of intensity with no difficulty. There was no evidence to show that a two-fold difference in 2-AP was great enough to be noticed by panellists, which might explain why there was no significant difference in popcorn odour, flavour or after-effect between the three fragrant rice samples. In addition, according to a log-log plot of 2-AP concentration and perceived popcorn odour intensity (**Figure 4.2b**), a decelerating relationship between 2-AP odour perception and 2-AP concentration may cause relatively more difficulty for panellists in discriminating higher 2-AP concentration samples and less difficulty in discriminating lower 2-AP concentration samples.

When 2-AP concentration and popcorn-like aroma of Sintanur, basmati and jasmine were plotted in **Figure 4.2b**, the coordinates of the three fragrant rice varieties were similar to that of the 100 µg/kg 2-AP standard (**Figure 4.6**). Hence, it appears that the relationship between 2-AP concentration and popcorn aroma in 2-AP standard can be applied to the fragrant rice varieties studied.

Limpawattana et al. (2008) reported that although 2-AP was the only contributor to popcorn-like note in boiled rice, there was no correlation between 2-AP concentration and perceived intensity of popcorn flavour. Their data showed that popcorn flavour had negative correlations with guaiacol and (*E,E*)-2,4-decadienal, which contributed smoky and fatty notes, respectively. They also reported that guaiacol was present in the popcorn they used for popcorn odour training, which might have affected the understanding of popcorn flavour. Guaiacol was not identified in any of the boiled rice samples in our study.

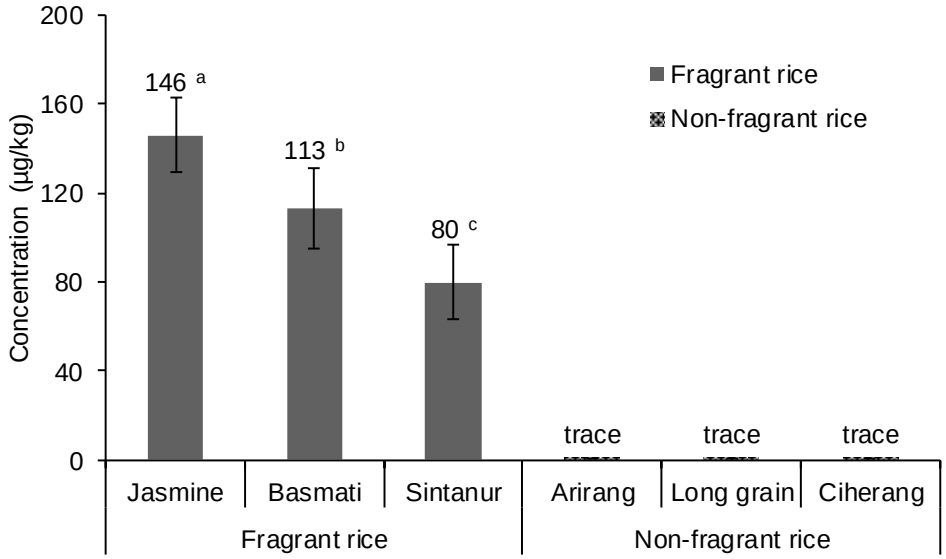

Figure 4.5: 2-AP concentrations in six boiled rice samples. Bars not sharing a common letter are significantly different ($p < 0.05$). Error bar represents standard deviations. ND: only trace level was found, concentration lower than 5 µg/kg.

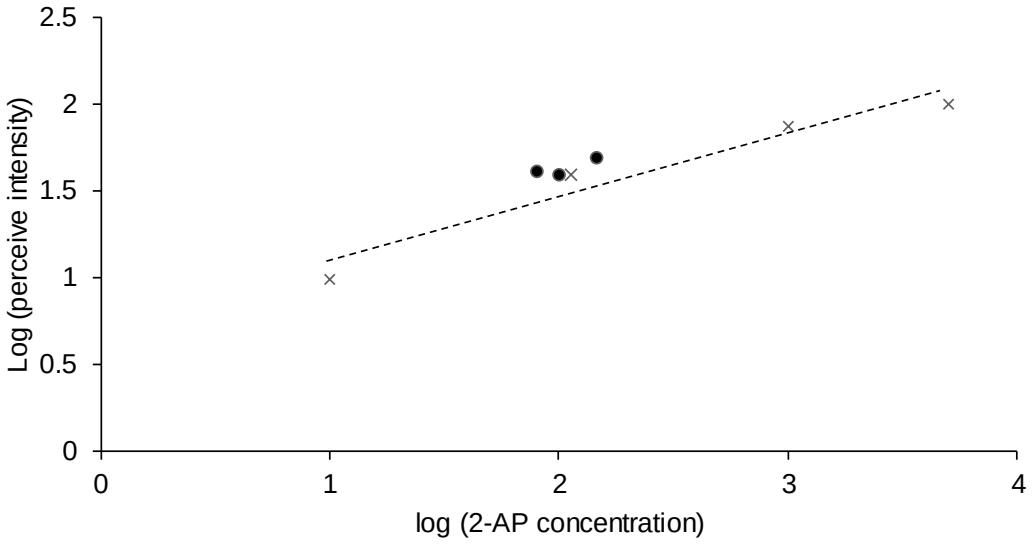

Figure 4.6: 2-AP concentration and perceived popcorn aroma intensity of boiled fragrant rice varieties fit in log stimulus vs log response plot of perceived intensities of odour of 2-AP standard references (10, 100, 1000 and 5000 µg/kg) from 11 panellists. Crosses represent 2-AP standards in training, points represent three fragrant rice varieties.

Yang et al. (2008) analysed 25 different odour-active compounds in five boiled fragrant rice samples and one boiled non-fragrant rice sample. They found that popcorn-like odour could be detected in both fragrant and non-fragrant rice varieties, and 2-AP was the only compound to

contribute to this odour. Another study also evaluated 25 aroma-active compounds in fragrant and non-fragrant long-grain and medium-grain Italian rice. Again, 2-AP was the only compound contributing popcorn-like odour (Griglione et al., 2015).

Compounds other than 2-AP may contribute roasty or popcorn-like aroma in popcorn, such as 6-acetyl-1,2,3,4-tetrahydropyridine and 2-propionyl-1-pyrroline (Schieberle, 1991). 2-Acetyl-2-thiazoline was reported to contribute to popcorn-like odour in boiled American-grown jasmine-style long-grain rice (Mahattanatawee & Rouseff, 2014). This compound has a similar aroma to 2-AP and is much more stable than 2-AP (Rey, Bel-Rhlid, & Juillerat, 2002). However, 6-acetyl-1,2,3,4-tetrahydropyridine, 2-propionyl-1-pyrroline and 2-acetyl-2-thiazoline were not detected in SPME extracts of boiled rice samples in the present study.

2-AP was detected in some non-fragrant rice varieties in previous studies using different extraction and quantification techniques; concentrations of 2-AP in non-fragrant rice have been reported from 0.6 µg/kg to 24.7 µg/kg (Buttery et al., 1983; Buttery, Turnbaugh, & Ling, 1988; Maraval et al., 2010). The lowest concentration of 2-AP standard that could be quantified by GC-MS in our study was 5 µg/kg (see calibration curve preparation in *Section 3.2.3*.). Trace levels of key 2-AP ions (*m/z* 68, *m/z* 83 and *m/z* 111) were detected in samples which contained less than 5 µg/kg 2-AP; these trace peaks could not be quantified. In our study, 2-AP levels in three non-fragrant rice varieties were lower than the limit of quantification (5 µg/kg), although peaks for the key ions of 2-AP can be observed (**Figure 4.5**). The odour thresholds of 2-AP are 0.1 nL/L in water (Buttery et al., 1983) and 0.02 ng/L in air (Schieberle, 1991), levels which are much lower than the quantification threshold of GC-MS in this study. Therefore, in order to confirm the presence of 2-AP in our non-fragrant rice samples, GC-olfactometry is likely to be a technique with higher sensitivity than GC-MS.

4.3.3. Detection of 2-acetyl-1-pyrroline in boiled rice by GC-olfactometry

2-AP levels with SPME in boiled non-fragrant rice were lower than the limit of quantitation (LOQ) in GC-MS, so this technique may not have been sensitive enough for GC-O. Although 2-AP levels in non-fragrant rice may be lower than its LOQ in GC-MS with solid-phase extraction (SPE), A larger sample size can be applied in SPE, and this can help to improve GC-O detection. Due to the gelation of starch, the supernatant from boiled rice-water solution could hardly pass through SPE sorbent. Yoshihashi (2002) reported that 2-AP cannot be formed during rice boiling. Therefore, raw milled Sintanur (fragrant) and Ciherang (non-fragrant) were extracted and analysed by GC-O.

The results from the GC-O analysis (four assessors analysing each rice extract in duplicate) showed that popcorn-like odour was only perceived over an LRI range between 1330 and 1347 in both raw Sintanur and Ciherang rice. The LRI value of 2-AP on the same stationary phase when used in the GC-MS analysis was 1333. Therefore, it seems likely that 2-AP was the sole contributor to perceived popcorn-like odour in both raw fragrant and non-fragrant rice. Sniffers rated aroma intensity from 0 (nil) to 10 (extreme) when compounds eluted from the GC column. Average perceived 2-AP intensity in Sintanur was 7.00 ± 0.50 and in Ciherang was 3.88 ± 0.93; Student's *t*-test showed that 2-AP intensity in Sintanur was around 2-fold higher than in Ciherang (p = 0.0001). In addition, all the sniffers scored popcorn intensity higher for Sintanur rice than Ciherang rice (**Appendix 1**).

The concentration of 2-AP in boiled fragrant rice was at least 15-fold higher than that in boiled non-fragrant rice (based on the LOQ of 2-AP) in our study; however, its perceived odour intensity in raw fragrant rice by GC-O was only two times higher than in raw non-fragrant rice. As discussed in *Section 3.3.1*, the odour perception of 2-AP fits Steven's law and shows a decelerating relationship with increasing concentration. Since the detection threshold of 2-AP is 0.02 ng/L in air (Schieberle, 1991), which is much lower than the LOQ of 2-AP, the difference in

2-AP perceived intensity between fragrant and non-fragrant rice is somewhat less than the difference in 2-AP concentration. When the concentration of 2-AP in boiled Sintanur rice (80 µg/kg) and LOQ of 2-AP in GC-MS (5 µg/kg, assumed as the possible highest concentration of 2-AP in non-fragrant rice) were substituted into the formula in **Figure 4.2a** (log 2-AP concentration *vs* log 2-AP perceived intensity of 2-AP sniff standard), the log perceived intensity of Sintanur and non-fragrant rice were 1.42 and 0.98, respectively, and perceived intensity were 26.4 and 9.5, i.e., the perceived intensity of Sintanur was no more than three times higher than non-fragrant rice, which is of a similar order to the values obtained by GC-O in our study.

Although 2-AP was not quantifiable by GC-MS of non-fragrant rice, GC-O provided clear evidence that a low concentration of 2-AP was present in raw Ciherang non-fragrant rice. Based on the sensory profiling of boiled non-fragrant rice, it can be concluded that 2-AP can also contribute popcorn-like odour to non-fragrant rice.

4.4. Conclusions

This study emphasised that 2-AP and popcorn-like attributes given by 2-AP (odour, flavour and after-effect) are the most important discriminators between fragrant and non-fragrant boiled rice. Sensory profiling showed that significant differences were observed in popcorn odour, flavour and after-effect when fragrant and non-fragrant rice samples were compared as two groups. 2-AP quantification results concluded that significant differences in 2-AP concentration between the three fragrant rice were too small to cause differences in their perceived popcorn-like aroma. Trace level of 2-AP ion chromatograms were found in non-fragrant rice by GC-MS, and its presence was confirmed by GC-O, but levels were lower than the limit of quantification. At least 15 times higher levels of 2-AP were found in fragrant rice than non-fragrant rice (based on the LOQ of 2-AP by GC-MS). Our study suggested that 2-AP is the most important aroma contributor in fragrant rice, and it is the discriminator for fragrant and non-fragrant rice. However, the

popcorn-like aroma of 2-AP can also be perceived in non-fragrant rice, although below the current levels of detection by GC–MS.

Chapter 5. 2-Acetyl-1-pyrroline Levels in Fragrant and Non-fragrant Rice (*Oryza sativa*) Baked at High Temperatures

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This chapter was presented at the 11th Wartburg Symposium as a poster presentation; the manuscript will be submitted to *Food Chemistry*.

Abstract

2-Acetyl-1-pyrroline (2-AP) was measured in milled rice and rice bran from fragrant and non-fragrant rice, both in uncooked ground rice and also in ground rice baked without water for 20 minutes at up to 220 °C. 2-AP concentrations were measured using solid-phase extraction, followed by gas chromatography-mass spectrometry. While 2-AP was found in both baked and unbaked fragrant rice and reached a maximum concentration (96.0 µg/kg) at around 100 °C, 2-AP was only found in non-fragrant rice heated above 140 °C and its concentration increased with increasing baking temperature, reaching a maximum (13.9 µg/kg) at 220 °C. 2-AP was only formed in milled rice during baking but cannot be generated in rice bran, even though 2-AP precursors (amino acids and reducing sugars) were at higher levels in rice bran compared with milled rice. 2-AP formation showed different trends in fragrant and non-fragrant rice when rice was baked over a range of temperatures. It could be seen that formation of 2-AP in heated rice may follow different routes at different baking temperatures. At temperatures above 140 °C, 2-AP can be formed by Maillard reaction in both types of rice. However, in fragrant rice, formation can occur at temperatures considered too low for Maillard-type reactions; a formation pathway other than Maillard reaction may occur in fragrant rice, which drive 2-AP generation at lower temperature.

Keywords: 2-acetyl-1-pyrroline, *Oryza sativa*, fragrant rice, non-fragrant rice, brown rice, white rice, solid-phase extraction, gas chromatography-mass spectrometry

5.1. Introduction

Rice (*Oryza sativa*) is one of the most important foods around the world; it provides calories for 25% of the world's population (FAO, 2002). Rice is categorised into two types depending on its flavour: fragrant rice and non-fragrant rice. 2-Acetyl-1-pyrroline (2-AP, IUPAC name: 1-(3,4-dihydro-2H-pyrrol-5-yl)ethanone, CAS number: 85213-22-5, FEMA number: 4249) is well-known as a key aroma compound, causing popcorn-like odour in cooked rice (Buttery et al., 1982). Much rice flavour research has focused on this polar volatile compound. The threshold of 2-AP in air is only 0.02 ng/L (Schieberle, 1991). In addition, this very low threshold compound is regarded as important in providing popcorn-like odour in many food products. It has been found in many cooked foods, such as popcorn (Schieberle, 1991) and bread (Schieberle and Grosch, 1987).

The difference in the levels of 2-AP between fragrant and non-fragrant rice is due to a recessive gene fragment (*fgr*) that can encode a betaine aldehyde dehydrogenase isoform, known as BADH2. Functional BADH2 in non-fragrant rice catalyses the oxidation of γ-aminobutyraldehyde to γ-aminobutyric acid (GABA; Chen, Yang, Shi, Ji, He & Zhang, 2008). A non-functional BADH2, which cannot catalyse the oxidation of γ-aminobutyraldehyde, is encoded in fragrant rice. The oxidation of γ-aminobutyraldehyde reduces the potential for 2-AP synthesis (Kovach, Calingacion, Fitzgerald & McCouch, 2009) because γ-aminobutyraldehyde exists in equilibrium with its cyclic form, 1-pyrroline (Struve and Christophersen, 2003); the latter is a precursor of 2-AP (Schieberle, 1990). Therefore, in fragrant rice 2-AP is accumulated, while in non-fragrant rice 2-AP is difficult to detect (Bradbury et al., 2005). Another biosynthetic pathway of 2-AP has been put forward by Huang, Teng, Chang, Chuang, Ho and Wu (2008) that does not involve BADH2. Higher levels of 1-pyrroline-5-carboxylate (P5C) synthetase enzyme, and hence increased amounts of P5C, occur in fragrant cultivars in comparison to non-fragrant cultivars. P5C can react with methylglyoxal (2-oxopropanal), giving rise to 2-AP.

Both pathways require 1-pyrroline in order to produce 2-AP. In a recent study where fragrant and non-fragrant rice callus were incubated with 1-pyrroline, a significant increase in 2-AP production was observed in both cases, proving 1-pyrroline to be a limiting and key intermediate of 2-AP biosynthesis (Poonlaphdecha, et al., 2016).

2-AP could be formed in rice heated above 100 °C from 1-pyrroline and methylglyoxal *via* the Maillard reaction (Schieberle, 1990); proline together with reducing sugars could be the precursors of this reaction. Hofmann and Schieberle (1998a) reported that 2-acetylpyrrolidine could oxidise to 2-AP; they proposed that 1-pyrroline and methylglyoxal formed 2-acetylpyrrolidine, which was then readily oxidised to 2-AP.

2-AP contributes popcorn-like aroma in fragrant and non-fragrant rice, and it is also a desirable aroma contributor of some rice products, such as rice cakes (Buttery, Orts, Takeoka and Nam, 1999). Therefore, investigation of 2-AP generation during thermal processing in rice is important, which could helpful for improving 2-AP content in rice products. The purpose of the research in this paper is to increase understanding of how 2-AP forms during the heating of fragrant and non-fragrant rice, by measuring its concentration in ground rice heated across a range of temperatures. In addition, the potential precursors of both Maillard-derived and biosynthesis-derived 2-AP are also quantified, in order to elucidate the correlation between 2-AP formation and precursors consumption.

5.2. Materials and Methods

5.2.1. Plant materials and chemicals

Two fragrant rice cultivars (basmati, Sintanur) and two non-fragrant rice cultivars (American long-grain and Ciherang) were studied. Basmati brown rice and long-grain brown rice were purchased from a branch of ASDA supermarket in the Reading area. Brown Sintanur and

brown Ciherang samples were provided by Indonesian Centre for Rice Research (ICRR), Sukamandi, Indonesia. All samples were stored at 4 °C before analysis.

HPLC-grade water, hydrochloric acid (36%), methanol (⩾99.8%) and dichloromethane (DCM, ⩾ 99.8%) were obtained from Fisher Scientific (Loughborough, UK); 2,4,6-trimethylpyridine (TMP) and trehalose were from Sigma-Aldrich (St Louis, MO).

5.2.2. Preparation of rice samples

Brown rice grains were milled in the laboratory using a Twinbird MR-E750 rice polisher; Twinbird Corporation, Tsubame City, Japan) in "70% white rice" mode; 200 g brown rice were polished for 160 s. Only around 70% of rice bran were removed from brown rice in this mode, to prevent contamination of the rice bran with white rice. Around 20 g of rice bran were prepared and collected from the brown rice. The remaining rice grain was then polished in "100% white rice mode" to remove the residual rice bran. Around 140 g of milled rice were collected from brown rice after the two polishing steps. Milled rice was ground using an MSE ATO Mix grinder; both milled rice and rice bran were sieved using an Endecotts Test sieve with 0.814-µm apertures (Endecotts Ltd, London, UK) before analysis, to keep the same particle size between milled rice and rice bran.

5.2.3. Rice high temperature baking

Rice flour (10 g ± 0.01 g) was put into 20-mL glass ampoules (Wheaton, Millville, NJ) for baking without water. All cultivars of rice bran and milled rice flour (ASDA basmati, ASDA long-grain, Sintanur and Ciherang) were baked in an oven at 180 °C for 20 min for 2-AP and precursors (amino acid and reducing sugar) analysis. Milled Sintanur and Ciherang rice were also baked for 20 min at different temperatures from 40 °C to 220 °C, at 20 °C intervals, for analysis of 2-AP. After initial analysis by GC-MS, concentrations of 2-AP around 100 °C showed an unexpected

increase; therefore, samples baked at 90 °C were added into the experiment to observe more specific changes of concentration. Finally, milled Sintanur and Ciherang were baked at 40 °C, 60 °C, 80 °C, 90 °C, 100 °C, 120 °C, 140 °C, 180 °C, 200 °C, and 220 °C. After baking, rice flour was cooled to room temperature before extraction.

5.2.4. Solid-phase extraction of 2-acetyl-1-pyrroline in rice

An accurately weighed (10.00 ± 0.01 g) rice flour or rice bran sample (uncooked or baked) was put into a 50-mL centrifuge tube and 35 mL HPLC-grade water were added. Then the tube was shaken for 20 min at 1700 rpm (Multi Reax; Heidolph, Schwabach, Germany), before centrifuging at 7000 rpm (5095 *g*) and 15 °C for 15 min (Sigma 3K10 laboratory centrifuge; Sigma, Osterode, Germany). A 20-mL aliquot of the supernatant was collected for solid-phase extraction (SPE). The Isolute ENV$^+$ cartridge (200 mg/6 mL; Biotage, Uppsala, Sweden) was firstly conditioned with 10 mL methanol, then with 10 mL HPLC-grade water. Then 20 mL rice supernatant was loaded onto the cartridge. After sample loading, the cartridge was washed with 10 mL HPLC-grade water. The washed cartridge was dried under vacuum for 30 min. Finally, compounds were eluted with 2 mL DCM and collected in a 2-mL autosampler vial (Supelco, Bellefonte, PA) with metal crimp cap. Five microlitres of 50 mg/kg 2,4,6-trimethylpyridine (TMP) solution in DCM were added to the collected sample before the sample was concentrated to around 100 µL with a nitrogen stream. Finally, the concentrated sample was transferred to a 200-µL glass insert (Thermo Scientific, Loughborough, UK), which was then sealed in a 2-mL autosampler vial with metal cap for GC-MS analysis.

5.2.5. Gas chromatography-mass spectrometry analysis of 2-acetyl-1-pyrroline

A Zebron™ ZB-Wax column (30 m length × 0.25 mm i.d. × 0.25 µm film thickness; Phenomenex, Torrance, CA) was used in this analysis. One microlitre of the extract was injected in splitless mode into the GC-MS system; the injector temperature was 250 °C, the splitter was

opened at 1 min. The carrier gas was helium at a constant column flow rate of 1 mL/min. The initial GC oven temperature was 60 °C, held for 35 min, and then the temperature increased to 240 °C at 5 °C/min; this temperature was finally held for 10 min. The total analysis lasted 66 min. Electronic ionisation (EI) mode was applied; ionisation energy was 70eV, and the electron multiplier was set at 2000 V. The full scan mode was used for analysis from *m/z* 10 to 200. A 50 mg/L standard of 2-AP in dichloromethane (aromaLAB GmbH, Planegg, Germany) was run under the same conditions as a reference compound.

5.2.6. Sugar analysis

The sugar analysis followed the method of Elmore, Koutsidis, Dodson, Mottram and Wedzicha (2005). Ground rice or rice bran (0.100 g) was mixed with 5 mL of 0.01 M hydrochloric acid; 500 µL of 20 mg/L trehalose were added as internal standard. The sample was stirred for 15 min at room temperature. Then the sample was allowed to settle for 30 minutes before 1.5 mL of supernatant were centrifuged at 12500 rpm (7200 *g*) for 10 min (Centrifuge MiniSpin®; Eppendorf, Hamburg, Germany). A 500-µL aliquot of centrifuged supernatant was transferred into a 2-mL glass vial through a 0.2-µm Minisart® PES filter disk (Sartorius, Epsom, UK).

An 8220i Dionex ion chromatography system (Dionex Corp; Sunnyvale, CA) was used for sugar analysis. Sugar extracts (25 µL) were injected onto a Carbopac PA1 column (Dionex). Solvent **A** was H_2O and solvent **B** was 400 mM NaOH; an 11-min isocratic program used 65% solvent **A** and 35% solvent **B** as the mobile phase. After data acquisition 50% solvent **A** was run for the next 7 min before the column was re-equilibrated with 65% of solvent **A**. A pulsed amperometric detector was used with the following settings: 420 ms at 0.05 V, 180 ms at 0.75 V, and 420 ms at –0.15 V. 0.2 mg/L, 2 mg/L, 20 mg/L and 100 mg/L of standards of glucose, fructose and sucrose were used to prepare calibration curves for quantification.

5.2.7. Free amino acids analysis

Following the method of Elmore et al. (2005), ground rice or rice bran (1.00 g) was mixed with 5 mL of 0.01 M hydrochloric acid and then stirred for 1 hour at room temperature. Then, the sample was allowed to settle for 45 min before 1.5 mL of supernatant were centrifuged at 12500 rpm (7200 *g*) for 20 min (Centrifuge MiniSpin®; Eppendorf, Hamburg, Germany). Finally, 300 µL of centrifuged supernatant were collected for analysis.

