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**EXTRACTION OF AROMA COMPOUND FROM
PANDAN LEAF AND USE OF THE COMPOUND TO
ENHANCE RICE FLAVOUR**

by

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Abstract

Supercritical carbon dioxide (SC-CO₂) and Soxhlet extraction using hexane as solvent were used to extract 2-acetyl-1-pyrroline (2-AP) from Pandan leaves. The effect of different extraction pre-treatments, such as particle size and drying on the extraction yield and concentration of 2-AP were investigated. The identification and quantification of 2-AP were carried out by gas chromatography-mass spectrometry and gas chromatography-flame ionization detector, respectively. This work aims to provide an understanding of the phenomena that occur during cooking and storage; typically on the changes of 2-AP absorption when cooking rice grains with Pandan leaves. The parameters investigated were cooking method of excess and optimal water conditions.

Even though low in yield, and the 2-AP concentration was obtained from supercritical carbon dioxide extraction, the extracts were pure without any contamination. The grinding and freeze-drying method revealed the best pre-treatments for supercritical extraction. The absorption of 2-AP during the cooking of rice grains did not smoothly increase with time. This unexpected result indicated that the phenomena occurring during cooking are quite complex. This work also quantified the potential of Pandan leaves to enhance the flavour of cooked rice, particularly under excess water conditions. Storage for 15 min at $24.0 \pm 1.0^\circ\text{C}$ is considered as the optimum time for obtaining cooked rice with a high quality of flavour.

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Contents

Chapter 1.0: Introduction	1
Chapter 2.0: Literature review	7
2.1 Rice	7
2.1.1 Rice starch	8
2.1.1.1 Starch gelatinization	10
2.1.1.2 Starch retrogradation	11
2.1.1.3 Differential scanning calorimetry	13
2.1.2 Cooking of rice	14
2.1.2.1 Excess water cooking	16
2.1.2.2 Optimal water cooking	17
2.1.3 Storage of cooked rice	17
2.1.4 Flavour of rice	18
2.1.5 Starch-flavour interaction	19
2.1.5.1 Polar interaction	20
2.1.5.2 Inclusion complexes of amylose-flavour	20
2.2 Headspace analysis	22

2.3 Gas chromatography	22
2.4 Partition coefficient	24
2.5 2-acetyl-1-pyrroline	25
2.6 Pandan leaf	29
2.7 Extraction process	31
2.7.1 Supercritical carbon dioxide extraction	31
2.7.2 Solvent extraction	33
2.7.3 Extraction pre-treatment	34
2.7.3.1 Particle size pre-treatment	34
2.7.3.2 Drying pre-treatment	35
Chapter 3.0: Materials and Methods	36
3.1 Extraction of 2-acetyl-1-pyrroline from Pandan leaves	36
3.1.1 Materials	36
3.1.2 Determination of moisture content of Pandan leaves	37
3.1.3 Extraction pre-treatment	38
3.1.4 Scanning electron microscopy	38
3.1.5 Supercritical carbon dioxide (SC-CO ₂) extraction	39
3.1.5.1 Modification of SC-CO ₂ collector system	42
3.1.6 Hexane extraction	44

3.1.7 Determination of total yield of Pandan leaves extracts	46
3.1.8 Gas chromatography-flame ionization detector	46
3.1.9 Gas chromatography-mass spectrometry	47
3.1.10 Quantitative analysis	48
3.2 Absorption of 2-acetyl-1-pyrroline during cooking of rice mixed with Pandan leaves	49
3.2.1 Materials	49
3.2.2 Determination of partition coefficient of 2-AP in water/air system	49
3.2.2.1 Phase ratio variation method	49
3.2.2.2 Determination of water/air partition coefficient	52
3.2.2.3 Gas chromatography-flame ionization detector	52
3.2.2.4 Analysis	53
3.2.3 Procedure of cooking	53
3.2.3.1 Excess water cooking	54
3.2.3.2 Optimal water cooking	56
3.2.4 Determination of moisture content of rice	56
3.2.5 Static headspace analysis	57
3.2.6 Gas chromatography-flame ionization detector	58
3.2.7 Differential scanning calorimetry	58
3.2.8 Selection of headspace analysis method	59

3.2.9 Effects of prolonged cooking time on the 2-AP under optimal water conditions	60
3.3 Changes of 2-acetyl-1-pyrroline absorption during storage of cooked rice	62
3.3.1 Materials	62
3.3.2 Determination of 2-AP amount during storage of cooked rice mixed with different quantities of Pandan leaves	62
3.3.3 Procedure of storage	63
3.3.3.1 Storage of excess water of cooked rice	63
3.3.3.2 Storage of optimal water of cooked rice	64
3.3.4 Measurement of temperature during storage of cooked rice	64
3.3.5 Determination of moisture content during storage of cooked rice	65
3.3.6 Quantification of 2-AP concentration during storage of cooked rice	65
3.4 Statistical analysis	65
Chapter 4.0: Extraction of 2-acetyl-1-pyrroline from Pandan leaves	66
4.1 Introduction	66
4.2 Results and discussion	68
4.2.1 Moisture content of Pandan leaves	68
4.2.2 Structure of Pandan leaves	69

4.2.3 Total yield of Pandan leaves extracts	72
4.2.3.1 Supercritical carbon dioxide extraction	72
4.2.3.2 Hexane extraction	75
4.2.4 Concentration of 2-AP in Pandan leaf extracts	78
4.2.4.1 Supercritical carbon dioxide extraction	80
4.2.4.2 Hexane extraction	83
4.2.5 Colour of Pandan leaf extracts	86
4.2.5.1 Supercritical carbon dioxide extraction	86
4.2.5.2 Hexane extraction	87

Chapter 5.0: Absorption of 2-acetyl-1-pyrroline during cooking of rice

mixed with Pandan leaves	89
5.1 Introduction	89
5.2 Results and Discussion	91
5.2.1 Partition coefficient of 2-AP	91
5.2.2 Excess water cooking	94
5.2.2.1 Temperature and moisture content of rice during cooking	94
5.2.2.2 Absorption of 2-AP during cooking of rice	97
5.2.2.3 Amount of 2-AP in cooking water	100

5.2.2.4 Release of 2-AP from Pandan leaves	102
5.2.3 Optimal water cooking	103
5.2.3.1 Temperature and moisture content of rice during cooking	103
5.2.3.2 Absorption of 2-AP during cooking of rice	106
5.2.3.3 Gelatinization endotherms of water-rice starch	109
5.2.3.4 Effect of different distance between Pandan leaves and rice sample on the absorption of 2-AP by rice	111
5.2.3.5 Amount of 2-AP in cooking water	112
5.2.3.6 Effect of different distance between Pandan leaves and water sample on the amount of 2-AP in water	113
5.2.3.7 Release of 2-AP from Pandan leaves	114
5.2.4 Effect of different headspace analysis on the absorption of 2-AP by rice	116
5.2.5 Effect of prolonged cooking time on the absorption of 2-AP by rice	117
Chapter 6.0: Changes of 2-AP absorption during storage of cooked rice	120
6.1 Introduction	120
6.2 Results and discussion	121
6.2.1 Effect of quantity of Pandan leaves added during cooking on the absorption of 2-AP during storage of cooked rice	122

6.2.2 Changes of temperature, moisture content and amount of 2-AP of excess water cooked rice at different storage times	125
6.2.3 Changes of temperature, moisture content and amount of 2-AP of optimal water cooked rice at different storage times	127
Chapter 7.0: Conclusions and further work	132
7.1 Introduction	132
7.2 Conclusions	133
7.2.1 Extraction of 2-AP from Pandan leaves	133
7.2.2 Absorption of 2-AP during cooking of rice mixed with Pandan leaves	134
7.2.3 Changes of 2-AP absorption during storage of cooked rice	136
7.3 Further work	137
References	141

List of Tables

Table 4.1	Total yield of hexane extraction of fresh Pandan leaves (with and without grinding) at different extraction times	76
Table 5.1	Data for aqueous solution of 2-acetyl-1-pyrroline in water/air system for 24 hours	91
Table 5.2	Amount of 2-acetyl-1-pyrroline in rice grains cooked with Pandan leaves under optimal water conditions at different cooking times.	107
Table 5.3	Amount of 2-acetyl-1-pyrroline in water during cooking of rice mixed with Pandan leaves under optimal water conditions at different cooking times.	113
Table 6.1	Amount of 2-AP during storage of rice cooked with different quantity of Pandan leaves under optimal water conditions	123
Table 6.2	Changes of temperature, moisture content and amount of 2-AP of excess water cooked rice at different storage times	125
Table 6.2	Changes of temperature, moisture content and amount of 2-AP of optimal water cooked rice at different storage times. Rice was cooked using a covered rice cooker.	128
Table 6.2	Changes of temperature, moisture content and amount of 2-AP of optimal water cooked rice at different storage times. Rice was cooked using an open rice cooker.	129

List of Figures

Figure 2.1	Scanning electron micrograph of rice starch granules with 500x magnification (left) and 1000x magnification (right)	9
Figure 2.2	Chemical structure of 2-acetyl-1-pyrroline	19
Figure 3.1	Picture of fresh Pandan leaves	37
Figure 3.2	Schematic diagram of SC-CO ₂ extraction system	41
Figure 3.3	Schematic diagram of SC-CO ₂ collector a) before modification and b) after modification	43
Figure 3.4	Experimental set up used for Soxhlet extraction	45
Figure 3.5	Picture of gas chromatography-flame ionization detector	47
Figure 3.6	Experimental set up used for excess water cooking	55
Figure 3.7	Picture of an aluminium cylinder	55
Figure 3.8	Experimental set up used for headspace analysis	60
Figure 3.9	Cooking of rice with Pandan leaves in optimal water conditions using a covered rice cooker and an open rice cooker at different cooking times	61
Figure 4.1	Scanning electron micrographs of surface structure (top row), disrupted epidermal cell (middle row) and papillae (bottom row) of fresh (a-c), oven-dried (d-f) and freeze-dried (g-i) ground Pandan leaves	70

Figure 4.2	Effect of various pre-treatments on the total yield of Pandan leaf extracts by supercritical carbon dioxide extraction	73
Figure 4.3	Effect of various pre-treatments on the total yield of Pandan extracts by hexane extraction	77
Figure 4.4	a) Mass spectrum of 2-AP peak; b) GC-FID chromatogram of Pandan leaf compounds obtained by hexane extraction	79
Figure 4.5	Effect of various pre-treatments on the 2-AP concentration in Pandan extracts by supercritical carbon dioxide extraction	81
Figure 4.6	Effect of extraction time on the 2-AP concentration of fresh hexane extracts samples (with and without grinding)	84
Figure 4.7	Effect of various pre-treatments on the 2-AP concentration in Pandan extracts by hexane extraction	85
Figure 4.8	Changes in colour of supercritical carbon dioxide extracts	87
Figure 4.9	Changes in colour of hexane extracts	88
Figure 5.1	Reciprocal of gas chromatographic peak area (A^{-1}) versus phase volume ratio (β) for 2-acetyl-1-pyrroline in water/air system at 25°C for 24 hours.	93
Figure 5.2	Effect of cooking time on the temperature and moisture content of rice grains during cooking under excess water conditions	95

Figure 5.3	Effect of cooking time on the absorption of 2-AP by rice grains cooked with Pandan leaves under excess water conditions	98
Figure 5.4	Effect of cooking time on the amount of 2-AP in water during cooking of rice mixed with Pandan leaves under excess water conditions	101
Figure 5.5	Effect of cooking time on the release of 2-AP from Pandan leaves during the cooking of rice mixed with Pandan leaves under excess water conditions	102
Figure 5.6	Effect of cooking time on the temperature and moisture content of rice grains during cooking under optimal water conditions	104
Figure 5.7	Rice cooked with fresh Pandan leaves at a) 5 minutes, b) 10 minutes, c) 15 minutes and d) 20 minutes of cooking under optimal water condition using an open electric rice cooker	105
Figure 5.8	Gelatinization thermograms of optimal water cooked rice	110
Figure 5.9	Effect of cooking time on the amount of 2-AP absorption by rice grains cooked with Pandan leaves in optimal water cooking at different collection points of rice sample	111
Figure 5.10	Effect of cooking time on the amount of 2-AP in the water during cooking of rice with Pandan leaves in optimal water conditions at different collection points of water samples	114
Figure 5.11	Effect of cooking time on the release of 2-AP from Pandan leaves during cooking of rice mixed with Pandan leaves under optimal water conditions	115

Figure 5.12	Effect of different equilibrium state of headspace (HS) analyses on the amount of 2-AP absorption by rice under excess water conditions	116
Figure 5.13	Changes of 2-AP concentration during prolonged cooking time under optimal water conditions	118
Figure 6.1	Changes in 2-AP concentration per 1 g Pandan leaves during storage of rice under optimal water conditions	124

Chapter 1

Introduction

Rice (*Oryza sativa* L.) is consumed as a staple food in half of the population in the world due to its nutrients, texture and aroma. Aromatic rice, such as Basmati rice and Jasmine rice (Khao Dawk Mali 105), is very popular in Asia and has gained wide acceptance in most countries (Laksanalamai and Ilangantileke, 1993; Hien *et al.*, 2006). Due to their superior grain qualities and distinct aroma (Sakthivel *et al.*, 2009; Sirisoontaralak and Noomhorm, 2006; Ahmed *et al.*, 2008), aromatic rice is sold at a premium price, which is four times higher than non-aromatic rice (Lopez, 2008). Since aromatic rice is in very high demand and commands a high price in the world market, it is subjected to heavy adulteration with non-aromatic rice in order to increase its value added thereby enabling it to be sold at an economic price without ignoring its quality.

The aroma of cooked rice is contributed by a mixture of several compounds (Widjaja *et al.*, 1996) in which 2-acetyl-1-pyrroline (2-AP) has been reported as the major compound that is significantly responsible for the characteristic aroma of cooked rice (Buttery *et al.*, 1983). However, this highly volatile compound is unstable and easy to lose during the cooking and storage process. Therefore, it is very challenging and impossible to keep cooked rice for long periods of time if the quality and physicochemical properties are to be maintained, especially its aroma (Sirisoontaralak and Noomhorm, 2006).

Besides being found in rice, 2-AP has been found in other raw plant materials, such as Pandan leaf (*Pandanus amaryllifolius* Roxb.) and Bread flowers (*Vallaris glabra* Ktze) (Buttery *et al.*, 1983; Wongpornchai *et al.*, 2003). Pandan leaf has been reported as one of the best natural sources of 2-AP (Laohakunjit and Kerdchoechuen, 2007). However, this unique leaf is not very well known. In South-East Asian countries, the usage of fresh Pandan leaf is an old practice when cooking non-aromatic rice in order to impart an aroma resembling that of cooked rice. For instance, in Malaysia and Indonesia, rice is cooked with coconut milk and Pandan leaf to make *nasi lemak* and *nasi kuning*. These two kinds of popular dishes are delicate even when eaten alone.

The lack of scientific information has been acknowledged concerning how the phenomena take place during cooking and the mechanism corresponding to the absorption of 2-AP by

rice grains. One successful attempt was achieved by Laohakunjit and Kerdchoechuen (2007), using 30% sorbitol-plasticized rice starch film containing 25% natural Pandan extract in order to increase the 2-AP of non-aromatic brown rice. However, this film coating could not prevent the loss of 2-AP during the storage of raw rice grains. In addition, Pandan leaves have also been used as flavouring and a colouring agent for various foods, such as bakery products, sweets, ice cream, yogurt, drinks and coconut jam (Loh *et al.*, 2005).

Several extraction methods of extracting 2-AP from Pandan leaves have been extensively used including Likens-Nikerson steam distillation-solvent extraction (Laksanalamai and Ilangantileke, 1993), solvent extraction (Laohakunjit and Noomhorm, 2004), or supercritical carbon dioxide (SC-CO₂) extraction (Laohakunjit and Noomhorm, 2004; Bhattacharjee *et al.*, 2005). Recently, the SC-CO₂ extraction method became popular to extract natural products due to its advantages, in as much as it uses CO₂, which is an inexpensive and highly pure substance, that is easy to remove from the extracts, and is non-flammable and non-harmful to humans (Laohakunjit and Noomhorm, 2004; Laohakunjit and Kerdchoechuen, 2007).

Studies on cooking and storage of cooked rice have also been previously reported (Hien *et al.*, 2006; Das *et al.*, 2006; Zhang and Sun, 2006; Leelayuthsoontorn and Thipayarat, 2006; Zheng *et al.*, 2009). Generally, the selection of cooking methods depends on the rice and

water ratio. For example, according to Chakkaravarthi *et al.* (2008) excess water cooking is a method of cooking in which the rice to water ratio is quite high (1:10-1:20) while optimal water cooking is a cooking of rice in a particular rice-water ratio (1:1.5-1:2.5). Consequently, different methods of cooking influence the texture, physical appearance and even the aroma of cooked rice (Leelayuthsoontorn and Thipayarat, 2006; Srikaeo *et al.*, 2006; Porrarud and Pranee, 2010). The cooking of rice is essentially the reaction of starch with elevated temperature (Kasai *et al.*, 2007), which normally results in changes to the microstructure, which contributes to the loss of the volatile compounds (Buttery *et al.*, 1983) during the cooking process. Thus, attention could be paid to the role of complex phenomena such as gelatinization and retrogradation, which promotes the interaction, particularly between the rice starch and the aroma compound, and enhances the aroma during cooking or the storage of cooked rice.

The aim of this research is to extract the 2-AP from Pandan leaves and to study the changes of 2-AP absorption during the cooking and storage of rice with Pandan leaves. The specific objectives of this research are as mentioned below:

- To extract the 2-AP from Pandan leaves by supercritical carbon dioxide and solvent (hexane) extraction.
- To investigate the effects of different particle sizes and drying pre-treatments on the total yield and 2-AP concentration of Pandan leaf extracts.

- To investigate the mechanism that occurs during the cooking of rice mixed with Pandan leaves under excess and optimal water conditions.
- To investigate the effects of moisture content and temperature on the changes of 2-AP absorption during the cooking of rice.
- To investigate the effects of moisture content and temperature on the changes of 2-AP absorption during the storage of cooked rice.

Publications and conferences

Publications

- Yahya, F., Lu, T., Santos, R. C. D., Fryer, P. J. and Bakalis, S. (2010). Supercritical carbon dioxide and solvent extraction of 2-acetyl-1-pyrroline from Pandan leaf: The effect of pre-treatment. *Journal of Supercritical Fluids*.55:200-207.
- Yahya, F., Fryer, P. J. and Bakalis, S. (2011). The absorption of 2-acetyl-1-pyrroline during cooking of rice (*Oryza sativa* L.) with Pandan (*Pandanus amaryllifolius* Roxb.) leaves. *Procedia Food Science*.1:722-728.

Conferences

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- Yahya, F., Fryer, P. J. and Bakalis, S. The absorption of 2-acetyl-1-pyrroline during cooking of rice (*Oryza sativa* L.) with Pandan (*Pandanus amaryllifolius* Roxb.) leaves. *11th International Congress in Engineering and Food*. Athens, Greece. 22-26 May 2011. Oral presentation.
- Yahya, F., Fryer, P. J. and Bakalis, S. The absorption of 2-acetyl-1-pyrroline during cooking of rice (*Oryza sativa* L.) with Pandan (*Pandanus amaryllifolius* Roxb.) leaves. *IFT 2011*. New Orleans, USA. 11-14 Jun 2011. Poster presentation.

Chapter 2

Literature review

2.1 Rice

Rice (*Oryza sativa* L.) can be categorized into aromatic/scented/fragrant rice and non-aromatic rice. Aromatic rice is a special rice, which is sold at a premium price in both the local and export market due to the superior grain quality, pleasant and distinct aroma (Tulyathan *et al.*, 2008). An example of aromatic rice is Basmati rice from India and Pakistan, Khoa Dawk Mali 105 or known as KDML-105 or Jasmine rice, which is a cultivar in Thailand and Hieru or Sawakaori rice from Japan (Itani *et al.*, 2004).

Aromatic rice possesses a characteristic odour that distinguishes it from ordinary rice. This odour results from a volatile component released from the rice (Wongpornchai *et al.*, 2004) in which the strength of the aroma of aromatic rice varies with the genetic and environmental conditions (Itani *et al.*, 2004).

As a source of carbohydrate, more than half the population in the world consume rice as a staple food. In addition to starch, rice also comprises protein and fat. According to Kasai *et al.* (2005) inner rice contains less protein and fat compared to whole grain.

2.1.1 Rice starch

Starch is the most common carbohydrate polymer in food and it occurs as water-soluble granules in a semi-crystalline structure (Ratnayake and Jackson, 2007; Liu *et al.*, 2002). The composition, morphology, thermal, rheological and retrogradation process of starch differs according to the plant source (Singh *et al.*, 2003).

Rice grain contains up to 90% of its dry weight as starch (Iturriaga *et al.*, 2004). Rice starch forms in pentagonal and angular shapes with a less smooth surface, as shown in Figure 2.1. Rice starch is the smallest starch in size (<20 μ m) compared to corn (<25 μ m), wheat (<30 μ m) and potato (<110 μ m) starch. In addition, Tari *et al.* (2003) also reported that the size of rice starch was found to be 3-10 μ m using Scanning Electron Microscopy (SEM). Rice starch develops in compact spherical bundles or clusters, also known as compound granules and is located in the endosperm cells.

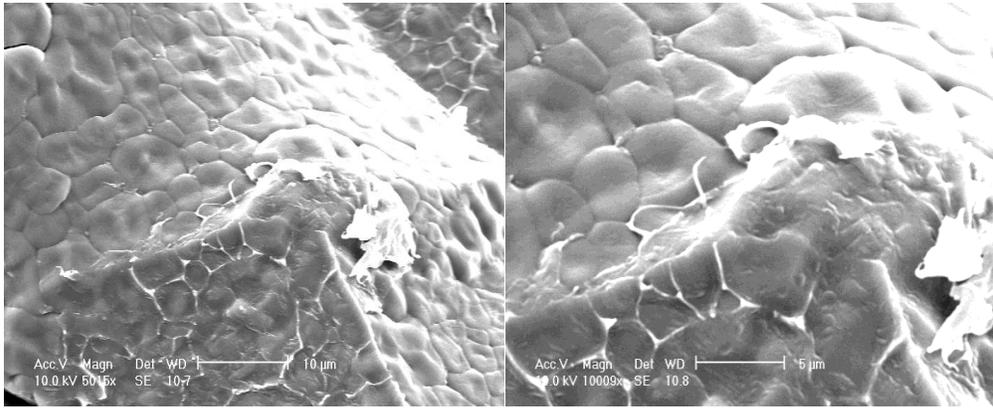


Figure 2.1: Scanning electron micrograph of rice starch granules with 500x magnification (left) and 1000x magnification (right).

It is well known that natural starches are mixtures of 10-20% of linear amylose and 80-90% of highly branched amylopectin (Itthisoponkul *et al.*, 2007; Miao *et al.*, 2010; Singh *et al.*, 2003). The ratio between amylose and amylopectin affects the physical properties of starch (Fredriksson *et al.*, 1998) and it may vary over a very wide range, depending on their botanical origin (Chen *et al.*, 2011). For instance, the high content of amylose in long grain rice makes the rice less sticky when compared to short grain rice, which contains more amylopectin (Kasai *et al.*, 2007). In addition, according to Singh *et al.* (2005), rice cultivars with high amylose content were observed to have a hard texture and require less cooking time.

Different types of starch contain different amounts of amylose. For example, the waxy starches contain less than 15% of amylose, normal starch has 20-35% and high amylose starch has more than 40% of amylose (Tester *et al.*, 2004). In hot pastes, soluble amylose

will affect the viscosity of the aqueous phase by leaching out from the granules while during cooling, the amylose tends to be associated with hydrogen bonding leading to the phenomena of retrogradation (Sirisoontaralak and Noomhorm, 2006).

2.1.1.1 Starch gelatinization

Starch gelatinization is one of the essential pre-requisites for understanding the cooking process of whole grains (Turhan and Gunasekaran, 2002; Liu *et al.*, 2002). Different types of starches have different gelatinization conditions. Generally, gelatinization happens when the starch granules are heated in the presence of water, through which the temperature rises to a critical value, and the birefringence of starch granules are lost (Li *et al.*, 2004). The critical value is referred to as the onset temperature of starch gelatinization, which is approximately 62°C (Watanabe *et al.*, 2001; Li *et al.*, 2004).

Gelatinization starts to occur through the absorption of water by starch granules and then facilitates an increase of starch granule mobility in the amorphous region, which is weak in hydrogen bonding (Liu *et al.*, 2002; Singh *et al.*, 2003). The swelling of the amorphous phase up to several times the original granules size may contribute to the disruption of the crystalline regions (Sagum and Arcot, 2000). Accelerated granular swelling reduces the energy requirement to disrupt the starch structure because gelatinization is a swelling-driven process (Ratnayake and Jackson, 2007). The increase in swelling also promotes the

leaching of amylose from the granules. The concentration of amylose in the remaining free water increases, thereby sharply increasing the viscosity of amylose solution (Mariotti *et al.*, 2009).

The gelatinization of starch is highly dependent on the type of starch, water content and the processing conditions used, such as pressure levels, temperature range and treatment time (Tan *et al.*, 2009). In the case of grains, the gelatinization takes place in parts of the whole grain where the water content is sufficiently high (Turhan and Gunasekaran, 2002). However, when the starch granules are subjected to different thermal conditions, it may result in the differing stages of partial and complete gelatinization of the starch granules of the grain (Miao *et al.*, 2010). Starch may also gelatinize even in cold water through the presence of alkali, urea, dimethyl sulphoxide or other reagents that can disrupt hydrogen bonds (Singh *et al.*, 2003). In addition, gelatinization occurs at high temperature when heating at atmospheric pressure. However, if the pressure is high, gelatinization can take place at room temperature.

2.1.1.2 Starch retrogradation

Retrogradation of starch is a term used for the changes that occur in gelatinized starch from an initially amorphous state to a more ordered and crystalline state (Gudmundsson, 1994; Chung *et al.*, 2006). This change may be promoted by decreasing the mobility of starch

chains (Banchathanakij and Suphantarika, 2009). The retrogradation process generally occurs during the storage and cooling of gelatinized starch molecules (Matalanis *et al.*, 2009), particularly amylose and amylopectin. According to Yu *et al.* (2009), rice starch begins to retrograde at a temperature of 80-95°C during the cooling process. Rice with high amylose is traditionally linked to a greater tendency for retrogradation (Singh *et al.*, 2003), as amylose retrogradation has been found to have a faster rate when compared to the amylopectin retrogradation (Matalanis *et al.*, 2009; Gudmundsson, 1994). Studies by Biliaderis (2009) and Yu *et al.* (2009) have also shown that amylose may retrograde within less than one day of ageing.

Molecular interaction happens involving the hydrogen bonding between starch chains; as a result it forms amylose double helices (Liu *et al.*, 2002; Singh *et al.*, 2003). Starch retrogradation usually has a transition temperature of 10-26°C lower and 60-80% smaller enthalpies than those for the gelatinization of starch granules (Singh *et al.*, 2003).

From the rheological point of view, the retrogradation process has a negative effect on the rice starch as it promotes an increase in the hardness of rice due to loss of the water holding capacity (Gudmundsson, 1994). The staling of bread and hardening of rice cakes also result from the retrogradation of starch (Chung *et al.*, 2006). However, retrogradation has a beneficial effect on the flavour retention of food based starch products (Banchathanakij and Suphantarika, 2009).

2.1.1.3 Differential scanning calorimetry

Besides microscopic analysis and viscosity-based measurement, differential scanning calorimetry (DSC) is currently used as a standard analytical tool to assess thermal analysis including the gelatinization temperature and heat of gelatinization. In addition, the retrogradation behaviour of starch has been extensively studied by previous researchers (Li *et al.*, 2004; Mariotti *et al.*, 2009; Chung *et al.*, 2006; Singh *et al.*, 2003). The degree of gelatinization is measured directly from gelatinization enthalpy (Li *et al.*, 2004).

In the DSC analysis, the scanning rate mainly affected the temperature and the shape of the endothermic peak. In general, the higher the heating rate, the higher the peak temperature and the narrower the endothermic DSC peak (Chung *et al.*, 2006). The DSC curves also commonly contain multiple endotherms under low water conditions. For instance, two endotherms were observed at 63°C and 71°C in the melting thermograms of complexes between potato starch-aroma compounds (Jouquand *et al.*, 2006).

Theoretically, the first heating of DSC scan refers to the formation of complexes during sample preparation while endothermic events during rescan indicate the reorganization of complexes. Moreover, complex reformation can be investigated by the exothermic event that appears during the cooling scan (Itthisoponkul *et al.*, 2007).

2.1.2 Cooking of rice

The cooking of rice mainly depends on the culture and traditional practice of each people around the world. For example, Americans prefer a semi-milled long grain rice or brown rice while Italians consume Bbaldo or Arborio rice, which has high amylopectin and short grain. The Japanese like sticky rice, Indians prefer well-milled white rice and Asians love to consume spicy and scented Basmati rice or Jasmine rice (Mohapatra and Bal, 2006).

The major purpose of cooking is to convert the raw rice grain into a palatable, digestible, and workable form through the gelatinization of starch (Turhan and Gunasekaran, 2002). Two different mechanisms occur, which are related to the cooking process—the diffusion of water from the surface to the core of the rice grains and the chemical and physical changes of the component in the rice (Dias *et al.*, 2010).

With the presence of heat treatment and enough water, rice granules absorb moisture, which promotes swelling to a size considerably larger than the original size (Bakalis *et al.*, 2009). As a result, the hydrogen bonds between the rice starch molecules are replaced by bonds between the starch and water molecules (Kasai *et al.*, 2005). The granules expansion causes rupture, and, hence, amylose leaching out from the granules. The amount of starch leaching and the fine structure of amylose and amylopectin at this particular time are

related to the texture of cooked rice (Yadav and Jindal, 2007). The mechanism that occurs is due to gelatinization of the rice starch.

The cooking of rice is associated with complete gelatinization of the starch, which implies significant changes in the physical, chemical and nutritional characteristics of starch as well as water and heat diffusion, swelling, rheological behaviour, viscosity, loss of crystalline order and deformation of original starch products by heat treatment in the presence of water (Das *et al.*, 2006).

The temperature at which rice starch begins and finishes gelatinization is important to measure as it is related to the cooking time. As well as cooking temperature, the moisture absorption is an important parameter for predicting optimum cooking conditions (Dias *et al.*, 2010). Some solid content of rice is dissolved into the cooking gruel. Moisture absorption by the rice kernel as well as solid loss during cooking vary among the different varieties of rice and are usually characterized by their physicochemical properties, such as amylose content, gel consistency, alkali spreading value, gelatinization temperature and protein content (Yadav and Jindal, 2007).

Besides changes in the physicochemical properties, flavour compounds are also continually formed and broken down through complex chemical reactions as a function of temperature,

moisture and pressure (Grimm *et al.*, 2001). Therefore slight changes in sample temperature, heating time or variation of moisture can and do affect the aroma of cooked rice (Buttery *et al.*, 1983; Grimm, *et al.*, 2001). According to Zeng *et al.* (2009), the volatile component during the early stage of cooking refers to uncooked rice while the volatile release promoted by steam evaporation during the last stage of cooking is representative of the volatiles of cooked rice.

2.1.2.1 Excess water cooking

In many western cultures, rice is boiled in an excessive amount of water until the centre of the grain is fully cooked (Leelayuthsoontorn and Thipayarat, 2006) and then the remaining water is discarded (Mihucz *et al.*, 2007). Generally, excess water cooking refers to a ratio of rice to water of 1:9 (w/v) (Dias *et al.*, 2010).

The water uptake of milled rice increases with an increasing rate in the beginning followed by a diminishing rate during cooking in excess water (Yadav and Jindal, 2007). In excess water, the interface of the gelatinized and ungelatinized region would then progress to the core, which is sustained by water diffusion through the gelatinized external layers. Starch gelatinization and water diffusion are active simultaneously within the sample (Riva *et al.*, 1994).

2.1.2.2 Optimal water cooking

In optimal cooking conditions, rice is generally cooked in an ample amount of water with a ratio of rice to water of 1:1 to 1:2 (Grimm *et al.*, 2001). This ratio is also used as normal domestic cooking generally, as per the recommended guidelines of the electric rice cooker manufacturer (Das *et al.*, 2006). At the end of cooking, all water is absorbed by the rice grains (Mihucz *et al.*, 2007).

2.1.3 Storage of cooked rice

The storage of cooked rice was performed in order to obtain the desired quality of rice, as rice is not consumed immediately after cooking is completed. The storage of rice generally includes a cooling process. However, during storage, changes of the physicochemical properties and flavour profile of cooked rice are expected to occur.

The flavour compound in cooked rice may be lost during the storage of rice with the rate of deterioration being dependant on the temperature and oxygen (Sirisoontaralak and Noomhorn, 2006). In addition, Laksanalamai and Ilangantileke (1993) also reported that the stability of the aromatic compound seems to decrease with the duration of storage of cooked rice.

2.1.4 Flavour of rice

Flavour is mainly related to the release in the gas phase of volatile aroma compounds present in the food (Martuscelli *et al.*, 2008). Compounds that elicit an aroma are volatile (Grimm *et al.*, 2001). Volatile compounds found in cooked food frequently occur in the raw foods although usually at a much lower concentration (Buttery *et al.*, 1983).

Rice is enjoyed by many people because of its flavour and texture (Bryant and McClung, 2011). From the several hundred compounds that have been observed in the headspace of cooked rice (Grimm *et al.*, 2001), only a few compounds have been found to make a significant contribution to the rice flavour. According to Widjaja *et al.* (1996), there is no single compound that could be judged as being responsible for the aroma of cooked rice. However, a volatile compound of 2-acetyl-1-pyrroline (2-AP) (Figure 2.2) plays the greatest role in the sensory quality of rice and measurement of its concentration can be used to differentiate between scented and non-scented rice (Buttery *et al.*, 1983). Although non-aromatic rice also accumulates 2-AP, its concentration is much lower than that found in aromatic rice (Fitzgerald *et al.*, 2009).

The level of 2-AP is controlled by a recessive gene encoding betaine aldehyde dehydrogenase 2 (BAD2), as reported by Lopez (2008), by using the polymerase chain reaction (PCR) method. The quantity of 2-AP in rice also depends on post-harvest treatment and storage duration (Widjaja *et al.*, 1996).

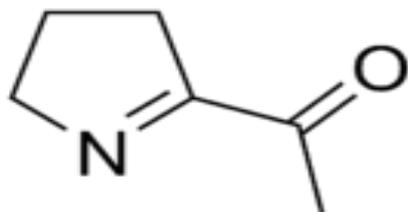


Figure 2.2: Chemical structure of 2-acetyl-1-pyrroline

As well as 2-AP, 2-acetyl-pyrrole, α -pyrrolidone and pyridine, (E,E)-deca-2,4-dienal, (E,Z)-deca-2,4-dienal, 4-vinylphenol, 2-aminoacetophenone were reported to be major contributors of scented rice aroma, which may enhance the acceptability of rice by the consumer (Sriseadka *et al.*, 2006; Maraval *et al.*, 2010). However, lipid oxidation products, such as hexanal, acetic acid, and pentanoic acid have been identified to have a negative effect on acceptability (Bryant and McClung, 2011).

2.1.5 Starch-flavour interaction

Interaction between aroma compounds and carbohydrates generally have a weak energy and mainly depend on the nature and concentration of aroma compound and carbohydrates (Seuvre *et al.*, 2007). Starch-flavour complexes can control retention and release of flavour compounds in which the complex formation might naturally occur in the plant or during its

processing to food (Wulff *et al.*, 2005). The interaction parameters are generally measured in the starch solution using the static headspace method.

2.1.5.1 Polar interaction

Polar interaction involves hydrogen bonds between hydroxyl groups of starch and aroma compounds. In the polar stationary phase, starch is capable of forming hydrogen bonds with aroma compounds. The aroma compound is less well retained by granular starch. Starch, which has lost its granular structure, gives higher specific retention volumes for aroma compound (Boutboul *et al.*, 2002). This interaction makes a significant contribution to the retention of hydrophilic compounds in the starch gel (Boland *et al.*, 2004).

2.1.5.2 Inclusion complexes of amylose-flavour

Formation of inclusion complexes leads to a flavour stabilization in food matrix. This interaction may promote the retention of aroma compound in the food matrix as well as decrease the diffusion of aroma compound with an increase in the viscosity (Jouquand *et al.*, 2006; Seuvre *et al.*, 2007).

The linear amylose fraction has the ability to form complexes or inclusion compounds with a variety of ligands including iodine, lipids, and aroma compound; alcohol, aldehydes, terpenes and lactones (Heinemann *et al.*, 2003; Tietz *et al.*, 2008; Nuessli *et al.*, 1995).

Generally, the hydrophobic parts of ligands with a linear structure may localize in the hydrophobic helical cavity of amylose while the cyclic structure of ligands can be placed between the helices (Itthisoponkul *et al.*, 2007; Wulff *et al.*, 2005; Heinemann *et al.*, 2003) correlating to the formation of inclusion complexes. This complex, known as V-type X-ray pattern, is one of the mechanisms of flavour retention in food containing starch (Nuessli *et al.*, 1995). The inclusion complex increases the stability of the helical structure of starch macromolecules, which enhances the absorption ability of starch to food flavours (Tolstoguzow, 2003).

The amylose complexes exist in two polymorphic forms, type I and type II. At low temperature ($\leq 60^{\circ}\text{C}$), the nucleation rate is observed to be very high where the amylose helix freezes rapidly with no crystallites existing (Gelders *et al.*, 2004). This type I polymorphic form melts at $< 100^{\circ}\text{C}$, which is about $10\text{-}30^{\circ}$ below that of type II. Type I is an amorphous state while type II has a crystalline structure (Itthisoponkul *et al.*, 2007). Type II has higher complexes temperature, ($\leq 90^{\circ}\text{C}$) which yield semi-crystalline order (Gelders *et al.*, 2004).

Complex inclusion between amylose and flavour may cause the amylose to minimize its interaction with water (Putseys *et al.*, 2010). The complexes are remarkably stable at room temperature with water activities below $a_w=0.5$ (Wulff *et al.*, 2005). Therefore, the release

of the complex can be manipulated by temperature, water activity or even by enzymatic action (Itthisoponkul *et al.*, 2007; Putseys *et al.*, 2010).

2.2 Headspace analysis

Headspace analysis is useful for quantifying the volatile component from complex matrices, such as food (Boutboul *et al.*, 2002), and it is one of the approaches of gas extraction in which sample headspace volatiles are brought directly to gas chromatography (Sriseadka *et al.*, 2006). Headspace analysis can be categorized to static and dynamic analysis. In static headspace analysis, the gas sample is sampled in thermodynamic equilibrium and the system closed (Cayot *et al.*, 2008). By heating, the sample would shift the concentration equilibrium towards the gas phase (Grimm *et al.*, 2001). While in the dynamic headspace, the equilibrium will not be reached, and the analysis will be carried out in an open system (Cayot *et al.*, 2008).

2.3 Gas chromatography

Gas chromatography (GC) is a chemical instrument used for separating and analysing compounds that can be vaporized from the matrix or other compounds in a sample mixture (McMahon, 2007). Two phases are involved in common GC, namely, the mobile phase and the stationary phase. The mobile phase is a carrier gas and normally an inert gas, such as helium, while the stationary phase is a microscopic layer of liquid. Inside the GC, there is a piece of glass or metal tubing called a column.

The gaseous compound being analysed interacts with the wall of the column, which is coated with different stationary phases. Therefore, each compound elutes at a different time, known as the retention time of a compound. The retention time of a compound will depend on the type and length of column, flow rate of mobile phase and temperature used during the GC analysis as well as the physicochemical properties of the compound.

A number of detectors are used in GC, such as thermal conductivity detector (TCD), mass spectrometric detector, nitrogen phosphorus detector (NPD) and flame ionization detector (FID). However, FID is the most common detector used with GC, particularly for essential oil, solvent and hydrocarbon analysis. This type of detector was originally used with hydrogen or a mixture of hydrogen and nitrogen as a carrier gas (Scott, 1998).

Besides being used for separating the different components of a mixture, GC may help in identifying a compound, and, normally for this purpose, the GC instrument is coupled with a mass spectrometer. The operation of the mass spectrometer involves creating ions in the gaseous phase, separating the ions in space or time based on their mass-to-charge-ratio (m/z) and measuring the quantity of ions of each mass-to-charge-ratio (McMahon, 2007). The identification analysis involves elucidation of the chemical and structural information of molecules through their molecular weight and distinctive fragmentation patterns (McMahon, 2007).

Basically, a mass spectrometer consists of a sample introduction system, an ion source, a mass-selective analyser, an ion detector and a computer. MS could provide reliable data for gas or volatile, liquid or even solid samples, which depends on the ionization and desorption technique used for each instrument. The electron impact (EI) is extensively used as the ion source in the ionization technique of MS. The result of the mass spectrum obtained gives excellent information concerning the structure and spectrum of a molecule, and can be compared to spectral libraries for definitive identification.

2.4 Partition coefficient

Flavour is mainly related to the release in the gas phase of volatile aroma compounds present in the food where kinetic and thermodynamic phenomena affect the partition of aroma compounds from a food to the gas phase. The knowledge of the way aroma compounds are distributed between the food matrix and the gas phase provides an understanding of the perception by consumers in the food system (Martuscelli *et al.*, 2008).

Partitioning is highly dependent on the polarity and solubility of the aroma compound in the phases (Tehrany *et al.*, 2007). The solubility of a volatile compound in liquid is normally expressed by the partition coefficient ($K_{w/a}$) and calculated by the ratio of the concentration of a compound in the liquid (w) to that in the gas (a) phase at equilibrium (Sato and Nakajima, 1979). Hydrophilic molecules typically have a high water/air partition

coefficient (Shojaei *et al.*, 2007). However, most flavour compounds partition more into oil than water (Pollien and Roberts, 1999).

The most accurate methods for partition coefficient measurement seem to be based on the equilibrium technique, such as equilibrium partitioning in closed systems (EPICS) and the vapour phase calibration (VPC) method. However these methods have many limitations, such as requirement for external calibration and exact knowledge of the initial concentration of the volatile compound in the solution (Atlan *et al.*, 2006). Therefore, the latest static headspace method known as phase ratio variation (PRV) method was used. The PRV method is based on the relationship between the reciprocal of the peak area and the phase ratio in the headspace vial containing the sample solution without the need of calibration and concentration measurement in both phases (Bakierowska and Trzeczynski, 2003; Juoquand *et al.*, 2004).

2.5 2-acetyl-1-pyrroline

2-acetyl-1-pyrroline (2-AP) is a volatile compound that is highly correlated with desirable aroma terms, such as *pandan-like* for the Orientals or *popcorn-like* for the non-Orientals. It can be found in a large variety of cereals, and vegetable-derived and animal-derived products (Adams and De Kimpe, 2006; Tulyathan *et al.*, 2008; Sirisoontaralak and Noomhorm, 2006). 2-AP is formed naturally in plants (Wongpornchai *et al.*, 2004) and becomes a major compound that significantly contributes to the flavour characteristic of

Pandan (*Pandanus amaryllifolius* Roxb.) leaves, bread flowers (*Vallaris glabra* Ktze) and aromatic rice (*Oryza sativa* L.) varieties, such as Jasmine rice and Basmati rice (Buttery *et al.*, 1988; Laohakunjit and Kendchoechuen, 2006; Wongpornchai *et al.*, 2004; Arikrit *et al.*, 2011).

The concentration of 2-AP in the plant matrix is influenced by genetic and environmental factors (Hien *et al.*, 2006.). It has been reported by Buttery *et al.* (1983) that the quantity of 2-AP present in Pandan leaves (of the order of 1 ppm) is more than ten times that found in aromatic milled rice like Basmati rice and 100 times more than that found in non aromatic rice. A concentration of 0.53 ppm was obtained from bread flower leaves by Wongpornchai *et al.* (2004). However, it is important to note that Pandan leaves are among the best natural sources of 2-AP (Laohakunjit and Kendchoechuen, 2006).

Besides occurring naturally in plants, the 2-AP compound can be generated from a reaction between amino acids and sugar on the surface of foods, such as rice and bread at high temperature (Fuganti *et al.*, 2007; Wongpornchai *et al.*, 2004). This reaction is known as non-enzymatic browning or Maillard reaction (Adams and De Kimpe, 2007; Yu *et al.*, 2009). Many researchers have attempted to synthesis the 2-AP compound by Maillard reaction. However, this procedure has drawbacks, such as high cost of reagent, length of reaction sequence, requirement of acid and base for final stages and being harmful for consumption (Fuganti *et al.*, 2007).

Microbial production of 2-AP by *Bacillus cereus* ATCC 27522 has been reported previously (Adams and De Kimpe, 2007). According to the authors, the production of 2-AP was proceeded via enzymatic acetylation of 1-pyrroline, a metabolic degradation product of the amino acids ornithine and proline. However, the specific *B. cereus* strains grown on plate count agar produced low and mostly irreproducible amounts of 2-AP (Adams and De Kimpe, 2007).

In addition, the synthetic formation of 2-AP by hydrogenation of 2-acetylpyrrole with rhodium on alumina, followed by oxidation of the resulting amino alcohol by means of an excess of silver carbonate, and absorbed on Celite in benzene has also been published by Buttery *et al.* (1983). However, Buttery and co-workers found that the procedure requires very expensive reagents (rhodium and silver carbonate) and toxic chemicals like benzene as well as the inaccessibility of 2-AP on a larger scale. Adams and De Kimpe (2006) also attempted a more straightforward procedure for the synthetic formation of 2-AP. However, it still has several complicated steps to follow in order to synthesis the 2-AP compound.

2-AP with IUPAC name 1-(3, 4-dihydro-2H-pyrrol-5-yl) ethanone is a substituted pyrroline and a cyclic imine. The pure standard of 2-AP is pale yellow in colour (Bhattacharjee *et al.*, 2005). This nitrogen-containing heterocyclic compound is generally low in concentration (Jianming, 2002). Furthermore, the 2-AP compound has been identified as a highly volatile

(vapour pressure of 25°C) hydrophilic compound (Fitzgerald *et al.*, 2009; Hien *et al.*, 2006; Maraval *et al.*, 2010).

It is interesting to note that 2-AP has an odour threshold value as low as 0.1 ppb in water (Buttery *et al.*, 1988) and has high solubility in water and alcohol with a log $K_{(o/w)}$ value of -1.27. Therefore, 2-AP can be considered as a hydrophilic compound because its log K (where the K is the partition coefficient of aroma compound between octanol and water) is less than 2, which is similar to ethyl acetate, ethyl butyrate, 2-hexanone and cis-3-hexenol (Fabra *et al.*, 2009). This compound is very pungent even at low concentrations and may mask other flavours.

Similar to other polar compounds, 2-AP presented more affinity for water than the oil phase (Seuvre *et al.*, 2007) and it is readily transferred to a gas phase, when subjected to an elevated temperature due to its low molecular weight (111.14 gmol^{-1}) (Sriseadka *et al.*, 2006). This compound has also been detected as a thermally labile compound and it can be very unstable under alkaline or acidic conditions as well as at ambient temperature conditions (Laohakunjit and Noomhorm, 2004; Widjaja *et al.*, 1996). Therefore, depletion of 2-AP in food products is probably due to its volatility (Tulyanthan *et al.*, 2008).