The free amino acids in 100 µL of the extract were then derivatised using the EZ-Faast amino acid analysis kit (Phenomenex, Torrance, CA) for analysis by GC-MS. The preparation of a sample for GC-MS began with the addition of 200 nM norvaline internal standard (100 µL in 0.01 M HCl) to the extract, followed by a solid-phase extraction clean-up and then a two-step derivatisation at room temperature.

The derivatised amino acids were analysed by GC-MS with a 10 m × 0.25 mm Zebron ZB-AAA capillary column, with 0.25 µm film thickness. Sample volume was 1 µL and samples were run in split mode with a split ratio of 20:1. The injection port temperature was 250 °C. The carrier gas was helium at a flow rate maintained at 1.1 mL/min; the oven temperature was initially at 110 °C, then increased at 35 °C/min to 310 °C then held for 1 min. The ion source was maintained at 220 °C. The mass spectrometer scanned from *m/z* 35 to *m/z* 500 at 3 scans per second. Amino acids were quantified using external calibration curves, prepared using 50 nmol/mL, 100 nmol/mL and 200 nmol/mL derivatised amino acid standards.

5.2.8. Data analysis

All samples were prepared in triplicate to obtain reproducible data. ANOVA was performed in XLSTAT (2012, Addinsoft, Paris, France), to examine the difference between rice samples in 2-AP, amino acids and sugars. Pearson's correlation tests were performed in XLSTAT

(2012, Addinsoft, Paris, France) to examine the relationships between proline, glutamic acid, ornithine and 2-AP during rice baking. Significance level (*p*-value) was set at 0.05.

5.3. Results and Discussion

5.3.1. 2-Acetyl-1-pyrroline in all raw and 180 °C baked rice cultivars

2-AP levels clearly increased after heating at 180 °C and 20 min in both milled fragrant (basmati and Sintanur) rice and non-fragrant (long-grain and Ciherang) rice (**Figure 5.1**). In milled fragrant rice, a significant difference in 2-AP concentration between baked rice and raw rice could be observed in both milled basmati ($p = 0.031$) and milled Sintanur ($p < 0.001$), 2-AP concentrations increased 56% and 129%, respectively.

Unsurprisingly, 2-AP was hardly detected in raw milled non-fragrant rice, but generation of small amounts of 2-AP were detected in 180 °C baked non-fragrant rice (**Figure 5.1**). The concentrations in non-fragrant varieties were relatively low compared with fragrant varieties; however, due to the low threshold of this compound, 0.02 ng/L in air, the popcorn-like aroma could also be noticed in raw non-fragrant rice by sensory panel and GC-O analysis (see **Chapter 4**).

The Maillard reaction can lead to 2-AP formation as the milled baked rice results indicated, and much research has focused on the reaction pathway. Proline and ornithine can form 2-AP with reducing sugars in a model system (Schieberle, 1990). Proline reacts with 1-deoxyosone, a reducing sugar fragment, to form important 2-AP intermediate, 1-pyrroline, and methylglyoxal *via* Strecker degradation (Schieberle, 1995); these intermediates can further react to give 2-acetylpyrrolidine, which oxidises to 2-AP (Hofmann & Schieberle, 1998a).

The rice samples were baked in a low-moisture closed system, these conditions may affect the generation of Maillard reaction products. Hofmann and Schieberle (1998a) proposed that 2-AP generation from 1-pyrroline and methylglyoxal may proceed by different pathways in hydrated

and anhydrous conditions, resulting in higher yield of 2-AP with the involvement of water than the reaction under anhydrous conditions. However, previous studies have not reported 2-AP increasing in boiled rice; it could be influenced by the gelation of amylose during boiling. The amylose gel could significantly affect volatile compounds release, as discussed in the previous chapter (*Section 4.3.2*).

Davidek, Festring, Dufossé, Novotny, and Blank (2013) reported 2-AP generation under similar conditions to the current study. 2-AP generation was detected in a model rice system with additional amino acids and reducing sugars during baking in a low moisture and sealed system at 135 °C for 20 min. Cooking in a closed system can affect Maillard reaction products. For example, it may prevent loss of low-boiling intermediates. Maillard formation of alkylthiazoles and alkyl-3-thiazolines were reported in pressure cooked beef steaks in closed system (Elmore, Mottram, Enser and Wood, 1997). The closed system prevented the loss of hydrogen sulfide and ammonia at the beginning of the reaction; hence, hydrogen sulfide and ammonia reacted with aldehydes and hydroxyl ketones, to generate alkylthiazoles and alkyl-3-thiazolines (Elmore & Mottram, 1997). The boiling points of the key intermediates of 2-AP are relatively low; the boiling point of methylglyoxal is 72 °C and the predicted boiling point of 1-pyrroline is 113.7 ± 13.0 °C (Scifinder). Therefore, rice cooked in a closed system may increase 2-AP yield by preventing vaporised intermediates loss during reaction. However, the high pressure in the closed system may affect the Maillard reaction. Depending on the pH of the reaction system, high pressure may accelerate or decelerate the formation and further degradation of Amadori rearrangement products (ARPs), and may also affect the further stages of the Maillard reaction (Moreno, Molina, Olano, & López-Fandiño, 2003). 2-AP yields during baking in open and closed system were not compared in previous studies directly, and research on 2-AP generation in model systems was conducted using open systems (Rewicki et al, 1993; Schieberle, 1995; Hofmann & Schieberle, 1998a).

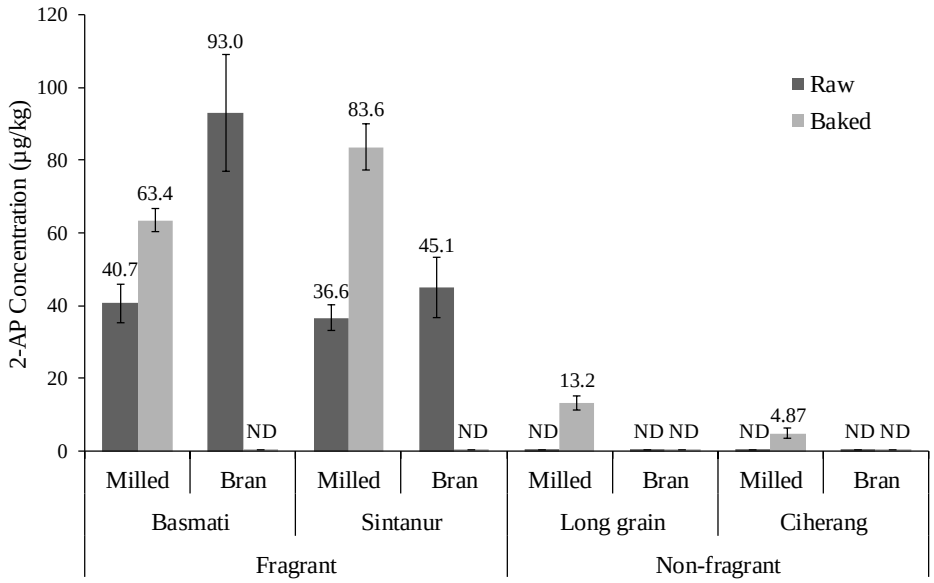

Figure 5.1: 2-AP concentrations (µg/kg) in raw (black bar) and 180 °C, 20 min baked (grey bar) milled and rice bran (n = 6); error bars show standard deviation (from six replicate analyses). ND means the compound cannot be detected by GC-MS.

2-AP content in rice bran is different from that in milled rice. 2-AP can be detected in raw fragrant rice bran; the concentration in raw basmati bran was around 1.3 folds higher than raw milled basmati (p = 0.0406). However, although 2-AP concentration was 23.2% higher in raw Sintanur bran than raw milled Sintanur, no significant difference between them was found (p = 0.151). 2-AP could not be quantified in uncooked non-fragrant rice bran nor in uncooked milled non-fragrant rice.

Buttery, Ling, Juliano and Turnbaugh (1983) identified 2-AP in boiled rice, and over two-fold higher 2-AP was detected in fragrant brown rice than milled rice. However, Bergman et al. (2000) indicated that distribution of 2-AP in rice bran and milled fragrant rice was not significantly different. Bounphanousay, Jaisil, Sanitchon, Fitzgerald and Sackville Hamilton (2008) found that brown rice contained more 2-AP than milled rice when 2-AP concentration in the rice variety was high, but less difference in 2-AP content was found between brown and milled rice when 2-AP concentration in the rice variety was low. They suggested that 2-AP distribution in rice bran and endosperm (milled rice) varied during 2-AP accumulation and that rice bran contains more 2-AP than endosperm. However, 2-AP levels in rice grain decreased during rice storage and its

distribution became uniform. In the current study, Sintanur and basmati samples were obtained from different suppliers; the harvest time and storage conditions of the basmati samples is unknown. Sintanur rice was harvested in autumn 2014 and the experiment was conducted between winter 2014 and spring 2015 but basmati was obtained in a local supermarket immediately prior to the experiment. It is therefore possible that the basmati may have been stored for a shorter time than the Sintanur rice, and hence 2-AP distribution in the Sintanur was more homogeneous than in the basmati. This may explain why 2-AP levels were different between raw bran and raw milled basmati but the difference was not observed between raw bran and raw milled Sintanur. No 2-AP was formed after 20 minutes baking at 180 °C in rice bran in this study (**Figure 5.1**). This is the first report showing that 2-AP cannot be formed during baking of rice bran, whereas it can be formed through baking milled rice.

5.3.2. Quantification of precursors in raw and baked rice sample

5.3.2.1. Quantification of free amino acids

Seventeen free amino acids were quantified in this study; concentrations of these free amino acids in raw rice and rice baked at 180 °C for 20 min are shown in **Table 5.1**. The 17 amino acids were detected in both raw and baked rice bran. Histidine was absent in all four varieties of milled rice, while ornithine was absent in milled basmati and milled long-grain rice. Arginine cannot be measured using this technique.

Raw rice bran has higher levels of amino acids compared with raw milled rice. The concentration of asparagine in raw rice samples was highest, followed by glutamic acid, aspartic acid, alanine and serine. Asparagine and glutamic acid have been previously reported as the major amino acids in both milled and brown rice (Moongngarm & Saetung, 2010; Rego et al., 2018). Over 10 mmol/kg of asparagine, glutamic acid and aspartic acid were quantified in raw rice bran and around 10-fold lower of these amino acids were found in milled rice. As amino acids are

involved in the Maillard reaction during baking, amino acids decreased after baking at 180 °C for 20 min in both rice bran and milled rice. The rate of amino acid decrease in rice bran was much higher than in milled rice. Total free amino acids in rice bran had a 73% reduction after baking compared with raw rice bran, whereas 37% reduction can be observed in milled rice.

Proline is reported to be the main precursor of 2-AP *via* Maillard reaction (Schieberle, 1990; Rewicki et al., 1993; Schieberle, 1995; Hofmann & Schieberle, 1998a). Raw rice bran (including fragrant and non-fragrant rice) contains around 1000 to 3000 µmol/kg of proline and raw milled rice contains 100 to 450 µmol/kg of this amino acid. Among the four milled varieties that can generate 2-AP *via* Maillard reaction, raw milled Sintanur and raw milled long-grain rice contain more than 400 µmol/kg proline (445 µmol/kg and 441 µmol/kg, respectively), whereas raw milled basmati and Ciherang rice had lower levels of proline, 205 µmol/kg and 96.8 µmol/kg, respectively. However, higher levels of 2-AP can be generated in baked milled fragrant rice (basmati and Sintanur), compared to its formation in baked milled non-fragrant rice (Ciherang and long-grain; **Figure 5.1**). Therefore, proline content in raw milled rice did not show a significant relationship with 2-AP generation ($r = 0.19$, $r^2 = 0.035$, $p = 0.76$).

Table 5.1 shows that proline concentration significantly decreased in all varieties of rice bran, from 1925 ± 851 µmol/kg in raw rice bran, to 460 ± 208 µmol/kg once baked (average of all fragrant and non-fragrant samples, $p < 0.0001$). This decrease was also detected in milled rice, although the change was not statistically significant. A difference in remaining levels of proline after baking was not found between milled fragrant (Sintanur and basmati) and milled non-fragrant (Ciherang and long-grain) rice. This indicated that consumption of proline in fragrant and non-fragrant rice during the Maillard reaction was similar, and this may lead to the same level of 2-AP increase in milled fragrant and non-fragrant rice after baking (**Figure 5.1**).

Ornithine is a precursor of 2-AP generation during rice cultivation *via* P5C accumulation (Huang et al., 2008). However, the levels of free ornithine are very low in both rice bran and milled

rice; indeed, one study has reported the absence of ornithine in rice (Kamara, Konishi, Sasanuma & Abe, 2010). Only 203 µmol/kg of ornithine was found in raw rice bran on average and less than 20 µmol/kg was found in milled rice, which was around 100 times lower than the levels of asparagine and glutamic acid. Levels of ornithine were too low to be detected in raw and baked milled basmati and long-grain rice. Only 8.98 µmol/kg of ornithine was detected in raw milled Ciherang rice, and its concentration was below its detection limit after baking. Ornithine contents in raw and baked milled Sintanur were 15.4 µmol/kg and 16.4 µmol/kg, respectively.

5.3.2.2. Quantification of reducing sugars

Reducing sugars, the other precursor of 2-AP in the Maillard reaction, were also quantified in this study. The concentrations of reducing sugars (glucose and fructose) and non-reducing sugar (sucrose) in raw and baked rice are shown in **Table 5.1**. Raw rice bran was found to have a higher level of reducing sugars and sucrose than raw milled rice. Raw rice bran contains 10- to 100-fold higher levels of glucose and around 10-fold higher levels of sucrose than milled rice. High levels of fructose were found in all raw rice bran samples. In raw milled rice samples, fructose was only detected in raw milled Sintanur rice, at a lower level than in milled Sintanur bran (**Table 5.1**). The decreases of glucose and fructose were found in most rice samples (basmati bran, Sintanur bran, Ciherang bran, long-grain bran, milled basmati and milled long-grain) after 20 minutes baking at 180 °C, which could be due to the consumption of reducing sugars (glucose and fructose) during Maillard reaction. However, glucose and fructose increased in Sintanur and Ciherang rice bran after baking. High contents of sucrose were found in these two rice brans. Sucrose cannot take part in the Maillard reaction directly because a reactive dicarbonyl cannot be formed, unless it can be hydrolysed to glucose or fructose initially (Parker, 2015). Handoko (2014) also indicated that heating of rice can cause degradation of sucrose to form glucose and fructose. This could explain the observation of reducing sugar increase in baked Sintanur and Ciherang rice bran. The levels of sucrose decreased in all varieties after baking.

Table 5.1: Mean value of free amino acids and sugars in raw and baked (180 °C for 20 min) milled rice and rice bran

			2-AP (nmol/kg)	free amino acids (µmol/kg)									
				alanine	glycine	valine	Leucine	isoleucine	threonine	serine	proline	asparagine	aspartic acid
milled	basmati	r*	366±47.9^{bc}	2641±210^{gh}	1959±263^{e}	209±30.3^{fgh}	157±75.3^{c}	143±70.2^{c}	218±27.7^{fg}	544±35.2^{e}	215±30.4^{ef}	2309±195^{fgh}	995±6.53^{f}
		b**	571±28.3^{b}	1714±125 ^{hij}	1326±67.8^{efg}	200±5.20^{fgh}	91.9±14.2^{c}	85.2±5.08^{c}	151±44.8^{g}	447±75.0^{e}	189±9.21^{ef}	1101±299^{h}	822±122^{f}
	Sintanur	r	329±31.7^{de}	2564±124 ^{gh}	1560±252^{ef}	590±54.2^{efg}	240±28.1^{c}	221±25.1^{c}	316±60.7^{fg}	967±164de	445±67.6^{def}	4718±361^{defgh}	1885±150^{ef}
	long grain	b	725±20.1^{cd}	2069±247$^{h\ i}$	1411±130^{ef}	507±55.4^{efgh}	166±17.6^{c}	162±46.7^{c}	260±40.7^{fg}	719±164de	335±20.9^{ef}	3019±595^{fgh}	2440±308^{def}
		r	nd	1485±107^{ijk}	1217±73.1^{efg}	510±36.5^{efgh}	297 ± 72.3 ^{c}	183±54.3^{c}	390±97.1^{fg}	746±218de	441±76.1^{def}	4180±417^{efgh}	1879±239^{ef}
		b	119±17.2^{de}	1013±108^{jk}	1000±6.68^{fgh}	370±32.0^{efgh}	129 ± 16.5 ^{c}	123±34.5^{c}	201±58.6^{fg}	311±103^{e}	226±71.3^{ef}	1285±240^{gh}	893±373^{f}
	Ciherang	r	nd	570±154^{k}	393±69.9^{h}	158±45.2^{gh}	76.0±21.5^{c}	94.2±12.9^{c}	116±36.3^{g}	237±72.2^{e}	96.8±11.6^{f}	1704±425^{fgh}	1216±529^{f}
		b	43.9±12.3^{e}	513±111^{k}	562±65.2^{gh}	146±19.9^{h}	89.9±45.6^{c}	71.0±25.8^{c}	107±36.9^{g}	248±62.4^{e}	122±21.0^{ef}	949±398^{h}	646±73.0^{f}
bran	basmati	r	837±144^{a}	13466±103^{a}	7597±216^{a}	1271±60.7^{d}	608±37.5^{b}	512±50.5^{b}	1777±83.7^{c}	5870±233^{a}	1194±24.9^{c}	30220±924^{c}	11867±692^{c}
		b	nd	5663±469^{e}	5474±266^{b}	286±47.2^{efgh}	92.8±36.4^{c}	108±21.2^{c}	591±38.0^{fg}	2870±256^{bc}	302±29.3^{ef}	7260±966^{defg}	3349±489^{def}
	Sintanur	r	406±73.9^{bc}	10383±682^{c}	5216±280^{b}	3025±153^{b}	1182±144^{a}	1131±126^{a}	2361±144^{b}	6962±513^{a}	2007±103^{b}	40836±3564^{b}	26260±2030^{ab}
	long grain	b	nd	3947±122^{f}	3716±53.9^{c}	601±37.0^{ef}	137±14.7^{c}	148±31.5^{c}	687±78.1^{ef}	3641±347^{b}	466±51.6^{de}	10034±214de	6743±421^{cde}
		r	nd	12021±452^{b}	7225±417^{a}	4005±194^{a}	1380±48.3^{a}	1255±67.0^{a}	3680±428^{a}	6543±455^{a}	3256±268^{a}	49741±3215^{a}	28037±4385^{a}
		b	nd	5103±100^{e}	5586±75.0^{b}	699±145^{e}	269±147^{c}	217±110^{c}	1180±56.3^{de}	3277±201^{b}	780±80.6^{d}	10610±671^{d}	7635±163^{cd}
	Ciherang	r	nd	7120±350^{d}	3247±432^{cd}	2075±343^{c}	770±107^{b}	524±144^{b}	1356±199^{cd}	2188±1443^{bcd}	1245±193^{c}	31089±4040^{c}	22277±2652^{b}
		b	nd	3539±166^{fg}	2834±134^{d}	405±49.3^{efgh}	74.6±28.1^{c}	117±30.4^{c}	512±104^{fg}	1580±136^{cde}	290±77.5^{ef}	7437±192^{def}	7388±234^{cd}

Mean values show average of 2-AP, free amino acids and sugars (n = 3) and standard deviation in each type of sample. Data not sharing the same letter are significantly different ($p < 0.05$) in each column. *r: raw rice. **b: baked rice. ***nd: not detected in chromatogram.

Table 5.1: continued

			free amino acids (µmol/kg)							Sugars (mmol/kg)		
			glutamic acid	phenylalanine	ornithine	lysine	histidine	tyrosine	tryptophan	glucose	fructose	sucrose
milled	basmati	r*	2153±169^{c}	122±52.6^{bc}	nd	87.6±42.9^{def}	nd	99.3±12.7^{d}	54.5±19.6^{d}	52.9±1.21^{g}	nd	1069±133^{def}
		b**	793±81.2^{c}	58.1±31.8^{bc}	0	13.6±12.2^{f}	nd	24.3±5.12^{d}	13.4±0.966^{d}	44.7±7.51^{g}	nd	387±039.4^{ef}
	Sintanur	r	2740±387^{c}	169±22.2^{bc}	15.4±4.11^{c}	79.4±34.7^{def}	nd	120±3.91^{d}	56.4±18.4^{d}	76.4±18.9^{g}	62.0 ± 30.5 ^{ef}	1361±59^{cde}
		b	2069±552^{c}	113±10.9^{bc}	16.4±6.65^{c}	147±24.0^{def}	nd	76.8±6.16^{d}	40.6±2.09^{d}	38.4±16.0^{g}	17.3 ± 2.88 ^{f}	390±81.4^{ef}
	long grain	r	2911±280^{c}	189±23.7^{bc}	nd	292±53.5^{cdef}	nd	202±30.1^{d}	124±11.9^{cd}	101±46.1^{g}	Nd	1112±81.4^{cdef}
		b	759±231^{c}	120±36.3^{bc}	nd	20.1±6.31^{ef}	nd	82.7±21.2^{d}	35.8±3.35^{d}	39.7±5.44^{g}	Nd	350±125^{ef}
	Ciherang	r	1666±441^{c}	46.9±31.3^{c}	8.98±6^{c}	51.8±10.6^{ef}	nd	44.6±8.56^{d}	32.0±1.74^{d}	20.4±9.54^{g}	Nd	613±256^{ef}
		b	725±290^{c}	52.2±16.8^{bc}	nd	57.1±17.8^{ef}	nd	52.2±29.9^{d}	22.6±12.6^{d}	29.8±11.0^{g}	Nd	202±31.3^{f}
bran	basmati	r	22656±940^{b}	429±43.4^{b}	172±17.9^{ab}	1811±147^{c}	1136±406^{a}	712±29.7^{c}	855±48.3^{b}	1437±113^{a}	1113±253^{a}	8623±95.6^{ab}
		b	2435±478^{c}	55.4±10.1^{bc}	63.8±12.1^{bc}	628±118^{cdef}	1015±112^{a}	96.8±21.4^{d}	47.3±25.9^{d}	782±74.7^{cde}	490±41.3^{cd}	1336±44.6^{cdef}
	Sintanur	r	31133±3059^{a}	884±179^{a}	287±47.6^{a}	4209±541^{ab}	2065±465^{a}	1311±95.9^{b}	1066±129^{b}	300±71.8^{fg}	316±47.2^{def}	9701±921^{a}
		b	2768±247^{c}	94.3±20.9^{bc}	122±18.6^{bc}	1670±261^{cde}	1082±243^{a}	95.4±11.1^{d}	37.7±8.90^{d}	507±234^{ef}	352±51.6^{cde}	1040±291^{def}
	long grain	r	32351±2884^{a}	1042±22.5^{a}	264±82.1^{a}	5650±1418^{a}	1551±648^{a}	2415±429^{a}	2540±472^{a}	964±91.9^{bc}	679±208^{bc}	8367±158^{b}
		b	2834±157^{c}	168±96.9^{bc}	168±43.3^{ab}	3519±447^{b}	1167±189^{a}	198±59.4^{d}	84.9±17.0^{d}	845±109^{cd}	170±6.86^{def}	1939±444^{cd}
	Ciherang	r	24943±3782^{b}	943±340^{a}	87.7±12.5^{bc}	1734±755^{cd}	1514±1034^{a}	668±39.4^{c}	601±188^{bc}	565±7.57^{g}	295±3.66^{def}	8956±59.7^{ab}
		b	2527±55.3^{c}	69.7±17.6^{bc}	41.4±18.9^{c}	843±54.2^{cdef}	877±103^{a}	95.0±10.1^{d}	37.2±13.9^{d}	1278±153^{ab}	895±99.8^{ab}	2250±199^{c}

Mean values show average of 2-AP, free amino acids and sugars (n = 3) and standard deviation in each type of sample. Data not sharing the same letter are significantly different ($p < 0.05$) in each column. *r: raw rice. **b: baked rice. ***nd: not detected in chromatogram.

5.3.2.3. Correlation between precursors and 2-acetyl-1-pyrroline

Ornithine, glutamic acid and proline have also been reported to generate 2-AP during rice grain growth *via* enzymatic formation (Huang et al., 2008; Bradbury, Gillies, Brusheet, Waters & Henry, 2008). Therefore, a correlation test was conducted for precursors decrease and 2-AP generation, the correlations are shown in **Table 5.2**. Significant negative correlations were only found between 2-AP generation and ornithine concentration change (r = –0.771, r^2 = 0.594, p = 0.025), and between 2-AP generation and sucrose change (r = –0.730, r^2 = 0.532, p = 0.040). Correlations did not exist between 2-AP generation and proline, glutamic acid glucose or fructose.