2.6 Pandan leaf

Pandan (*Pandanus amaryllifolius* Roxb.) is a tropical plant of the family Pandanaceae in the screw pine genus. Pandan leaf, often known as screw pine, because they resemble the Ananas plant (pineapple) with the spiral arrangement of long, narrow and strap-shaped green leaves (Wongpornchai, 2006). Even though the Pandanaceae family comprises approximately 600 species, there are only *Pandanus amaryllifolius* Roxb. and *Pandanus odoratissimus* Linn that have fragrant leaves and flowers, respectively (Wakte *et al.*, 2007).

The extracts of Pandan leaf consist of a number of volatile compounds in a group of alcohols, aromatics, carboxylic acids, ketones, aldehydes, esters, hydrocarbons, furans, furanones and terpenoids. 2-AP was found as the compound mainly responsible for the scent of Pandan leaves. Besides 2-AP, other volatile compounds found in Pandan extracts include ethyl formate, 3-hexanol, 4-methylpentanol, 3-hexanone, 2-hexanone, trans-2-heptenal, β -damascenone, 4-ethylguaiacol and 3-methyl-2-(5H)-furanone (Wongpornchai, 2006).

The storage site of 2-AP has been detected in the papillae, which are located in the abaxial surface of the lower epidermis cells of Pandan leaves (Wakte *et al.*, 2007; Wakte *et al.*, 2010). These papillae were found to be distributed parallel to the leaf length and make the

lower epidermal surface velvety compared to the upper epidermal surface of Pandan leaves (Wakte *et al.*, 2007).

Fresh Pandan leaves contain a higher amount of 2-AP than those at the dried stage, although they hardly smell. Mature Pandan leaves contain a higher concentration of 2-AP compared to young leaves (Wakte *et al.*, 2010) as young leaves might take some time to attain the 2-AP levels of mature leaves. 2-AP dissolves in the fresh leaf tissue where the percentage of water is relatively high. When the leaves are withering and the water content is reduced, the compound is then forced to partition into the gas phase, resulting in the pleasant smell continually released from the withering leaves (Wongpornchai, 2006).

The sweet and delightful flavour of Pandan leaves, which is well-known as a source of natural flavouring, is widely used in various parts of South-East Asian countries including India, Thailand, Indonesia and Malaysia. For example, Pandan leaves are commonly used when preparing rice dishes as a means of enhancing flavour. In addition, Pandan leaves are also used in making other food, such as desserts, sweets, coconut jam and ice cream. Due to the high chlorophyll content, Pandan leaves are also a popular green colourant for food (Loh *et al.*, 2005).

2.7 Extraction process

Extraction techniques have been widely investigated to obtain valuable natural compounds from plants (Wang and Weller, 2006) including steam distillation, solvent, Liken-Nikerson and supercritical carbon dioxide extraction. Differences in the quantity of analyte found in different extractions were mainly due to the differences in the solubility and polarity of the analyte in the extraction media (Laohakunjit and Noomhorm, 2004).

2.7.1 Supercritical carbon dioxide extraction

The supercritical state is achieved when the temperature and the pressure of the substance are raised over its critical value (Wang and Weller, 2006). For example, the critical state of carbon dioxide fluid is at a temperature of 31.1°C and pressure of 7.38 MPa (Wang and Weller, 2006). This critical value makes CO₂ an ideal solvent for thermally labile compounds that are capable of degradation (Lang and Wai, 2001; Araujo and Sandi, 2006; Dokeret *et al.*, 2010; Balachandran *et al.*, 2006). Supercritical fluids also possess liquid-like solvating power as well as gas-like transport and these phases combination make them suitable for an extraction from the matrices where diffusion can be controlled, such as in plant tissues (Nik Norulaini *et al.*, 2008).

During SC-CO₂ extraction, four stages of process including desorption of the compound from the matrix, subsequent diffusion into the matrix, solubilisation of the analyte by supercritical fluid and sweeping out of the extraction cell by fluid occur (Pourmortazavi and Hajimirsadeghi, 2007). Therefore, the SC-CO₂ method mainly depends on the extraction step including the nature of supercritical fluids, raw material-solvent ratio, and method of solvent feeding as well as the choice of extraction parameter, such as pressure, temperature, flow rate and time. The condition of the raw material matrix, for which pre-treatment is recommended, such as particle size and moisture content, must also be taken into consideration (Pourmortazavi and Hajimirsadeghi, 2007; Adasoglu *et al.*, 1994).

The usage of SC- CO₂ for isolation of active compounds has many advantages compared to organic solvents, especially in the food, pharmaceutical and cosmetic industries (Zizovic *et al.*, 2007). Besides being suitable for heat sensitive compounds, CO₂ is easily removed from the extract when the pressure and temperature are reduced below its critical condition (Wei *et al.*, 2009), which makes it advantageous over the conventional solvent extraction (Ozkal *et al.*, 2005). It is also an inert, inexpensive, odourless, tasteless and environment-friendly solvent (Nik Norulaini *et al.*, 2008; Araujo and Sandi, 2006). Therefore, no solvent residues would be present in the extracts, as CO₂ has low latent heat of evaporation and high volatility of the solvent.

2.7.2 Solvent extraction

Solvent extraction using Soxhlet is a generally well-established conventional technique (Wang and Weller, 2006). Organic solvent extraction with hexane was commonly used for extracting the extracts (Araujo and Sandi, 2006). Besides hexane, isopropanol, ethanol, hydrocarbons and water are the alternative solvents used in Soxhlet extraction (Wang and Weller, 2006). The total yield and composition of extracts are depends on the solvent used. In addition, the extraction occurs at the boiling point of the solvent used. For example, hexane has a boiling point range of approximately 63-69°C and it is an excellent oil solvent in terms of oil solubility and ease of recovery (Wang and Weller, 2006).

Organic solvent extraction has disadvantages, such as long extraction time, requires relatively large quantities of solvent and an additional concentration step (Araujo and Sandi, 2006; Sanal *et al.*, 2004; Wang and Weller, 2006). However, Soxhlet extraction is a very cheap and simple manipulation and may maintain a relatively high extraction temperature with heat from the distillation flask (Wang and Weller, 2006). Sample fresh solvent contact during the whole extraction process is also one of the advantages of the Soxhlet extraction.

2.7.3 Extraction pre-treatment

In most studies, the extraction yield increases with a decrease in the moisture content and particle size of sample. Matrix characteristics and particle size, which are related to internal diffusion, may affect the extraction process (Wang and Weller, 2006), and the change in the aroma compounds during drying depend on the drying method as well as the type of plant material (Pourmortazavi and Hajimirsadeghi, 2007). Damage to plant cell walls can result in the compound being more easily extracted at supercritical condition.

2.7.3.1 Particle size pre-treatment

Particle size is one of the most important parameters affecting the extraction (Lang and Wai, 2001). The yield of extracts was significantly influenced by the particle size of sample, such as coffee beans (Araujo and Sandi, 2006) and valerian root (Zizovic *et al.*, 2007). Decreasing the particle size of solid matrix through grinding treatment leads to a higher surface area, making extraction more efficient (Pourmortazavi and Hajimirsadeghi, 2007). In as much as large particles might prolong the extraction time due to the diffusion-controlled process (Wang and Weller, 2006). However, excessive grinding results in a form of fine powder of the sample, which is not suitable to use in the extraction process. These kinds of sample will promote difficulty in controlling the proper flow rate of extraction and results in re-adsorption of analytes onto the matrix surface and pressure drop inside the extraction chamber (Sanal *et al.*, 2004; Pourmortazavi and Hajimirsadeghi, 2007).

2.7.3.2 Drying pre-treatment

Generally, drying treatment refers to the removal of moisture from a substance (Ratti, 2001). In the extraction of natural products, water activity would be a critical factor affecting all component interactions (Sanal *et al.*, 2004). The moisture of food materials may interfere with the extraction of desired components from the sample matrix (Sanal *et al.*, 2004). Therefore, drying pre-treatment is needed in order to completely or partly remove water molecules from the matrix. The drying procedures for a plant material could affect the yield and quality of the extracts (Lang and Wai, 2001).

The high moisture content of raw materials may cause mechanical difficulties, such as restrictor clogging due to ice formation (Wang and Weller, 2006). In addition, higher moisture content would result in low efficiency of SC-CO₂, which is due to the water-soluble solute preferring to partition into the aqueous phase compared to the gas phase. However, water may also function as a modifier for the supercritical fluid extraction of certain compounds such as flavonoids (Lang and Wai, 2001).

Chapter 3

Materials and Methods

This chapter is divided into three main sections. Section 3.1 describes in detail the materials and methods used in the extraction of 2-AP from Pandan leaves using supercritical carbon dioxide and solvent (hexane) extraction. While sections 3.2 and 3.3 cover the methods used for the study of the changes of 2-AP absorption during the cooking and storage of rice cooked with Pandan leaves, respectively.

3.1 Extraction of 2-acetyl-1-pyrroline from Pandan leaves

3.1.1 Materials

Fresh Pandan leaves (Figure 3.1) were purchased from a local supplier (Sing Fat Ltd.) in Birmingham, United Kingdom. They were grown in Thailand. Only leaves with a moisture content of $85.0\pm 3.0\%$ were selected for further analysis. Hexane (HPLC grade) used for Soxhlet extraction, acetone (HPLC grade), and 2,4,6-trimethylpyridine (TMP, 99.0% purity), used as an internal standard in gas chromatography-flame ionization detector (GC-

FID) analysis, were all purchased from Sigma-Aldrich Co. Ltd., United Kingdom. High purity carbon dioxide (CO₂): CP grade, 99.995% pure, liquid withdrawal; helium (99.9% purity), used as the carrier gas, and dry ice were supplied by BOC, United Kingdom.



Figure 3.1: Picture of fresh Pandan leaves

3.1.2 Determination of moisture content of Pandan leaves

The moisture content of fresh Pandan leaves was determined by gravimetric analysis. The mass of approximately 5 g leaves was measured before being subjected to 105 °C, 24 h of oven drying method, which was similar to the method used by Arslan and Musa Ozcan (2008). The loss of mass was assumed to be equal to the mass of water in the sample. Five replicates were carried out for each measurement.

3.1.3 Extraction pre-treatment

In this study, the effects of different particle sizes of Pandan leaves and drying pre-treatment were investigated. The particle size of Pandan leaves was altered either by grinding using a grinder (model PB-323, Pensonic, Malaysia) for 30 s to obtain the leaf scrap of around 0.5-1.0 mm² or intact leaves without grinding of around 5 mm². Samples were then subjected to drying. Two drying methods were investigated:

- i. Oven drying in a forced convection conventional oven: 30 °C for 48 h.
- ii. Freeze drying using a freeze dryer (Virtis Advantage 2.0, Bench-top Freeze dryer, Gardiner, USA) with the following temperature programme: freezing at -50 °C for 15 h, then drying at 0 °C for 3 h and followed by post drying at 20 °C for 2 h.

3.1.4 Scanning electron microscopy

Scanning electron microscopy (SEM) was used to investigate the structural features of pre-treated Pandan leaves before the extraction process. The specimens were attached to SEM stubs using double-sided adhesive tape and then coating with gold using a sputter coater. The surfaces of the leaves were scanned using an analytical scanning electron microscopy (Philips XL30, England) operating at an accelerating voltage of 10.0 kV.

3.1.5 Supercritical carbon dioxide (SC-CO₂) extraction

The whole SC-CO₂ extraction system was assembled in house and all the items of equipment were purchased from Baskerville Reactors and Autoclaves Ltd., Trafford Park, Manchester, UK and Swagelok Co., Solon, Ohio, USA. Batch mode extraction was used throughout the experiments. In this study, Pandan leaves were extracted using SC-CO₂ at 50 °C, with a pressure of 20 MPa for 2 h of extraction time. The flow rate of CO₂ was fixed at 3 ml/min in all experiments. The parameter conditions were selected based on previous experiments carried out by Laohakunjit and Noomhorm (2004). Due to the high volatility of 2-AP, a very low amount of 2-AP concentration was obtained below 1 h of extraction time; therefore, we believe that the 2 h extraction is the optimum time for SC-CO₂ to extract this compound from Pandan leaves. As the concentration of 2-AP in all raw materials and products is far from its saturated value, the static mode of extraction was not applied in this study.

Figure 3.2 shows the schematic diagram of the SC-CO₂ extraction system. The methodology used in the present study was as follows. An air bath was used to control the temperature ($\pm 0.5^\circ\text{C}$) of the whole extraction system. Before placing the sample inside the extraction vessel (1), the air bath was allowed to reach the desired temperature; 50 g (wet weight) of Pandan leaves were then charged into the vessel. Mesh filters were fitted on both the top and bottom of the vessel with Pandan leaves placed in the middle to avoid any

material loss in the extractor. Then the liquid CO₂ was drawn from cylinder (2), as solvent. After passing through a check valve and a 15 µm filter (3), the CO₂ was cooled to a temperature of 0-5°C in a cold bath (4) located between the cylinder outlet and the air driven liquid pump (PowerStar4 Liquid Pump, model 4F-64, 0-6400 psig) from Sprague Products. This low temperature condition was used to ensure that the CO₂ was in a liquid state, hence preventing pump cavitations'. The CO₂ was then pumped into the system using a liquid pump (6) until the required pressure (\pm 0.05 MPa) was obtained. A backpressure regulator (7) from Go Products (model UP66) was used to set the system pressure.

When the extraction conditions reached the desired temperature and pressure, the SC-CO₂ flow was provided continuously through the extractor, contact with packed bed and extract the extractable material from the bed. The CO₂ loaded with extracts flowed to the collector through valves (8) and (10) and was separated from the extracts by dropping the pressure to ambient. The duration of the extraction was 2 h. The Pandan leaf extracts were collected in collectors (11) and (12). The less volatile extracts were deposited in collector (11) while the high volatile components passed through it and entered collector (12).

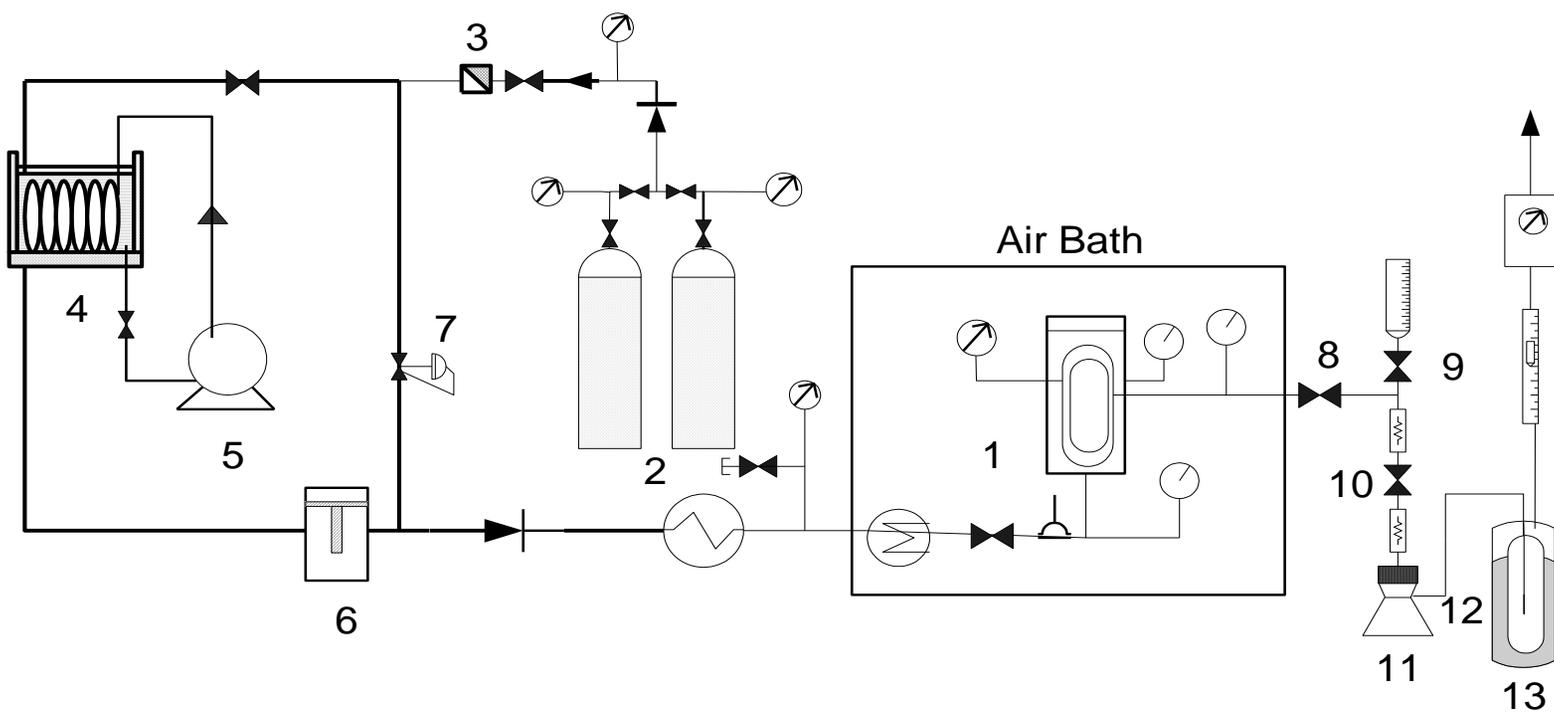


Figure 3.2: Schematic diagram of SC-CO₂ extraction system:1, extraction vessel; 2, CO₂ cylinder; 3, filter; 4, cold bath; 5, chilling pump; 6, PowerStar4 air driven liquid pump for CO₂; 7, backpressure regulator; 8, 9 and 10, valves; 11 and 12, collectors; 13, cooling bath.

In order to ensure a good degree of contact between the gas and liquid phases, a minimum of 20 ml of acetone was used as an absorbent. Collector (12) was pre-charged with this volume of acetone and was immersed in a cooling bath (13) containing a dry ice-acetone mixture at -82 °C. The CO₂ stream was bubbled through the pre-charged acetone and the very volatile components were absorbed by acetone in this collector. The extracts collected in collector (11) were washed out by acetone and added to those obtained from collector (12), so the entire extract solution was obtained.

The acetone was then removed from the extracts using a rotary-evaporator (Rotavapor-RE, BuchiOrme Scientific Ltd., England) under reduced pressure, at a bath temperature of 40 °C for up to 10 min until the solvent free extracts were obtained. The extracts were diluted with 10 ml of acetone and transferred to screw-capped glass vials, which were then stored in a refrigerator at 4 °C for further analysis. All the experiments were carried out in triplicate.

3.1.5.1 Modification of SC-CO₂ collector system

As a preliminary study, before the application of two collectors (11) and (12), only one collector (11) was practically used to collect the whole extracts of Pandan leaves (Figure 3.3a). By using one collector, most of the highly volatile compounds spread out to the open

air during the extraction process. Therefore, it was decided to add an extra collector (12), which was located in the cooling bath (13) to entrap the maximum amount of volatile compounds from the Pandan leaf extracts (Figure 3.3b).

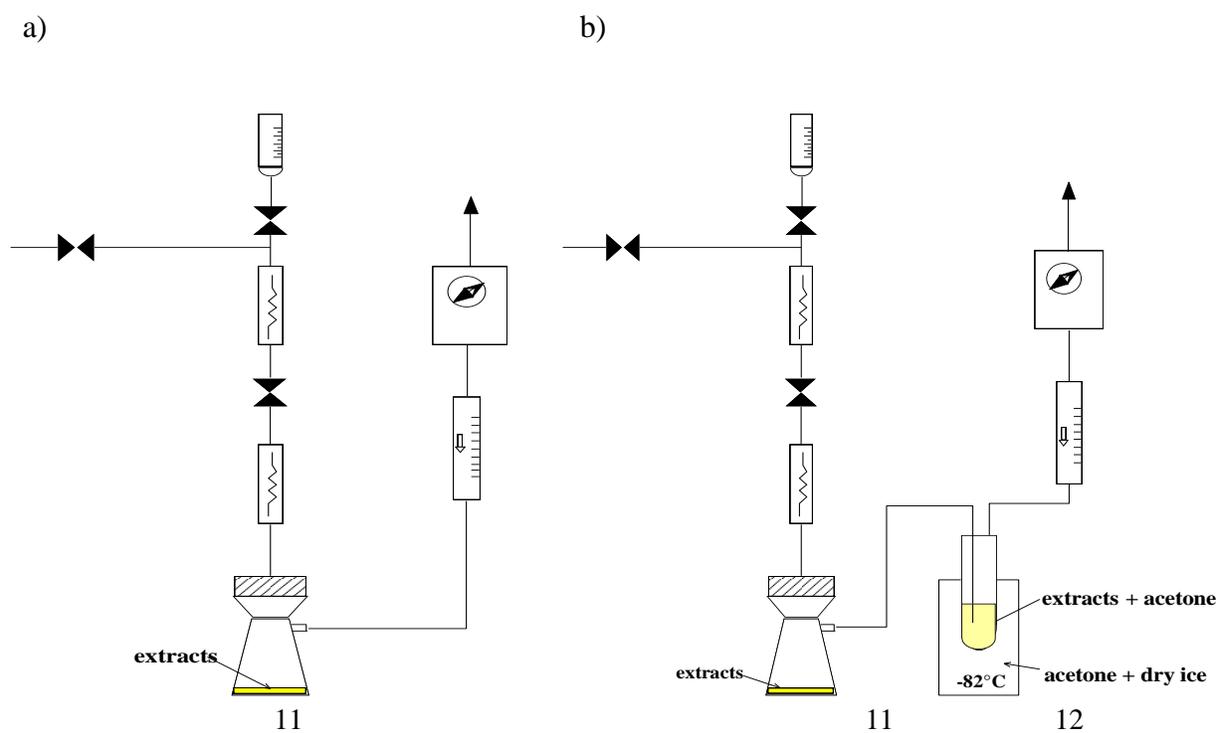


Figure 3.3: Schematic diagram of SC-CO₂ collector; a) before modification and b) after modification.

3.1.6 Hexane extraction

Organic solvent extraction was carried out in the Soxhlet apparatus (Figure 3.4) using hexane as a solvent. Five grams of Pandan leaves (wet weight) were packed into a cellulose thimble in the Soxhlet chamber, which was then transferred into the Soxhlet extractor. Approximately 300 ml of hexane was placed in a round bottom distillation flask and then used in a typical run. Water was allowed through the condenser at a constant rate of 1 l/min and the round bottom flask was heated with a heating mantle. When the temperature in the round flask reached 68 °C, the hexane vapour travelled up a distillation arm, and flooded into the chamber housing the Pandan leaves thimble.

During heating, the chamber containing the Pandan leaves was slowly filled with warm hexane. Some of the desired compound was then dissolved in the warm hexane and when the Soxhlet chamber was almost full, the chamber was automatically emptied by a siphon side arm, where the warm hexane ran back down to the distillation flask. This continual re-charging of the flask resulted in a continuous extracting process, which promotes the concentration of the desired compound (2-AP) in the distillation flask. The total reflux time was up to 4 h after which it was allowed to cool for 1 h. For concentrating purposes, the solvent was evaporated using a rotary evaporator under reduced pressure, at 40°C bath temperature until dryness, while the non-soluble portion of extracted Pandan leaves remained in the thimble and was discarded. The concentrated extracts were then diluted in 10 ml of acetone and submitted to analysis by gas chromatography-flame ionization

detector (GC-FID) and gas chromatography-mass spectrometry (GC-MS). All Soxhlet extractions were done in triplicate.

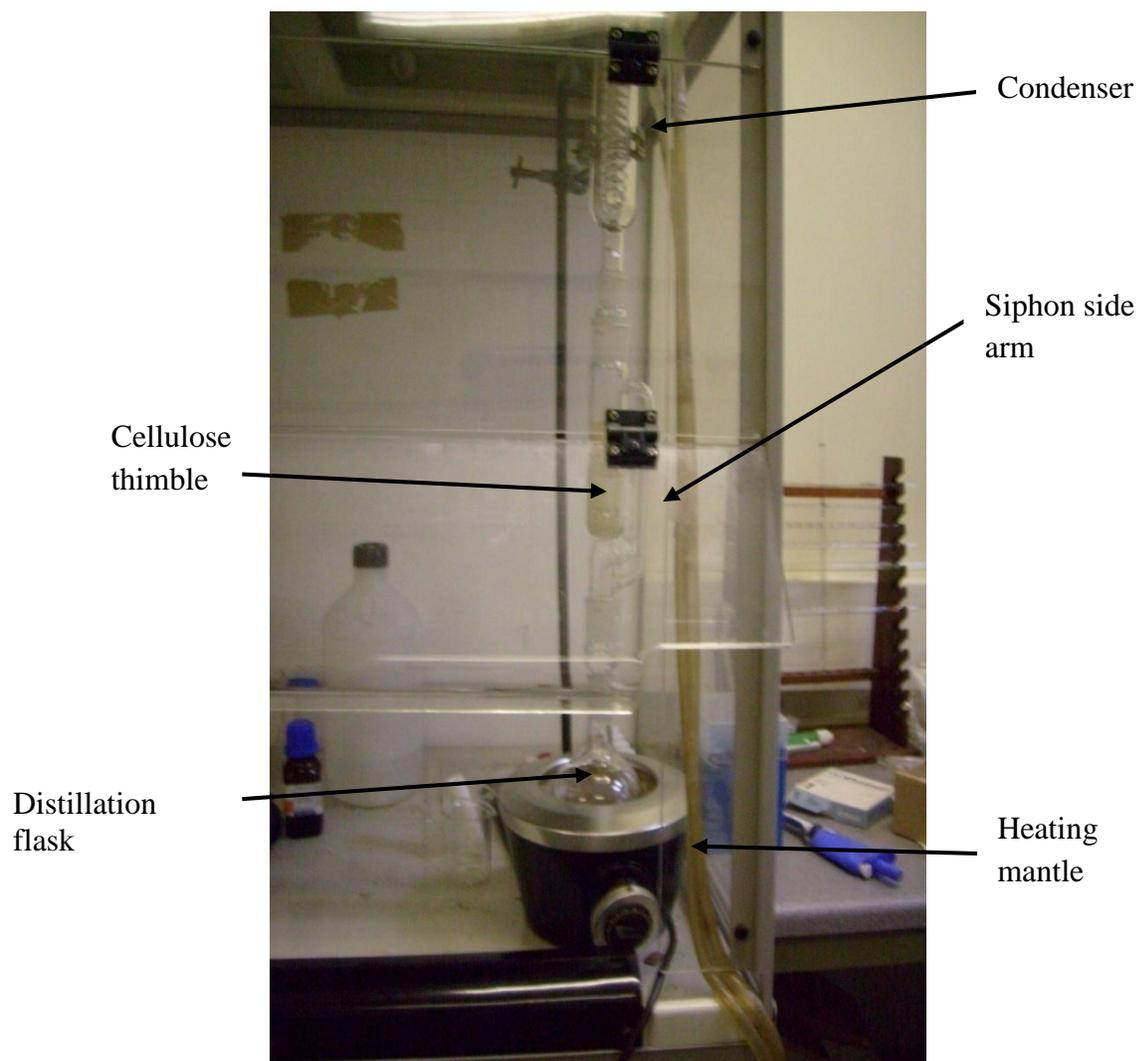


Figure 3.4: Experimental set up used for Soxhlet extraction

3.1.7 Determination of total yield of Pandan leaves extracts

The total amount of yield of extracts was determined gravimetrically after collection. The extraction yield is expressed as the percentage ratio of the mass of extracted material to the mass of dried Pandan leaves loaded in the extraction vessel (SC-CO₂) or cellulose thimble (hexane extraction), as follows:

$$\text{Extraction yield (\%)} = m_1/m_2 \times 100$$

m_1 = mass of extracted material, (g); m_2 = mass of dried Pandan leaves, (g)

3.1.8 Gas chromatography-flame ionization detector

Pandan extracts obtained by SC-CO₂ and solvent extraction were analysed in triplicate. The quantitative analysis of extracts was performed on an HP 6850 gas chromatograph equipped with a flame ionization detector (FID) (Agilent Technologies), as shown in Figure 3.5. A 3 μ l aliquot of extracts containing the 2, 4, 6-trimethylpyridine (used as an internal standard) was then injected into the GC-FID using an auto sampler injection with the split ratio of 1:80. A DB-5 (5% phenyl, 95% methyl silicone) capillary column (J & W Scientific, USA) with a dimension of 30.0 m length \times 0.25 mm inner diameter \times 0.1 μ m film thickness was used. The oven temperature was programmed starting at 50 $^{\circ}$ C for 2 min and ramped at 15 $^{\circ}$ C/min up to 200 $^{\circ}$ C. The injector and detector temperatures for GC-FID

system were set at 170 °C and 250 °C, respectively. Helium at a flow rate of 1.3 ml/min was used as the carrier gas.

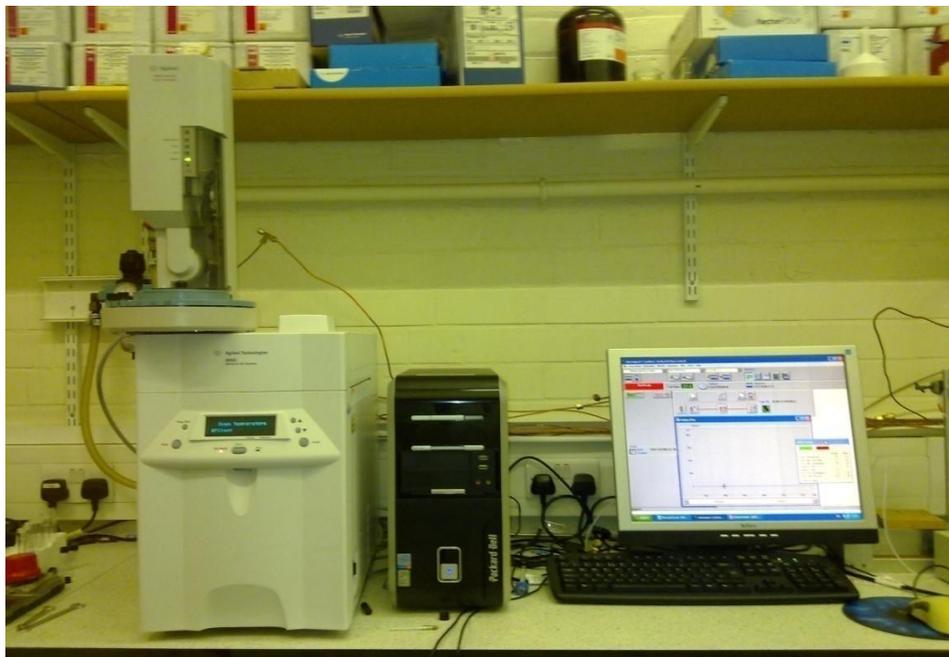


Figure 3.5: Picture of gas chromatography-flame ionization detector

3.1.9 Gas chromatography-mass spectrometry

The extracts obtained were submitted to GC analysis in a HP 7890A gas chromatography equipped with a GCT Premier mass spectrometer and a capillary column of DB-5MS (J & W Scientific, USA) with a dimension of 30.0 m length \times 0.25 mm inner diameter \times 0.25 μ m film thickness. A 1 μ l aliquot of extract sample was injected for analysis. The programme started at 50 °C for 1 min, then increased at a rate of 25 °C/min from 50 °C to

280 °C and was held for 5 min. Other operating conditions were as follows: injector temperature, 250 °C; carrier gas, helium at a flow rate of 1.0 ml/min operated in the split mode 1:5. The mass spectrometry parameters were: electrons impact, 70 eV; ion source temperature, 200 °C; line of transference, 250 °C; electron multiplier voltage, 2500 V; scan rate, 5 scan/s; mass interval, 35-600 m/s.

3.1.10 Quantitative analysis

In order to minimize errors occurring when an accurate weight or volume of samples was required, an internal method was used as previously reported by other researchers who used 2, 4, 6-trimethylpyridine (TMP) in the quantitative analysis of 2-AP by GC-FID (Laohakunjit and Kerdchoechuen, 2007; Laohakunjit and Noomhorm, 2004; Wakte *et al.*, 2007). A stock solution of 250 ppm TMP was prepared by adding 10 µl of TMP in 40 ml of acetone. This stock solution was diluted to 50 ppm before adding it to each 0.1 ml of the extracts and then 3 µl samples were injected to GC-FID. Quantification of 2-AP was calculated using the following:

Amount of 2-AP (µl/ml) =

$$\frac{\text{Concentration of TMP } (\mu\text{l/ml}) \times \text{Injection volume of TMP (ml)}}{\text{TMP GC area}} \times \frac{\text{2-AP GC area}}{\text{Injection volume of sample (ml)}}$$

3.2 Absorption of 2-acetyl-1-pyrroline during cooking of rice mixed with Pandan leaves

3.2.1 Materials

Easy cook long-grain pre-fluffed rice, Sea Isle Limited, (Moorcroft Park Wednesbury, United Kingdom) was purchased from a local supermarket (Tesco, Birmingham) and stored in the laboratory at room temperature until used. The required quantity of rice was taken out when necessary for experimental work. Fresh Pandan leaves were purchased (Sing Fat Limited, Birmingham). Pandan leaves were cleaned, swabbed with tissue paper, cut into small pieces (≈ 3.0 to 5.0 mm^2 in size) and tied in muslin gauze before use.

3.2.2 Determination of 2-AP partition coefficient in water/air system

Partition coefficient of 2-AP in water/air system was measured by using phase ratio variation method.

3.2.2.1 Phase ratio variation method

The initial concentration of 2-AP in fresh Pandan leaves was C_i while the concentration of 2-AP in stock solution was C_p . By using a sample solution with C_p and testing increasing volumes (V_p) in separate headspace vials, we could assume that C_p and partition

coefficient, K , were the same in each vial at a temperature of 25°C . Therefore, the concentration of 2-AP introduced in the sample vial can be expressed as mass per volume:

$$C_p = m_p/V_p \quad (1)$$

$$C_p = C^*_p + C^*_q$$

Where C^*_p is the concentration of the 2-AP in the liquid phase at equilibrium, while C^*_q is the concentration of the 2-AP in gas phase at equilibrium.

Phase volume ratio (β) of sample vial containing the 2-AP is

$$\beta = V_q/V_p \quad (2)$$

The partition coefficient, K can be expressed as

$$K = C^*_p/C^*_q \quad (3)$$

$$C^*_p = m^*_p/V_p \quad (4)$$

$$C^*_q = m^*_q/V_q \quad (5)$$

Partition coefficient can be expressed as

$$K = C^*_p/C^*_q = (m^*_p/m^*_q)(V_q/V_p) = (m^*_p/m^*_q)\beta \quad (6)$$

$$m_p = m^*_p + m^*_q \quad (7)$$

$$m_p/V_p = m^*_p/V_p + m^*_q/V_p \quad (8)$$

From eq. (2), $V_p = V_q/\beta$, therefore

$$m_p/V_p = m^*_p/V_p + (m^*_q/V_q) \beta \quad (9)$$

$$C_p = C^*_p + (C^*_q) \beta \quad (10)$$

From eq. 3, $C^*_p = K (C^*_q)$, by substituting it in eq. 10, therefore

$$C_p = K (C^*_q) + (C^*_q) \beta = C^*_q (K + \beta) \quad (11)$$

$$C^*_q = C_p / (K + \beta) \quad (12)$$

Taking the reciprocals of both sides, eq. (12) becomes

$$1/ C^*_q = K/C_p + (1/C_p) (\beta) \quad (13)$$

In headspace analysis, the peak area is proportional to equilibrium concentration in the headspace vials, A ;

$$A = f_i (C^*_q) \quad (14)$$

From eq. 14; $C^*_q = A/f_i$, therefore

$$1/A = K/ (C_p \cdot f_i) + (1/ (C_p \cdot f_i))(\beta) \quad (15)$$

$$1/A = a + b (\beta) \quad (16)$$

$$a = K/(C_p \cdot f_i) \quad , \quad b = 1/ (C_p \cdot f_i)$$

$$K = a/b \quad (17)$$

K was calculated from the values of a and b , which was obtained by plotting A^{-1} against β , which established its slope, b and intercept, a .

3.2.2.2 Determination of water/air partition coefficient

Fresh Pandan leaves (40 g) were introduced into 100 ml of distilled water and then blended using a blender (model PB-323, Pensonic, Malaysia) for 2 min. The liquid solution was separated from the ground Pandan leaves through filtration using a Whatman Grade No. 1 filter paper. Increasing volumes (2, 3, 5, and 7 ml) of the solution were poured into 20 ml headspace vials. The vials were immediately sealed tightly with silicone septa and were placed in an incubator at 25 °C for 24 h to achieve an equilibrium partition between the liquid and the headspace. Then a 1 ml sample of headspace was injected manually into gas chromatography by a gas tight syringe.

3.2.2.3 Gas chromatography-flame ionization detector

A HP- Innowax capillary column (J & W Scientific) with a dimension of 25.0 m length × 0.2 mm inner diameter × 0.4 µm film thickness was used to analyse the 2-AP concentration in the gas phase of the headspace vial. The conditions for gas chromatograph were as follows: oven temperature programme: starting at 50 °C for 2 min and ramped at 15 °C/min up to 200 °C and then hold for 15 min. The carrier gas was purified helium at a flow rate of 1.3 mL/min with split ratio 1:80. The injector and detector temperatures were set at 170 °C and 250 °C, respectively. For flame ionization detector (FID), air and H₂ flow rates were 450 and 40 ml/min.

3.2.2.4 Analysis

Linear regression analysis was performed according to eq. 16 where $x = \beta$ and $y = A^{-1}$. The partition coefficient was calculated using eq. 17.

3.2.3 Procedure of cooking

In the present study, long grain white rice was cooked with fresh Pandan leaves in two different methods –excess and optimal water cooking. The differences between the methods are the rice to water ratio and the temperature used at the start of cooking. The excess water condition is selected as the typical cooking method in Western countries (Leelayuthsoontorn and Thipayarat, 2006; Grimm *et al.*, 2001), where rice is boiled in excess water until the centre of the grain is fully cooked. The rice to water ratio is quite high (1:10 to 1: 20) and used as a standard laboratory test procedure (Chakkaravarthi *et al.*, 2008). The optimal water cooking experiments were performed generally as per the recommended guidelines of the rice cooker manufacturer and according to the general practice in South-East Asian countries. Optimal water cooking is a cooking of rice in a particular rice-water ratio and can vary from 1:1.5 to 1:2.5 largely depending on the type of rice (amylose content) (Chakkaravarthi *et al.*, 2008). The experiments were conducted in triplicate.

3.2.3.1 Excess water cooking

The procedure for cooking rice in excess water condition was adopted from Leelayuthsoontorn and Thipayarat, (2006) and the experimental set up is shown in Figure 3.6. The rice was washed three times with 800 ml of tap water and rinsed prior to cooking. A known quantity of boiling tap water (800 ml) was added in an aluminium cylinder (diameter of 10 cm and height of 11.5 cm) (Figure 3.7) before being placed on a hot plate (Jenway 1000, Bibby Scientific Ltd., Exssex, UK). The aim of using an aluminium cylinder was to achieve a constant temperature of 100 °C, and, for the same reason, aluminium foil was used to cover the cylinder during cooking. A constant temperature of 100 °C was selected as it is typical heating applied in domestic excess water cooking.

When the temperature of water reached 100 °C, the rice grains (240 g) and tied fresh Pandan leaves (5 g) were added to the covered cylinder. The temperature and time of cooking were recorded. Samples of rice (5 g), water (10 ml) and Pandan leaves (5 g) were obtained at intervals of 5, 10, 15 and 20 min of cooking and subjected to headspace analysis. The experiments were conducted in triplicate.

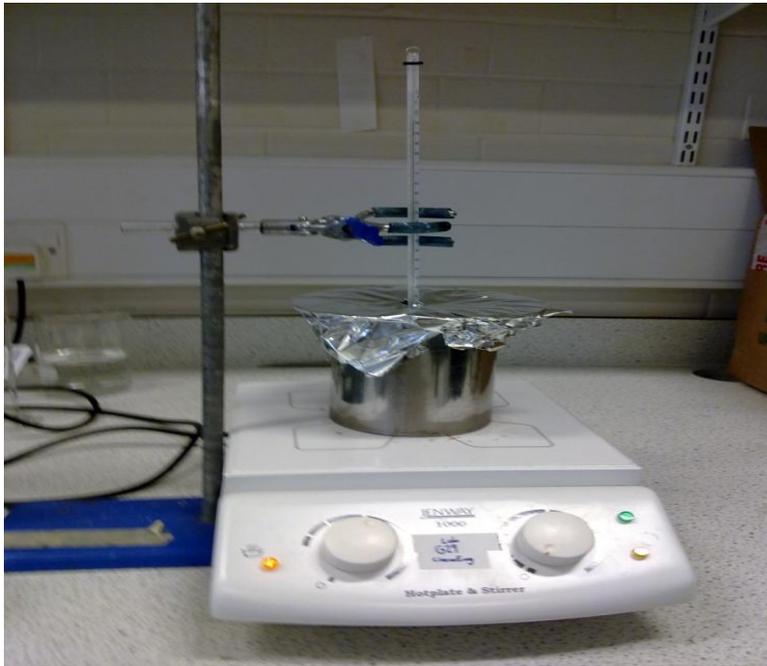


Figure 3.6: Experimental set up used for excess water cooking.

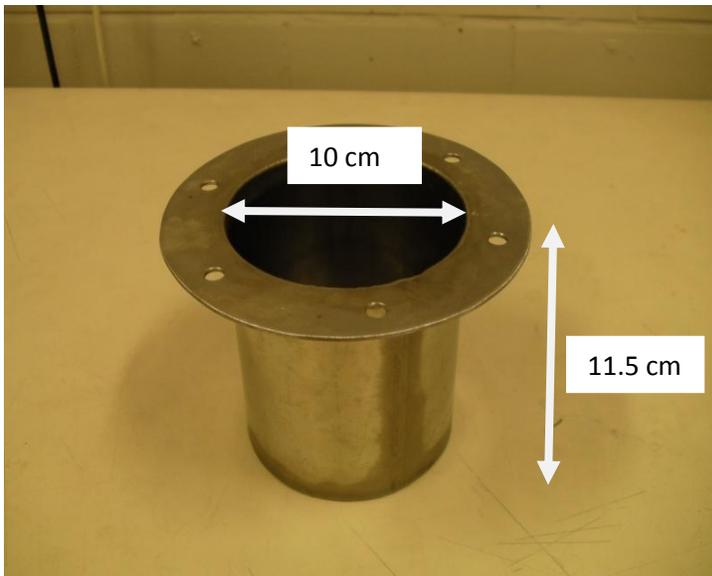


Figure 3.7: Picture of an aluminium cylinder

3.2.3.2 Optimal water cooking

Rice was washed and rinsed three times with tap water prior to cooking and without any soaking. Approximately 240 g of non-aromatic long grain white rice was added into an open electric rice cooker that was filled with 800 ml of tap water. Before cooking started, 5 g of tied fresh Pandan leaves was placed in the middle of rice cooker. The cooking system was started by switching on the power supply of the electric rice cooker. The temperature of the water cooking was measured at a fixed location by thermometer. Samples of rice, water and Pandan leaves were taken out from the cooker at cooking times of 5, 10, 15 and 20 min. The temperature was recorded as a function of time. All the experiments were performed in triplicate.

3.2.4 Determination of moisture content of rice

The moisture content of rice cooked in excess and optimal water conditions was measured after being drawn at different times (at intervals of 5 min up to 20 min). A sample of rice (5 g) was taken out from cylinder (excess water) or rice cooker (optimal water) and placed on filter paper before the weight of the sample in the aluminium Petri dish was measured by an electronic balance. Moisture contents were measured using the oven drying method at 105 °C for 24 h, as suggested by Das *et al.* (2006) and Chakkaravarthi *et al.* (2008). This moisture content (w.b.) is defined as the ratio of the water weight to the initial total weight of the rice. The experiments were performed in triplicate.

3.2.5 Static headspace analysis

The absorption of 2-AP during the cooking of rice mixed with fresh Pandan leaves in both excess and optimal water conditions was determined by static headspace analysis. The method was adopted from previous literature (Grimm *et al.*, 2001; Itani *et al.*, 2004) with some modification. At each cooking time, rice samples were taken from the cooker and were ground after cooking by hand using a mortar and pestle for 30 s. Approximately 5 g of ground rice was added to 10 ml of distilled water and sealed in 20 ml of headspace vial (22.5 mm × 75 mm, Supelco, USA) fitted with a PTFE/silicone septum and secured by an aluminium pressure release crimp cap. After being tightly capped, the sample vial was shaken using a vortex for 30 s and was then incubated in a water bath at 100 °C for 30 min. The headspace of rice sample (1 ml) at a position of 5 mm above the sample surface was taken out from the vial using a gas tight syringe and subjected to the GC-FID analysis.

The same procedure for analysing 2-AP content in headspace of rice sample was applied for Pandan leaves. In the headspace vial, a 5 g sample of Pandan leaves from the rice cooker was mixed with 10 ml of distilled water prior to 30 min incubation in a water bath at 100 °C. For the cooking water, a 10 ml sample was taken out directly from the cooker and placed in a headspace vial without the addition of distilled water and then directly injected into GC-FID after incubation. Only one headspace injection was made per vial, and three vials were analysed for each sample.

3.2.6 Gas chromatography –flame ionization detector

Headspace (1 ml) of rice, cooking water and pandan leaves were submitted to GC analysis in a HP 6850 gas chromatograph equipped with a flame ionization detector (FID) (Agilent Technologies) and a capillary column of HP-Innowax Poly-ethylene Glycol (J&W Scientific, USA) with dimensions of 25.0 length \times 0.20 mm inner diameter \times 0.40 μ m film thickness using a gas tight syringe. The programme started at 50 °C for 1 min, then increased at a rate of 7 °C/min from 50 °C to 170°C and was held for 5 min. The injector and temperatures for the GC-FID system were set at 170 °C and 250 °C, respectively. Helium at a flow rate of 1.3 ml/min was used as a carrier gas. Quantitative analysis was performed in triplicate. The retention time of 2-AP was identified at 5.47 min and the internal standard of 2, 4, 6-trimethylpyridine (TMP) with a retention time of 5.7 min was used in the quantifying of 2-AP by GC-FID. The quantification of 2-AP based on its peak area for each cooked rice, cooking water and Pandan leaf samples at different cooking times and were calculated, as mentioned previously in section 3.1.10.

3.2.7 Differential scanning calorimetry

The thermal properties of the rice starch during cooking in optimal and excess water conditions were determined by using a Differential Scanning Calorimetry (DSC) Diamond (Pelkin Elmer, USA) equipped with a thermal analysis data station of Pyris software. A

DSC analyser was calibrated using indium and an empty stainless steel pan was used as a reference according to the specification of the manufacturer. Raw ground rice grain (3.8 mg, dry weight) was loaded into a 50 μ l high pressure stainless steel pan (Mettler, ME-27331) and distilled water was added to achieve the ratio of ground rice-water of 1:3. The samples were hermetically sealed and allowed to stabilize for 1 h at room temperature prior to subjecting them to DSC. A thermal scan was conducted from 20 to 120 $^{\circ}$ C at the heating rate of 10 $^{\circ}$ C/min. The DSC measurements were performed in duplicate. Thermal transitions of rice starch were measured in terms of the onset temperature (T_o). The enthalpy (ΔH) associated with starch gelatinization was evaluated as the total peak area of the endotherm by using Pyris software.

3.2.8 Selection of headspace analysis method

Before the absorption of 2-AP during storage by cooked rice was determined, two different methods of static headspace analysis were selected either by incubation using a water bath at 100 $^{\circ}$ C for 30 min or 80 $^{\circ}$ C for 15 min (Figure 3.8) to get the optimum 2-AP concentration in cooked rice during cooking under excess water conditions. Only the absorption of 2-AP by cooked rice was taken into consideration. The best method of static headspace was chose for determination of 2-AP during the storage of cooked rice.