Table 5.2: Pearson correlation coefficients (r) between change in precursors[a] and change in 2-AP[b] after 180 °C and 20 min baking

	2-AP	proline	glutamic acid	ornithine	glucose	fructose	sucrose
2-AP	—	−0.386	−0.599	−0.771*	−0.432	−0.451	−0.730*
proline	—	—	0.925***	0.779*	−0.0289	0.334	0.802*
glutamic acid	—	—	—	0.890**	−0.125	0.177	0.963***
ornithine	—	—	—	—	0.105	0.327	0.923**
glucose	—	—	—	—	—	0.921**	−0.0807
fructose	—	—	—	—	—	—	0.146
sucrose	—	—	—	—	—	—	—

[a]precursors change: amino acid change (μmol/kg) = mean value of amino acid in raw sample – mean value of amino acid in baked sample, sugar change (mmol/kg) = mean value of sugar in raw sample – mean value of sugar in baked sample; [b]2-AP change (nmol/kg) = mean value of 2-AP in baked sample – mean value of 2-AP in raw sample; *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$.

Amino acids can generate various volatile compounds during Maillard reaction: proline and ornithine not only can form 2-AP, but they also play important roles in 6-acetyl-1,2,3,4-tetrahydropyridine (ATHP) generation (Schieberle, 1990). In addition, as proline contains a pyrrolidine ring, nitrogen heterocycles can be produced *via* Maillard reaction (Mottram, 1994). Therefore, the relationship between consumption of amino acids and sugars and the formation of aroma compounds such as 2-AP in the Maillard reaction is complex. Hence, correlation tests may not be able to provide useful information regarding the relationship between 2-AP generation and single amino acid or reducing sugar consumption.

Amino acids and reducing sugar contents in raw rice bran were much higher than in raw milled rice, and the reduction of these precursors in rice bran was also more than in milled rice after baking, which indicated that more Maillard-derived compounds were generated in rice bran than milled rice under the same cooking conditions. However, **Figure 5.1** showed that the same level of 2-AP was generated in milled fragrant and non-fragrant rice; in contrast, 2-AP had not been generated in baked rice bran. Moreover, ATHP, the other Maillard-derived compound from proline was absent in baked rice bran, whereas, this compound was detected in baked milled rice.

The composition of rice bran could be the reason for 2-AP and ATHP reduction after baking. **Table 5.3** showed the average values of main compositions in brown rice, milled rice and rice bran (Juliano, 2016). It can be seen that rice bran contains around double the protein, 50-fold higher fat and 30-fold higher fibre than milled rice.

Table 5.3: Comparison of nutrient composition of brown rice, milled rice, and rice bran (Juliano, 2016).

	Amounts (per 100 g)		
Property	Brown rice	Milled rice	Rice bran
Moisture (g)	14.0	14.0	14.0
Crude protein (g)	7.1–8.3	6.3–7.1	11.3–14.9
Crude fat (g)	1.6–2.8	0.3–0.6	15.0–19.7
Crude fibre (g)	0.6–1.0	0.2–0.5	7.0–11.4
Crude ash (g)	1.0–1.5	0.3–0.8	6.6–9.9
Available carbohydrates (g)	73–87	77–89	34–62

Fat contributes around 2% by mass in brown rice. However, the distribution of fat in brown rice is not uniform. Fat content in milled rice is 0.61–0.95% and in rice bran is 15–22% (Zhou, Robards, Helliwell & Blanchard, 2002; Sharif, Butt, Anjum & Khan, 2014). Parker (2015) indicated that interaction of other components can significantly affect the Maillard reaction in food. Fat can interact with the Maillard reaction and hence change final volatile compound composition in food systems. Nursten (2005) indicated that the involvement of fats in the Maillard reaction can modify heterocyclic compound formation and produce heterocyclic compounds with long alkyl

side-chains. The presence of thiazoles and thiophenes with long alkyl chains in the 2-position was reported in cooked products with high lipid content (Mottram, 1994; Whitfield, Mottram, Brock, Puckey & Salter, 1988). Whitfield (1992) evaluated heterocyclic compounds with long alkyl side-chains in the lipid-containing Maillard systems; pyrazines, pyridines, furans, thiophenes and thiazoles with long alkyl side-chains were increased due to the presence of lipids in different Maillard systems. In our study, **Table 5.4** showed that 2-pentylfuran and 2-heptylfuran were detected in both milled and rice bran, however, the concentrations of both furans in rice bran were significantly higher than milled rice (the relative peak area of 2-pentylfuran in milled rice was 15.2 and in rice bran was 92.7, $p = 0.018$; the relative peak area of 2-heptylfuran in milled rice was 15.9 and in rice bran was 44.5, $p < 0.001$). Hence, presence of high concentrations of lipids could be a potential explanation of the reduction of 2-AP and ATHP formation during baking in rice bran. However, since no research has investigated how lipids affect Maillard reaction in bran or similar systems during baking, this would need to be researched in a future study.

Table 5.4: heterocyclic compounds with long alkyl side-chains in 180 °C baked milled rice and rice bran

	Peak area relative to 2,4,6-trimethylpyridine (peak area = 100)							
	Fragrant rice				**Non-fragrant rice**			
	Sintanur		**basmati**		**Ciherang**		**Long grain**	
	milled	**bran**	**milled**	**bran**	**milled**	**bran**	**milled**	**bran**
2-pentylfuran	12.0	79.4	20.8	147	10.1	72.9	17.8	71.6
2-heptylfuran	20.6	55.4	19.6	46.4	11.2	36.5	12.3	39.5

Phenolic compounds can affect Maillard reaction yield in model system and food systems. The influence of hydroxycinnamic acids (HCAs, one type of phenolic compound, including caffeic acid, ferulic acid and sinapinic acid) on Maillard reaction products in model systems and food matrix were researched by Moskowitz and Peterson (2010). They indicated that addition of HCAs can alter Maillard reaction products in an aqueous model system by trapping Maillard reaction precursors, such as two- and three-carbon sugar fragments (glyoxal and methylglyoxal) and amino

acid moieties. Further research on bread crust showed that 2-AP formation was significantly affected by the content of HCAs. 2-AP was measured and compared in refined bread crust, whole wheat bread crust and refined bread crust with ferulic acid (one of the HCAs) addition. Refined bread crust contained the highest level of 2-AP (10.4 µg/kg), 2-AP in whole wheat bread crust (2.6 µg/kg) was around 5 folds lower than refined bread crust, and only 0.94 µg/kg 2-AP was detected in ferulic acid addition refined bread crust (Moskowitz, Bin, Elias, & Peterson, 2012). This result suggested that phenolic compounds especially HACs can affect 2-AP generation in food matrix during baking.

Phenolic compounds were found in both rice bran and milled rice, and rice bran contained a much higher level of phenolic compounds than milled rice (Iqbal, Bhanger & Anwar, 2005; Walter & Marchesan, 2011). Tian, Nakamura and Kayahara (2004) compared phenolic compounds in milled rice and brown rice, and indicated that 2.8 mg/kg total soluble phenolic compounds and 57.7 mg/kg total insoluble phenolic compounds were measured in milled rice, 21.7 mg/kg soluble and 184.5 mg/kg insoluble total phenolic compounds were measured in brown rice. Among these phenolic compounds, ferulic acid was present at the highest concentration: 0.7 mg/kg soluble and 52.6 mg/kg insoluble ferulic acid were detected in milled rice, 3.2 mg/kg soluble and 151.9 mg/kg insoluble ferulic acid were detected in brown rice (Tian et al., 2004). Since 8–10% of brown rice is rice bran and the rest is milled rice (Singh et al., 2000), the amount of phenolic compounds in rice bran could be estimated as being 100-fold higher than in milled rice. Rice bran could contain 192–239 mg/kg soluble and 1643-1661 mg/kg insoluble total phenolic compounds, 25.7–31.2 mg/kg soluble and 1050–1290 mg/kg insoluble ferulic acid. Therefore, high concentrations of phenolic compounds, especially ferulic acid, could be an important contributor to 2-AP reduction in rice bran during baking.

2-AP loss in baked basmati and Sintanur bran (**Figure 5.1**) also indicated that 2-AP in rice was degraded during baking at 180 °C, since raw basmati and Sintanur bran contained 93.0 µg/kg

and 45.1 µg/kg of 2-AP, respectively, but levels were reduced to undetectable after baking. When Buttery et al. (1982) first identified 2-AP in fragrant rice, the instability of this compound was noticed, and they assumed that polymerisation could be the main reason for 2-AP reduction. An equilibrium exists between monomer and trimer 1-pyrroline in liquid solution (Baker, Heath, & Millar, 1992); Fang and Cadwallader (2014) proposed that condensation of 1-pyrroline trimer may form polymeric 2-AP. NMR and high resolution mass spectrometry (HR-MS) analysis on 2-AP showed the presence of complex polymeric 2-AP during 2-AP degradation in methanol and water. The polymeric forms and dehydrated polymeric forms (loss of one H_2O molecule) were both measured during 2-AP polymerisation. However, based on the NMR and HR-MS result, the structures of these polymers could not be proposed (Hausch & Cadwallader, 2018). There have been no studies reporting the detection of polymeric 2-AP through GC–MS, and evidence of polymeric 2-AP was not observed in GC–MS chromatograms in the current study. Moreover, Fang and Cadwallader's work on 2-AP polymerisation was carried out using pure 2-AP or 2-AP solution with high concentration at room temperature. Obviously, 2-AP concentration is much lower in rice than in a model system; it would be difficult for 2-AP molecules to polymerise with each other in the presence of, for example, other volatile compounds, lipids and phenolic compounds, with which 2-AP could also react.

5.3.3. Milled Sintanur and Ciherang baked for different times and at different cooking temperatures

Basmati and American long-grain rice samples were obtained from the local ASDA supermarket, and information regarding the growth region and harvest time was not available. It has been reported that the growth region, climate, harvest time and storage time could influence 2-AP concentration in rice (Wongpornchai, Dumri, Jongkaewwattana & Sirri, 2004; Yoshihashi, Nguyen & Kabaki, 2004). In addition, 2-AP concentrations in different basmati cultivars can vary, from 60 µg/kg to 434 µg/kg in milled basmati (Wei et al., 2017). As previously mentioned, the

Indonesian Centre for Rice Research provided the Sintanur and Ciherang samples, which were harvested in Sukamandi, autumn 2014, where climate, soil, growth and harvest conditions were monitored. Therefore, milled Sintanur and Ciherang rice were selected for 2-AP formation analysis when rice was baked for different times and at different temperatures.

Since 2-AP was detected in both fragrant and non-fragrant rice after the rice was baked for 20 min at 180 °C, 180 °C was selected to research the effect of cooking time on 2-AP generation in rice. **Figure 5.2** shows 2-AP concentrations in Sintanur and Ciherang rice after they were baked for different times at 180 °C. 2-AP was detected in unbaked Sintanur (34.3 µg/kg), and then 2-AP level increased with increasing baking time. 2-AP level achieved a maximum when rice was baked for 20 min (69.8 µg/kg). When the baking time was longer than 20 min, 2-AP concentration decreased in Sintanur rice; 2-AP concentration reached the same level as in unbaked Sintanur when it was baked for 30 min (32.2 µg/kg), and the concentration was fell below that in unbaked Sintanur when rice was baked for 40 min (21.4 µg/kg).

The concentration of 2-AP in Ciherang after baking was only 1/10 of that in Sintanur. 2-AP was not detected in unbaked Ciherang. Trace levels of 2-AP were detected in 5-min baked Ciherang, 2-AP was clearly observed in ion chromatograms, but the level was too low to be quantified. 2-AP level increased to quantifiable amounts at baking times longer than 10 min, and the 2-AP level in Ciherang rice increased to a maximum when it was baked for 20 min. Then 2-AP concentration was decreased with the increasing of baking time and reached significant difference when Ciherang was baked for 40 min ($p = 0.048$).

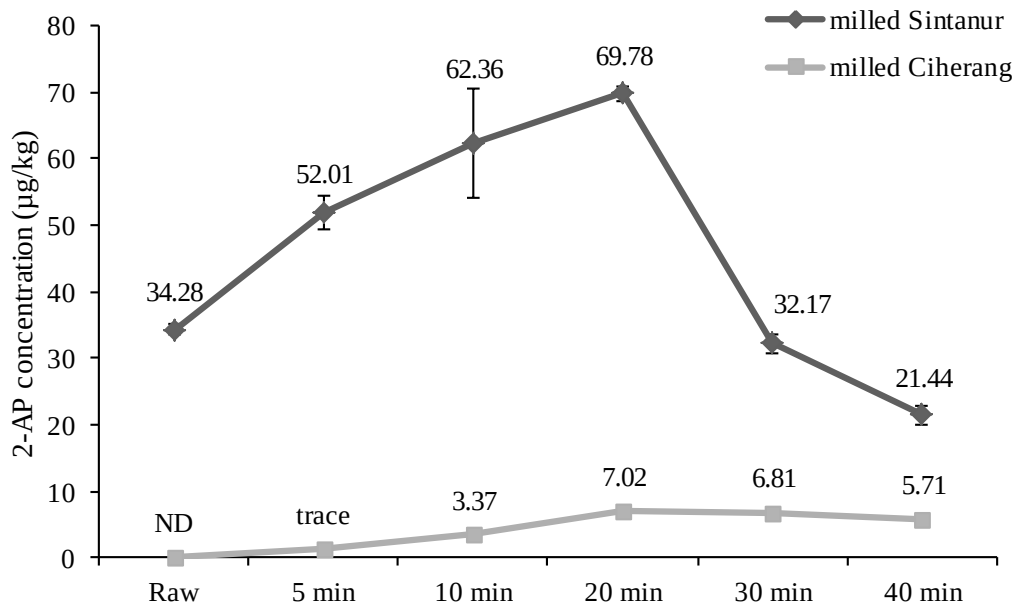

Figure 5.2: 2-AP concentration (µg/kg) in milled Sintanur (a) and Ciherang (b) rice baked for different durations at 180 °C (*n* = 3). Error bar shows standard deviation of triplicate analyses. ND – 2-AP not detected, trace – trace level of 2-AP was detected but under LOQ.

Blank, Devaud, Matthey-Doret and Robert (2003) reported that 2-AP increased with extending of cooking time at 100 °C in a model glucose/proline aqueous system with phosphate buffer; 2-AP level did not decrease until 4 hours, the maximum experimental duration. However, **Figure 5.2** shows the different thermal kinetic trends under dry baking condition. The 2-AP level in both Sintanur and Ciherang rice increased with increasing baking time at 180 °C until 20 min. Then 2-AP concentration decreased with further baking. Generation of 2-AP during heating was caused by Maillard reaction; it was reported that 1-pyrroline and methylglyoxal were the key intermediates in the reaction, 2-AP was formed by the methylglyoxal acetylation of 1-pyrroline (Hofmann & Schieberle, 1998a).

The kinetic modelling of methylglyoxal generation in a dry system was reported by Kocadağlı and Gökmen (2016). They showed that longer baking time and higher baking temperature can lead to more methylglyoxal generation in a dry wheat system. The kinetic study of 1-pyrroline generation through Maillard reaction was not previously reported because 1-

pyrroline was difficult to determine especially at the natural concentrations as an intermediate in a food matrix. Hofmann and Schieberle (1998a) reported that a higher ratio of methylglyoxal to 1-pyrroline can lead to high yield of 2-AP through Maillard reaction in both aqueous and dry model systems. Hence, considering more methylglyoxal could be involved in the reaction with 1-pyrroline when cooking time was extended, 2-AP yield should be increased. However, 2-AP-cooking time curve showed a decrease of 2-AP when cooking time was longer than 20 min; the instability of 2-AP may play an important role in 2-AP reduction. The instability of 2-AP has been reported in both pure 2-AP solution and in fragrant rice. Pure 2-AP will turn red and degrade within 10 minutes at room temperature (Fang & Cadwallader, 2014), 2-AP in fragrant rice was reduced by 40–50% after three months' storage (Widjaja et al., 1996a).

Concentrations of 2-AP in Sintanur and Ciherang rice baked at different temperatures were measured and results are shown in **Figure 5.3**. 2-AP can be detected and quantified in both raw and baked Sintanur (fragrant rice) at all temperatures, but can only be detected in baked Ciherang rice (non-fragrant rice) above 140 °C. Yoshihashi (2002) measured 2-AP level in five fragrant rice grains (around 100 mg) after 90 °C incubation for 8, 10, 12 and 14 min with and without water. This study indicated that 2-AP level is slightly decreased after incubation; however, the cooking condition was not clearly stated.

Figure 5.3 shows that maximum 2-AP formation in Sintanur was between 100 °C and 120 °C, and 2-AP concentration increased in samples heated above 40 °C. Above 120 °C, 2-AP concentration showed a decreasing trend and at 220 °C, the concentration of 2-AP was lower than in the raw sample. In Ciherang no 2-AP was detected at 140 °C or below, with concentrations rising from 3.03 μg/kg at 160 °C up to 13.9 μg/kg at 220 °C. At 240 °C, a strong burnt odour was smelled in both baked samples and the colour changed to dark brown, which prevented analysis above 220 °C.

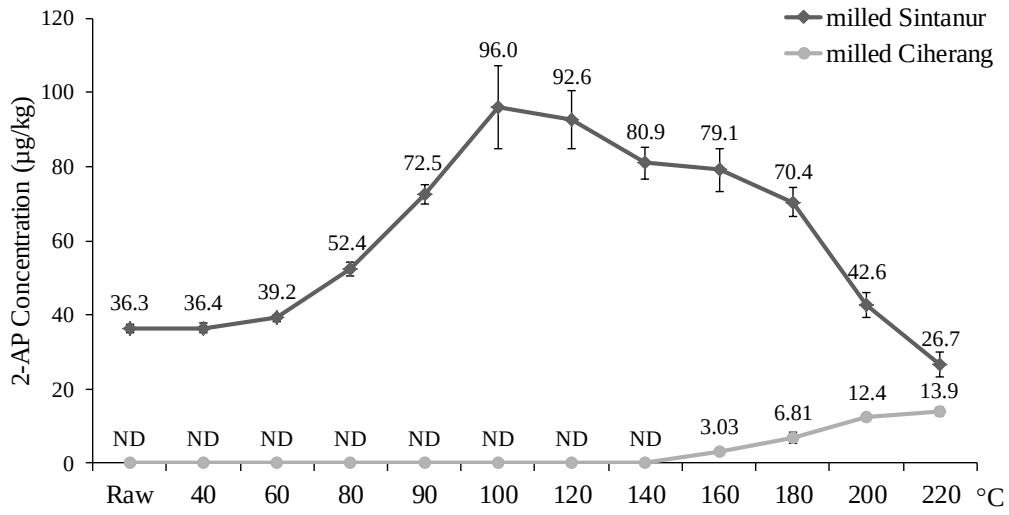

Figure 5.3: 2-AP concentration (µg/kg) in milled Sintanur (black line) and Ciherang (grey line) rice baked for 20 min at different temperatures (n=3). Error bar shows standard deviation of triplicate analyses. ND – 2-AP not detected.

Previous research suggested that 2-AP could be formed *via* both Maillard reaction during cooking (Schieberle, 1990) and enzymatic reaction during grain growth in fragrant rice but this enzymatic pathway was present to a much lower extent in non-fragrant rice (Bradbury, Fitzgerald, Henry, Jin & Waters, 2005; Huang et al., 2008). In this study both milled fragrant and non-fragrant rice can form 2-AP. Since *Section 5.3.2* showed there was no significant differences in precursors (amino acids and reducing sugars) between Sintanur and Ciherang rice varieties, the difference in 2-AP formation at different temperatures may be due to their enzymatic differences. The presence of non-functional BADH2 (Chen et al., 2008) and higher levels of 1-pyrroline-5-carboxylate and methylglyoxal in fragrant rice (Huang et al., 2008) during rice cultivation can lead to 2-AP generation during fragrant rice growth, and the reaction between these compounds on heating could be the reason for additional 2-AP generation.

Hofmann and Schieberle (1998a) suggested that 1-pyrroline and methylglyoxal could be the key intermediates of 2-AP generation in a model system of glucose and proline *via* the Maillard reaction. They found that 2-AP can be generated when heating 1-pyrroline and methylglyoxal, and

higher proportions of methylglyoxal to 1-pyrroline gave increased yields of 2-AP under aqueous conditions at 100 °C (**Figure 2.4** in **Chapter 2**). 1-Pyrroline also plays an important role in the biosynthesis of 2-AP during rice planting. Poonlaphdecha et al. (2016) reported that 1-pyrroline could be the key limiting factor for 2-AP formation in biosynthesis. When 1000 mg/kg 1-pyrroline was added into fragrant calli culture after 12 hours incubation at 28 °C, a 5-fold increase of 2-AP was detected in rice calli with 1-pyrroline added, compared with rice calli with no additional 1-pyrroline. Moreover, when 1000 mg/kg 1-pyrroline was added to non-fragrant rice calli, similar increases in 2-AP to those in fragrant rice callus were achieved after incubation. Their study suggested that 1-pyrroline is the compound which can limit 2-AP generation during rice cultivation; inducing 1-pyrroline formation during non-fragrant rice cultivation can also result in 2-AP accumulation. The enzymatic differences between fragrant and non-fragrant rice cannot influence 2-AP accumulation from 1-pyrroline. Therefore, 1-pyrroline could be the potential limiting factor, which influences 2-AP generation in Sintanur and Ciherang rice during baking over a range of temperatures. 1-Pyrroline was reported as a compound that exists in equilibrium between its mono and trimer form in liquid solution and this compound tends to present as mono form when it is vaporised (Baker, Heath & Millar, 1992; Zhang et al., 2017). Hence, addition of 1-pyrroline into rice before baking could be an efficient way to investigate 2-AP generation in both rice types.

5.4. Conclusions

Formation of 2-AP was detected in both milled fragrant and non-fragrant rice when they were baked without water at 180 °C for 20 min. Raw fragrant rice bran contains more 2-AP than raw milled fragrant rice under these conditions. Maillard reaction precursors of 2-AP (amino acids and reducing sugars) were also quantified in this study; no significant differences in precursors were found between the rice species Sintanur and Ciherang, and due to the complexity of Maillard reaction, no correlation was found between 2-AP generation and precursor consumption. When milled Sintanur and Ciherang were baked over a range of different temperatures, different 2-AP

formation pathways were observed. Based on previous studies about 2-AP Maillard reaction and biosynthesis, a hypothesised formation pathway of 2-AP in both fragrant and non-fragrant rice is proposed. The Maillard reaction occurs in both fragrant and non-fragrant rice after high temperature baking without additional water; proline reacts with reducing sugars to form the key intermediates, 1-pyrroline and methylglyoxal, and then 2-AP is generated from these intermediates. In fragrant rice, because of the presence of non-functional BADH2, γ-aminobutyraldehyde, which is generated from ornithine, is catalysed to 1-pyrroline. In addition, 1-pyrroline can also be enzymatically formed from proline, glutamic acid and ornithine *via* the intermediate, P5C. Finally, 1-pyrroline in fragrant rice can follow either the enzyme-catalysed pathway or Maillard reaction pathway to form 2-AP. Therefore, differences in 1-pyrroline generation mechanisms in milled fragrant and non-fragrant rice may lead to different levels of 2-AP in the baked product.

Chapter 6. The importance of 1-pyrroline and methylglyoxal in 2-acetyl-1-pyrroline formation during rice baking

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The manuscript will be submitted to *Food Chemistry*.

Abstract

2-Acetyl-1-pyrroline (2-AP) contributes desirable popcorn aroma to fragrant rice. Free amino acid compositions in uncooked fragrant and non-fragrant rice were measured alongside 2-AP contents. Concentrations of the amino acids reported in the literature as 2-AP precursors or intermediates, i.e. glutamic acid, proline and γ-aminobutyric acid (GABA), were higher in non-fragrant rice than in fragrant rice. Baking at 100 °C was found to enhance GABA in both fragrant and non-fragrant rice and enhance 2-AP in fragrant rice. The effects of adding intermediates of 2-AP to fragrant and non-fragrant rice were also investigated. 1-Pyrroline addition in fragrant and non-fragrant rice enhanced 2-AP generation during baking at 180 °C. But both additional 1-pyrroline and methylglyoxal inhibited 2-AP formation at 100 °C. In contrast, addition of GABA did not influence 2-AP formation at 100 °C in either rice type. This study suggested that 2-AP formation at 180 °C in non-fragrant rice is solely driven by the Maillard reaction, whereas 2-AP enhancement in fragrant rice may be due to formation of 1-pyrroline by biosynthesis, followed by 2-AP generation from 1-pyrroline reaction with methylglyoxal *via* the Maillard reaction.