Figure 3.8: Experimental set up used for headspace analysis

3.2.9 Effects of prolonged cooking time on the absorption of 2-AP under optimal water conditions

In order to get fully cooked rice under optimal water conditions, rice was cooked for another 20 min, thereby amounting to a total cooking time of 40 min. In this study, the optimal water cooking of rice was divided into two different methods by using an open rice cooker or a covered rice cooker, as illustrated in Figure 3.9. Only the headspace of rice sample was taken into consideration. The absorption of 2-AP by rice grains was determined at 0, 10, 20, 30 and 40 min.

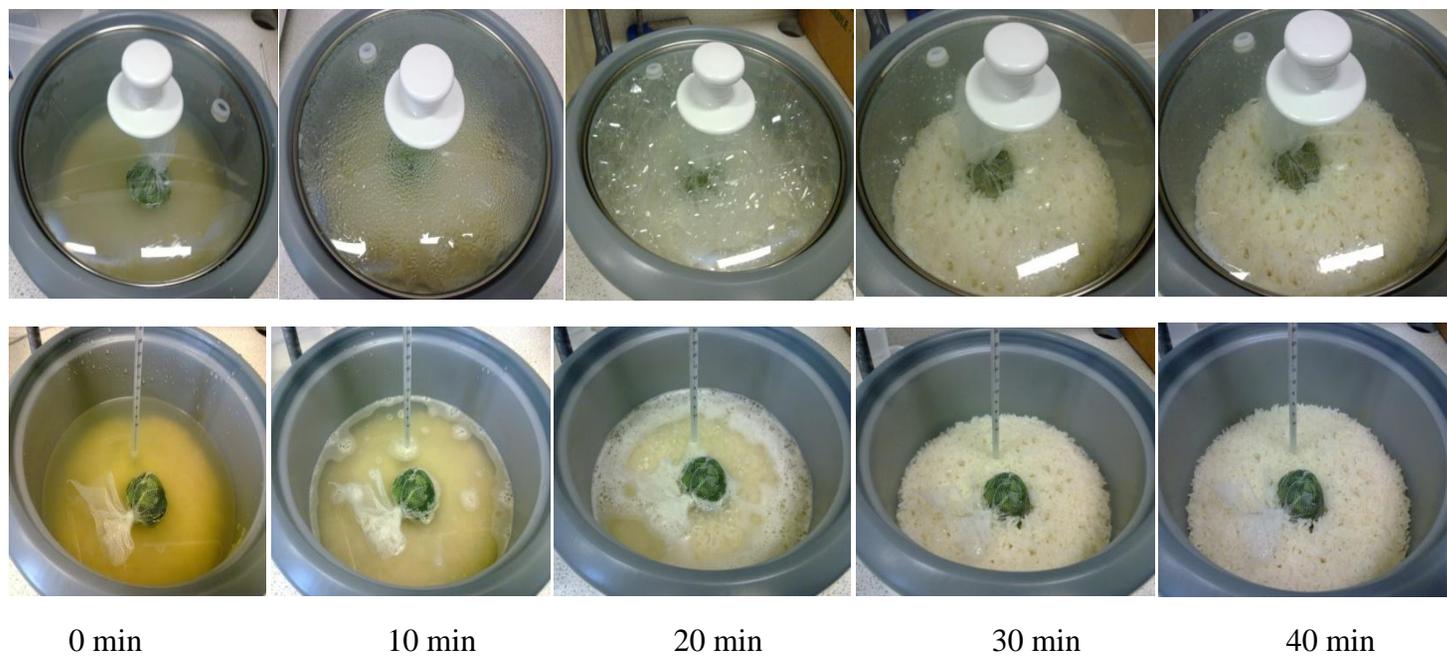


Figure 3.9: Cooking of rice with Pandan leaves in optimal water condition using a covered rice cooker (top row) and an open rice cooker (bottom row) at different cooking times.

3.3 Changes of 2-acetyl-1-pyrroline absorption during storage of cooked rice

3.3.1 Materials

All the materials used in the absorption of 2-AP during the storage of cooked rice were similar to the materials used in the study of absorption of 2-AP by rice during cooking, as described earlier in section 3.2.1.

3.3.2 Determination of 2-AP amount during storage of cooked rice mixed with different quantities of Pandan leaves

Before determining the absorption of 2-AP during the storage of cooked rice, the effect of adding different masses of Pandan leaves during the cooking of rice in optimal water condition was performed in order to obtain the highest 2-AP absorption by aged cooked rice. The amounts of 5 g and 10 g of Pandan leaves were selected and mixed with rice (240.0 g) and water (800 ml). The rice was cooked for 40 min using an open rice cooker and the procedure of cooking was similar to that mentioned in section 3.2.3.2.

The time of storage was started after 40 min cooking at which time the electricity power supply was switched off. During storage, the cooked rice and Pandan leaves remained in the rice cooker. The absorption of 2-AP by cooked rice was measured after 15, 30, 45 and 60 min of storage. For each storage time, 5 g of cooked rice was ground by hand

with a pestle and mortar for 30 s before being added into a headspace vial filled with 10 ml of distilled water. The vial was then sealed tightly with an aluminium pressure cap and PTFE/silicon septum, and was shaken with a vortex for 30 s before being incubated in the water bath at 80 °C for 15 min. The headspace above the cooked rice (1 ml) of each vial was subjected to GC-FID analysis by a gas tight syringe. The method used for quantifying the 2-AP absorption by cooked rice during storage was similar to the 2-AP quantification during cooking of rice as previously mentioned in section 3.2.6. The experiments were carried out in triplicate.

3.3.3 Procedure of storage

The storage study of cooked rice was investigated by storing cooked rice for 15, 30, 45 and 60 min after the cooking process of excess and optimal water conditions at a temperature of 24.0 ± 1.0 °C. The optimal water cooking was performed using an open and a covered rice cooker. Rice was cooked mixed with tap water (800 ml) and tied Pandan leaves (10 g).

3.3.3.1 Storage of excess water cooked rice

In the case of excess water condition, non-aromatic rice (60 g) was cooked with water (800 ml) and 10 g of tied Pandan leaves (~3.0 to 5.0 mm² in size) for 20 min in an aluminium cylinder, as previously mentioned in section 3.2.3.1. Aluminium foil was used to cover the cylinder in order to maintain the constant temperature of 100 °C

during the whole process of cooking. The electricity power of the hot plate was switched off immediately after 20 min of cooking. After the cooking was done, the water was removed from the cylinder while the cooked rice and Pandan leaves were taken out, and placed together in an open plastic container and left at $24.0\pm 1.0^{\circ}\text{C}$ for further analysis. The experiments were conducted in triplicate.

3.3.3.2 Storage of optimal water cooked rice

Rice was cooked in optimal water conditions using a covered and an open rice cooker for 40 min and the procedure of cooking is similar to that mentioned in section 3.2.3.2. The storage study of cooked rice started immediately after the power supply of the rice cooker was switched off, after cooking for 40 min. The cooked rice and Pandan leaves remained in the rice cooker until the end of storage, 60 min.

3.3.4 Measurement of temperature during the storage of cooked rice

The temperature of the cooked rice during storage was measured using a thermometer. The experiments were performed in triplicate.

3.3.5 Determination of moisture content during the storage of cooked rice

For each storage time of both excess and optimal water, the moisture content of aged cooked rice was determined by the oven drying method at 105°C for 24 h using a similar procedure to that mentioned in section 3.2.4. The experiments were conducted in triplicate.

3.3.6 Quantification of 2-AP concentration during the storage of cooked rice

The quantification of 2-AP concentration during storage of cooked rice was similar to the method described earlier in section 3.2.5 and 3.2.6. In this present study, static headspace analysis using 80°C for 15 min was selected prior to GC-FID analysis.

3.4 Statistical analysis

Data are reported for each parameter as mean value \pm standard deviation. Analysis of variance (ANOVA) and Fisher's least significant difference (LSD) at $P < 0.05$ were conducted. The statistical analyses of data were performed using a MINITAB (Release 14) (Minitab Inc., USA) statistical software package.

Chapter 4

Extraction of 2-acetyl-1-pyrroline from Pandan leaves

4.1 Introduction

Pandanus amaryllifolius Roxb. has been widely used as a flavour source in food, especially in South-East Asian countries. The major compound contributing to the flavour characteristics of Pandan leaves is 2-AP. This hydrophilic compound has an odour threshold value as low as 0.1 ppb in water (Buttery *et al.*, 1988). 2-AP significantly contributes to the flavour of rice varieties, such as Basmati rice and Jasmine rice (Laohakunjit and Kendchoechuen, 2006). It has been reported by Buttery *et al.* (1983) that the quantity of 2-AP present in Pandan leaves (of the order of 1 ppm) is more than ten times that found in scented milled rice like Basmati rice and 100 times more than that found in common rice. According to Wongpornchai *et al.* (2003), 2-AP also occurs naturally in fresh *Vallaris glabra* Ktze (Bread flowers) with a concentration of 0.53 ppm. However, it is important to note that Pandan leaves are one of the best natural sources of 2-AP (Laohakunjit and Kendchoechuen, 2006).

Several extraction methods of extracting 2-AP from Pandan leaves have been used extensively, for example, using ethanol extraction, 2.77 ppm of 2-AP has been obtained from ground fresh Pandan leaves (Laohakunjit and Noomhorm, 2004). The yield of 1 ppm of 2-AP was attained by using Likens-Nikerson steam distillation-solvent extraction (Laksanalamai and Ilangantileke, 1993). SC-CO₂ is the latest extraction method reported in the extraction of 2-AP from Pandan leaves. For instance, a yield of 2-AP of 0.72 ppm was obtained using supercritical extraction at 20 MPa pressure, 50 °C temperature and 20 min contact time by Laohakunjit and Noomhorm (2004). Bhattacharjee *et al.* (2005) extracted the highest concentration of 2-AP (7.16 ppm) in Pandan leaf extracts by using supercritical conditions of 45 MPa, 60 °C and 3 h extraction time. However, the authors quantified the major compound of Pandan leaves based on the rapid densitometric assay method, besides GC-FID, the common analytical method used for quantitative purposes.

There is a range of process parameters and steps that can affect the effectiveness of oil extraction from natural products, such as cleaning, dehulling, drying and grinding (Romanik *et al.*, 2007; Gutierrez *et al.*, 2008). As the aromatic plant contains a high level of moisture, the water content of samples during extraction has been found to be a critical parameter while the particle size may affect mass transfer (Park *et al.*, 2007). Both these parameters, together with extraction time, pressure and temperature, are important factors in extracting oil. However, the pre-treatment and extraction methods used may significantly affect (partially or totally) the quality of a product (Ratti, 2001; Arslan and Musa Ozcan, 2008).

The present study aims to assess the influence of various pre-treatment and extraction time on the extraction yield and concentration of 2-AP from Pandan leaf extracts by supercritical carbon dioxide and solvent (hexane) extraction. Scanning electron micrographs of pre-treated ground Pandan leaves before the extraction processes were observed. The extracts obtained were submitted to gas chromatography analysis coupled to a flame ionization detector (GC-FID) and identified by gas chromatography-mass spectrometry (GC-MS), and the results were discussed.

4.2 Results and Discussion

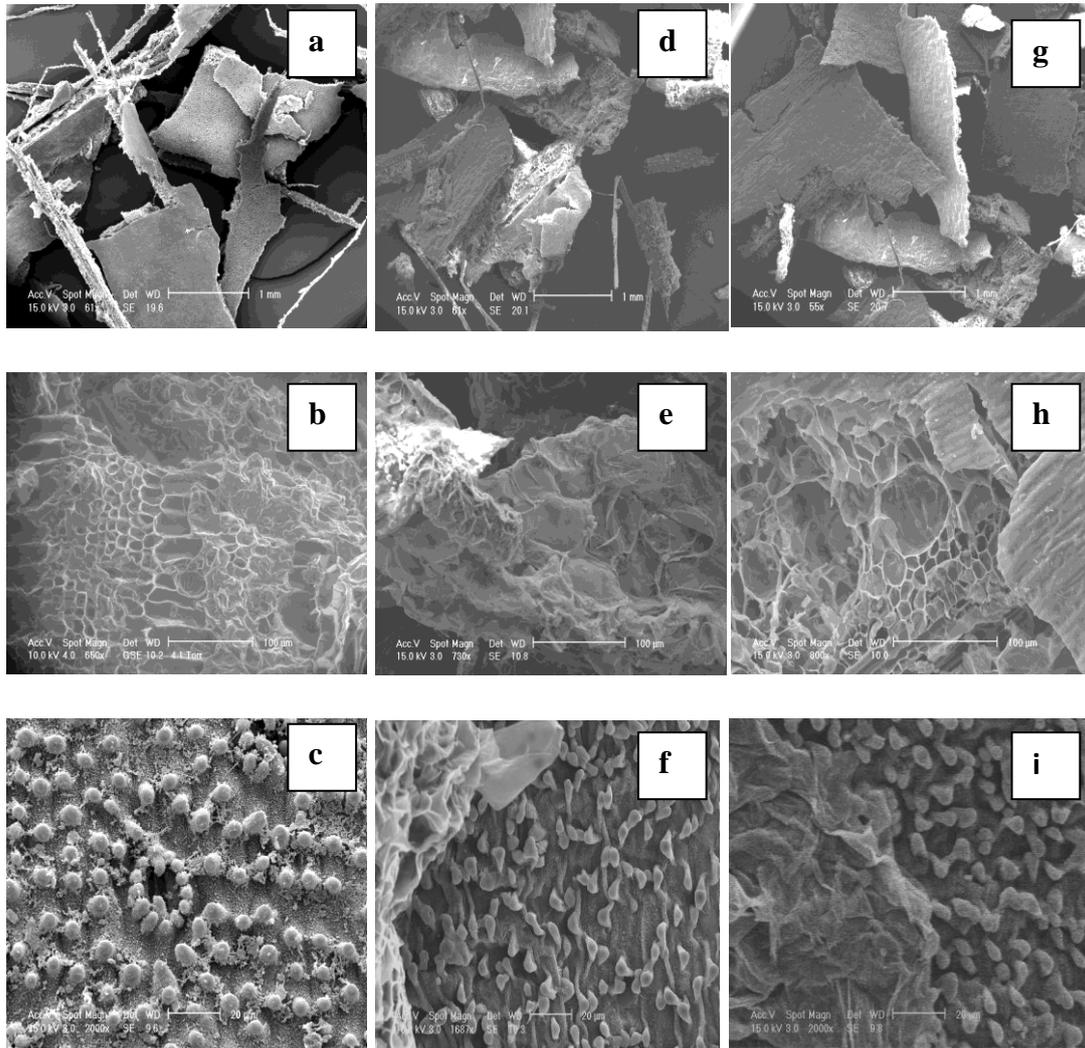
4.2.1 Moisture content of Pandan leaves

Moisture content determination was conducted to evaluate the influence of the drying pre-treatment on the yield and concentration of 2-AP in Pandan leaf extracts. The moisture content of fresh Pandan leaves was $86.5\pm 0.3\%$ measured by oven-drying at $105\text{ }^{\circ}\text{C}$ for 24 h. The drying pre-treatment procedures included either oven-drying at $30\text{ }^{\circ}\text{C}$ for 48 h or freeze-drying at specific conditions (as mentioned in section 3.1.3). As we expected, the moisture content of Pandan leaves decreased significantly to $17.7\pm 0.2\%$ (oven-drying) and $17.9\pm 0.3\%$ (freeze-drying). These results are typical for plant materials where about 80% of water is removed, which is in good agreement with Venskutonis (1997) who found that the moisture content of sage was 11.2% after oven drying at $30\text{ }^{\circ}\text{C}$ for 63 h, which was slightly lower compared to freeze-dried leaves at $-50\text{ }^{\circ}\text{C}$ for 42 h (12.5%). While after 24 h of drying operations, Gutierrez *et al.* (2008) found that the average moisture content of air-dried and freeze-dried quebec buckthorn

pulp was 2.6% and 1.5%, respectively compared to fresh pulp (87.6%). Arslan and Musa Ozcan (2008) also reported that the moisture content (dry weight basis) of fresh rosemary leaves was 13.2% and for dried leaves after 9 h of oven-drying, it was 0.04%. From these results, it shows that the moisture content removed from leaves depends on the method of drying, temperature and operation time used, as well as the biological nature of the plant itself.

4.2.2 Structure of Pandan leaves

Figure 4.1 shows scanning electron micrographs of both fresh ground Pandan leaves and those obtained after different drying pre-treatment prior to the extraction process. Scanning electron microscopy (SEM) is one of the effective methods that provide direct information concerning the size and the structure of leaves. All the leaves were ground for 30 s and a leaf size of $\approx 0.5-1.0 \text{ mm}^2$ was obtained prior to SEM measurement. Micrographs of fresh Pandan leaves are shown in the left column (Figures 4.1a, 4.1b and 4.1c), while micrographs of oven-dried and freeze-dried ground Pandan leaves are shown in the middle (Figures 4.1d, 4.1e and 4.1f) and right column (Figures 4.1g, 4.1h and 4.1i), respectively.



Fresh ground Pandan leaves

Oven-dried ground Pandan leaves

Freeze-dried ground Pandan leaves

Figure 4.1: Scanning electron micrographs of surface structure (top row), disrupted epidermal cell (middle row) and papillae (bottom row) of fresh (a-c), oven-dried (d-f) and freeze-dried (g-i) ground Pandan leaves.

These SEM studies show the comparison between the surface structure of Pandan leaves (Figure 4.1a, 4.1d, and 4.1g), structure of disrupted epidermis cell (Figure 4.1b, 4.1e and 4.1h) and structure of papillae (Figure 4.1c, 4.1f and 4.1i) of fresh, oven-dried and freeze-dried ground Pandan leaves, respectively. There are no differences observed in the structure of any of the Pandan leaves due to the drying pre-treatment. However, the pre-treatment involving grinding destroys the epidermis cells of the Pandan leaves, which makes the oil more accessible to the solvent used in the extraction process.

The drying pre-treatment has a greater effect on the structure of the papillae of Pandan leaves. The papillae, located on the lower epidermis cell, are a storage site for 2-AP (Wakte *et al.*, 2007; Nadaf *et al.*, 2006). The presence of papillae is the main indicator in distinguishing *Pandanus amaryllifolius* Roxb. from other *Pandanus* species (Wakte *et al.*, 2007). The size and shape of fresh (Figure 4.1c), oven-dried (Figure 4.1f) and freeze-dried (Figure 4.1i) Pandan leaf papillae are clearly shown. Similar to the study of Wakte *et al.* (2007), some papillae were found surrounding the stomata forming a necklace-like structure (Figure 4.1c). There are also two longer papillae over the stomatal pore.

One can see that the size of the oven-dried papillae (5.0 μm) is smaller (Figure 4.1f) than fresh (Figure 4.1c) and the freeze-dried papillae (Figure 4.1i). Oven-drying causes breakage and destruction of the cell walls including the papillae, and the reduction in size is due to the water being removed from the papillae during the drying operation. However, the papillae of freeze-dried leaves did not differ much from that of the fresh

sample with the sizes being 6.5 μm and 6.7 μm , respectively. This might be due to the solid state of water during freeze-drying, which may protect the primary structure and shape of the papillae (Aguilera *et al.*, 2003). These results are also in good agreement with Ratti (2001) who found the shrinkage of berries during freeze-drying to be minimal (5 to 15%).

4.2.3 Total yield of Pandan leaf extracts

The total yield of pre-treated Pandan leaves extracted by supercritical carbon dioxide and hexane extraction refers to the percentage ratio of the mass of extracted material to the mass of dried Pandan leaves used in the extraction process, as previously mentioned in section 3.1.7. The particle sizes of Pandan leaves were altered by grinding with the size of $\sim 0.5\text{-}1.0\text{ mm}^2$ and intact leaves without grinding ($\sim 5\text{ mm}^2$). Both particle sizes of Pandan leaves were then subjected to drying pre-treatment either using oven-drying or freeze-drying prior to the extraction process. The results obtained from the extracts of dried samples were compared to fresh extracts.

4.2.3.1 Supercritical carbon dioxide extraction

Figure 4.2 shows the effect of various pre-treatments on the total yield of Pandan leaves extracted by SC-CO₂. It clearly shows that a decrease of Pandan leaf size from approximately $\sim 5\text{ mm}^2$ (without grinding) to $\sim 0.5\text{-}1.0\text{ mm}^2$ (with grinding) resulted in a

50% increase in the yield of supercritical extracts from both the dried samples. The larger the surface area of the leaves, the easier the oil is accessible to the solvent. These results are in good agreement with the yields found in other systems, such as caffeine removal from green tea leaves (Park *et al.*, 2007) and extraction of coriander seeds (Grosso *et al.*, 2008), sesame seed (Doker *et al.*, 2010), silkworm pupae (Wei *et al.*, 2009) and valerion root (Zizovic *et al.*, 2007).

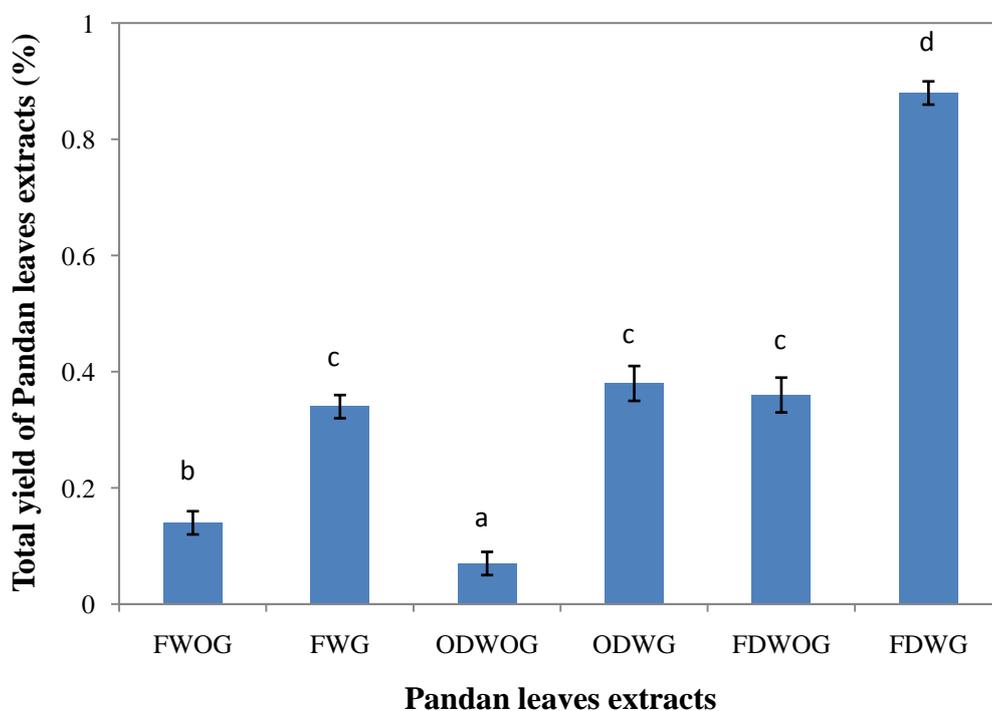


Figure 4.2: Effect of various pre-treatments on the total yield of Pandan leaf extracts by supercritical carbon dioxide extraction. Error bars represent the standard deviation of the means of the total yield of Pandan leaf extracts (n=3). Means with different letters are significantly different ($P<0.05$).

FWOG - fresh leaf extract without grinding pre-treatment; FWG - fresh leaf extract with grinding pre-treatment; ODWOG - oven-dried extract without grinding pre-treatment; ODWG - oven-dried extract with grinding pre-treatment; FDWOG - freeze-dried extract without grinding pre-treatment; FDWG - freeze-dried extract with grinding pre-treatment.

A significant ($P < 0.05$) relationships were observed among the drying pre-treatment and the yield of the extracts. In this study, the freeze-drying pre-treatment can significantly increase the Pandan leaf extracts yield up to 45% compared to fresh ones. A yield of $0.88 \pm 0.06\%$ was obtained from freeze-dried ground Pandan leaves extracted by SC-CO₂ extraction.

Ratti (2001) and Asami *et al.* (2003) found that freeze-drying treatment resulted in high extraction efficiency due to ice crystals forming within the plant matrix. These ice crystals can then rupture the structure allowing the solvent to have better access to the cellular components, and, consequently, better extraction. In contrast, no significant differences were noted for fresh and oven-dried ground Pandan leaf extracts.

The range of total yield of SC-CO₂ extraction from different pre-treated Pandan leaves was 0.18-0.88%. These results indicate that low yield was obtained by SC-CO₂ extraction and this finding is in agreement with Kotnik *et al.* (2007) who also found a low yield (3.81%) of supercritical extracts of chamomile flower heads. Interestingly, according to Donelian *et al.* (2008), and Porto *et al.* (2009) low yields of SC-CO₂ extraction are due to the high selectivity nature of this solvent, which offers the advantage that extracts obtained from supercritical extraction are devoid of unwanted compounds. Although modifiers, such as methanol can enhance the yield of extracts, as previously reported by Lang and Wai (2001), it will require high temperatures to reach

the supercritical state, which could be a disadvantage for thermally labile compounds, such as 2-AP. In addition, modifiers can also cause poor selectivity (Wang and Weller, 2006), which we tried to avoid in this study in order to obtain a solvent free product.

4.2.3.2 Hexane extraction

In order to study the influence of extraction time on the total yield, hexane extracts of fresh Pandan leaves with and without grinding pre-treatment were performed before the comparison of total yield extracts between fresh and dried samples were investigated. Hexane extraction was conducted at 4, 8 and 12 h. The hexane extraction yield of Pandan leaves, with and without grinding pre-treatment at different extraction time is reported in Table 4.1. The total yield of fresh ground Pandan leaves was compared with untreated ones. For both Pandan leaf extracts, with or without grinding pre-treatment, a significantly ($P < 0.05$) increased yield was obtained from 4 to 8 h of extraction. As can be observed, the grinding pre-treatment seems to have resulted in up to a 40% increase in yield compared to untreated ones. Grinding pre-treatment increased the surface area of the leaves and led to rupturing of a large number of epidermal cells, as previously observed in Figure 4.1b, therefore resulting in good oil recovery.

Table 4.1: Total yield of hexane extraction of fresh Pandan leaves (without and with grinding) at different extraction times.

Solvent extraction time (hour)	Total yield of Pandan leaf extracts (%)	
	Without grinding	With grinding
4	1.62 ±0.01 ^a	2.73±0.08 ^a
8	1.76±0.07 ^b	3.49±0.02 ^b
12	1.93±0.03 ^c	3.52±0.04 ^b

Values correspond to mean data, ± correspond to standard deviation (n=3). Means with different letters in the same column are significantly different ($P<0.05$).

As expected, prolonging the contact time increases the yield; the longer the extraction time, the more solvent can penetrate into the cells, and at the same time, increase the surface contact, which allows a greater quantity of compound to be extracted. Chan and Ismail (2009) reported a similar finding when extracting oil from kenaf seed. Although in the present study shows a significant increase in yield ($P<0.05$) with the extraction time, 4 h of extraction time was selected and used for further experiments after considering the oil yield, production cycle and run time.

As can be seen in Figure 4.3, the yield of Pandan leaves extracts was significantly affected by grinding and drying pre-treatment. For instance, the oven-dried extracts without grinding pre-treatment obtained the lowest yield ($1.18 \pm 0.04\%$) while freeze-dried ground samples obtained the highest ($5.23 \pm 0.22\%$) ones. In contrast, Arabhosseini *et al.* (2007) found that drying tarragon leaves at a low temperature of 45°C resulted in the smallest quality changes in terms of essential oil content when compared to fresh samples.

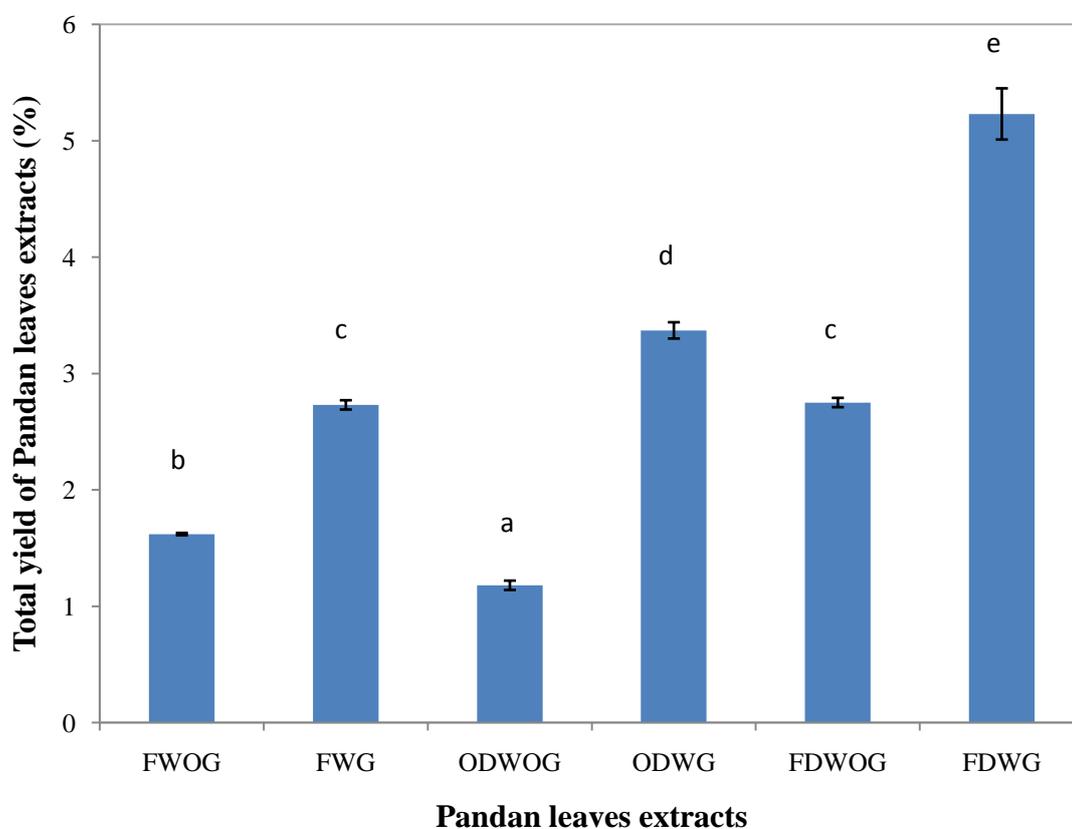


Figure 4.3: Effect of various pre-treatments on the total yield of Pandan extracts by hexane extraction. Error bars represent the standard deviation of the means of the total yield of Pandan leaf extracts (n=3). Means with different letters are significantly different ($P < 0.05$).

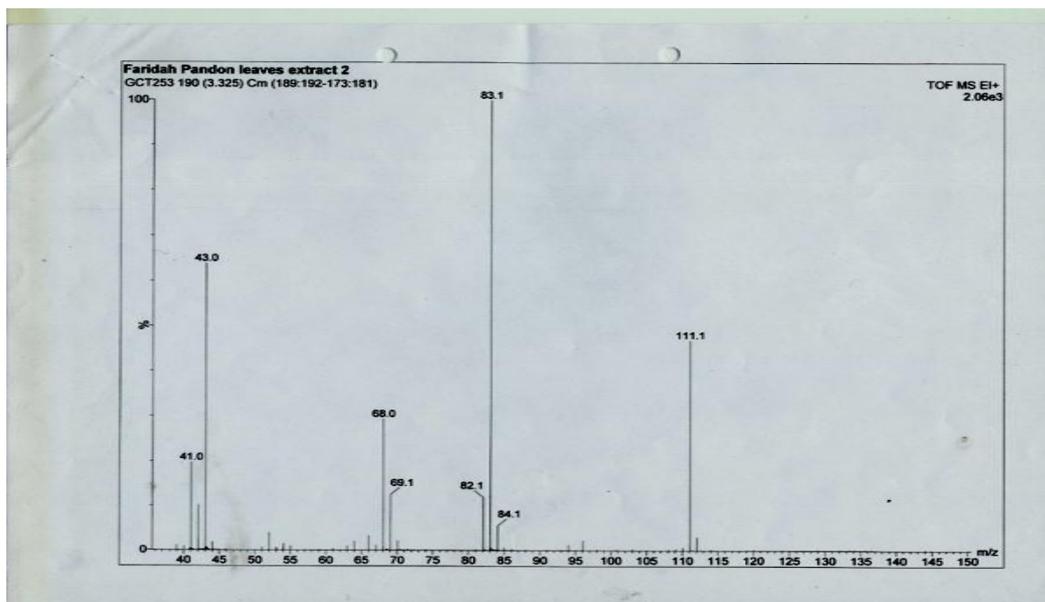
FWOG - fresh leaf extract without grinding pre-treatment; FWG - fresh leaf extract with grinding pre-treatment; ODWOG - oven-dried extract without grinding pre-treatment; ODWG - oven-dried extract with grinding pre-treatment; FDWOG - freeze-dried extract without grinding pre-treatment; FDWG - freeze-dried extract with grinding pre-treatment.

4.2.4 Concentration of 2-AP in Pandan leaf extracts

Identification of 2-AP isolated by SC-CO₂ and hexane extraction was based on the retention time data obtained and mass spectra. The peak of 2-AP was confirmed by mass spectral data of GC-MS with their mass of molecular ion at 41, 43, 68, 69, 82, 83 and 111 (Figure 4.4a). Identification results were compared with data presented in the literature (Buttery *et al.*, 1983; Laohakunjit and Noomhorm, 2004; Laksanalamai and Ilangantileke, 1993) and were found to correspond well.

When Pandan extracts were subjected to quantitative analysis by GC-FID, a similar pattern of component separation was achieved for all samples. Under these GC conditions, chromatographic peaks identified as 2-AP and TMP, which appeared at retention times of 2.79 and 3.65 min, respectively, without any interfering peak. Figure 4.5b shows one example of a GC chromatogram of Pandan leaf extracts. As explained previously, a number of volatile components exist in Pandan leaves (Laohakunjit and Noomhorm, 2004; Bhattacharjee *et al.*, 2005). However, detection of 2-AP was the major focus of the present work, and, therefore, other volatile compounds in the chromatogram were not taken into consideration.

a)



b)

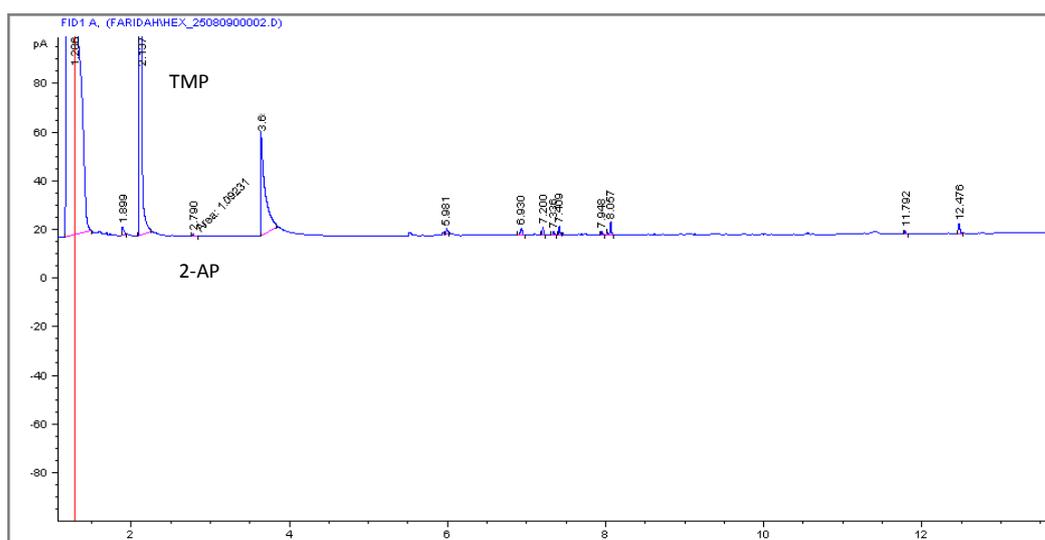


Figure 4.4: a) Mass spectrum of 2-AP peak; b) GC-FID chromatogram of the Pandan leaf compounds obtained by hexane extraction. 2-AP is 2-acetyl-1-pyrroline, TMP is 2, 4, 6-trimethylpyridine.

4.2.4.1 Supercritical carbon dioxide extraction

The concentration of 2-AP of pre-treated Pandan leaves by using SC-CO₂ extraction is presented in Figure 4.5. A low amount of 2-AP was quantified by GC-FID analysis, as a small 2-AP peak area was obtained when compared to other peaks. The amount of 2-AP in SC-CO₂ extracts was in the range of 0.04±0.01 to 0.18±0.01 ppm (Figure 4.5). These findings are lower than those reported by Laohakunjit and Noomhorm (2004) who studied Pandan leaf extracts using the same conditions of SC-CO₂ (20 MPa, 50 °C) with 20 min contact time for the leaves while 2 h extraction time was applied in this present work. The authors reported findings of 0.72 ppm of 2-AP in extracts of fresh Pandan leaves. A high value of 7.16 ppm of supercritical extracts was obtained by Bhattacharjee *et al.* (2005). However, these authors used a high pressure (45 MPa) for a longer time (3 h) and they were unable to identify the 2-AP by GC-MS. Although Wongpornchai *et al.* (2003) reported that the highest value of 2-AP concentration was obtained from fresh Pandan leaf extracts (10.26 ppm), they did not mention which extraction method was used.

Grinding pre-treatment increased the concentration of 2-AP in Pandan leaf extracts as more leaf cells are destroyed during the treatment resulting in a higher amount of extracts being obtained. However, no significant differences were noted between samples either with or without grinding pre-treatment. These results are not in good agreement with previous studies concerning the concentration of oleoresin in ginger (Balachandran *et al.*, 2006), Spanish essential oil (Langa *et al.*, 2009) and Turkish

lavender flowers (Adasoglu *et al.*, 1994) by supercritical extraction. The 2-AP concentration of supercritical carbon dioxide extracts was not significantly affected by the reduction of Pandan leaf particle size from approximately $\sim 5 \text{ mm}^2$ to $\sim 0.5\text{-}1.0 \text{ mm}^2$ by grinding.

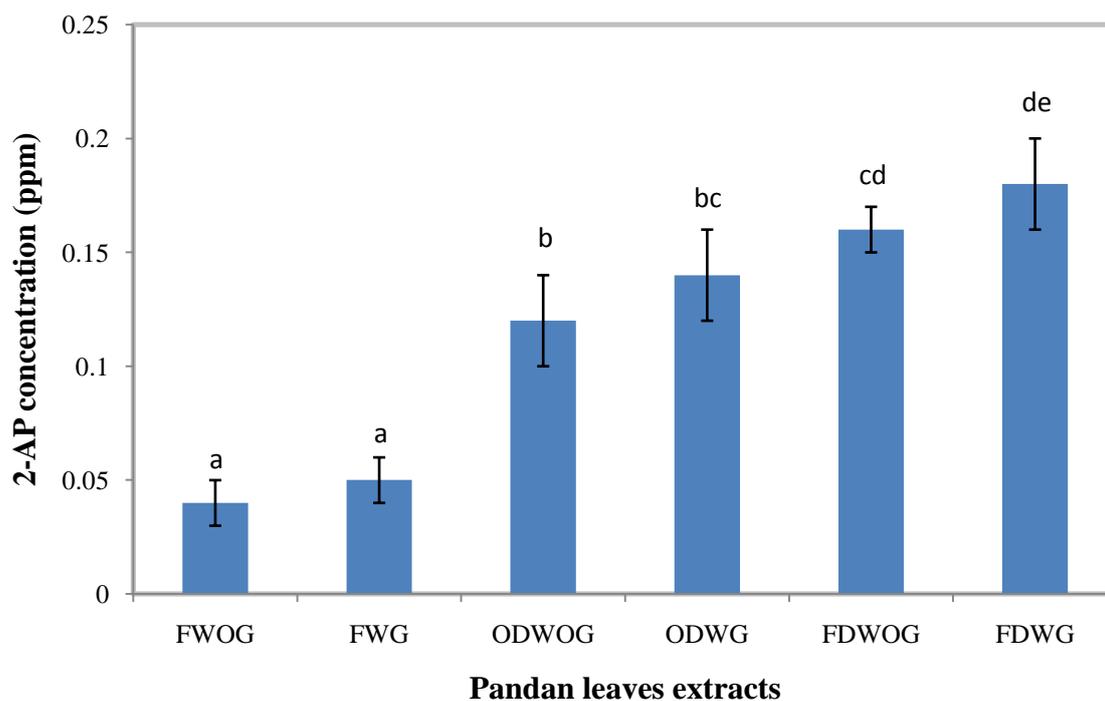


Figure 4.5: Effect of various pre-treatments on the 2-AP concentration in Pandan extracts by supercritical carbon dioxide extraction. Error bars represent the standard deviation of the means of the 2-AP concentration (n=3). Means with different letters are significantly different ($P < 0.05$).

FWOG - fresh leaf extract without grinding pre-treatment; FWG - fresh leaf extract with grinding pre-treatment; ODWOG - oven-dried extract without grinding pre-treatment; ODWG - oven-dried extract with grinding pre-treatment; FDWOG - freeze-dried extract without grinding pre-treatment; FDWG - freeze-dried extract with grinding pre-treatment.

Drying pre-treatments were found to significantly ($P < 0.05$) influence the 2-AP concentration when SC-CO₂ extraction was used. As can be seen in Figure 4.5, Pandan leaf extracts from dried samples obtained higher 2-AP concentration compared to fresh

extracts. Freeze-dried samples of supercritical extracts had the highest 2-AP concentration (0.16 ± 0.04 to 0.19 ± 0.01 ppm) followed by oven-dried (0.12 ± 0.01 to 0.14 ± 0.02 ppm) and then fresh extracts (0.04 ± 0.01 to 0.05 ± 0.01 ppm), which means that an increase of up to four times of 2-AP concentration was obtained from freeze-dried samples isolated by supercritical extracts compared to intact ones.

These results can be explained by damaging Pandan leaf cells during drying pre-treatment, which resulted in the easy extraction of 2-AP by supercritical extraction, and is in good agreement with the essential oil obtained from dried Rosemary leaves, as studied by Pourmortazavi and Hajimirsadeghi (2007). These results also suggest that freeze-drying is the best pre-treatment for SC-CO₂ extraction due to requiring the mildest temperature being applied in this drying treatment. Therefore, less aroma loss is expected with this pre-treatment. These findings are also in line with those of Kaminski *et al.* (1986) who found that the percentage loss of carrot volatiles was lower by freeze-drying (69%) compared to other drying treatments such as by hot air drying (75%) and microwave hot air drying (84%). Houpalathi *et al.* (1985) also reported that the reduction of the flavour extracts after the drying of dill herb was lower from freeze-drying (3.9 times) than air-drying (11.2 times).

Similar results were obtained by Venskutonis (1997), who found that the amount of β -caryophyllenein in oven-dried thyme at 30 °C and in the freeze-dried herb increased by 29% and 37%, respectively. Chin *et al.* (2008) also found that most volatile components of fresh durian pulp decreased when freeze-drying was used (71.5 to 97.2%) but that

these values were lower compared to the 98% to 99% amount of volatiles lost from spray dried durian pulp. Even though the freeze-drying technique required high cost, the results obtained provide the advantage of this drying pre-treatment, especially in the extraction of natural materials like Pandan leaves.

4.2.4.2 Hexane extraction

Figure 4.6 shows the effect of solvent extraction time on the concentration of 2-AP samples (with and without grinding) of hexane extracts and one can see that when the grinding pre-treatment was used, there is a significant increase of 60% on the 2-AP concentration compared to extracts from the leaves without grinding pre-treatment (Figure 4.6). At the beginning of extraction, the concentration of 2-AP increased sharply but then continued to increase slowly until the end of extraction. During the early stage of extraction, 2-AP is easily accessible to the hexane, especially at the outer surface of the leaves or in broken cells. By increasing the extraction time, most of the easily accessible 2-AP is removed. Therefore, at this period of time, the rate of extraction depends on the diffusion from the interior to the surface of the leaves (Han *et al.*, 2009). The results showed a slow increase of 2-AP concentration when the extraction time was prolonged. This study also indicated that the longer the extraction time, the higher the 2-AP concentration obtained. The concentration of 2-AP from extracted fresh Pandan leaves (without and with grinding) at 4, 8 and 12 h of hexane extraction time were within the range of 0.21 ± 0.01 to 0.33 ± 0.03 ppm, 0.28 ± 0.02 to 0.44 ± 0.01 ppm and 0.34 ± 0.02 to 0.52 ± 0.02 ppm, respectively.

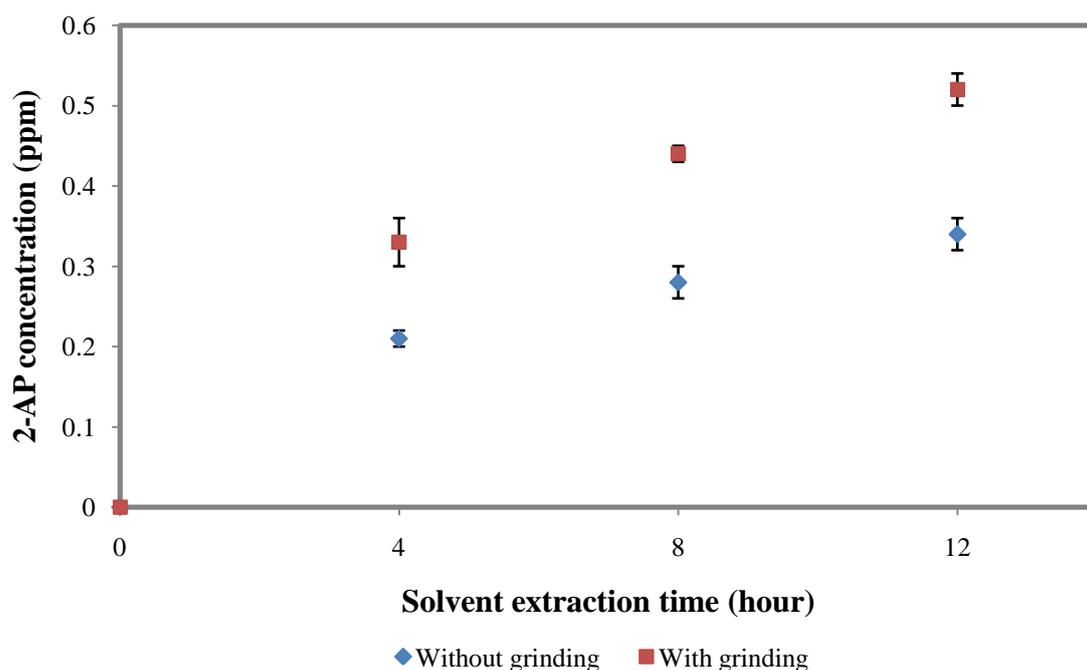


Figure 4.6: Effect of extraction time on the 2-AP concentration of fresh hexane extract samples (with and without grinding). Error bars represent the standard deviation of the means of the 2-AP concentration (n=3).