Keywords: 2-acetyl-1-pyrroline, 2-AP, flavour, rice, Maillard reaction, biosynthesis, 1-pyrroline, γ-aminobutyric acid, GABA, gas chromatography-mass spectrometry.

6.1. Introduction

Since Buttery, Ling and Juliano (1982) first detected 2-acetyl-1-pyrroline (2-AP) in boiled fragrant rice, this compound has been regarded as the most important contributor to popcorn-like aroma in fragrant rice. Relatively high concentrations of 2-AP can be detected in fragrant rice, whereas 2-AP levels in non-fragrant rice are much lower. Its detection threshold is very low: only 0.02 ng/L in air (Schieberle, 1991) and 0.1 ng/L in water (Buttery et al., 1982).

Popcorn-like aroma is the main characteristic that distinguishes fragrant from non-fragrant rice, and its presence in fragrant rice adds value, when compared with non-fragrant rice (Wei et al., 2017). The difference in 2-AP concentrations between fragrant and non-fragrant rice has been shown to be due to different enzymatic pathways occurring in the rice grain. A mutated gene *badh2* is only present in fragrant rice, the mutation due to the deletion of eight base pairs in exon 7 (Bradbury, Fitzgerald, Henry & Waters, 2005) or deletion of seven base pairs in exon 2 (Amarawathi et al., 2008). Bradbury et al. (2005) indicated that gene *badh2* encodes an enzyme BADH2 in non-fragrant rice, which can catalyse the transformation of γ-aminobutyraldehyde (GABald) to γ-aminobutyric acid (GABA). In fragrant rice the mutated gene *badh2* encodes a non-functional BADH2; GABald is oxidised to generate 1-pyrroline, which can then degrade to form 2-AP (Bradbury, Gillies, Brusheet, Waters & Henry, 2008).

Another possible pathway to 2-AP formation in fragrant rice involves the accumulation of 1-pyrroline-5-carboxylate (P5C), which was found to correlate with 2-AP formation (Huang, Teng, Chang, Chuang, Ho, & Wu, 2008). Higher activities of proline dehydrogenase (PDH), 1-pyrroline-5-carboxylate synthetase (P5CS) and ornithine aminotransferase (OAT) in fragrant rice catalyse the conversion of proline, glutamic acid and ornithine to P5C (Hu, Delauney & Verma, 1992; Delauney & Verma, 1993). P5C undergoes a reaction with methylglyoxal, giving rise to 2-AP, either directly or *via* degradation to 1-pyrroline (Huang et al., 2008). Both 2-AP formation pathways during fragrant rice cultivation were presented in **Figure 2.1** (**Chapter 2**). 1-Pyrroline

could be the limiting factor for 2-AP biosynthesis in the above formation pathways. When rice callus was incubated in a culture containing 1-pyrroline, 2-AP was formed in both fragrant and non-fragrant rice (Poonlaphdecha et al., 2016).

2-AP can also be formed *via* the Maillard reaction during cooking. Research focusing on single amino acid and sugar aqueous model systems suggested that proline and a reducing sugar can be the precursors of 2-AP in the Maillard reaction (Schieberle, 1990). Proline can react with α-dicarbonyl compounds, such as 1-deoxyglucosone or methylglyoxal, to give several intermediates, including 1-pyrroline. 1-Deoxyglucosone is a typical product from fructose degradation (Velíšek, 2014) and methylglyoxal can be formed by cleavage of the C3-C4 bond of 1-deoxyglucosone (Hollnagel & Kroh, 1998). In addition, methylglyoxal can also be generated during the reaction of proline and 1-deoxyglucosone. 2-AP is then synthesised from 1-pyrroline and methylglyoxal *via* oxidation of 2-acetylpyrrolidine (Hofmann & Schieberle, 1998a).

2-AP could be generated in both fragrant and non-fragrant rice when it was baked at 180 °C without water addition (**Chapter 5**). In addition, 2-AP could be generated at lower temperature in fragrant rice, reaching a maximum concentration at 100 °C (around 3-fold higher than in uncooked fragrant rice), whereas 2-AP could only be detected in non-fragrant rice when baked above 160 °C (**Chapter 5**). Hence, 2-AP generation in fragrant and non-fragrant rice may follow different routes.

In this study, 2-AP and free amino acids were quantified in 13 fragrant rice and 8 non-fragrant rice samples, to find out the relationship between precursors and 2-AP content in uncooked rice. In addition, 1-pyrroline and methylglyoxal were added into selected varieties of fragrant and non-fragrant rice, which were baked under different conditions; 2-AP and amino acid concentrations were monitored, aiming to understand the differences in the 2-AP formation mechanism of fragrant and non-fragrant rice.

6.2. Materials and Methods

6.2.1. Materials

Twenty-one different uncooked milled rice samples were analysed in this study: 13 of them were fragrant rice and 8 of them were non-fragrant rice. The fragrant rice samples included six basmati rice samples (Badshah and Tilda brands; four supermarket own-brands: ASDA, Morrison's, Morrison's Organic and Sainsbury's), five jasmine rice samples (Double Elephant, Royal Umbrella and Tilda brands; two supermarket own-brands: ASDA and Morrison's), Sintanur (obtained from Indonesian Centre for Rice Research (ICRR)) and Xiangdaowan (bought from a Chinese supermarket in Reading). Seven of the eight non-fragrant rice samples were obtained from supermarkets, three branded (Everyday Rice from Tilda, Nishiki medium-grain rice, Laila pudding rice) and four supermarket own-brands (two bags of Arborio risotto rice from Sainsbury's and Tesco, long-grain rice from Sainsbury's and Spanish paella rice from ASDA). Ciherang non-fragrant rice was supplied by ICRR. Both Sintanur and Ciherang were harvested in autumn 2016, in ICRR, Sukamandi, Subang, Indonesia. The rice paddy was dried by hot air at 50 °C for 12 hours. Then the husks were removed, to give brown rice, which was then posted to the University of Reading and stored at 4 °C before milling and analysis.

The following chemicals were used in this study. Acetonitrile, diethylenetriaminepentaacetic acid (DETAPAC), *o*-phenylenediamine (98%), 4-aminobutyraldehyde diethyl acetal (90%), pyrrolidine (99%), methylglyoxal (40% in water) and potassium carbonate (99%), were purchased from Sigma-Aldrich (St Louis, MO). Hydrochloric acid (32%), anhydrous sodium sulfate (99%), ethanol (99%), dichloromethane (99%) and HPLC-grade water (99%) were purchased from Fisher Scientific (Loughborough, UK). 2-AP (30000 mg/kg in dichloromethane) and deuterated 2-AP (2-AP-d_2, 30000 mg/kg in dichloromethane) were purchased from AromaLAB (Planegg, Germany).

EZ-Faast (Phenomenex, Torrance, CA) was used for free amino acid analysis. Calibration curves of free amino acids were prepared for quantification. They included γ-aminobutyric acid (GABA, 99%, Sigma-Aldrich) and two mixed standards (Standard 1 and Standard 2) contained in the EZ-Faast kit. Standard 1 (200 nmol/mL each in 0.01 M hydrochloric acid) included α-aminobutyric acid, alanine, aspartic acid, cysteine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, ornithine, phenylalanine, proline, serine, threonine, tyrosine, and valine. Standard 2 (200 nmol/mL each in water) included asparagine, glutamine and tryptophan.

6.2.2. 1-Pyrroline preparation and GC-MS analysis

1-Pyrroline was prepared from acid hydrolysis of γ-aminobutyraldehyde diethyl acetal, using the method of Poonlaphdecha et al. (2016). One millilitre of γ-aminobutyraldehyde diethyl acetal was added in a 50-mL pear-shaped flask and cooled in an ice-water bath for 10 min. Then 12 mL of ice-cold 2 M hydrochloric acid was added into the cold flask. Potassium carbonate (18 mL; 2 M) was slowly added to the reaction at 0 °C with stirring (800 rpm). The stirring continued for 15 min, resulting in a final pH > 12.

The reaction mixture was allowed to reach room temperature, and then 1-pyrroline was extracted by reduced pressure fractional distillation (**Figure 6.1**). The extraction was carried out for 1 hour at 35 °C, after which the distillate was collected and dissolved in 15 mL of dichloromethane. After 15 min stirring at 800 rpm, the extract was dried over anhydrous sodium sulfate and concentrated gently by nitrogen gas to approximately 100 µL; ethanol was then added to 500 µL. This solution was stored at –15 °C and used within 3 days.

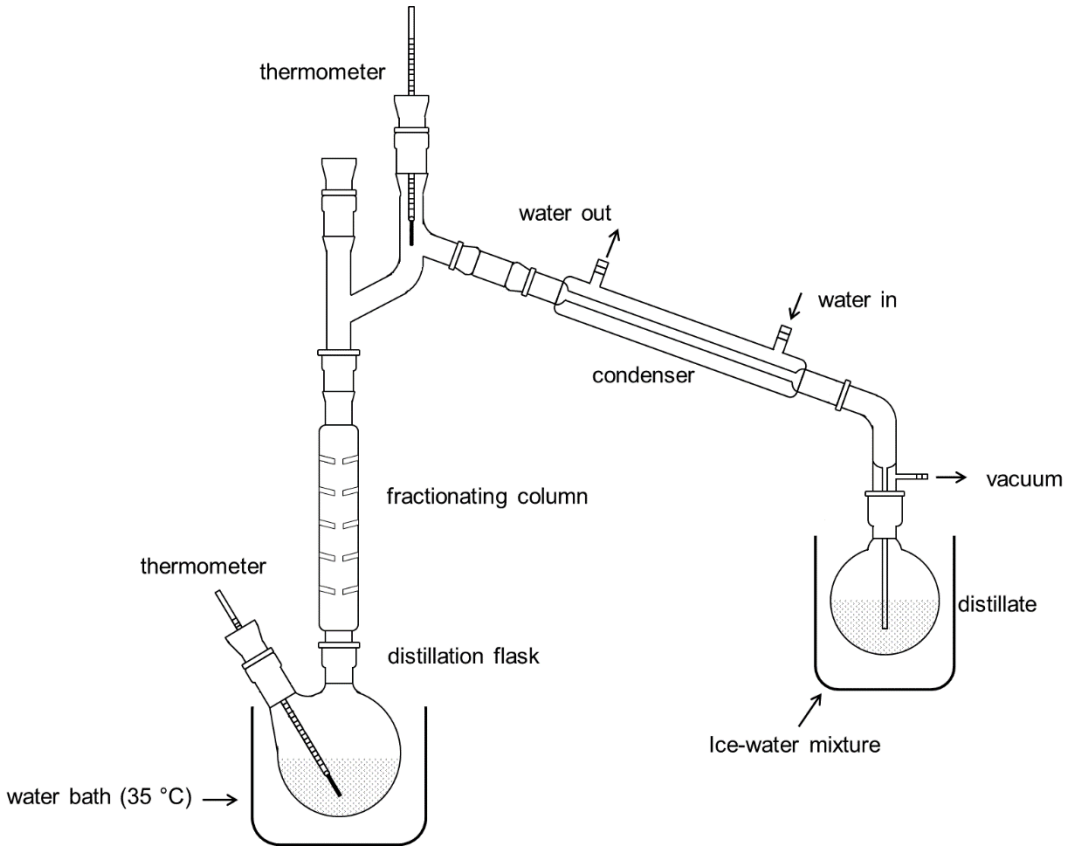

Figure 6.1: Reduced pressure fractional distillation for 1-pyrroline extraction.

To provide an approximate value for the concentration of the 1-pyrroline solution, 10,000 mg/kg pyrrolidine in ethanol was used. Equal volumes of pyrrolidine and prepared 1-pyrroline solutions were mixed and diluted 100 times with ethanol, then transferred to a 2-mL GC vial for analysis by gas chromatography-mass spectrometry (GC-MS). A Restek Rtx-5 Amine column (30 m × 0.25 mm; 0.25 µm film thickness; Restek, Bellefonte, PA) was used for analysis. One microlitre of sample was injected in split mode into the 6890 gas chromatograph with 5975 mass spectrometer (Agilent); the split ratio was 10:1 and the injector temperature was 250 °C. The carrier gas was helium at a constant column flow rate of 1 mL/min. The initial GC oven temperature was 40 °C, held for 1 min, and then the temperature increased at 2 °C/min to 60 °C, then increased at 6 °C/min to 250 °C, where the temperature was held for 10 min. Electronic ionisation (EI) mode was applied; ionisation energy was 70 eV, and the electron multiplier was set at 2235 V. Full scan mode was used for analysis, from *m/z* 20 to *m/z* 350. The mass spectra of 1-

pyrroline and pyrrolidine are shown in **Figure 5.2**; *m/z* 41 for 1-pyrroline and *m/z* 43 for pyrrolidine were used for 1-pyrroline quantification.

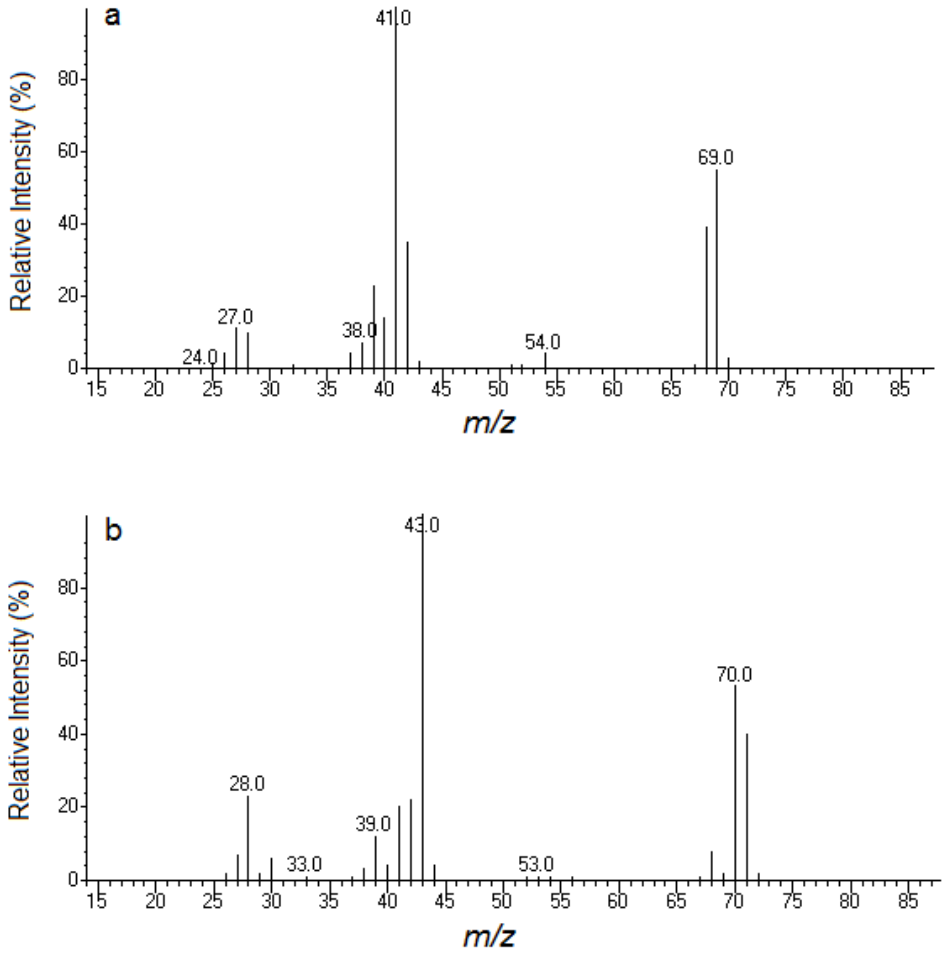

Figure 6.2: Mass spectrum of (a) 1-pyrroline, (b) pyrrolidine.

6.2.3. Rice sample preparation

A Twinbird MR-E750 rice polisher (Twinbird, Tsubame, Japan) was used for Sintanur and Ciherang rice milling. Two-hundred grams of brown rice were milled in '100% white rice mode' to remove all the rice bran for around 3 min until the polisher automatically stopped. Around 140 g milled rice were obtained. The milled Sintanur and Ciherang were collected.

All milled rice samples were ground to rice flour using a De'Longhi grinder (KG40; De'Longhi). Deuterated 2-AP (2-AP-d_2) was used as an internal standard. Two hundred microlitres of prepared 2-AP-d_2 solution (approximately 200 mg/kg in dichloromethane) were evaporated by nitrogen gas to remove solvent, and then 300 mL of HPLC grade water was added to dissolve the 2-AP-d_2. Accurately weighed rice samples (1.000 ± 0.001 g) were put in 20-mL solid-phase microextraction (SPME) glass vials and capped with metal screw-caps with PTFE-faced silicone septa; 1.5 mL of internal standard solution were added to the vial before analysis.

6.2.4. Reaction of 1-pyrroline, methylglyoxal and GABA with rice

Milled Sintanur (fragrant rice variety) and Ciherang (non-fragrant rice variety) were selected to react with 1-pyrroline. 1-Pyrroline is an important intermediate of 2-AP synthesis in rice; it may enhance 2-AP content in both fragrant and non-fragrant rice during rice cultivation (Poonlaphdecha et al., 2016).

Accurately weighed ground rice flour sample (1.000 ± 0.001 g) was spread on a 10 cm × 15 cm foil tray; to ensure the rice flour was flat on the surface of the foil tray. Then 1 mL of intermediate (1000 mg/kg 1-pyrroline or methylglyoxal in ethanol; or 1 µmol/5 µmol GABA in ethanol–water solution) was gently sprayed on the surface of the tray using a plastic spray bottle, to ensure all the flour sample was in contact with the solution. Because GABA is hard to dissolve in pure ethanol, GABA is initially dissolved in water and then diluted with ethanol. Accurately weighed 5 mmol (0.515 g) of GABA was dissolved in 10 mL water, and then 1 mL GABA/water solution was transferred and diluted with ethanol to 100 mL; the concentration of this solution was 5 µmol/mL. 1 µmol/mL GABA solution was made up from 5 µmol/mL GABA solution through 5-fold dilution.

The rice flour and 1-pyrroline or GABA solution formed a thin and uniform layer in the foil tray. Then the wetted rice flour layer was dried by nitrogen gas stream gently at room

temperature for 10 min to remove ethanol from rice flour. All of the dried, treated rice flour was transferred into 20-mL SPME glass vials and capped with metal screw-caps with PTFE-faced silicone septa. It can be observed that the dried flour did not attach to the side wall of the glass vial. Baking without water at 100 °C and 180 °C were selected as the heating conditions. Rice with addition of 1-pyrroline or GABA was baked in a GC oven at 100 °C or 180 °C for 20 min for reaction under dry heating conditions. Rice samples were allowed to cool to room temperature before internal standard addition and HS-SPME-GC-MS analysis. In the set of samples where 2-AP intermediates were added, pure ethanol was added to the control group (untreated rice) and dried in the same manner as the treated samples, in order to allow for the possible influence of ethanol on 2-AP formation.

6.2.5. Headspace solid-phase microextraction and GC-MS analysis for 2-acetyl-1-pyrroline quantification

SPME extraction and GC-MS analysis followed the method in **Chapter 3** (*Section 3.2.3*), and was modified from the method of Hopfer, Jodari, Negre-Zakharov, Wylie and Ebeler (2016). Untreated uncooked and baked rice samples and those treated with 1-pyrroline or GABA were extracted using an SPME autosampler (GC Sampler 120; Agilent, Santa Clara, CA) attached to a 6890 gas chromatograph with 5975 mass spectrometer (Agilent). Rice samples were pre-incubated with magnetic shaking for 10 min at 40 °C, and then extracted with a 1-cm Supelco 75 µm divinylbenzene/Carboxen™/polydimethylsiloxane (DVB/CAR/PDMS) SPME fibre for 1 hour. The extraction was followed by GC-MS analysis; a Zebron ZB-Wax column (30 m × 0.25 mm and 1 µm film thickness; Phenomenex, Torrance, CA) was used. The SPME fibre was desorbed in a GC injector at 250 °C for 20 min in splitless mode. The carrier gas was helium at a constant column flow rate of 0.9 mL/min and the splitter was opened at 0.75 min. The initial GC oven temperate was 40 °C for 2 min, then increased to 60 °C at the rate of 2 °C/min, and then the rate increased to 6 °C/min and held for 35 min after the temperature reached 250 °C. Electron ionisation

(EI) mode was applied; ionisation energy was 70 eV, and the electron multiplier was set at 2824 V. Selected ion monitoring (SIM) was used alongside full scan analysis, using SIM/scan data acquisition. The mass spectrometer scanned from *m/z* 30 to 280, while the ions *m/z* 68, *m/z* 83 and *m/z* 111 were selected for 2-AP in SIM mode. The dwell time of the monitored ions was set at 100 ms/ion.

A pre-prepared 2-AP standard solution (30000 mg/kg in dichloromethane) was used for 2-AP calibration. Non-fragrant rice was used as the matrix for the calibration curve, in order to allow for the effect of food matrix on 2-AP release. 2-AP standard was diluted to 5 mg/kg, 2 mg/kg, 1 mg/kg, 0.5 mg/kg and 0.1 mg/kg with dichloromethane; 50 µL of 2-AP standard solution at each concentration were added into 1 g uncooked ground rice (Sainsbury's American long-grain) rice flour, followed by 1.5 mL 2-AP-d_2 aqueous solution. Samples and standards were prepared and analysed in triplicate.

6.2.6. Free amino acids analysis

EZ-Faast amino acid derivatisation technique (Phenomenex, Torrance, CA) was used for free amino acid quantification in all of the 42 rice samples, using the method of Elmore et al. (2005). Sample (1.000 ± 0.001 g) was mixed with 5 mL of 0.01 M hydrochloric acid and then stirred for 1 hour at room temperature. Then, the sample was allowed to settle for 45 min before 1.5 mL of supernatant were centrifuged at 7200 *g* for 20 min. Finally, 100 µL of centrifuged supernatant were collected for derivatisation.

Free amino acids in 100 µL of the extract were then derivatised using EZ-Faast amino acid analysis kit for GC-MS. Preparation of a sample for GC-MS began with the addition of 200 nM norvaline internal standard (100 µL in 0.01 M HCl) to the extract, followed by a solid-phase extraction clean-up and then a two-step derivatisation at room temperature using propyl chloroformate.

The derivatised amino acids were analysed by GC-MS with a 10 m × 0.25 mm Zebron ZB-AAA capillary column. Sample volume was 1 µL and samples were run in split mode with a split ratio of 20:1. The injection port temperature was 250 °C. The carrier gas was helium at a flow rate of 1.1 ml/min; the oven temperature was initially at 110 °C, then increased at 35 °C/min to 310 °C then held for 1 min. The ion source was maintained at 220 °C. The mass spectrometer scanned from *m/z* 35 to *m/z* 500 at 3 scans per second. Amino acids were quantified using external calibration curves, prepared using derivatised amino acid standards (5, 10, 20, 50, 100, 200 nmol/mL). All samples and standards were prepared and analysed in triplicate. Arginine cannot be measured using EZ-Faast.

6.2.7. Measurement of methylglyoxal in rice

The methylglyoxal extraction and measurement were modified from Kocadağlı and Gökmen (2016). Accurately weighed ground rice flour sample (1.000 ± 0.001 g) was mixed with 2 mL of HPLC-grade water, the rice and water mixture were shaken for 20 min at 1700 rpm (Multi Reax; Heidolph, Schwabach, Germany) to extract methylglyoxal from rice. Then the extract was centrifuged at 5000 *g* for 15 min. A 0.5-mL aliquot of the supernatant of the extract was mixed with an equal volume of acetonitrile and centrifuged at 7000 g for 5 min; 0.5 mL of this supernatant was collected for derivatisation. The derivatisation was conducted through adding 150 µL 0.1 M pH 7 phosphate buffer and then 150 µL 0.2% *o*-phenylenediamine in 10 mM DETAPAC. The mixture was filtered into a 2-mL HPLC vial using a syringe filter and the reaction conducted at room temperature for 2 h, protected from light prior to analysis.