Figure 4.7 shows the effect of various pre-treatment on the concentration of 2-AP in Pandan leaf extracts by hexane. The concentrations of 2-AP were significantly ($P < 0.05$) difference among pre-treatments. A range of 0.06 ± 0.01 to 0.45 ± 0.01 ppm of 2-AP was obtained from hexane extracts. This amount was 6 times lower than that obtained by Laohakunjit and Noomhorm (2004), who used ethanol extraction and reached about 2.77 ppm of 2-AP in their Pandan leaf extracts. The quantity of extracted component depends on its stability in the solvent used. The stability of 2-AP is low in hexane. These findings are in good agreement with the stability studies by Rungsardthong and Noomhorm (2005). The authors found that after 14 days storage, 92% of 2-AP remained present in ethanol compared to only 40% in hexane. Apart from growth conditions, it should also be considered that the 2-AP level differs according to the species, variety and origin of

the plant, which indicates that it is under genetic control (Laohakunjit and Kendchoechuen, 2006; Arslan and Musa Ozcan, 2008).

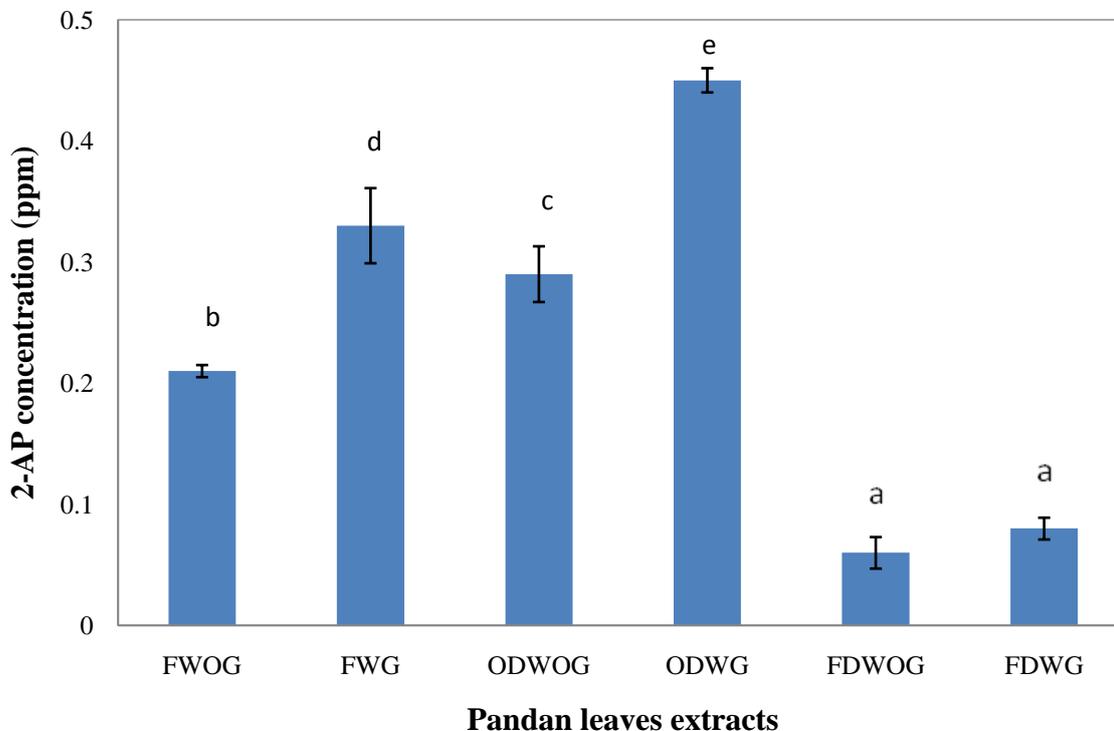


Figure 4.7: Effect of various pre-treatments on the 2-AP concentration in Pandan extracts by hexane extraction. Error bars represent the standard deviation of the means of the 2-AP concentration (n=3). Means with different letters are significantly different ($P<0.05$).

FWOG - fresh leaf extract without grinding pre-treatment; FWG - fresh leaf extract with grinding pre-treatment; ODWOG - oven-dried extract without grinding pre-treatment; ODWG - oven-dried extract with grinding pre-treatment; FDWOG - freeze-dried extract without grinding pre-treatment; FDWG - freeze-dried extract with grinding pre-treatment.

The low concentration of 2-AP of freeze-dried Pandan leaves extracted by hexane was obtained, and no significant difference was observed between without and with grinding pre-treatment samples as illustrated in Figure 4.7, while the ground oven-dried sample showed the highest amount of 2-AP (0.45 ± 0.01 ppm) compared to others. Oven-drying

is another possible alternative pre-treatment for the extraction of volatile compounds, such as 2-AP, especially through solvent extraction. This might be due to the low oven temperature (30 °C) used. These results are similar to the previous study conducted by Venskutonis (1997) who stated that oven-drying at 30 °C did not deplete volatiles in sage (*Salvia officinalis* L.) and thyme (*Thymus vulgaris* L.). Diaz-Maroto *et al.* (2002) also found that oven-drying at 45 °C was better at preserving the sensory characteristics of bay leaf and closest to the intact samples.

4.2.5 Colour of Pandan leaf extracts

In order to evaluate the effect of the extraction method on the colour, extracts of ground Pandan leaves were selected and a comparison was made between fresh and dried samples in both SC-CO₂ and hexane extraction, as illustrated in Figure 4.8 and 4.9, respectively.

4.2.5.1 Supercritical carbon dioxide extraction

As can be seen in Figure 4.8, the grinding pre-treatment gave light yellowish extracts when SC-CO₂ was used. The physical appearance of the SC-CO₂ extracts depends on the drying pre-treatment and, interestingly, it was observed that the pure standard 2-AP (Bhattacharjee *et al.*, 2005) is similar in colour to the freeze-dried sample. These results are in agreement with Laohakunjit and Noomhorm (2004) who found that SC-CO₂ had

superior quality extracts. The yellow colour of extracts is due to the oxidation of carotenoid pigments since carotenoid is soluble while chlorophyll is very insoluble in this solvent (Moyler, 1983). A light yellow colour was also found when Pandan leaf extracts with a chloroform-methanol mixture were loaded on dry column chromatography as well as on the thin layer chromatography (Teng *et al.*, 1979).



Figure 4.8: Changes in colour of supercritical carbon dioxide extracts. Fresh, oven-dried and freeze-dried ground Pandan leaf extracts are on the left, middle and right of the column, respectively.

4.2.5.2 Hexane extraction

From a qualitative point of view, all Pandan leaf extracts obtained by hexane extraction are dark green in colour, which is probably due to the large amount of chlorophyll pigments (Figure 4.9). From these observations, it can be suggested that the colour of the extracts did not depend on the different drying pre-treatments but were significantly influenced by the different extraction methods. Thus, the extracts using the hexane

extraction method contained more undesirable impurities compared to the SC-CO₂ extracts. These observations are supported by Quispe-Condori *et al.* (2005) who reported that organic solvent extraction contains a large amount of contaminants, such as chlorophyll, which decrease the extract purification yield. The darkness of extracts can be the result of the high molecular weight of the compounds that are extracted in solvent extraction.



Figure 4.9: Changes in colour of hexane extracts. Fresh, oven-dried and freeze-dried ground Pandan leaf extracts are on the left, middle and right of the column, respectively.

Chapter 5

Absorption of 2-acetyl-1-pyrroline during cooking of rice mixed with Pandan leaves

5.1 Introduction

Rice (*Oryza sativa* L.) is a staple food for about half of the population in the world. The cooking process and the choice of cooked rice texture differ from place to place. For example, consumers in Western countries prefer long grain, fluffy or slightly dry individual kernels of rice having cooked flavour by cooking in excess water whereas most Asian people's preference is for medium grain with fluffy, light individual kernels of rice with cooked flavour and without a hard core from optimal water cooking (Das *et al.*, 2006).

Aroma is one of the most important cooked rice sensory characteristics and is a result of the combination of hundreds of compounds (Widjaja *et al.*, 1996; Grimm *et al.*, 2001).

Aromatic rice is considerably more expensive than common rice (Buttery *et al.*, 1983). The chemical compound that contributes mostly to the aroma profile of cooked aromatic rice; 2-AP can be found to occur naturally in Pandan leaves, as reported by many researchers (Buttery *et al.*, 1983; Bhattacharjee *et al.*, 2005; Wakte *et al.*, 2007) and as proved by our previous experiments through supercritical carbon dioxide and hexane extraction (Yahya *et al.*, 2010). The details of which were discussed in Chapter 4.

Cooking rice is essentially a reaction of starch with elevated temperature (Kasai *et al.*, 2007) whereas the method of cooking affects the texture and physical appearance of cooked rice (Leelayuthsoontorn and Thipayarat, 2006), which is probably induced by water diffusion during starch gelatinization (Srikaeo *et al.*, 2006; Kasai *et al.*, 2007). The rice starch microstructure changes mainly contribute to the flavour retention of cooked rice through the interaction between starch and volatile flavour compounds (Srikaeo *et al.*, 2006; Porrarud and Pranee, 2010).

In the normal cooking of rice, the amount of volatile compounds would mostly be lost (Buttery *et al.*, 1983). A few studies have been published to improve the quality of the cooked rice flavour. For instance, by blending 5-10% aromatic rice with non-aromatic cultivars, as reported by Itani and Yamazaki (1994), and coating brown rice with its flour. In addition, the adding of fresh Pandan leaves during cooking is an old practice in South-East Asian countries to enrich the flavour profile of non-aromatic rice. However, there has been little work done on how the phenomena take place during cooking or how the mechanism that occur corresponds to the absorption of 2-AP by the rice grains.

Therefore, the aim of this study is to investigate the phenomena occurring during the cooking of rice and the absorption of 2-AP by rice grains when incorporating fresh Pandan leaves in the cooking process under excess and optimal water conditions.

5.2 Results and Discussion

5.2.1 Partition coefficient of 2-AP

The partition coefficient of 2-AP in water/air system was measured at 25°C by the Phase Ratio Variation (PRV) method. This method allows the measurement of water/air partition coefficient of 2-AP without the need for direct measurement of the concentration of 2-AP in either the liquid phase (water) or the gas phase (air), by determining the gas chromatographic peak area of 2-AP from both phases. The headspace of the aqueous solution was analysed from closed vials with different phase ratios. Linear regression analysis was performed according to equation (16) where $y=A^{-1}$ and $x=\beta$ (section 3.2.2.1). The water/air partition coefficient of 2-AP is representative of the volatility of 2-AP in water. The behaviour of volatile compounds in water can be explained by their physicochemical properties, such as vapour pressure, solubility in water and molecular weight. Table 5.1 shows the gas chromatographic peak area, the results of linear regression analysis and the calculation of the partition coefficient value for 2-AP in the water/air system. As one can see in Figure 5.1, the increase in phase ratios increased the reciprocal of the chromatographic peak area of 2-AP. As expected, an increase in the volume of liquid in the headspace vial (V_p) increased the GC peak area of 2-AP (A).

Table 5.1 Data for aqueous solution of 2-acetyl-1-pyrroline in water/air system for 24 hours.

Temperature, (°C)	Volume of liquid in headspace vial (ml)	Phase ratio, β	Peak area, A	Intercept, a	Slope, b	Correlation coefficient, R^2	Partition coefficient, K
25	2	9.00	0.265	0.362	0.398	0.978	0.909
	3	5.67	0.350				
	5	3.00	0.630				
	7	1.86	1.125				

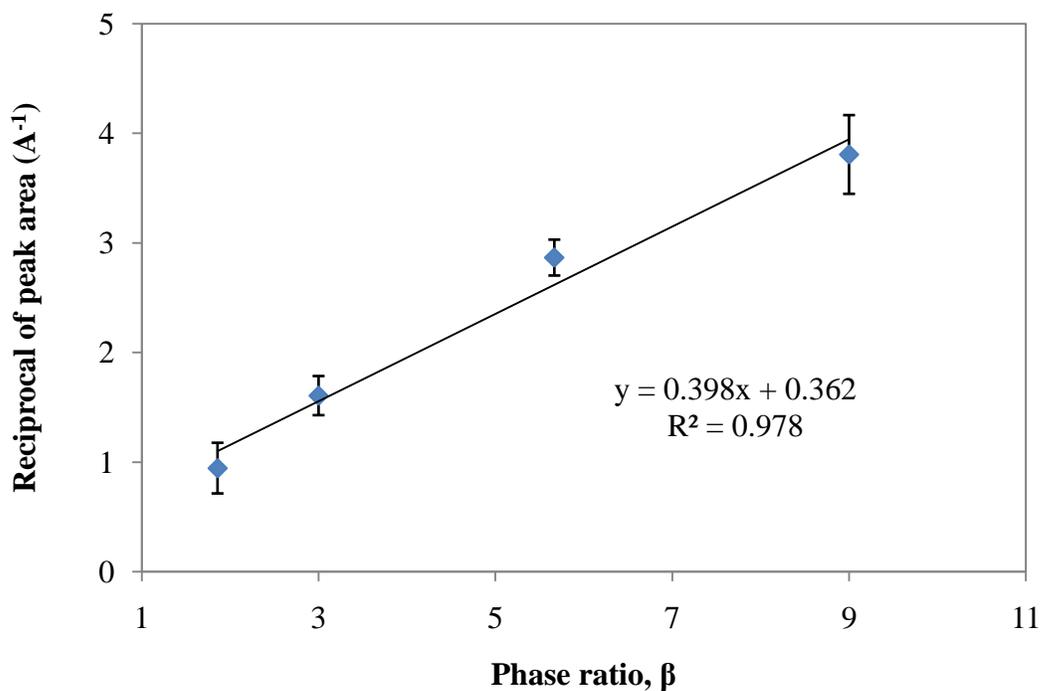


Figure 5.1: Reciprocal of gas chromatographic peak area (A^{-1}) versus phase volume ratio (β) for 2-acetyl-1-pyrroline in water/air system at 25°C for 24 hours.

These results are in good agreement with Tehrany *et al.* (2007) who found that an increase in the phase ratios increased the reciprocal of the chromatographic peak area of ethyl acetate for water, 10% ethanol, 3% acetic acid and air. The partition coefficient of 2-AP between water and air at 25°C was 0.909. According to Bakierowska and Trzeszczynski (2003), 2-AP has a higher partition coefficient compared to Cyclopentene (0.567) and Cyclohexene (0.836) but lower than Toluene (4.048) and Benzene (4.444). This result indicates that at 25°C, the 2-AP compound partitions more into air than into water. However, it has a low water/air partition coefficient when compared to other aromatic hydrocarbons.

5.2.2 Excess water cooking

5.2.2.1 Temperature and moisture content of rice during cooking

The temperature and moisture content during the cooking of rice grains were determined in order to understand the mechanism of water absorption by rice starch and how it corresponds to the absorption of 2-AP compound. The temperature and moisture content of rice grains during cooking under excess water conditions is presented in Figure 5.2. Before cooking started, the temperature of boiling water in an aluminium cylinder was measured as 100 °C by placing the cylinder on the hot plate. As expected, a slight decrease in temperature was observed when rice and Pandan leaves were added to the system, even though the mass of rice was significantly smaller when compared to the mass of the water. In order to keep the temperature of the system maintained at 100 °C, the heating scale of the hot plate was adjusted until the temperature of the system reached boiling point. After 2-3 min the rice and Pandan leaves were added, the temperature remained constant until the end of 20 min. Aluminium foil was used to cover the cylinder to prevent heat loss during the cooking process.

As presented in Figure 5.2, the initial moisture content obtained for long grain white rice was $11.82 \pm 0.56\%$ (wet basis), which is in agreement with the values found in the literature (Tulyathan *et al.*, 2008; Grimm *et al.*, 2001; Leelayuthsoontorn and Thipayarat, 2006; Chen, 2003; Das *et al.*, 2006). As expected, the moisture content of rice grains increased with cooking time. A sharp increase in the moisture content was observed at the beginning

of the process (5 min of cooking). Since rice is a dried matter and a high temperature of water was applied to the system, diffusion occurs in a short time resulting in the quick absorption of water to the rice grains.

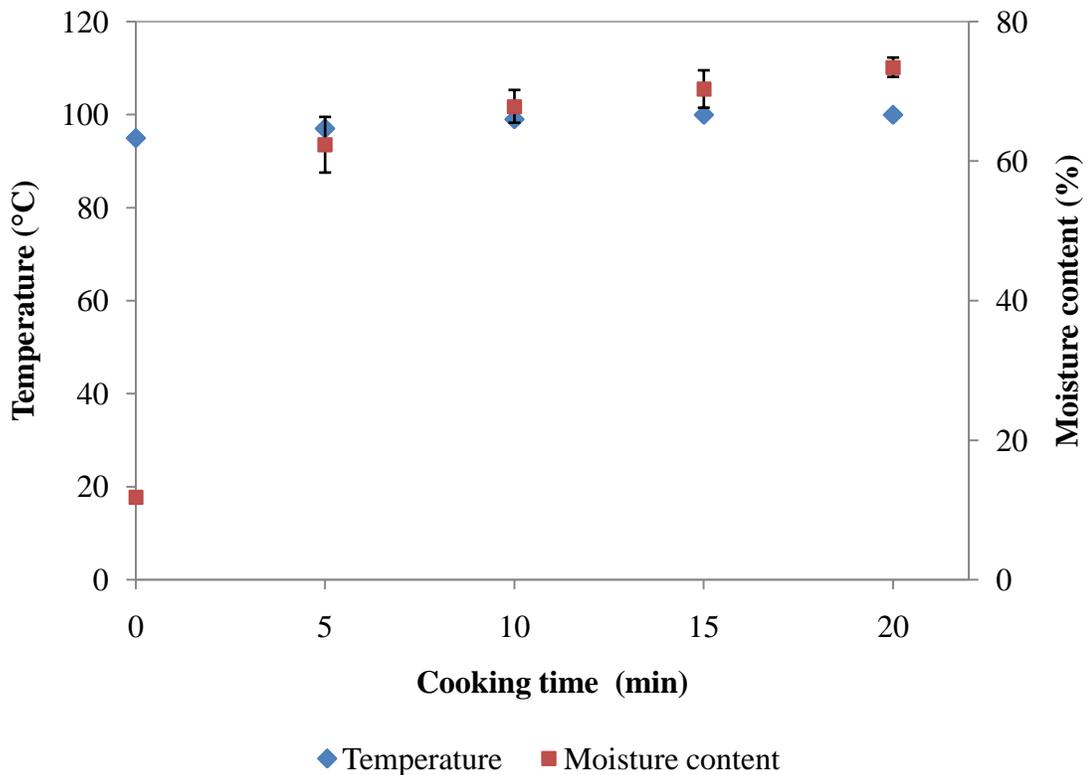


Figure 5.2: Effect of cooking time on the temperature and moisture content of rice grains during cooking under excess water conditions.

Figure 5.2 also illustrates that a slow increase of rice moisture content started at 10 min and reached a plateau by prolonging the cooking time to 20 min. These results suggest that the equilibrium moisture content of long grain rice cooked with excess water is obtained after

10 min of cooking. At this cooking time, water diffusion was promoted and the distribution of water in the rice grains became homogeneous with a small change of moisture content in the grains. The results are in good agreement with previous literature for a similar system (Kasai *et al.*, 2005).

During cooking, the absorption of water occurs from outside to the inside of rice grains. As the boiling temperature is applied continuously in the excess water system, water interacts with starch, which results in the hydrogen bonds between the rice starch molecules being replaced by the hydrogen bonds of the starch and water molecules (Kasai *et al.*, 2005). This interaction induces structural changes in whole rice grains.

Under excess water conditions, rice grains cooked for 20 min can be defined as fully cooked rice, as the soft texture of the grains was observed and was in good agreement with the moisture content value (73.5%) obtained at this point of cooking time. This finding is also in line with previous literature (Leelayuthsoontorn and Thipayarat, 2006; Das *et al.*, 2006). The long grain white rice took 20 min for cooking, and, during the process, the moisture content increased from 11.8% to 73.5%. However, cooked rice still remains separate without aggregating with others and is less sticky when touched by hand, which is probably due to the use of long grain rice with a high amount of amylose.

5.2.2.2 Absorption of 2-AP during cooking of rice

Absorption of 2-AP during the cooking of rice was analysed by static headspace at 100 °C for 30 min and GC-FID analysis. Rice was cooked in excess water conditions mixed with fresh Pandan leaves. Pandan leaves were used as a source of 2-AP. In Figure 5.3, the plot of 2-AP absorption by rice versus cooking time is shown. The concentration of 2-AP in the rice was calculated as a ratio of the GC peak area of 2-AP with the GC peak area of the internal standard, TMP per dry mass of the rice. The initial amount of 2-AP in raw rice was 0.32 µg/g. After 5 min of cooking (temperature of water was 100°C at this point), the amount of 2-AP appeared to be lower than its initial value. However, as can be seen, the amount of 2-AP increased after a further 10 minutes and remained relatively unchanged at 15 min until the end of cooking.

The changes in the amount of 2-AP in cooked rice could be mainly contributed by the temperature and moisture content of rice as well as the changes of starch microstructure during the cooking process (Srikaeo *et al.*, 2006). The perception of 2-AP absorption by rice grains can be defined as the interaction between the 2-AP compound and the starch molecules, especially with the linear fraction of amylose. After cooking rice in boiling excess water for five minutes, a moisture content of 61% was obtained. At this cooking time, rice granules started to swell, which promotes the melting of crystallites as well as the disruption of the structure of the granules, hence amylose leached out from the granules (Fredriksson *et al.*, 1998; Leelayuthsoontorn and Thipayarat, 2006). However, at this point

of cooking, we can assume that the leaching process had only just begun. Therefore, no or little interaction between the 2-AP and amylose molecules was occurring in the grains.

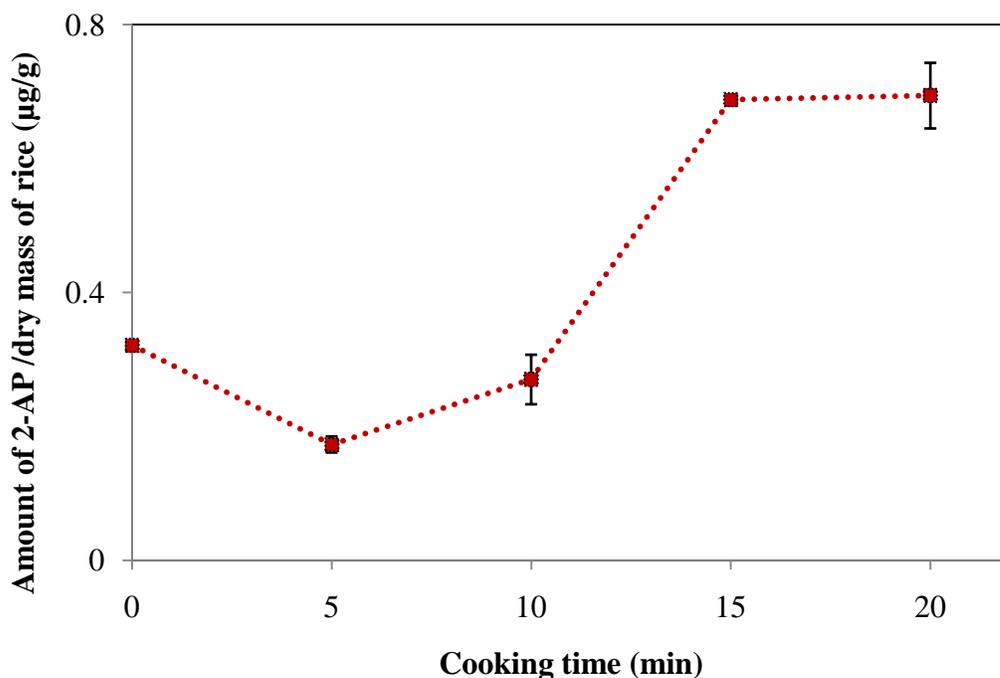


Figure 5.3: Effect of cooking time on the 2-AP absorption by rice grains cooked with Pandan leaves under excess water conditions.

At the same cooking time, the molecules of 2-AP compound started to diffuse slowly from the tissue cells of the Pandan leaves to the water. Water acted as a transporter in transferring the 2-AP from the Pandan leaves to the rice grains, and, therefore, may promote the interaction between 2-AP molecules with the starch. A decrease in the 2-AP amount might be due to the release of the existence of 2-AP from the inside to the outer surface of the rice grains during the disruption of the structure of the starch granules. It can also be explained by the high temperature being applied to the system.