High-performance liquid chromatography-diode array detection system (HPLC-DAD) was used for the methylglyoxal measurement. The separation was performed by Kinetex 2.6 µm C18 100A column (100 mm × 4.6 mm; Phenomenex, Torrance, CA) and a mixture of (**A**) 1% formic acid in water and (**B**) 1% formic acid in acetonitrile was used as the mobile phase. The flow rate was set at 0.8 mL/min at 30 °C. A volume of 10 µL was injected into the HPLC system; the mobile

phase started from 30% **B** and increased to 60% **B** in 10 min, and then decreased to 30% in 2 min and remained at 30% for 3 min. The total run time was 15 min. Methylglyoxal was detected at 3.99 min in the chromatogram.

6.2.8. Data analysis

All samples were analysed in triplicate to obtain reproducible data. ANOVA was performed in XLSTAT (2012, Addinsoft, Paris, France) to examine the difference of 2-AP and amino acids between rice samples. A correlation test (Pearson's) in XLSTAT (2012, Addinsoft, Paris, France) was used to study the relationships between free amino acid and 2-AP concentrations in 13 uncooked milled fragrant rice samples. A significance level (*p*-value) was set at 0.05.

6.3. Results and Discussion

6.3.1. 2-AP and free amino acids quantification for uncooked milled rice

During 2-AP calibration, 5 µg/kg (45 nmol/kg) (50 µL of 0.1 mg/kg 2-AP in non-fragrant matrix) was the lowest concentration which could be detected after HS-SPME; hence, the limit of quantification (LOQ) was 5 µg/kg (45 nmol/kg) in our study. 2-Acetyl-1-pyrroline was detected and quantified in all 13 fragrant rice varieties (see **Table 6.1**); trace levels of 2-AP were detected in 8 non-fragrant varieties, lower than the limit of quantification in our study.

Xiangdaowan rice contained the highest 2-AP level (1050 nmol/kg, 117 µg/kg) among the 13 fragrant rice varieties and Morrison's jasmine rice contained the lowest 2-AP (363 nmol/kg, 40.4 µg/kg). Six basmati and five jasmine samples from different brands were analysed in this study. Of the six basmati samples Morrison's organic contained significantly more 2-AP than the others ($p < 0.05$). For jasmine rice, Tilda, Double Elephants and ASDA contained significantly higher 2-AP level than Morrison's jasmine. 2-AP level in Tilda jasmine was significantly higher

than Tilda basmati ($p < 0.05$); however, no significant differences were found in 2-AP concentration between basmati and jasmine in the other supermarket brands (Morrison's and ASDA).

Sixteen free amino acids (alanine, glycine, valine, leucine, isoleucine, threonine, γ-aminobutyric acid (GABA), serine, proline, asparagine, aspartic acid, glutamic acid, phenylalanine, lysine, tyrosine, and tryptophan) were quantified in 21 uncooked milled rice samples (**Table 6.1**). The average values of free amino acids in all rice samples showed that the concentrations of asparagine (2516 μmol/kg) and alanine (2429 μmol/kg) were highest among the 16 amino acids, followed by glutamic acid (1706 μmol/kg), aspartic acid (1526 μmol/kg) and glycine (1254 μmol/kg). Concentrations of proline, GABA, valine, leucine, isoleucine, threonine, serine, and phenylalanine were between 100 μmol/kg and 1000 μmol/kg. Contents of lysine, tyrosine and tryptophan were lowest in all rice samples, lower than 100 μmol/kg. When samples were separated into fragrant and non-fragrant rice, levels of proline, GABA, glutamic acid, threonine, serine, aspartic acid and lysine in non-fragrant rice were significantly higher than in fragrant rice ($p < 0.05$). No free amino acids were present at significantly higher concentrations in fragrant rice.

Table 6.1: 2-AP (nmol/kg) and free amino acids (μmol/kg) in 21 different rice samples.

			Free amino acids (μmol/kg)						
	Rice Varieties	**2-AP (nmol/kg)**	**Proline**	**GABA**	**glutamic acid**	**alanine**	**glycine**	**valine**	**leucine**
Fragrant	basmati *Badshah*	545 ± 72.7 ^{bc}	213 ± 5.88 ^{hi}	100 ± 2.53 ^{de}	1608 ± 65.6 ^{defg}	2358 ± 5.48 ^{efg}	1710 ± 71.1 ^{e}	300 ± 8.33 ^{fgh}	308 ± 36.0 ^{c}
	basmati *ASDA*	441 ± 34.7 ^{cd}	210 ± 7.67 ^{hi}	182 ± 30.0 ^{bc}	1731 ± 54.1 ^{def}	2925 ± 98.1 ^{cde}	2215 ± 51.4 ^{d}	272 ± 7.70 ^{gh}	306 ± 15.3 ^{c}
	basmati *Morrison*	435 ± 26.6 ^{cd}	265 ± 8.32 ^{fghi}	216 ± 16.7 ^{b}	2189 ± 239 ^{cde}	3218 ± 76.9 ^{cd}	2526 ± 65.0 ^{c}	441 ± 7.74 ^{cdefg}	646 ± 61.3 ^{c}
	basmati *Morrison organic*	647 ± 77.2 ^{b}	410 ± 14.2 ^{cd}	177 ± 10.0 ^{bc}	1976 ± 194 ^{de}	6837 ± 282 ^{a}	3818 ± 166 ^{a}	613 ± 43.7 ^{abc}	637 ± 13.9 ^{c}
	basmati *Sainsbury*	429 ± 10.9 ^{cd}	104 ± 4.53 ^{k}	16.6 ± 7.63 ^{f}	1322 ± 198 ^{efgh}	952 ± 18.8 ^{j}	303 ± 63.4 ^{k}	243 ± 18.5 ^{h}	148 ± 16.8 ^{c}
	basmati *Tilda*	416 ± 28.5 ^{cd}	317 ± 12.8 ^{ef}	181 ± 22.6 ^{bc}	2435 ± 155 ^{bcd}	5975 ± 98.5 ^{b}	3099 ± 37.0 ^{b}	649 ± 32.9 ^{ab}	521 ± 14.7 ^{c}
	jasmine *ASDA*	578 ± 22.1 ^{bc}	214 ± 56.7 ^{hi}	31.8 ± 2.69 ^{ef}	713 ± 186 ^{gh}	1848 ± 383 ^{ghi}	601 ± 143 ^{ij}	522 ± 124 ^{abcde}	2137 ± 568 ^{a}
	jasmine *Double Elephants*	587 ± 60.2 ^{bc}	198 ± 53.3 ^{ij}	39.3 ± 6.99 ^{def}	700 ± 257 ^{h}	1978 ± 491 ^{fg}	699 ± 114 ^{hi}	440 ± 142 ^{cdefg}	1687 ± 696 ^{a}
	jasmine *Morrison*	363 ± 31.6 ^{d}	198 ± 9.41 ^{ij}	33.9 ± 1.64 ^{def}	553 ± 70.4 ^{h}	1120 ± 51.4 ^{j}	518 ± 36.6 ^{ij}	367 ± 27.5 ^{efgh}	808 ± 129 ^{bc}
	jasmine *Royal Umbrella*	524 ± 40.6 ^{bcd}	233 ± 18.8 ^{ghi}	47.2 ± 7.97 ^{def}	705 ± 73.2 ^{h}	2084 ± 143 ^{fg}	728 ± 40.7 ^{hi}	373 ± 12.7 ^{efgh}	518 ± 64.5 ^{c}
	jasmine *Tilda*	639 ± 57.1 ^{b}	208 ± 31.6 ^{ij}	51.2 ± 12.7 ^{def}	941 ± 113 ^{fgh}	1235 ± 170 ^{ij}	618 ± 41.1 ^{ij}	414 ± 45.4 ^{defgh}	1682 ± 358 ^{a}
	Sintanur *ICRR*	425 ± 25.3 ^{cd}	127 ± 7.21 ^{jk}	15.2 ± 3.18 ^{f}	1409 ± 219 ^{efgh}	802 ± 64.4 ^{j}	346 ± 24.2 ^{jk}	281 ± 17.5 ^{gh}	208 ± 5.08 ^{c}
	Xiangdaowan	1050 ± 63.3 ^{a}	203 ± 9.57 ^{ij}	77.8 ± 13.6 ^{def}	1631 ± 230 ^{def}	1001 ± 36.3 ^{j}	349 ± 36.6 ^{jk}	279 ± 21.4 ^{gh}	516 ± 58.8 ^{c}
Non-fragrant	Arborio risotto *Sainsbury*	ND	349 ± 16.9 ^{de}	109 ± 9.95 ^{cd}	2324 ± 207 ^{a}	1300 ± 44.5 ^{hij}	414 ± 35.4 ^{jk}	350 ± 34.1 ^{efgh}	340 ± 8.60 ^{c}
	Arborio risotto *Tesco*	ND	433 ± 18.0 ^{c}	210 ± 52.9 ^{b}	3338 ± 711 ^{bcd}	1822 ± 255 ^{ghi}	767 ± 121 ^{hi}	435 ± 64.7 ^{cdefg}	525 ± 28.5 ^{c}
	Ciherang *ICRR*	ND	293 ± 3.88 ^{efgh}	46.5 ± 9.48 ^{def}	1031 ± 30.2 ^{fgh}	1278 ± 44.8 ^{ij}	739 ± 18.6 ^{hi}	481 ± 13.3 ^{bcdef}	654 ± 22.1 ^{c}
	Everyday *Tilda*	ND	432 ± 8.22 ^{c}	237 ± 12.9 ^{b}	2035 ± 190 ^{de}	3544 ± 71.5 ^{c}	2104 ± 28.2 ^{d}	691 ± 14.7 ^{a}	1592 ± 50.2 ^{ab}
	long grain *Sainsbury*	ND	218 ± 8.97 ^{ghi}	93.7 ± 4.44 ^{de}	855 ± 97.3 ^{fgh}	1971 ± 85.0 ^{fgh}	1280 ± 60.1 ^{f}	406 ± 27.6 ^{defgh}	499 ± 44.0 ^{c}
	medium grain *Nishiki*	ND	298 ± 20.0 ^{efg}	220 ± 14.0 ^{b}	3190 ± 337 ^{ab}	2880 ± 121 ^{cde}	948 ± 32.9 ^{gh}	480 ± 10.8 ^{bcdef}	1605 ± 115 ^{ab}
	pudding rice *Laila*	ND	754 ± 21.9 ^{a}	321 ± 46.5 ^{a}	2961 ± 252 ^{abc}	2561 ± 117 ^{def}	1168 ± 46.7 ^{fg}	576 ± 16.0 ^{abcd}	486 ± 40.3 ^{c}
	Spanish paella *ASDA*	ND	587 ± 7.90 ^{b}	205 ± 15.8 ^{b}	2169 ± 63.4 ^{cde}	3319 ± 23.8 ^{c}	1391 ± 76.5 ^{f}	412 ± 8.48 ^{defgh}	240 ± 8.63 ^{c}
	fragrant rice	545 ± 178	223 ± 78.2 ^{b}	89.9 ± 71.4 ^{b}	1378 ± 620 ^{b}	2487 ± 1841 ^{a}	1349 ± 1150 ^{a}	400 ± 139 ^{a}	779 ± 672 ^{a}
	non-fragrant rice	ND	420 ± 165 ^{a}	180 ± 88.2 ^{a}	2238 ± 924 ^{a}	2334 ± 827 ^{a}	1102 ± 487 ^{a}	479 ± 106 ^{a}	742 ± 510 ^{a}
	mean	—	298 ± 14.2	124 ± 13.3	1706 ± 142	2429 ± 123	1254 ± 39.6	430 ± 35.5	765 ± 185

ND means the compound cannot be detected in GC-MS chromatogram. Data that do not share the same letter in each column are significantly different ($p < 0.05$).

Table 6.1 continue

		Free amino acids (µmol/kg)								
	Rice Varieties	**isoleucine**	**threonine**	**serine**	**asparagine**	**aspartic acid**	**phenylalanine**	**lysine**	**tyrosine**	**tryptophan**
Fragrant	basmati *Badshah*	203 ± 17.1 ^{cd}	134 ± 6.08 ^{bcde}	226 ± 14.8 ^{cde}	3343 ± 185 ^{bc}	1134 ± 43.4 ^{ghij}	198 ± 5.21 ^{efg}	29.3 ± 6.09 ^{b}	50.7 ± 5.37 ^{ef}	10.3 ± 3.05 ^{cd}
	basmati *ASDA*	173 ± 18.2 ^{cd}	148 ± 16.6 ^{bcde}	391 ± 52.8 ^{bc}	3693 ± 166 ^{b}	1139 ± 10.8 ^{fghi}	194 ± 25.8 ^{efg}	35.3 ± 4.94 ^{b}	34.8 ± 4.36 ^{f}	13.4 ± 0.465 ^{bcd}
	basmati *Morrison*	262 ± 7.90 ^{bcd}	172 ± 11.0 ^{bcd}	439 ± 20.0 ^{ab}	3456 ± 246 ^{bc}	1394 ± 30.7 ^{efgh}	380 ± 38.8 ^{cdefg}	79.9 ± 14.5 ^{b}	71.1 ± 6.60 ^{def}	27.9 ± 0.957 ^{abcd}
	basmati *Morrison organic*	327 ± 27.6 ^{abcdF}	323 ± 14.6 ^{a}	435 ± 4.78 ^{ab}	3732 ± 579 ^{b}	1455 ± 44.3 ^{defg}	397 ± 41.5 ^{bcdef}	61.6 ± 7.37 ^{b}	93.9 ± 7.89 ^{cdef}	22.9 ± 4.01 ^{abcd}
	basmati *Sainsbury*	148 ± 16.0 ^{d}	24.7 ± 6.18 ^{f}	18.7 ± 9.57 ^{f}	2538 ± 135 ^{cdef}	1633 ± 122 ^{defg}	88.7 ± 18.8 ^{g}	4.69 ± 1.88 ^{b}	33.1 ± 6.01 ^{f}	16.4 ± 4.93 ^{abcd}
	basmati *Tilda*	257 ± 27.2 ^{cd}	288 ± 8.67 ^{a}	364 ± 57.9 ^{bcd}	4897 ± 67.8 ^{a}	2307 ± 57.4 ^{c}	354 ± 23.6 ^{cdefg}	82.5 ± 3.29 ^{b}	71.5 ± 9.67 ^{def}	32.2 ± 8.61 ^{abc}
	jasmine *ASDA*	448 ± 122 ^{ab}	146 ± 44.2 ^{bcde}	92.1 ± 22.0 ^{ef}	1544 ± 358 ^{gh}	624 ± 87.9 ^{jk}	684 ± 225 ^{ab}	26.5 ± 4.88 ^{b}	175 ± 53.7 ^{ab}	28.1 ± 15.8 ^{abcd}
	jasmine *Double Elephants*	349 ± 155 ^{abc}	137 ± 52.4 ^{bcde}	106 ± 63.6 ^{ef}	1418 ± 303 ^{gh}	722 ± 155 ^{ijk}	560 ± 234 ^{abcd}	27.0 ± 16.3 ^{b}	192 ± 26.3 ^{a}	32.6 ± 12.2 ^{ab}
	jasmine *Morrison*	327 ± 6.41 ^{abcd}	82.9 ± 2.11 ^{ef}	71.6 ± 15.0 ^{ef}	1266 ± 23.9 ^{h}	527 ± 19.1 ^{k}	251 ± 22.1 ^{efg}	17.9 ± 5.34 ^{b}	41.5 ± 12.3 ^{ef}	10.2 ± 2.39 ^{cd}
	jasmine *Royal Umbrella*	252 ± 24.5 ^{cd}	101 ± 8.23 ^{def}	62.2 ± 31.4 ^{ef}	1374 ± 160 ^{gh}	561 ± 58.3 ^{k}	209 ± 20.8 ^{efg}	23.4 ± 13.9 ^{b}	46.8 ± 8.08 ^{ef}	9.85 ± 2.28 ^{d}
	jasmine *Tilda*	281 ± 36.2 ^{abcd}	162 ± 33.8 ^{bcde}	208 ± 51.5 ^{de}	1555 ± 132 ^{gh}	848 ± 77.3 ^{ijk}	472 ± 93.8 ^{abcde}	46.8 ± 2.85 ^{b}	104 ± 13.2 ^{cde}	24.6 ± 2.38 ^{abcd}
	Sintanur *ICRR*	148 ± 25.0 ^{d}	104 ± 18.5 ^{def}	119 ± 26.9 ^{ef}	1836 ± 280 ^{efgh}	1678 ± 72.5 ^{de}	125 ± 16.9 ^{fg}	27.3 ± 8.47 ^{b}	36.4 ± 5.41 ^{f}	14.7 ± 5.50 ^{bcd}
	Xiangdaowan	167 ± 31.6 ^{cd}	82.5 ± 6.80 ^{ef}	198 ± 10.2 ^{de}	1639 ± 132 ^{fgh}	1651 ± 105 ^{def}	239 ± 8.36 ^{efg}	9.91 ± 2.64 ^{b}	52.4 ± 22.0 ^{ef}	11.9 ± 6.10 ^{bcd}
Non-fragrant	Arborio risotto *Sainsbury*	238 ± 15.4 ^{cd}	111 ± 2.77 ^{cde}	227 ± 28.0 ^{cde}	1347 ± 48.9 ^{h}	1612 ± 42.3 ^{defg}	150 ± 26.2 ^{fg}	93.0 ± 15.4 ^{b}	62.4 ± 5.25 ^{def}	14.6 ± 1.32 ^{bcd}
	Arborio risotto *Tesco*	287 ± 57.7 ^{abcd}	185 ± 32.2 ^{bc}	397 ± 120 ^{bc}	1681 ± 444 ^{fgh}	2356 ± 451 ^{c}	289 ± 14.0 ^{defg}	415 ± 112 ^{a}	79.2 ± 13.8 ^{def}	26.8 ± 2.18 ^{abcd}
	Ciherang *ICRR*	284 ± 6.38 ^{abcd}	160 ± 6.59 ^{bcde}	167 ± 8.95 ^{ef}	2294 ± 157 ^{defg}	847 ± 73.3 ^{ijk}	295 ± 37.6 ^{defg}	52.9 ± 7.66 ^{b}	71.5 ± 13.3 ^{def}	14.1 ± 1.52 ^{bcd}
	Everyday *Tilda*	457 ± 19.6 ^{a}	297 ± 25.0 ^{a}	361 ± 11.6 ^{bcd}	5315 ± 217 ^{a}	1943 ± 63.6 ^{cd}	701 ± 27.8 ^{a}	83.2 ± 7.78 ^{b}	151 ± 14.7 ^{abc}	29.6 ± 1.85 ^{abcd}
	long grain *Sainsbury*	281 ± 22.1 ^{abcd}	103 ± 8.32 ^{def}	97.0 ± 21.7 ^{ef}	1993 ± 170 ^{efgh}	923 ± 61.1 ^{hijk}	234 ± 17.0 ^{efg}	16.4 ± 5.80 ^{b}	40.3 ± 2.41 ^{ef}	9.77 ± 3.32 ^{d}
	medium grain *Nishiki*	308 ± 41.5 ^{abcd}	194 ± 11.0 ^{b}	349 ± 14.5 ^{bcd}	3173 ± 237 ^{bcd}	3753 ± 186 ^{a}	623 ± 22.9 ^{abc}	62.0 ± 12.2 ^{b}	123 ± 19.3 ^{bcd}	20.0 ± 2.98 ^{abcd}
	pudding rice *Laila*	284 ± 16.9 ^{abcd}	306 ± 20.7 ^{a}	586 ± 116 ^{a}	2700 ± 188 ^{cde}	2983 ± 208 ^{b}	348 ± 25.5 ^{cdefg}	96.9 ± 25.1 ^{b}	86.6 ± 7.02 ^{def}	37.8 ± 8.02 ^{a}
	Spanish paella *ASDA*	213 ± 13.1 ^{cd}	198 ± 6.43 ^{b}	450 ± 12.3 ^{ab}	2049 ± 52.8 ^{efgh}	1951 ± 63.5 ^{cd}	162 ± 11.8 ^{fg}	67.4 ± 1.66 ^{b}	52.2 ± 8.18 ^{ef}	27.5 ± 3.68 ^{abcd}
	fragrant rice	257 ± 105 ^{a}	146 ± 81.4 ^{b}	210 ± 149 ^{b}	2484 ± 1180 ^{a}	1206 ± 525 ^{b}	319 ± 194 ^{a}	36.3 ± 25.2 ^{b}	77.1 ± 53.6 ^{a}	19.6 ± 10.8 ^{a}
	non-fragrant rice	294 ± 74 ^{a}	194 ± 72.3 ^{a}	329 ± 161 ^{a}	2569 ± 1187 ^{a}	2046 ± 942 ^{a}	350 ± 193 ^{a}	111 ± 125 ^{a}	83.3 ± 36.5 ^{a}	22.5 ± 9.60 ^{a}
	mean	271 ± 36.4	165 ± 13.6	255 ± 32.1	2516 ± 131	1526 ± 93.6	331 ± 62.3	64.7 ± 22.8	79.4 ± 11.0	20.7 ± 3.78

Data that do not share the same letter in each column are significantly different ($p < 0.05$).

Previous research reported that when ^{15}N-labelled proline or glutamic acid was added during rice seedling incubation, the ^{15}N isotope was also detected in 2-AP (Yoshihashi, Huong, & Inatomi, 2002; Huang, et al., 2008), suggesting that proline and glutamic acid can be the nitrogen source in 2-AP generation. In our study on 21 rice samples, non-fragrant rice contained significantly higher proline ($p < 0.0001$) and glutamic acid ($p < 0.0001$) than fragrant rice samples (**Table 6.1**), which could imply that proline and glutamic acid are consumed to form 2-AP during fragrant rice growth. Ornithine was reported as another important precursor of 2-AP in rice plants (Huang, et al., 2008). However, only trace levels of ornithine were detected in the uncooked rice samples.

GABA can be formed from proline, ornithine and glutamic acid (Bouché & Fromm, 2004; Bradbury, et al., 2008). GABA is formed by functional BADH2 in non-fragrant rice from GABald (the intermediate of putrescine, which is formed from ornithine and proline metabolism); hence cyclisation of GABald to 1-pyrroline is limited. Non-functioning BADH2 in fragrant rice allows the cyclisation of GABald, which finally leads to 2-AP accumulation (Bradbury, et al., 2008). Moreover, GABA can be generated in plant tissue from residual glutamic acid *via* glutamic acid decarboxylase (GAD) (Bouché & Fromm, 2004). Our study found that GABA positively correlated with glutamic acid ($r = 0.741$; $p < 0.001$) and proline ($r = 0.786$; $p = 0.006$), because routes to GABA exist for both amino acids. In addition, mean values of GABA in fragrant rice were lower than in non-fragrant (89.9 μmol/kg and 180 μmol/kg, respectively), possibly due to GABA formation in non-fragrant rice *via* BADH2 catalysis.

Table 6.2: Pearson's correlation coefficient (r) of 16 amino acids and 2-AP in 13 fragrant rice samples.

	Pro	GABA	Glu	Ala	Gly	Val	Leu	Ile	Thr	Ser	Asn	Asp	Phe	Lys	Tyr	Trp	2-AP
proline	—	0.718**	0.490nc	0.914***	0.860***	0.809***	0.064nc	0.408nc	0.912***	0.728**	0.558*	0.176nc	0.369nc	0.730**	0.192nc	0.371nc	0.123nc
GABA	—	—	0.829***	0.775**	0.907***	0.425nc	-0.285nc	-0.060nc	0.703**	0.963***	0.845***	0.434nc	0.043nc	0.801**	-0.144nc	0.276nc	-0.075nc
glutamic acid	—	—	—	0.663*	0.767**	0.267nc	-0.537nc	-0.404nc	0.573*	0.823***	0.895***	0.845***	-0.226nc	0.678*	-0.339nc	0.208nc	0.002nc
alanine	—	—	—	—	0.950***	0.780**	-0.126nc	0.228nc	0.931***	0.760**	0.790**	0.420nc	0.214nc	0.772**	0.096nc	0.432nc	-0.101nc
glycine	—	—	—	—	—	0.628*	-0.237nc	0.096nc	0.877***	0.895***	0.874***	0.425nc	0.109nc	0.817***	-0.038nc	0.341nc	-0.164nc
valine	—	—	—	—	—	—	0.424nc	0.686**	0.857***	0.435nc	0.389nc	0.138nc	0.673*	0.726**	0.531nc	0.738**	-0.061nc
leucine	—	—	—	—	—	—	—	0.837***	0.116nc	-0.210nc	-0.455nc	-0.565*	0.925***	0.026nc	0.894***	0.592*	0.189nc
isoleucine	—	—	—	—	—	—	—	—	0.362nc	-0.052nc	-0.228nc	-0.547nc	0.885***	0.207nc	0.802**	0.553*	-0.024nc
threonine	—	—	—	—	—	—	—	—	—	0.766**	0.665*	0.335nc	0.421nc	0.838***	0.272nc	0.561*	-0.017nc
serine	—	—	—	—	—	—	—	—	—	—	0.797**	0.447nc	0.102nc	0.816***	-0.087nc	0.309nc	0.017nc
asparagine	—	—	—	—	—	—	—	—	—	—	—	0.680nc	-0.147nc	0.707**	-0.252nc	0.259nc	-0.271nc
aspartic acid	—	—	—	—	—	—	—	—	—	—	—	—	-0.357nc	0.399nc	-0.371nc	0.176nc	0.046nc
phenylalanine	—	—	—	—	—	—	—	—	—	—	—	—	—	0.323nc	0.932***	0.771**	0.188nc
lysine	—	—	—	—	—	—	—	—	—	—	—	—	—	—	0.120nc	0.618*	-0.244nc
tyrosine	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	0.747***	0.202nc
tryptophan	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	-0.069nc
2-AP	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, nc: no correlation, ≥ 0.05

Proline and glutamic acid can also be converted to each other via the key intermediate 1-pyrroline-5-carboxylate (P5C). In plants and other eukaryotes, glutamic acid is catalysed to glutamic-γ-semialdehyde (GSA) by 1-pyrroline-5-carboxylate synthetase (P5CS), GSA is converted to P5C by spontaneous cyclisation, and then P5C is reduced to proline by P5C reductase (P5CR) (Hu et al., 1992). Proline accumulation normally happens as a result of osmotic stress in plants, and 2-AP can be enhanced through inducing osmotic stress during fragrant rice cultivation (Yoshihashi, Nguyen & Kabaki, 2004; Itani, Tamaki, Hayata, Fushimi & Hashizume, 2004; Szabados & Savouré, 2010; Poonlaphdecha et al., 2012; Li et al., 2016, Mo et al., 2017). Once osmotic stress no longer exists, proline is oxidised to P5C by catalysation of proline dehydrogenase (PDH), and then P5C can convert back to glutamic acid through P5C dehydrogenase (P5CDH) (Delauney & Verma, 1993). However, P5C can be consumed in rice to generate 1-pyrroline and finally lead to 2-AP accumulation, and P5C level in rice positively correlates with 2-AP (Huang, et al., 2008). Our study (**Table 6.2**) showed that glutamic acid does not correlate strongly with proline ($r = 0.483$, $p = 0.089$). The effect of P5C may reduce the correlation between glutamic acid and proline.

2-AP was not correlated with any of the amino acids in fragrant rice (**Table 6.2**). 2-AP level in fragrant rice is affected by many factors, not only by rice variety, soil and climate, but also affected by postharvest processing, especially drying, and storage conditions (Wei et al., 2017). Wongpornchai, Dumri, Jongkaewwattana and Sirri (2004) reported that drying significantly influenced 2-AP loss during fragrant rice storage. They indicated that more 2-AP was lost in sun-dried rice during storage compared with air-dried rice, because of a longer drying time of sun drying. When atmospheric pressure storage and reduced pressure storage were compared, 2-AP level was reduced by 40–50% after 3 months in both cases (Widjaja, Craske & Wootton, 1996a). Moreover, generation of GABA and reduction of glutamic acid in rice bran during storage have been reported (Kim, Lee, Lim & Han, 2015). Due to the activity of GAD during storage, an increase in GABA of over 9-fold was observed when 30% moisture content rice bran was stored

at 40 °C for 5 hours. Therefore, different postharvest conditions in different rice samples may affect 2-AP levels, which cannot be reflected by initial free amino acid composition. Although there was no correlation between the amino acids involved in 2-AP generation (glutamic acid, proline and GABA) and 2-AP content in fragrant rice, these amino acids could still reflect 2-AP generation when 1-pyrroline was added into rice samples. Sintanur (fragrant rice) and Ciherang (non-fragrant rice) were selected for this further analysis, due to their known planting and postharvest conditions (*Section 6.2.1.*).

6.3.2. 2-AP in Sintanur and Ciherang rice after 1-pyrroline or methylglyoxal addition and baking at 100 °C

2-AP quantification in raw, 100 °C and 180 °C baked rice in this Chapter followed the same trend as it was in **Chapter 5**, but the 2-AP concentrations were around double than **Chapter 5** under the same condition. Different batch of rice samples (2016 autumn for **Chapter 6** and 2014 autumn for **Chapter 5**) were used in two chapters are the main reason cause this difference. As it was previous reported, 2-AP and precursors especially proline were accumulated at different level under different climate conditions even for the same variety (Itani, Tamaki, Hayata, Fushimi, & Hashizume, 2004; Rhodes, Handa, & Bressan, 1986; Yoshihashi et al., 2004; Poonlaphdecha et al., 2012). Because Sintanur and Ciherang were obtained in the open field without temperature and moisture control, this variation is difficult to be avoided.

2-AP generation reached a maximum at a baking temperature of around 100 °C in Sintanur (fragrant rice). However, 2-AP concentration in Ciherang (non-fragrant rice) above the limit of quantification (LOQ) was only measured when the baking temperature was higher than 160 °C. This result indicated that 2-AP generation routes in fragrant and non-fragrant rice may be different. In this study, 1-pyrroline, an important 2-AP intermediate, was added into both Sintanur and Ciherang rice, which were then baked at 100 °C and 180 °C. 2-AP was quantified, in order to determine whether 1-pyrroline was involved in reactions in both fragrant and non-fragrant rice.

In our study, 2-AP generation in rice not treated with 1-pyrroline (**Figure 6.3**) showed similar trends to those reported in **Chapter 5**. 2-AP was quantified in unbaked fragrant Sintanur rice (49 µg/kg), but not quantifiable in uncooked non-fragrant Ciherang. When both varieties were baked at 100 °C, 2-AP level increased to around 4-fold higher than in uncooked Sintanur rice (180 µg/kg), whereas 2-AP was still below its LOQ in Ciherang rice. After 180 °C baking, 2-AP level in Sintanur rice dropped to 106 µg/kg in Sintanur rice, and 24.3 µg/kg 2-AP was detected in Ciherang rice. This indicated that ethanol added in rice during 1-pyrroline addition did not influence 2-AP formation during baking, and also showed that the change of 2-AP in 1-pyrroline addition samples were affected by intermediates rather than their solvent (ethanol).

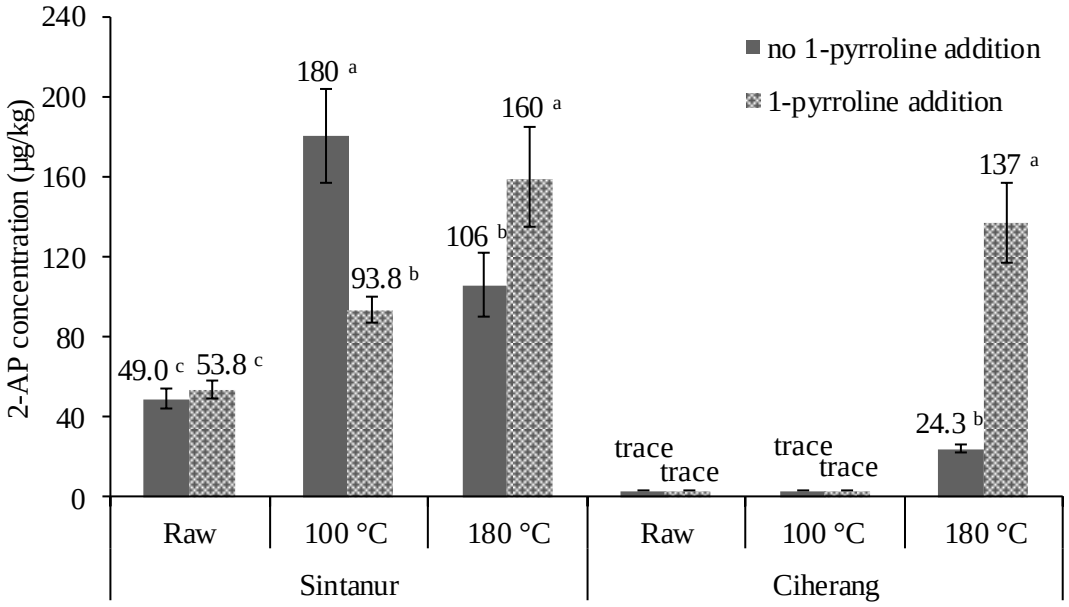

Figure 6.3: 2-acetyl-1-pyrroline concentrations in uncooked, 100 °C, and 180 °C baked Sintanur and Ciherang rice with and without 1-pyrroline addition (µg/kg). Data not sharing the same letter are significantly different ($p < 0.05$) in each rice variety. Error bar shows the standard deviation of triplicate analyses. 'Trace' means concentration of the compound is lower than LOQ of 5 µg/kg.

Figure 6.3 shows that, when 1-pyrroline was added to the rice before cooking, values of 2-AP in 180 °C Ciherang and 180 °C Sintanur are not significantly different (137 µg/kg and 160 µg/kg, respectively) and they are both significantly higher than unbaked Ciherang and Sintanur. Hence, 2-AP generation can be enhanced at 180 °C in both Sintanur and Ciherang rice when 1-pyrroline is added to rice before baking. Because this enhancement was observed in both rice

varieties, it is assumed that 2-AP generation in both Sintanur and Ciherang occurred through the Maillard reaction *via* acetylation of 1-pyrroline at 180 °C, and the level of 1-pyrroline could be a limiting factor for 2-AP generation. Hofmann and Schieberle (1998a) suggested that 1-pyrroline is the important intermediate in 2-AP generation. When 1-pyrroline reacts with the α-dicarbonyl compound methylglyoxal, 2-AP can be generated *via* oxidation of 2-acetylpyrrolidine under both wet and dry conditions during heating.

Methylglyoxal is formed from reducing sugars at the beginning of the Maillard reaction; therefore, the amount of methylglyoxal is limited in rice. Hence, the addition of 1-pyrroline in the current experiment affected the generation of other Maillard-derived products at 180 °C. Because large amounts of 2-AP were generated from the reaction of methylglyoxal and additional 1-pyrroline, the consumption of extra methylglyoxal by additional 1-pyrroline caused the reduction of other Maillard-derived products derived from methylglyoxal. Methylglyoxal is involved in the generation of pyrazines with methyl groups, including methylpyrazine, isomers of dimethylpyrazine and trimethylpyrazine. The correlation between methyl pyrazines and methylglyoxal was proposed by Weenen, Tjan, de Valois, Bouyer, Pos, and Vonk (1994); they reported that the addition of methylglyoxal can lead to dimethylpyrazines and trimethylpyrazine generation in model asparagine system; addition of methylglyoxal and glycoaldehyde can lead to methylpyrazine, dimethylpyrazines and trimethylpyrazine generation; addition of methylglyoxal and glyceraldehyde can lead to dimethylpyrazines and trimethylpyrazine generation. **Table 6.3** showed methylpyrazines in 180 °C baked untreated rice and 1-pyrroline addition rice. Significant reductions of methyl pyrazines were observed in all the rice when 1-pyrroline was added before baking. This degradation conformed that methylglyoxal was consumed by the additional 1-pyrroline, and 2-AP generation at 180 °C was caused by Maillard reaction.

Table 6.3: Methylpyrazines in untreated and 1-pyrroline added rice after baking at 180 °C

	Peak area relative to 2-acetyl-1-pyrroline-d_2 (peak area = 100)			
	Sintanur		Ciherang	
	untreated	1-pyrroline addition	Untreated	1-pyrroline addition
methylpyrazine	425±6.62[a]	311±1.05[b]	368±13.65[a]	312±10.09[b]
2,5-dimethylpyrazine	101±1.66[a]	64.9±3.25[b]	79.0±3.07[a]	57.9±3.50[b]
2,6-dimethylpyrazine	114±9.05[a]	79.8±3.84[b]	83.8±0.06[a]	67.1±2.16[b]
2,3-dimethylpyrazine	140±5.89[a]	92.8±1.60[b]	101±1.82[a]	85.6±3.33[b]
trimethylpyrazine	74.0±2.09[a]	47.3±0.16[b]	58.2±5.42[a]	40.7±5.33[b]

Data are mean value of triplication. Data not sharing the same letter are significantly different ($p < 0.05$) in each rice variety and each row.

However, **Figure 6.3** shows that additional 1-pyrroline did not enhance 2-AP generation at 100 °C in either Sintanur or Ciherang rice. In fact, 2-AP was not quantified in 1-pyrroline-treated Ciherang rice after baking at 100 °C, while 2-AP level decreased to 93.8 μg/kg in Sintanur baked at 100 °C after 1-pyrroline addition, from 180 μg/kg in untreated Sintanur baked at 100 °C ($p = 0.018$). These results indicate that 2-AP generation at 100 °C may be reduced by additional 1-pyrroline, but this reduction was not present at 180 °C, when 1-pyrroline can form 2-AP *via* Maillard reaction.

According to Hofmann and Schieberle (1998a), methylglyoxal is another important limiting factor in 2-AP generation from 1-pyrroline *via* Maillard reaction. They found that heating of 1-pyrroline and methylglyoxal at 100 °C for 30 min in a phosphate buffer generated a substantial amount of 2-AP, especially when methylglyoxal was present at relatively high molar concentrations relative to 1-pyrroline. When the molar ratio of methylglyoxal to 1-pyrroline was 5:1, the yield of 2-AP was 28.7%. In contrast, higher amounts of 1-pyrroline than methylglyoxal inhibited 2-AP generation; a 1:5 ratio of methylglyoxal to 1-pyrroline yielded only 0.3% 2-AP (Hofmann & Schieberle, 1998a). As an α-dicarbonyl compound, the formation of methylglyoxal from glucose and fructose is significantly affected by cooking temperature. It was found that higher baking temperature and longer baking time can significantly increase the formation of short chain

α-dicarbonyl compounds (e.g. glyoxal, methylglyoxal and diacetyl), while the content of long chain α-dicarbonyl compounds was reduced (Kocadağlı & Gökmen, 2016) in a dry wheat flour system when the baking temperature was between 160 °C and 200 °C. As the melting temperature of glucose is 146 °C and of fructose is 107 °C, the reducing sugar ring could not be opened in the dry wheat flour system, degradation of reducing sugars could not commence, and α-dicarbonyl compounds could not be generated when baking temperature was lower than 107 °C. Because the baking conditions of the rice flour are similar to those of the dry wheat flour system, the result of Kocadağlı and Gökmen (2016) suggested that, on one hand, a large amount of methylglyoxal can be generated at 180 °C from reducing sugars and hence can react with 1-pyrroline or proline to form 2-AP *via* Maillard reaction. On the other hand, α-dicarbonyl compounds including 1-deoxyglucosone and methylglyoxal cannot be generated in 100 °C baked rice *via* Maillard reaction.

A previous study reported the presence of methylglyoxal in rice (Kaur, Ghosh, Pareek, Sopory & Singla-Pareek, 2015); this compound is a by-product in several metabolic reactions in active plant cells (Hoque et al., 2016). The reaction between photosynthetic intermediates, glyceraldehyde-3-phosphate (G3P) and dihydroxyacetone phosphate (DHAP) in active plant cells can produce methylglyoxal, and this reaction is considered to be the main route to methylglyoxal formation in plants (Yadav, Singla-Pareek, Reddy & Sopory, 2005; Takagi, Inoue, Odawara, Shimakawa, & Miyake, 2014; Kaur et al., 2015). Moreover, methylglyoxal can also be produced from deprotonation and elimination of triose phosphates (Richard, 1993), and enzymatic hydrolysis of G3P and DHAP (Phillips & Thornalley, 1993). However, because of the strong cytotoxicity of methylglyoxal, several detoxification pathways are present in plant cells and these maintain methylglyoxal at a low level (30–75 μM) in normal plant cells (Hoque et al., 2016). Using a similar method to Kocadağlı and Gökmen (2016), methylglyoxal was measured in raw and 100 °C baked Sintanur and Ciherang rice, and could not be quantified in any of these samples. HPLC-DAD was used and the lowest quantifiable concentration of methylglyoxal standard was 1 mg/L. Although methylglyoxal concentration in both raw and 100 °C baked rice was lower than

the limit of quantification, this measurement confirmed that methylglyoxal cannot be generated from reducing sugars during rice baking without water at 100 °C.

Methylglyoxal was also added into rice, before baking at 100 °C. 2-AP values are shown in **Figure 6.4**. 2-AP increase in methylglyoxal-treated Sintanur was not significant after 100 °C baking. 2-AP concentration in 100 °C baked untreated Sintanur was around 3 folds higher than in baked methylglyoxal-treated rice. In non-fragrant Ciherang rice, 2-AP concentrations were always lower than LOQ. 2-AP generation was reported in methylglyoxal and proline aqueous model system; methylglyoxal can be the precursor to 1-pyrroline through its reaction with proline, and then it can react with 1-pyrroline to form 2-AP (Hofmann & Schieberle, 1998a). In **Figure 6.4**, 2-AP was not increased in methylglyoxal-treated rice; this indicated that the Maillard reaction between methylglyoxal and proline cannot be carried out during 100 °C baking without water in the rice matrix.

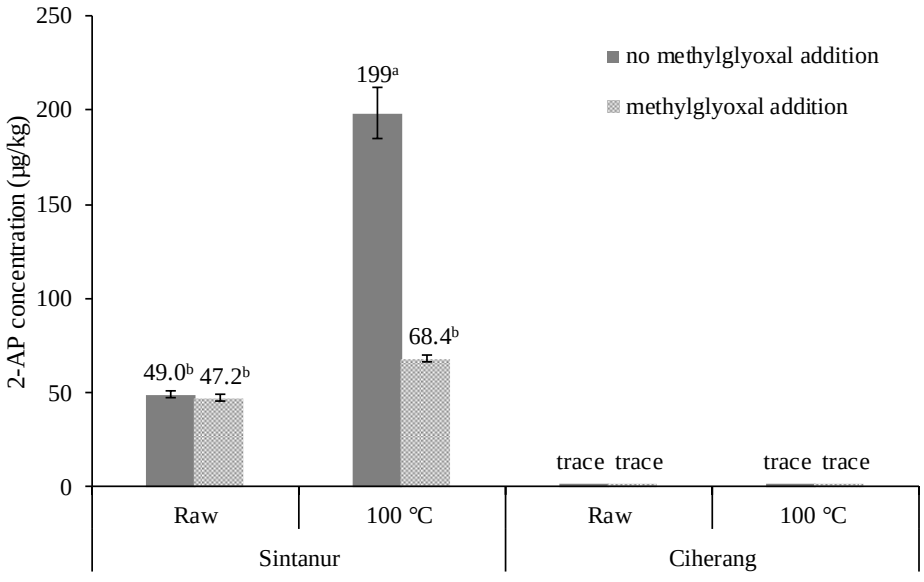

Figure 6.4: 2-Acetyl-1-pyrroline concentrations (µg/kg) in uncooked and 100 °C baked Sintanur and Ciherang rice with and without methylglyoxal addition (n = 3). Data not sharing the same letter are significantly different ($p < 0.05$) in each rice variety. Error bar shows the standard deviation of triplicate analyses. 'Trace' means concentration of the compound is lower than LOQ of 5 µg/kg.

2-AP concentration in 1-pyrroline addition rice (**Figure 6.3**) and methylglyoxal-treated rice (**Figure 6.4**) showed the same trend; 2-AP did not increase during baking at 100 °C when either 2-AP Maillard precursor was added while baking. These results indicated that the external precursors cannot be involved in 2-AP generation at 100 °C baking; moreover, the addition of these intermediates can cause the reduction of 2-AP. As discussed in **Chapter 5**, 2-AP increase was observed in 100 °C baked Sintanur extracted by SPE, a solvent-based extraction at room temperature. There is no doubt that 2-AP increase during 100 °C baking was caused by generation rather than release from rice matrix during heating. Previous research has confirmed the presence of 1-pyrroline in fragrant rice and its absence in non-fragrant rice during cultivation (Bradbury et al., 2008); 1-pyrroline is the limiting factor of 2-AP biosynthesis in fragrant rice (Poonlaphdecha et al., 2016). Because 2-AP increased in 100 °C baked fragrant rice but was absent in non-fragrant rice, the increase could correlate to the presence of 1-pyrroline. As addition of either Maillard precursor cannot lead to 2-AP increase during baking at 100 °C, 2-AP increase in fragrant rice at 100 °C may not be due to Maillard reaction.

Biosynthesis could be a potential explanation of 2-AP increase in fragrant rice during baking at 100 °C. 1-Pyrroline was generated and accumulated in fragrant rice through BADH2-dependent (Bradbury et al, 2008) and BADH2-independent (correlation with P5C; Huang et al., 2008) pathways. 2-AP was formed from acetylated 1-pyrroline by acetyltransferase in fragrant rice during cultivation; since non-fragrant rice cannot produce 1-pyrroline, 2-AP is absent in non-fragrant rice. Although previous studies indicated that heating at around 100 °C can reduce acetyltransferase activity to a low level (Poonlaphdecha et al., 2016; Gardner et al., 2006), the contribution of acetyltransferase to 2-AP generation at 100 °C in fragrant rice may still be significant, since Gardner et al. (2006) indicated that acetyltransferase retained 15% activity after heating at 100 °C and 60 min, and baking at 100 °C for 20 min in the current study may not denature all of the acetyltransferase. Research on the source of the acetyl group may increase our understanding of the contribution of acetyltransferase in 1-pyrroline acetylation during baking. It

is clear that the acetyl group is contributed by methylglyoxal, when 2-AP is formed through Maillard reaction (Hofmann & Schieberle, 1998a). However, there are a few different hypotheses proposed for acetyl group donors in 2-AP biosynthesis in fragrant rice through acetyltransferase catalysis. Methylglyoxal and its precursor (pyruvic acid) were regarded as candidates for donating the acetyl group in 2-AP biosynthesis (Huang et al., 2008); increase of 2-AP was observed when pyruvic acid was added into the incubation of rice calli with 1-pyrroline (Poonlaphdecha et al., 2016). It is also reported that acetyl-CoA can contribute an acetyl group for 1-pyrroline in rice (Suprasanna, Ganapathi, Ramaswamy, Surendranathan, & Rao, 1998) and *L. hilgardii* bacterium (Costello & Henschke, 2002); this was confirmed from labelling studies (Poonlaphdecha et al., 2016). Therefore, further research could focus on the study of the key intermediates and enzymes which correlate to 2-AP biosynthesis in rice, in order to investigate 2-AP generation in fragrant rice during baking at lower temperatures where 2-AP formation by Maillard reaction is unlikely to take place.

6.3.3. Key 2-AP related amino acids in 100 °C baked rice

Proline, glutamic acid and GABA were quantified in uncooked and 1-pyrroline treated and untreated Sintanur and Ciherang rice samples baked at 100 °C (**Figure 6.5**). For both Sintanur and Ciherang rice, no significant differences in proline and glutamic acid were found between uncooked, 100 °C baked treated and untreated samples. However, a significant difference in GABA was found in both Sintanur and Ciherang rice. GABA in untreated baked Sintanur was significantly higher than in uncooked Sintanur ($p = 0.042$) and 1-pyrroline-treated baked Sintanur ($p = 0.021$). Moreover, GABA in untreated baked Ciherang was also significantly higher than in uncooked Ciherang ($p = 0.039$) and 1-pyrroline-treated baked Ciherang ($p = 0.022$). However, no significant difference was observed between uncooked and 1-pyrroline-treated baked rice samples. Therefore, it appears that GABA can be enhanced during 100 °C baking in both Sintanur and Ciherang rice, but that additional 1-pyrroline can reduce this enhancement.

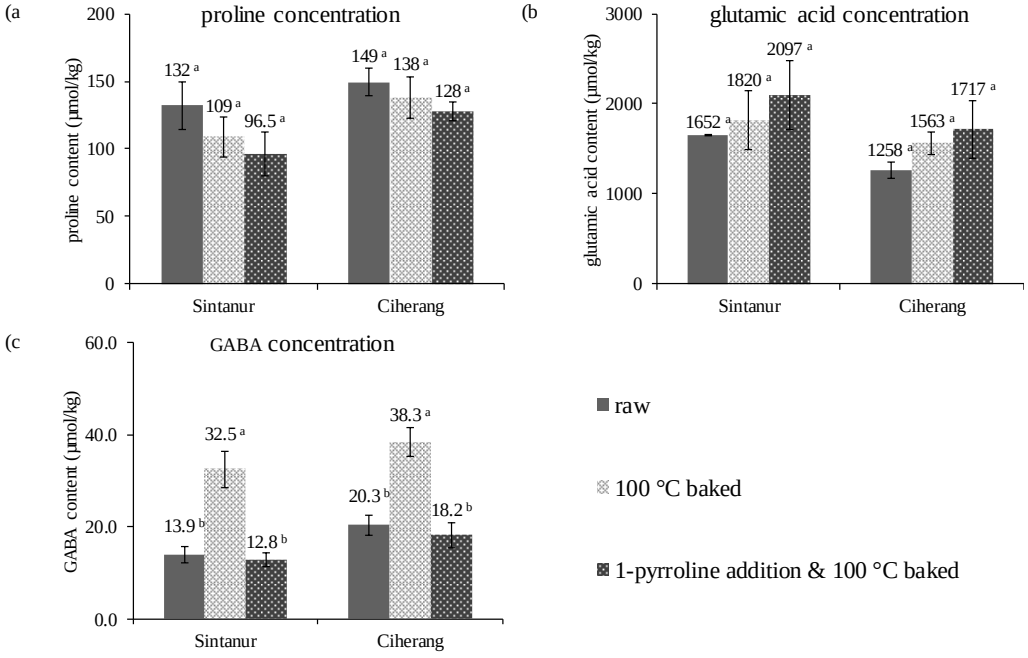

Figure 6.5: (a) proline, (b) glutamic acid, and (c) GABA concentrations (µmol/kg) in uncooked, 100 °C and 100 °C Sintanur and Ciherang rice with 1-pyrroline addition (n=3). Data not sharing the same letter are significantly different ($p < 0.05$) in each rice variety. Error bars show standard deviation of triplication.

It has been reported that induction of stress on glutamic acid metabolism can influence glutamic acid decarboxylase (GAD) activity and finally enhance GABA in rice. Bouché and Fromm (2004) concluded that enzyme GAD is a Ca^{2+}-dependent calmodulin (CaM)-binding protein and it is regulated by CaM. This means that Ca^{2+} concentration change in cytoplasm can result in a rapid change of GAD activity, and various stress situations (*in vitro* and *in vivo*) can lead to cytosolic Ca^{2+} concentration change and hence improve GAD activity. GABA enhancement studies in rice found that ultrasonication of red rice seed during soaking or germination can enhance GABA formation after rice germination; a two-fold increase of GABA was observed in rice seed sonicated for 5 min during soaking or germination compared with untreated samples (Ding et al., 2018). Kim et al. (2015) reported that activation of GAD is not restricted to rice cultivation; it can even be enhanced in rice bran flour. Anaerobic storage during heating at appropriate moisture content (20–50%) in their study induced a metabolic stress on glutamic acid and caused a rapid increase of GABA. In addition, a significant reduction of glutamic

acid was found during GABA enhancement (Kim et al., 2015). In our study, GABA enhancement was detected after rice was baked in a sealed ampule or value, which may provide similar conditions to those of Kim et al. (2015). Therefore, activation of GAD may play an important role in the GABA increase, which was observed in our study. In addition, when rice was baked at a high temperature (180 °C), an increase of GABA was not observed in either Sintanur or Ciherang rice (**Figure 6.6**). GABA may be decomposed, and GAD can also be inactivated during 180 °C baking. These results also suggest that the GABA increase at 100 °C may be through GAD catalysis from glutamic acid.

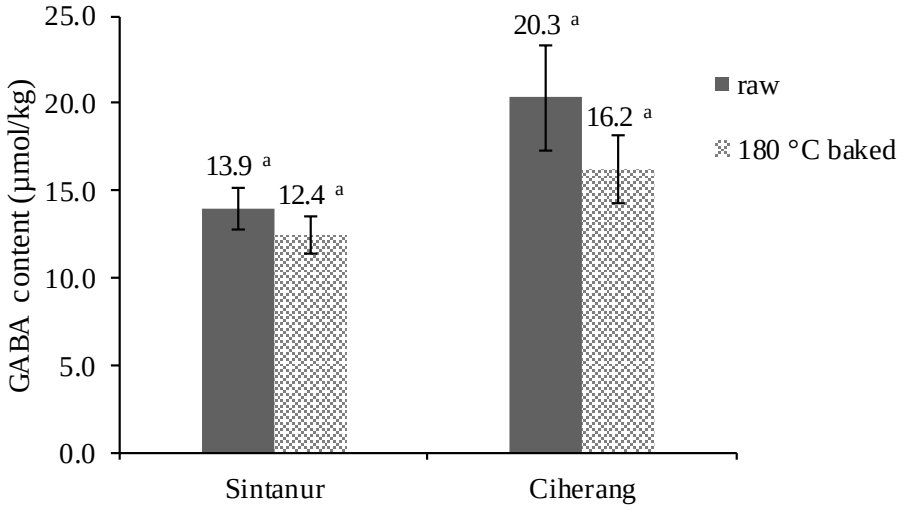

Figure 6.6: GABA concentration (µmol/kg) in uncooked and 180 °C baked Sintanur and Ciherang rice (n=3). Data not sharing the same letter are significantly different ($p < 0.05$) in each rice variety. Error bars show standard deviations of triplicate analyses.

2-AP increased in untreated Sintanur and remained undetected in Ciherang after baking at 100 °C. However, baking did not lead to an increase of either 2-AP or GABA in 1-pyrroline-treated rice. Like 2-AP, formation of GABA was also reduced by 1-pyrroline addition. Bradbury et al. (2008) reported that GABA was generated *via* functional BADH2 catalysis from GABald in non-fragrant rice; at the same time, GABald can also be cyclised to 1-pyrroline in fragrant rice. However, the mechanism of GABA formation inhibition by 1-pyrroline is not clear. Since a change of GABA occurred alongside a change in 2-AP in our study, especially in Sintanur rice, a

correlation between GABA and 2-AP may occur during 2-AP generation in fragrant rice. In order to verify this potential correlation, two different levels of GABA (1 µmol and 5 µmol) were added into 1 g of both fragrant and non-fragrant varieties. GABA and 2-AP levels were measured in uncooked and baked Sintanur and Ciherang samples.

Table 6.4: GABA concentration in untreated and GABA treated raw and 100 °C baked Sintanur and Ciherang rice (µmol/kg).

	GABA concentration (µmol/kg)					
	untreated		1000 nmol GABA addition		5000 nmol GABA addition	
	raw	baked	raw	baked	raw	baked
Sintanur	13.9 ± 3.75 [b]	32.5 ± 4.72 [a]	569 ± 50.8 [a]	524 ± 34.5 [a]	3192 ± 232 [a]	2787 ± 159 [a]
Ciherang	20.3 ± 8.62 [b]	38.3 ± 3.17 [a]	582 ± 40.0 [a]	513 ± 51.5 [a]	2983 ± 437 [a]	3006 ± 622 [a]

Data are mean value of triplicate (n = 3). Data not share same letter in each treatment are significantly different (p < 0.05).

Table 6.4 showed that GABA contents in uncooked Sintanur and Ciherang samples were 13.9 µmol/ kg and 20.3 µmol/ kg, respectively. After baking, around a 2-fold increase of GABA was observed. When 1 mL of 1 µmol GABA was added into 1 g uncooked rice, 569 µmol/kg GABA was quantified in Sintanur rice and 582 µmol/kg GABA was quantified in Ciherang rice. GABA contents in 5 µmol-treated Sintanur and Ciherang were 3192 µmol/ kg and 2983 µmol/kg, respectively. After 1 µmol/g and 5 µmol/g GABA addition in uncooked rice, GABA increased by more than 20-fold and 100-fold. GABA levels were not significantly changed in GABA treated samples after 100 °C baking. 2-AP was not detected in uncooked Ciherang and GABA-treated Ciherang at either level. 2-AP concentration in Sintanur rice is shown in **Figure 6.7**. Similar levels of 2-AP can be seen in uncooked untreated, 1 µmol and 5 µmol-treated Sintanur (48.6 µg/kg, 42.5 µg/kg and 41.3 µg/kg, respectively), and no significant difference was observed between them. Around three-fold increases of 2-AP were found in baked samples compared with uncooked samples. No significant differences were found between baked Sintanur samples (133 µg/kg in untreated Sintanur, 125 µg/kg in 1 µmol-treated Sintanur and 155 µg/kg in 5 µmol-treated Sintanur). This result indicated that 2-AP can only be increased by baking, and a high level of

GABA addition cannot influence the formation of 2-AP in 100 °C baked Sintanur rice; a correlation between GABA and 2-AP formation during baking at 100 °C may not present.

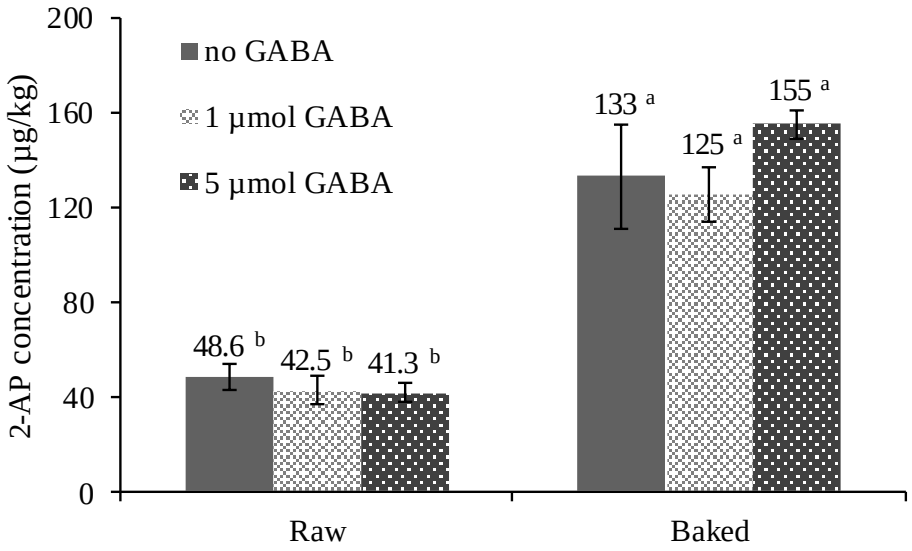

Figure 6.7: 2-AP concentration (µmol/kg) in uncooked and 100 °C baked Sintanur rice with different level of GABA addition (n=3). Data not sharing the same letter are significantly different ($p < 0.05$).

6.3. Conclusions

In this study, 2-AP and free amino acids were quantified in 21 varieties of uncooked milled rice (including 11 fragrant rice and 8 non-fragrant rice samples). Although correlation between amino acids and 2-AP was not found, higher amounts of glutamic acid, proline and GABA were found in non-fragrant rice varieties than fragrant rice.

GABA levels significantly increased when both fragrant and non-fragrant rice were baked at 100 °C because a metabolic stress was induced on glutamic acid, which increased GAD activity, whereas, an increase of 2-AP was only observed in fragrant rice. 1-Pyrroline is regarded as the most important intermediate in 2-AP generation. Additional 1-pyrroline can enhance 2-AP generation in 180 °C baked fragrant and non-fragrant rice *via* Maillard reaction. However, this enhancement was not observed after 100 °C baking. Moreover, 2-AP generation in 100 °C baked rice was inhibited by 1-pyrroline addition. Methylglyoxal is the other intermediate which react

with 1-pyrroline to form 2-AP in Maillard reaction, however, addition of methylglyoxal cannot enhance 2-AP generation at 100 °C in fragrant rice. GABA formation during 100 °C baking was also inhibited by additional 1-pyrroline. An experiment on GABA-enhanced samples showed that 2-AP concentration was not influenced by GABA addition.

This study suggested that 2-AP generation in fragrant rice when rice was baked at 100 °C followed a different formation pathway to the Maillard reaction at 180 °C; either additional 1-pyrroline or methylglyoxal cannot improve 2-AP generation yield in fragrant rice, but also cause a lower 2-AP generation yield than untreated fragrant rice. As the other 2-AP generation route, biosynthesis in fragrant rice need to be further investigated.

In future work, activities of the key enzymes in 2-AP biosynthesis (e.g. BADH2, acetyltransferase) and intermediates of 1-pyrroline biosynthesis, such as P5C and GABald need to be monitored. P5C can be quantified by high-performance liquid chromatography (Huang et al., 2008); and nuclear magnetic resonance spectroscopy has been used to detect GABald (Struve & Christophersen, 2003). ^{15}N-labelling techniques could be applied to examine intermediates, especially the effects of 1-pyrroline, methylglyoxal and GABA addition. In the current study, high concentrations of 1-pyrroline (1000 mg/kg), methylglyoxal (1000 mg/kg) and GABA (100 and 500 times higher than GABA content in uncooked rice) were used in addition experiments. In further experiments, the quantities of additional intermediates need to be adjusted; a wider range of concentrations should be considered, in order to avoid the inhibition that was observed by Hofmann and Schieberle (1998a). The mechanism of 2-AP and GABA generation still needs to be explored. Since GABA formation can be inhibited by 1-pyrroline addition, although the mechanism is unknown, the equilibrium of GABA formation from glutamic acid or from GABald may be influenced by 1-pyrroline.

Chapter 7. General discussion

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7.1. Introduction

2-Acetyl-1-pyrroline (2-AP) is regarded as the key contributor of popcorn-like aroma in fragrant rice. 2-AP can be generated in baked rice *via* Maillard reaction and in fragrant rice during rice cultivation. 2-AP biosynthesis in fragrant rice is influenced by many factors, such as climate and soil conditions (Yoshihashi, Nguyen, & Kabaki, 2004; Itani, Tamaki, Hayata, Fushimi, & Hashizume, 2004; Szabados & Savouré, 2010; Poonlaphdecha, et al., 2012), but previous research suggested that 2-AP cannot be enhanced after rice harvest (Yoshihashi, 2002). However, Handoko (2014) found 2-AP increasing in fragrant rice after 180 °C baking, which indicated that 2-AP could be enhanced post-harvest.

This PhD thesis is a continuation of the research initiated by Handoko (2014), with an overarching aim to develop techniques to enhance 2-AP content in fragrant and non-fragrant rice. The scientific hypothesis in this thesis is that 2-AP can be generated in fragrant and non-fragrant rice during baking without water, and hence the formation routes to enhancing 2-AP in rice has been investigated.

In **Chapter 3,** the optimised 2-AP extraction and detection techniques were developed.

In **Chapter 4**, quantitative descriptive analysis was used to evaluate sensory attributes in boiled rice, and GC-MS and GC-O were used to measure 2-AP levels in rice.

In **Chapter 5,** the effects of the baking of milled rice and rice bran at 180 °C were examined. 2-AP generation and precursor changes were measured. A range of cooking time and temperatures was selected to maximise 2-AP generation in fragrant and non-fragrant rice.

In **Chapter 6,** free amino acids and 2-AP in 21 raw, milled rice varieties were measured, and 1-pyrroline, methylglyoxal and GABA were added into selected rice varieties before baking, to explore potential mechanisms of 2-AP formation in fragrant and non-fragrant rice during baking.

7.2. Key Findings

7.2.1. Detection and differentiation of popcorn-like characteristics in boiled fragrant and non-fragrant rice by a trained panel

Sensory profiling of six varieties of boiled rice (three fragrant rice and three non-fragrant rice) using 13 trained panellists indicated that popcorn-like attributes were found in odour, flavour and after-effect. Significant differences in the intensity of popcorn-like attributes were found between fragrant and non-fragrant rice types; however, significant differences could not be observed between different rice varieties.

GC-MS analysis of the six boiled rice varieties showed that 2-AP could only be quantified in the three fragrant rice varieties. However, although the concentration was very low, the presence of 2-AP has been reported in non-fragrant rice (Buttery, Ling, Juliano, & Turnbaugh, 1983; Buttery, Turnbaugh, & Ling, 1988; Maraval et al., 2010). The GC-O analysis of raw non-fragrant rice in this study also confirmed the presence of 2-AP.

Popcorn-like odour intensity rating on 2-AP standards showed that perceived odour intensity of 2-AP and its concentration present a decelerating relationship (**Figure 3.2a**). This relationship fits Steven's law: log of 2-AP odour intensity *versus* log of 2-AP concentration shows a linear regression (**Figure 3.2b**). It suggested that assessors find it relatively more difficult to notice the change of 2-AP level at higher concentrations compared to at lower concentrations. A

two-fold difference in 2-AP concentration existed between the fragrant rice varieties (80 µg/kg in Sintanur rice and 146 µg/kg in jasmine), whereas such levels were over 10-fold higher than the limit of quantification (LOQ) of GC-MS analysis (5 µg/kg), and all substantially higher than the 2-AP concentrations of the non-fragrant varieties, which were lower than the LOQ. Hence, fragrant and non-fragrant rice can be differentiated based on popcorn-like attributes, whereas the two-fold differences between fragrant varieties do not appear to lead to perceivable differences.

7.2.2. Exploration of 2-acetyl-1-pyrroline formation in fragrant and non-fragrant rice during baking without water

When Sintanur (fragrant rice) and Ciherang (non-fragrant rice) were baked across a range of temperatures in **Chapter 5**, formation of 2-AP showed different trends with temperature. 2-AP increased in fragrant rice from 40 °C and its level achieved a maximum around 100 °C. On the other hand, 2-AP could only be detected in non-fragrant rice when baking temperature was higher than 140 °C. 2-AP levels in non-fragrant rice were always lower than in fragrant rice when rice was baked at the same temperature.

100 °C and 180 °C were selected as baking temperatures in **Chapter 6** to explore 2-AP formation mechanisms in two rice varieties. 2-AP was generated in both fragrant and non-fragrant rice at 180 °C. 2-AP generation in a proline and α-dicarbonyl compounds model system through Maillard reaction was proposed by Hofmann and Schieberle (1998a). Proline reacts with 1-deoxyglucosone or methylglyoxal to give 1-pyrroline and 1-hydroxy-2-propanone during the early stages. Then, as the important intermediate, 1-pyrroline can react with methylglyoxal to form 2-AP and react with 1-hydroxy-2-propanone to form 6-acetyl-1,2,3,4-tetrahydropyridine (ATHP) (Hodge, Mills, & Fisher, 1972; Hofmann & Schieberle, 1998a). Although it was not quantified, ATHP was detected in both 180 °C baked fragrant and non-fragrant rice but absent in raw rice; this indicated that 2-AP generation in 180 °C baked rice was driven by the Maillard reaction. Maillard formation of 2-AP at 180 °C was also confirmed by a 1-pyrroline addition experiment in

Chapter 6; when 1-pyrroline was added in both fragrant and non-fragrant rice and then baked at 180 °C, 2-AP was generated in both rice varieties. Moreover, concentrations of methylglyoxal-related Maillard-formed compounds (methyl pyrazines) were decreased, since more methylglyoxal was consumed by extra 1-pyrroline.

When both rice varieties were baked at 100 °C, 2-AP was significantly increased in fragrant rice but absent in non-fragrant rice. Precursor analysis in **Chapter 5** and **Chapter 6** showed a similar level of proline (precursor of 2-AP *via* Maillard reaction) in both types of rice varieties. As the precursor of 2-AP Maillard formation, methylglyoxal was formed from degraded reducing sugars, Kocadağlı & Gökmen (2016) pointed out that glucose melts at 146 °C in a dry glucose/flour system and fructose melts at 104 °C in a dry fructose/flour system, opening the cyclic structure and causing degradation. The methylglyoxal measurement experiment in **Chapter 6** showed that methylglyoxal was not detected in either raw and 100 °C baked rice, which confirmed that methylglyoxal cannot be produced in dry baked rice system at 100 °C. Moreover, when methylglyoxal was added into rice, 2-AP was not enhanced after 100 °C baking. This suggested that Maillard reaction between methylglyoxal and proline does not occur in rice matrix during baking at 100 °C without water. The 1-pyrroline addition experiment showed that 2-AP can be enhanced by additional 1-pyrroline at 180 °C through Maillard reaction, but cannot be enhanced at 100 °C. In addition, addition of 1-pyrroline or methylglyoxal can cause the reduction of 2-AP generation in fragrant rice at 100 °C. Therefore, 2-AP increase in fragrant rice at 100 °C baking without water was due to a pathway other than the Maillard reaction. This reaction could be sensitive to 1-pyrroline and methylglyoxal, addition of either compound can reduce 2-AP generation yield.

7.3. Limitations and Future Works

7.3.1. Sensory evaluation of 2-acetyl-1-pyrroline enhanced rice

In this study, baking at around 100 °C without water addition tripled the 2-AP level in fragrant rice. Therefore, in future work, sensory evaluation could be conducted on the difference between raw rice and 2-AP-enhanced samples. Sensory evaluation would focus on the difference of odour and flavour other than popcorn-like aroma, and the texture difference, and the change of other sensory aspects during the 2-AP enhancement would be measured. With the assistance of sensory evaluation, optimised enhancement conditions could be developed which could obtain a significant increase of popcorn-like aroma but minimise the changes in other attributes.

7.3.2. Development of extraction technique

SPE was selected for GC-O in **Chapter 4** because more samples can be loaded for a single extraction compared with SPME. However, SPE was difficult to apply for boiled rice analysis. Therefore, GC-O was only conducted on raw rice in **Chapter 4**. Calibration of 2-AP for SPE and SPME (in **Chapters 4, 5** & **6**) showed that the LOQ of 2-AP with SPE and SPME were the same (5 µg/kg). This indicated that replacing SPME with SPE did not significantly improve the sensitivity of 2-AP detection. An automated SPME sampler was not available for GC-O in our laboratory; hence, manual SPME is likely to be the method of extraction for boiled rice GC-O in future work. Aroma extraction dilution analysis (AEDA) could be applied to 2-AP in both boiled fragrant and non-fragrant rice, to compare the 2-AP intensities in these two rice types. Because SPME is a headspace extraction technique, the dilution in AEDA can be conducted through increasing the split ratio during sample injection.

Both SPME and SPE were applied in this project for 2-AP extraction in unbaked and baked rice, and their advantages were discussed in earlier chapters. However, due to the weakness of each technique, the extraction technique still requires further development to get a better extraction

of 2-AP in rice. Theoretically, SPE can provide a more accurate extraction than SPME. Depending on the different stationary phase of SPE cartridge, this technique can extract compounds based on their polarity, or molecule size. Furthermore, protein, fat and salt can also be extracted with the application of specific SPE cartridges (Sigma-Aldrich Co., 1998; Waters Corporation, 2016). However, in order to exclude free fatty acids, protein and non-volatile compounds, multiple sorbents and complex procedures need to be used during extraction, which could multiply the cost of extraction.

Therefore, a method that combines SPE and SPME is being developed in our laboratory (SPE-SPME). The sample is extracted using the SPE method in **Chapter 5**. The extract after elution is transferred into a 10 mL SPME vial, and then it is gently enveloped by an N_2 stream to remove all solvent; the extracted material forms a film in the bottom of the SPME vial. Then the SPME extraction method in **Chapter 6** is applied for further extraction and analysis.

Figure 7.1 shows selective ion chromatograms (*m/z* 68, 83, 111) of SPE-SPME (red) and SPME (black) for raw Sintanur (fragrant rice); the peaks at 14.49 min are due to 2-AP. It can be seen that much more 2-AP can be extracted by SPE-SPME than SPME. Hence, the sensitivity of 2-AP detection can be improved, and the profile of the whole chromatogram would not be influenced by the solvent. Calculations of 2-AP concentration in raw Sintanur using SPE-SPME and SPME in triplicate were close, 49.9 ± 5.5 μg/kg and 49.1 ± 5.0 μg/kg, respectively. These same concentrations from each method showed the reliability of both extraction techniques. The new technique was not widely applied in this project; however, SPE-SPME could be a better extraction technique than SPE or SPME separately. Compared with SPE, the absence of solvent makes it easier for the 2-AP to enter the headspace and therefore the fibre. Compared with SPME, SPE-SPME can extract a higher quantity of compounds to improve the LOQ of 2-AP in future studies.

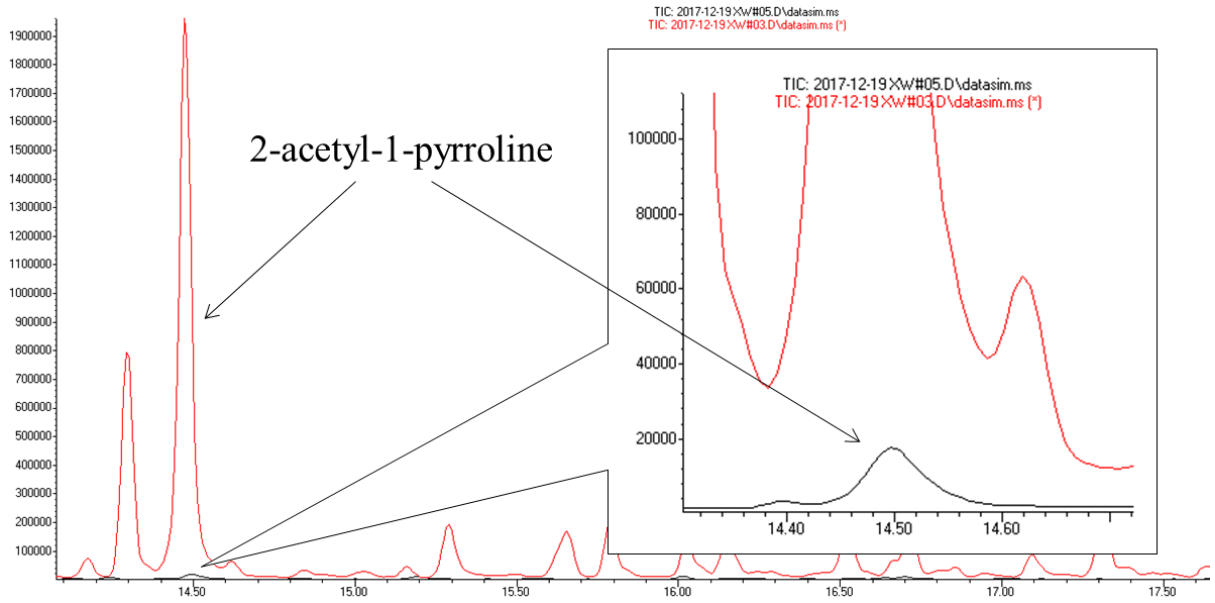

Figure 7.1: Comparison of combined selective ion chromatogram (*m/z* 68, 83, 111) of SPE & SPME combination extraction and SPME for raw Sintanur rice. Red ion chromatogram represents extraction with SPE & SPME combination, black ion chromatogram represents extraction with SPME.

Quadrupole-time-of-flight mass spectrometry (Q-TOF-MS) is a technique where the third quadrupole (Q3) in a triple quadrupole is replaced with a TOF mass spectrometer. Q-TOF-MS is regarded as a technique that has 'high sensitivity, mass resolution and mass accuracy'; it can particularly improve the sensitivity of 'scan' mode in both MS and MS/MS (Chernushevich, Loboda, & Thomson, 2001). Current analysis methods in our study obtained a relatively high LOQ of 2-AP and it is higher than the 2-AP content in non-fragrant rice and the 2-AP odour detection threshold. The application of Q-TOF-MS in 2-AP quantification could be considered in future work to improve LOQ of 2-AP in rice; moreover, high-resolution Q-TOF could separate *m/z* 111 for 2-AP from *m/z* 111 for 6-methyl-5-hepten-2-one, compounds that co-eluted in some studies (Paule & Powers, 1989; Tanchotikul & Hsieh, 1991; Seitz et al., 1993). Chemical ionisation (CI) could also be another option to improve detection sensitivity as it is a softer ionisation technique than electron ionisation (EI), as mentioned in *Section 2.7.3.* A few studies applied positive CI in 2-AP quantification and reported a better LOQ of 2-AP than EI (Maraval et al., 2010; Poonlaphdecha et al., 2012).

7.3.3. 2-Acetyl-1-pyrroline formation pathway in 100 °C baked fragrant rice

Because both intermediates (1-pyrroline and methylglyoxal) cannot increase 2-AP generation in fragrant rice at 100 °C, further investigation could focus on the other 2-AP generation route: biosynthesis. Mutation of *badh2* gene in fragrant rice is regarded as the main reason for 2-AP synthesis in fragrant rice during cultivation and this is also the main genetic difference between fragrant and non-fragrant rice (Bradbury, Fitzgerald, Henry, Jin, & Waters, 2005; Arikit et al., 2011; Fitzgerald, Waters, Brools, & Henry, 2010; Kovach, Calingacion, Fitzgerald, & McCouch et al., 2009; Siddiq, Vemireddy, & Nagaraju, 2012). A functional BADH2 enzyme is encoded by *badh2* gene, and this enzyme can catalyse the oxidation of γ-aminobutyraldehyde (GABald) to γ-aminobutyric acid (GABA), whereas the mutated *badh2* gene in fragrant rice encodes a non-functional BADH2 enzyme. The absence of functional BADH2 leads to the cyclisation of GABald to 1-pyrroline, and then 2-AP is generated from the acetylation of 1-pyrroline (Bradbury, Gillies, Brusheet, Waters, & Henry, 2008). On the other hand, the level of 2-AP was found to correlate to that of 1-pyrroline-5-carboxylate (P5C) in rice; P5C level in fragrant rice was significantly higher than in non-fragrant rice (Huang et al., 2008). Huang et al. (2008) proposed that the high level of P5C led to 2-AP accumulation in fragrant rice through the formation of 1-pyrroline. Numerous studies have indicated that the presence of 1-pyrroline in fragrant rice is the key reason for 2-AP generation during cultivation.

1-Pyrroline in fragrant rice, which leads to 2-AP generation during baking at 100 °C, may be accumulated in rice before baking, or it may be generated during baking. 1-Pyrroline was detected by GC-MS in this project; however, this compound was not found in either raw or baked rice. Therefore, in order to investigate the complete mechanism of 1-pyrroline generation during 100 °C baking in fragrant rice, intermediates and the activity of several key enzymes that are involved in 2-AP generation pathways need to be measured. There are two routes which may produce 1-pyrroline through biosynthesis: presence of non-functional BADH2 and a high level of

P5C. Ornithine, glutamic acid and proline are regarded as the precursors of 2-AP *via* biosynthesis. However, the **Chapter 6** study showed that the content of amino acids in rice is more than a thousand-fold higher than 2-AP, and the increase of 2-AP during baking can hardly be reflected in the change of precursors. Therefore, exploration of 1-pyrroline formation routes can only be pursued through tracking of intermediates and enzyme activity change during baking.

In the pathway through P5C, P5C is produced from proline through catalysis of proline dehydrogenase (PRODH), from glutamic acid through catalysis of 1-pyrroline-5-carboxylate synthetase (P5CS) and from ornithine through ornithine aminotransferase (OAT) (Huang, et al., 2008; Kishor et al., 2005; Bradbury et al., 2008). Measurement of PRODH, P5CS and OAT activities was introduced in several studies focusing on 2-AP biosynthesis (Huang et al., 2008; Li et al., 2016; Mo et al., 2017). Hence, the change in activity of P5C synthesis enzymes can be measured. Moreover, P5C can be measured by the method of Mezl and Knox (1976), through the reaction with *o*-aminobenzaldehyde and analysis by HPLC, and a P5C extraction technique has been developed for soybean (Wu, Chou, Wu, Chen, & Huang, 2009). In the non-functional BADH2 pathway, GABald is catabolised from proline *via* putrescine oxidation; GABald is cyclised to 1-pyrroline in the absence of functional BADH2 (Bradbury et al., 2008). Direct estimation of GABald has not been carried out in previous studies, however, addition of ^{13}C-labelled GABald during baking is possible; tracking of labelled carbon in 2-AP would be helpful to confirm the hypothesis of 2-AP formation in fragrant rice during baking.

Acetyltransferase activity can be measured by a commercial kit (Enzo Life Sciences, Exeter, UK). There are a few potential acetyl group donors for 2-AP. Methylglyoxal and its precursor pyruvic acid could be candidates as acetyl group donors (Huang et al., 2008). In addition, acetyl-CoA was also regarded as a potential acetyl group donor in rice (Suprasanna, Ganapathi, Ramaswamy, Surendranathan, & Rao, 1998) and the bacterium *L. hilgardii* (Costello & Henschke, 2002). Poonlaphdecha et al (2006) showed a labelling experiment to track these potential donors.

Kocadağlı and Gökmen (2016) reported a derivatisation and detection technique to measure methylglyoxal in dry baked wheat flour-glucose system; they used HPLC-MS for the detection, and the calibration curve was established using methylglyoxal standard over a range between 0.1 mg/L and 5 mg/L, and although it had not been state in their article, the minimal concentration of methylglyoxal can be quantified in the dry wheat flour-glucose system was lower than 0.01 μmol/g according to their figures. However, derivatised methylglyoxal could not be quantified in either raw or 100 °C baked rice in **Chapter 6** by using HPLC with diode array detection. The lowest concentration of methylglyoxal standard detected by the HPLC-DAD method was 1 mg/L and it converts to around 0.05 μmol/g in rice. Therefore, the higher resolution detecting instrument such as HPLC-MS suggested by Kocadağlı & Gökmen (2016) can be used of the further measurement of methylglyoxal.

7.4. Conclusions

In this project, the importance of popcorn-like aroma and 2-AP in fragrant rice was confirmed. In fact, 2-AP was also shown to be important in non-fragrant rice using GC-olfactometry. However, its presence in non-fragrant rice was much lower than in fragrant rice and below the limit of quantification of the GC-MS system used. A simple method was found to enhance 2-AP level in both milled fragrant and non-fragrant rice: baking of rice without additional water at a high temperature (> 140 °C) was found to generate 2-AP *via* Maillard reaction.

Although high level of 2-AP was present in raw fragrant rice bran, this project found that baking reduced 2-AP in bran. This project also investigated 2-AP formation at different temperatures: when rice was baked at around 100 °C, 2-AP concentration was doubled in fragrant rice but 2-AP increase was only observed at baking temperatures higher than 140 °C in non-fragrant rice. Although the pathway remains inconclusive, enzymes may be involved in 2-AP

increase in fragrant rice at around 100 °C. Through 1-pyrroline and methylglyoxal addition, it was shown that the mechanism of formation of 2-AP at 100 °C was different to the mechanism *via* Maillard reaction at 180 °C; external 1-pyrroline and methylglyoxal did not increase 2-AP generation at 100 °C but additional 1-pyrroline can significantly increase 2-AP at 180 °C.

From the results of this study, this research hypothesise that, at lower cooking temperatures, 2-AP may be produced with the involvement of enzyme catalysis, and this reaction is sensitive to the amounts of 1-pyrroline and methylglyoxal present; at higher cooking temperature (>140 °C), 2-AP can be generated from the Maillard reaction between amino acids and reducing sugars. Future work should focus on tracking key intermediates (P5C and GABald) and measuring the activities of enzymes (PRODH, P5CS, OAT and Acetyltransferase), in order to clarify the complete reaction pathway.

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Appendix 1: Perceive intensity of popcorn-like odour in GC-O analysis of raw Sintanur and Ciherang rice.

	Sintanur		Ciherang	
Sniffer	LRI	Perceived intensity (0-10)	LRI	Perceived intensity (0-10)
1	1335	8	1342	3
	1339	7	1342	5
2	1333	7	1347	3
	1338	6	1344	3
3	1335	7	1338	5
	1336	7	1342	5
4	1333	7	1334	3
	1330	7	1341	4
Average		7 ± 0.5		3.88 ± 0.93

Appendix 2: Publications

Wei, X., Handoko, D. D., Pather, L., Methven, L., & Elmore, J. S. (2017). Evaluation of 2-acetyl-1-pyrroline in foods, with an emphasis on rice flavour. *Food Chemistry*, *232*, 531-544.

Wei, X., Methven, L., & Elmore, J. S. (2018). Comparison of the Sensory Properties of Fragrant and Non-Fragrant Rice, Focusing on the Role of the Popcorn-like Aroma Compound 2-Acetyl-1-pyrroline. *Food Science: Proceedings of the XV Weurman Flavour Research Symposium.* Siegmund, B., & Leitner, E. (eds). Verlag der Technischen Universität Graz. 427-432.

Comparison of the sensory properties of fragrant and non-fragrant rice: The role of the popcorn-like aroma compound 2-acetyl-1-pyrroline

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Abstract

2-Acetyl-1-pyrroline (2-AP) has been widely reported as being a key contributor to the popcorn-like aroma of fragrant rice. To understand more about the contribution of 2-AP to the aroma of fragrant rice and to highlight the sensory differences between fragrant and non-fragrant rice, quantitative descriptive analysis was conducted, to examine the sensory properties of six boiled rice samples (three fragrant rice and three non-fragrant rice) by 11 panellists, with emphasis on popcorn-like odour and flavour. The results showed perceived intensity of popcorn odour and flavour in fragrant rice were higher than in non-fragrant rice ($p = 0.016$, $p = 0.026$, respectively). However, the panellists could not differentiate between fragrant boiled rice varieties based on popcorn odour or flavour. 2-AP was extracted from the six boiled rice samples by headspace solid-phase microextraction and quantified by gas chromatography-mass spectrometry. 2-AP was found in fragrant rice samples (146 µg/kg in Jasmine, 113 µg/kg in Basmati and 80 µg/kg in Sintanur) but could not be quantified in non-fragrant varieties (below 5 µg/kg). These results suggested that although 2-AP is a key contributor to popcorn-like notes in fragrant rice, the differences in level of 2-AP content between different boiled fragrant rice samples may be too small to cause perceptual discrimination. In addition, popcorn-like notes were perceived in non-fragrant rice samples, despite levels of 2-AP being below detection limits.

Introduction

2-Acetyl-1-pyrroline (2-AP) is a volatile compound with a popcorn-like odour and a low detection threshold (0.1 µg/kg in water). It was firstly identified in boiled fragrant rice [1]. When popcorn odour intensities in several fragrant rice varieties were ranked, 2-AP was considered as the most important contributor to this odour [2]. However, Yang et al. [3] reported that popcorn-like note may not be the only important characteristic in boiled fragrant rice odour. In addition, Limpawattana et al. [4] reported no correlation between popcorn flavour and 2-AP. Moreover, 2-AP has been reported to be generated only during fragrant rice growth and not during other postharvest procedures or cooking [5].

Lexicons of rice descriptors have been established in several studies, especially for fragrant rice [6-8]. The selection of descriptors depends on the panellists' culture and familiarity with the samples [9]. However, no rice lexicon has previously been reported using a UK sensory panel. In this study, different boiled rice varieties were evaluated using quantitative descriptive analysis (QDA). A lexicon was developed for both boiled fragrant and non-fragrant rice varieties using a UK-based panel. Differences in flavour and odour between fragrant and non-fragrant rice were evaluated. In addition, the relationship between popcorn flavour/odour and 2-AP content in boiled fragrant and non-fragrant rice was examined.

B. Siegmund & E. Leitner (Eds): Flavour Sci., 2018, Verlag der Technischen Universität Graz
DOI: 10.3217/978-3-85125-593-5-62, CC BY-NC-ND 4.0

Experimental

Materials

Six varieties of white rice were obtained in summer 2016, including three fragrant rice varieties (Basmati and Thai Jasmine from ASDA supermarket; Sintanur from Indonesia Centre of Rice Research) and three non-fragrant rice varieties (American long grain from ASDA supermarket; Arirang from Korea Foods Company Limited; and Ciherang from Indonesia Centre of Rice Research). 2-AP and deuterated 2-AP (2-AP-d2) standards were used for 2-AP quantification (both 30,000 ppm in dichloromethane (DCM), Aroma Lab, Germany).

Quantitative Descriptive Analysis (QDA) in boiled rice

Milled rice (200 ± 1 g) was weighed and then boiled using 300 mL mineral water in a rice cooker (0.8 L, Lloytron, UK). Cooking proceeded for 20 min before the rice cooker automatically turned to warm mode. The samples were kept warm (65 °C) for 20 min before serving to panellists for evaluation.

Quantitative descriptive analysis (QDA) was conducted for six rice samples, using 11 trained UK panellists. A vocabulary was developed for appearance, odour, taste, flavour, mouthfeel and after-effect. A pre-heated ceramic cup (50 mL) filled with boiled rice (20 g) covered by foil was served to panellists for developing odour attributes and another 20-g sample was then served in the same manner for developing all the other attributes. The scoring for each attribute of sample was conducted in individual booths in duplicate on separate days, and samples labelled with three-digit codes were presented randomly in a balanced order. Data were collected using Compusense at-hand (Canada).

2-Acetyl-1-pyrroline quantification in boiled rice

Rice samples (1 g ± 0.001 g) and 1.5 mL HPLC-grade water were added to 20-mL SPME glass vials with metal screw-caps and PTFE-faced silicone septa. Vials were then heated in a GC oven at 100 °C for 20 min. A 1.5-mL aliquot of 2-AP-d_2 aqueous solution (approximately 100 µg/kg, prepared by replacing DCM with HPLC-grade water) was added into the vials after they were cooled to room temperature. 2-AP in boiled rice was extracted from these samples by automated SPME (GC Sampler 120, Agilent). Samples were incubated with magnetic shaking for 10 min at 40 °C, and then extracted with a Supelco DVB/CAR/PDMS SPME fibre for 1 hour at 40 °C. After extraction, the extracts were analysed by gas chromatography-mass spectrometry (GC-MS) using a 7890 GC with 5975C MS (both Agilent). The SPME fibre was desorbed in the GC injector at 250 °C for 20 min, in splitless mode, onto the front of a Zebron ZB-Wax column (30 m × 0.25 mm; 1 µm film thickness; Phenomenex). The carrier gas was helium at a constant column flow rate of 0.9 mL/min. The initial GC oven temperature was 40 °C and held for 2 min, then increased to 60 °C at the rate of 2 °C/min; then the rate increased to 6 °C/min until the temperature reached 250 °C. Electron ionisation (EI) mode was used at 70 eV. Full scan mode was used for analysis from m/z 30 to 280. Simultaneous selective ion monitoring was also applied: ions *m/z* 68, *m/z* 83 and *m/z* 111 were monitored for 2-AP; *m/z* 86 and *m/z* 114 were monitored for 2-AP-d_2. The dwell time of monitored ions was set at 100 ms/ion.

Results and discussion

Quantitative Descriptive Analysis (QDA) in boiled rice

Thirty-seven attributes (covering appearance, odour, taste, flavour, mouthfeel and after-effect) were found in six boiled rice samples by 11 trained UK panellists. Significant differences between samples were found in all appearance attributes ($p < 0.0001$), popcorn odour ($p = 0.028$) and cohesive mouthfeel ($p < 0.0001$). Popcorn-like attributes were not only found in fragrant rice, but also in non-fragrant rice. When the six samples were grouped into fragrant (Jasmine, Basmati and Sintanur) and non-fragrant rice (long grain, Arirang and Ciherang), the perceived intensities of popcorn odour and flavour in fragrant rice were found significantly higher than in non-fragrant rice ($p = 0.016$, $p = 0.026$, respectively; Figure 1a). Although a significant difference in perceived popcorn odour was observed by ANOVA between different rice varieties (Figure 1b, $p = 0.028$), this difference was caused by a difference between Jasmine and Ciherang ($p < 0.05$); differences between other rice varieties were not observed. Jasmine and Sintanur tended to show higher perceived popcorn flavour than other samples, but no significant differences in popcorn flavour were found between rice varieties (Figure 1b, $p = 0.134$). These results indicated that although the panellists could not detect a difference in popcorn odour and flavour between individual boiled rice varieties, fragrant and non-fragrant rice could be categorised based on popcorn odour or flavour.

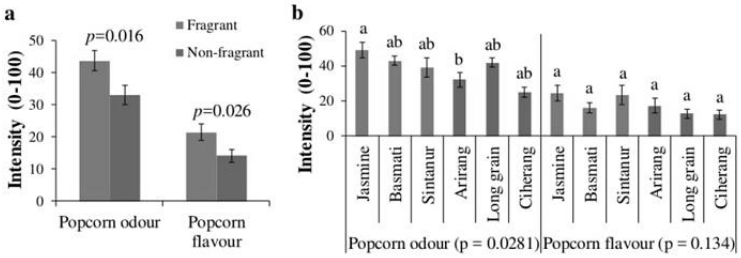

Figure 1: popcorn odour and flavour in fragrant and non-fragrant rice (a); perceived popcorn odour and flavour among six rice samples (b). Bars not sharing a common letter are significant different ($p<0.05$). Error bar represents standard error. Blue bars represent fragrant rice; red bars represent non-fragrant rice.

Quantification of 2-acetyl-1-pyrroline in boiled rice

Concentrations of 2-AP in six boiled rice samples are shown in Figure 2. Significant difference in 2-AP concentrations was observed between the three boiled fragrant rice samples ($p = 0.028$). The concentrations of references (four levels of 2-AP standards) used for popcorn odour in QDA training were 5 or 10-fold in difference, and the trained panellists could rank these samples in order of intensity with no difficulty. However, the two-fold difference in 2-AP (Jasmine *vs* Sintanur) was not great enough to be noticed by panellists, which might explain why there was no significant difference in popcorn odour or flavour between fragrant rice samples. Limpawattana et al. [4] reported that although 2-AP was the only contributor to popcorn-like note in boiled rice, this compound did not correlate with popcorn flavour. Therefore, as rice contains numerous volatile compounds, the interaction of other compounds with 2-AP might affect the perception of popcorn odour and flavour. In three non-fragrant rice varieties, although traces of 2-AP were detected in the GC-MS chromatograms, the concentration in these samples was too low to be quantified (Figure 2). The lowest concentration of 2-AP that could be quantified in

this study was 5 µg/kg. However, these concentrations may be still about 50-fold higher than the 2-AP detection threshold (0.1 µg/kg in water), which could be the reason that popcorn-like attributes in non-fragrant rice were also detected by panellists.

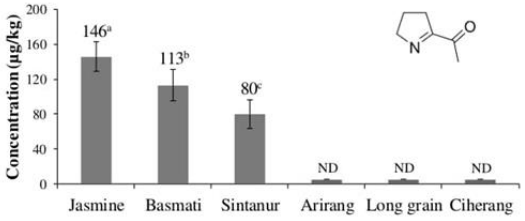

Figure 2: 2-acetyl-1-pyrroline concentrations in six boiled rice samples. Bars not sharing a common letter are significantly different ($p < 0.05$). Error bar represents standard deviation. ND: not detected, concentration lower than 5 µg/kg. Blue bars represent fragrant rice; red bars represent non-fragrant rice.

Yang et al. [3] analysed odour-active compounds in five boiled fragrant rice and one boiled non-fragrant rice samples. They found that 2-AP was detected in all six rice varieties, and popcorn-like odour was also detected in the non-fragrant rice variety. However, no other compounds that contributed popcorn-like odour were detected in their study. As no other compounds known to possess popcorn aroma were found in the current study, this suggests that trace levels of 2-AP in the non-fragrant varieties may be responsible for their popcorn-like aroma.

Conclusion

A lexicon was developed by a trained UK panel to describe six boiled rice varieties (three fragrant and three non-fragrant rice types). Popcorn odour and flavour were found in both fragrant and non-fragrant rice, but it was difficult to differentiate all six boiled rice varieties based on these attributes. However, significant differences were observed in both popcorn odour and popcorn flavour when fragrant and non-fragrant rice were compared by t-test. Significant differences in 2-AP concentration were found between the three fragrant rice varieties, although such differences were too small to cause a significant perceptual difference. Much higher levels of 2-AP were found in fragrant rice than non-fragrant rice. However, trace levels of 2-AP may contribute to popcorn attributes in non-fragrant rice varieties.

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Appendix 3: Titles of presentations in conferences

Wei, X., Methven, L., & Elmore, J. S. (2015). 2-Acetyl-1-Pyrroline in Fragrant and Non-fragrant Rice after High Temperature Heating. In 3rd Nursten Postgraduate Flavour Symposium, Newcastle, UK, presented as an oral presentation.

Wei, X., Methven, L., & Elmore, J. S. (2016). Research of 2-Acetyl-1-pyrroline in Cooked Fragrant and Non-fragrant Rice. In 11th Wartburg Symposium on Flavour Chemistry & Biology, Eisenach, Germany, presented as a poster presentation.

Wei, X., Sun, Q., Methven, L., & Elmore, J. S. (2017). Comparison of the Sensory Properties of Six Rice Varieties, Focusing on the Role of the Popcorn-like Aroma Compound 2-Acetyl-1-pyrroline. In 5th Nursten Postgraduate Flavour Symposium, Belfast, UK, presented as an oral presentation.

Wei, X., Sun, Q., Methven, L., & Elmore, J. S. (2017). Comparison of the Sensory Properties of Fragrant and Non-Fragrant Rice, Focusing on the Role of the Popcorn-like Aroma Compound 2-Acetyl-1-pyrroline. In 15th Weurman Flavour Research Symposium, Graz, Austria, presented as a poster presentation.