However, with continuous cooking for another five minutes, an increase in the amount of 2-AP in the cooked rice was obtained as more amylose diffused from the swollen granules. The amylose formed three-dimensional networks on the surface of the starch granules, which may entrap or bind with the 2-AP molecules (Porrarud and Pranee, 2010). Due to the hydrophilic characteristics of 2-AP, interaction between the amylose and 2-AP molecules may occur probably through the hydrogen bond, which is similar to the interaction of the starch-water molecules.

At 15 and 20 min of cooking, the absorption of 2-AP appeared to remain constant (0.7 $\mu\text{g/g}$ of dry rice mass), which might correlate to the constant value of moisture content (70%) obtained within these periods of cooking. At this stage, we can assume that the maximum absorption of 2-AP from the Pandan leaves by rice grains had occurred as the ceiling moisture content of gelatinized starch had been reached. These results suggest that the absorption of 2-AP by rice grains under excess water conditions was controlled by the leaching out of the amylose from the granules due to the melting of the crystallite structure during the gelatinization of rice starch.

It is interesting to note that the incorporation of Pandan leaves in the excess water cooking of rice could significantly contribute to the 2-AP absorption, and as a result may enrich the flavour of cooked long grain white rice. This is in good agreement with Buttery *et al.* (1983) who found that the concentration of 2-AP in Pandan leaves is more than 100 times

that obtained in common rice. Therefore, the results obtained in this study provide good evidence for the sufficient transfer of the 2-AP compound from Pandan leaves to rice grains in order to impart the aromatic rice aroma to the common rice. This finding also revealed that rice cooked with Pandan leaves was detected to be higher in flavour profile when compared to the raw rice even though 2-AP is not the only compound responsible for the cooked rice aroma, as previously reported by Widjaja *et al.* (1996).

5.2.2.3 Amount of 2-AP in cooking water

The effect of cooking time on the 2-AP amount in water is presented in Figure 5.4. As can be seen, the 2-AP content in water increased smoothly with cooking time. This was expected as 2-AP is a hydrophilic compound with high solubility in water. The boiling of excess water promotes the release of 2-AP from Pandan leaves, which then transfers the 2-AP molecules to the water. The temperature of the water significantly affects the interaction between 2-AP and water molecules, which, consequently, influences the partition behaviour of 2-AP. The hydroxyl group of 2-AP molecules may be capable of binding with water molecules through the hydrogen bond (Boutboul *et al.*, 2002). In addition, the gelatinization process of rice starch, which was found to occur after as little as five minutes of cooking, was accompanied by leaching out of some amylose to the water, which also contributed to the increase in the amount of 2-AP in the water.

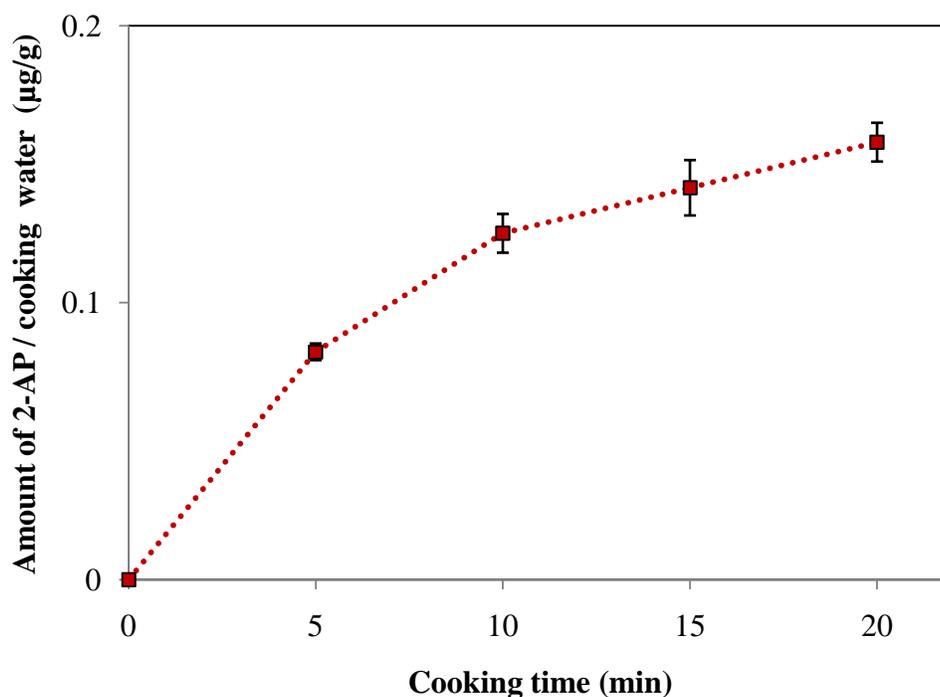


Figure 5.4: Effect of cooking time on the amount of 2-AP in the water during the cooking of rice mixed with Pandan leaves under excess water conditions.

By prolonging the cooking time from 15 to 20 min, a slow increase of 2-AP in water was observed. This may be because most 2-AP molecules have already bound with water molecules in the covered cooking system. However, due to the boiling temperature, some water molecules as well as the 2-AP compound evaporated into the air. The use of aluminium foil only seems to control the temperature of boiling but does not completely avoid the loss of aroma during the cooking process as the cylinder used was not tightly sealed with the foil.

5.2.2.4 Release of 2-AP from Pandan leaves

A decrease in the amount of 2-AP in Pandan leaves was expected to occur during cooking with rice in excess water. The initial amount of 2-AP in Pandan leaves was 0.908 $\mu\text{g/g}$. The release of 2-AP from Pandan leaves was calculated as the percentage of differences between the initial amount of 2-AP and the amount 2-AP for each cooking time, was then divided by the initial amount of 2-AP in Pandan leaves. The plot of the percentage of 2-AP release from Pandan leaves against cooking time as illustrated in Figure 5.5. There was a rapid increase in the release of 2-AP (74%) at the beginning of the cooking process, while during the prolonged cooking time, a slight increase in the release of 2-AP was observed until the cooking ended at 20 min.

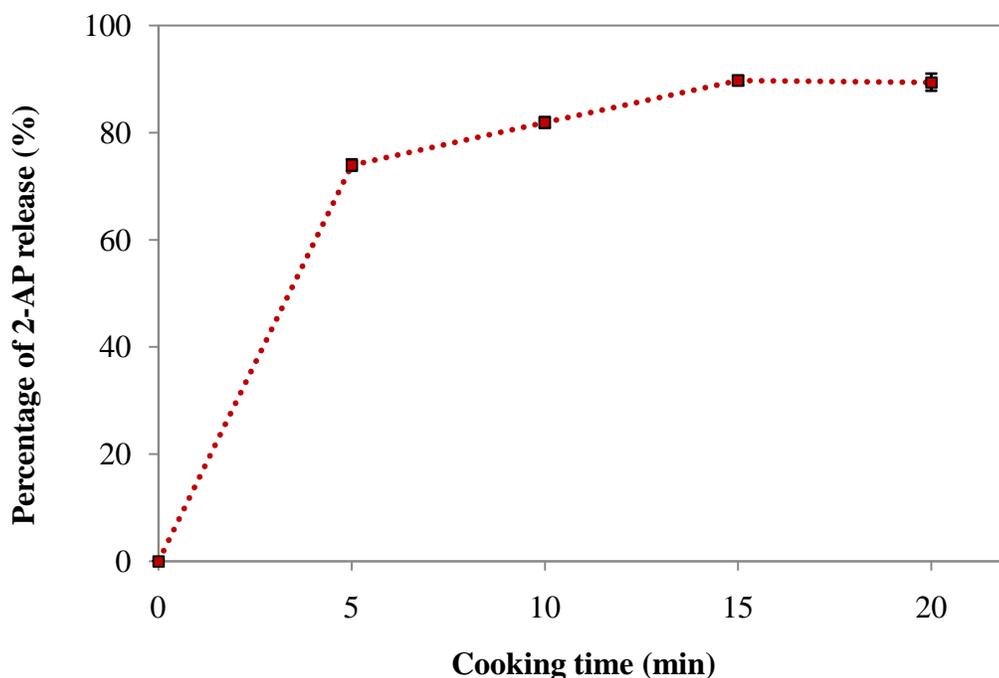


Figure 5.5: Effect of cooking time on the release of 2-AP from Pandan leaves during the cooking of rice mixed with Pandan leaves under excess water cooking conditions.

The release rate of 2-AP from Pandan leaves decreased with cooking time. The high temperature of 100 °C significantly influences the release of 2-AP in the excess water system. This can be explained by the damage of the Pandan leaf structure, which promotes the extraction of 2-AP and moves out from its storage site in the Pandan leaves, known as the papillae (Figure 4.1), to the water and the rice grains. It can be assumed that some 2-AP evaporates directly to the air.

After cooking for 20 min, the release of 2-AP from Pandan leaves only reached 90%. This might be due to the distribution and contact of Pandan leaves with water, which is not homogenous for each individual piece of Pandan leaf as they were tied together in the muslin gauze to form a pouch. Therefore, the 2-AP of the Pandan leaves located in the centre of the pouch still remain inside the papillae of the leaves with no or little disturbance from the high temperature of the system.

5.2.3 Optimal water cooking

5.2.3.1 Temperature and moisture content of rice during cooking

As clearly shown in Figure 5.6, the temperature of the water and moisture content of rice under optimal conditions were measured and appeared to smoothly increase with cooking time. The trend of temperature increase was expected as an electric rice cooker was used

and automatic heating was provided. The cooking system was started by switching on the electricity power of an open rice cooker. When rice and Pandan leaves were added to the cooker and filled with tap water, the temperature of the sample was measured as 25°C. The adding of rice and Pandan leaves to the cooker did not affect the temperature of the initial water used as heating had only just been applied to the system at that time.

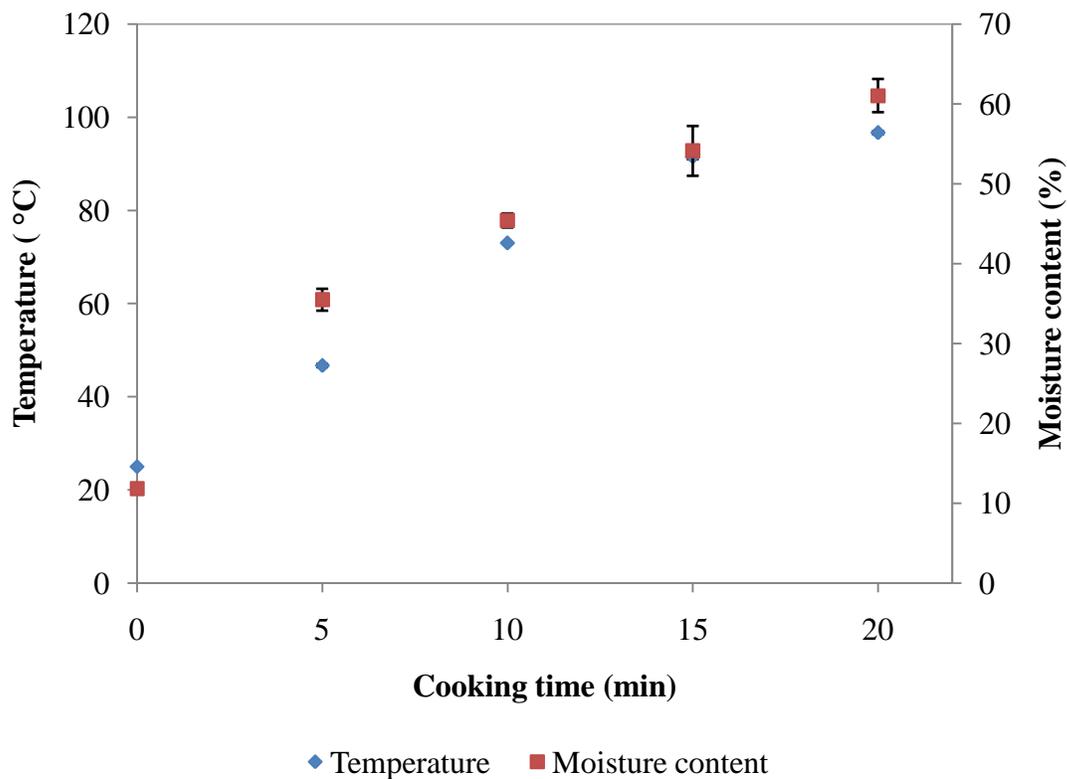


Figure 5.6: Effect of cooking time on the temperature and moisture content of rice grains during cooking under optimal water conditions.

After cooking for five minutes, the temperature of the system was twice as high compared to that observed at the beginning of cooking. This can be explained by the use of an automatic rice cooker in which the heat is regulated by a thermostat (Das *et al.*, 2006).

After prolonging the cooking time for a further five minutes, the temperature had increased to 73°C. However, this temperature only contributed to a slight increase in the moisture content of rice grains. Interestingly at 15 min of cooking, the temperature had gradually increased to 92°C with more bubbles forming on the surface of the water in the rice cooker, as shown in Figure 5.7c.

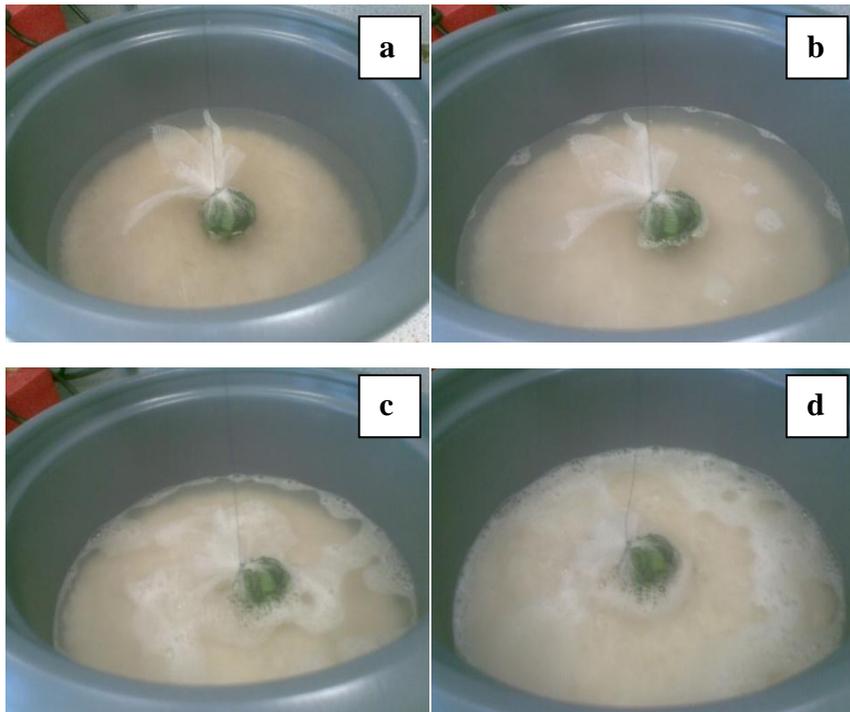


Figure 5.7: Rice cooked with fresh Pandan leaves at a) 5 minutes, b) 10 minutes, c) 15 minutes and d) 20 minutes of cooking under optimal water conditions using an open electric rice cooker.

After cooking for 20 minutes, the long rice grains seemed to begin to absorb more water and swell slightly, even though the sample temperature was approximately 96.7°C; an average moisture content of 61.1% was obtained. These findings can be explained by the

fact that rice grains become coated with a gelatinized layer of unstable crystallites of starch, which probably reduces the heat and mass transfer into the grain, resulting in half-boiled rice. Therefore, at this cooking time, the equilibrium state of moisture had still not been reached. Based on observation, bubbles were distributed across most of the surface area of the water in the system (Figure 5.7d) and more vapour began to evaporate into the air. However, there was still water present on top of rice. This result also revealed that 20 min of cooking is not enough to cook long grain rice under optimal water conditions as the moisture content obtained was less than 73%; the value considered as being the optimum moisture content used to examine fully cooked rice samples, as reported by Das *et al.* (2006).

This finding was expected, as the normal practice for optimal water cooking in Asian countries is carried out for 40 min using a closed rice cooker. Therefore, at this stage we could not conclude that the adding of Pandan leaves did not contribute in flavour enrichment of cooked rice, as the process of optimal water cooking had still not been completed. Further investigation on this purpose is described in details in section 5.2.4.

5.2.3.2 Absorption of 2-AP during cooking of rice

Table 5.2 shows the amount of 2-AP absorbed by rice cooked with Pandan leaves in optimal water conditions at different cooking times. Significant effects ($P < 0.05$) of the

cooking time were observed on the amount of 2-AP in rice during cooking. However, the profile obtained for the absorption of 2-AP was not a smooth increase. After five minutes of cooking, an increase of 2-AP absorption was obtained. This can be explained by the diffusion of 2-AP from Pandan leaves to the rice grains. It was assumed that the 2-AP inside the rice had not leached out from the grains because the temperature of the system at this point was low (46.7°C).

Table 5.2: Amount of 2-AP in rice grains cooked with Pandan leaves under optimal water conditions at different cooking times.

Cooking time (min)	Amount of 2-AP/dry mass of rice (µg/g)
0	0.32 ± 0.01 ^a
5	0.72 ± 0.07 ^c
10	0.41 ± 0.02 ^{ab}
15	1.59 ± 0.02 ^d
20	0.49 ± 0.12 ^b

Values correspond to mean data, ± correspond to standard deviation (n=3). Means with different letters in the same column are significantly different ($P < 0.05$).

However, as previously obtained in the study of 2-AP extraction from Pandan leaves, as described in detail in Chapter 4, this low temperature is enough to disrupt the storage site – papillae of 2-AP in Pandan leaves. We found that the 2-AP concentration decreased when the sample of Pandan leaves (without grinding) was treated with oven-drying at 30°C and it was obviously decreased in the total yield of the Pandan leaf extracts. Therefore, at this

cooking time, more 2-AP molecules had leaked from the Pandan leaves due to the disruption of the papillae structure thereby promoting the rice grains to absorb the 2-AP molecules. An unexpected result was obtained at ten minutes of cooking where the absorption of 2-AP was significantly ($P<0.05$) decreased from 0.72 to 0.41 $\mu\text{g/g}$ of dry mass of rice. This result suggested that almost all the 2-AP absorbed at five minutes of cooking was released either to the air or to the water. The amount of 2-AP at this cooking time was no significant difference with the amount of 2-AP of raw rice (0 min of cooking time). These results are possibly due to the changes in the micro molecule structure of rice starch. At this cooking time, rice grains absorb more water, which resulted in 45.4% of moisture content being obtained at a temperature of 73°C. With these conditions, the unstable crystallites of the rice starch had started to melt. Presumably, the melting promotes the cracks in the surface of the rice grains, and, therefore, some of the 2-AP inside the grains moves out from the granules. At the same time, the leaching of amylose as well as the low molecular weight of amylopectin (Leelayuthsoontorn and Thipayarat, 2006) started to occur.

By prolonging the cooking time for another five minutes, the temperature and moisture content of the sample increased to 91°C and 54%, respectively, which was likely to contribute to the leaking of amylose to the surface of the rice granules. The more amylose that moves to the surface of granules, the more the interaction between the molecules of 2-AP and amylose, which possibly occurs as a result of the increase in the absorption of 2-AP by the whole rice grains.

Interestingly, a gradual decrease of 2-AP absorption was observed at 20 min of optimal water cooking and only a small amount of 2-AP per dry mass of rice ($0.49 \pm 0.11 \mu\text{g/g}$) remained inside the rice grains. This is particularly due to the melting of stable crystallites of rice starch. As the temperature reached 97°C , the excessive damage of rice grains associated with the breaking apart of the 2-AP-amylose structure occurred.

5.2.3.3 Gelatinization endotherms of water-rice starch

In order to better understand the interaction of water-rice starch, which was expected to reflect the gelatinization mechanism during the cooking of rice under optimal water, a sample of cooked rice was thermally scanned by differential scanning calorimetry (DSC), as previously mentioned in section 3.2.7. Bi-phasic endotherms were seen during the gelatinization of rice starch at low water level, as clearly illustrated in Figure 5.8. The first endothermic transition was observed at a temperature of 74.5°C with onset temperature (T_o) and end temperature (T_e) 73.9°C and 86.0°C , respectively. Another high peak was also noticed at high temperature (96.13°C) with onset temperature (95.6°C) and end temperature of 97.3°C , respectively. The first and second enthalpies were calculated as 0.026 J/g and 0.131 J/g , respectively.

An explanation for the endotherm peaks could be suggested by consideration of the phase transition of regions within rice starch granules during hydration. It was indicated that the hydration was optimum to melt the unstable crystallites at first transition. However, at low

moisture contents of rice grains, more stable crystallites melted at high temperature (second transition). These results also revealed that high energy is needed to melt the stable crystallites of rice starch when compared to unstable crystallites, as high enthalpy was observed for the second endothermic transition of cooked rice of optimal water.

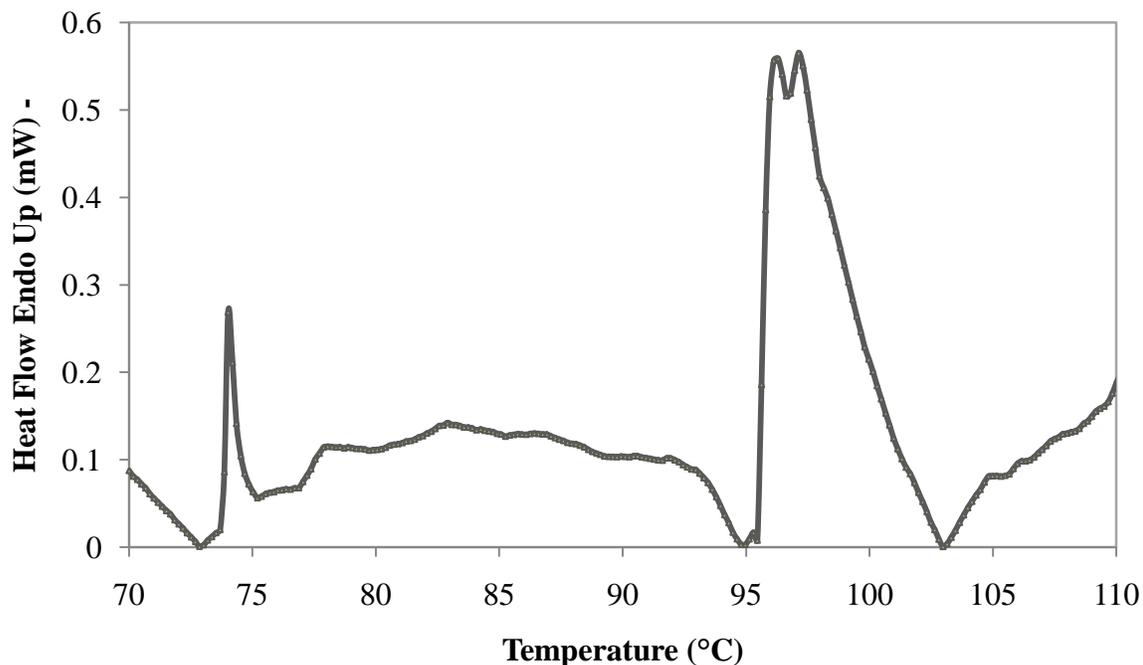


Figure 5.8: Gelatinization thermograms of optimal water cooked rice.

5.2.3.4 Effects of different distance between Pandan leaves and rice sample on the absorption of 2-AP by rice

In order to investigate the effect of distance between Pandan leaves and rice on the absorption of 2-AP during cooking, a sample of rice cooked in optimal water content was taken out from the rice cooker at two different collection points, 1 cm (RN) and 7 cm (RF) from the location of the Pandan leaves and the results were compared. As described previously, the tied Pandan leaves were located in the middle of the rice cooker during the cooking of rice under optimal water conditions.

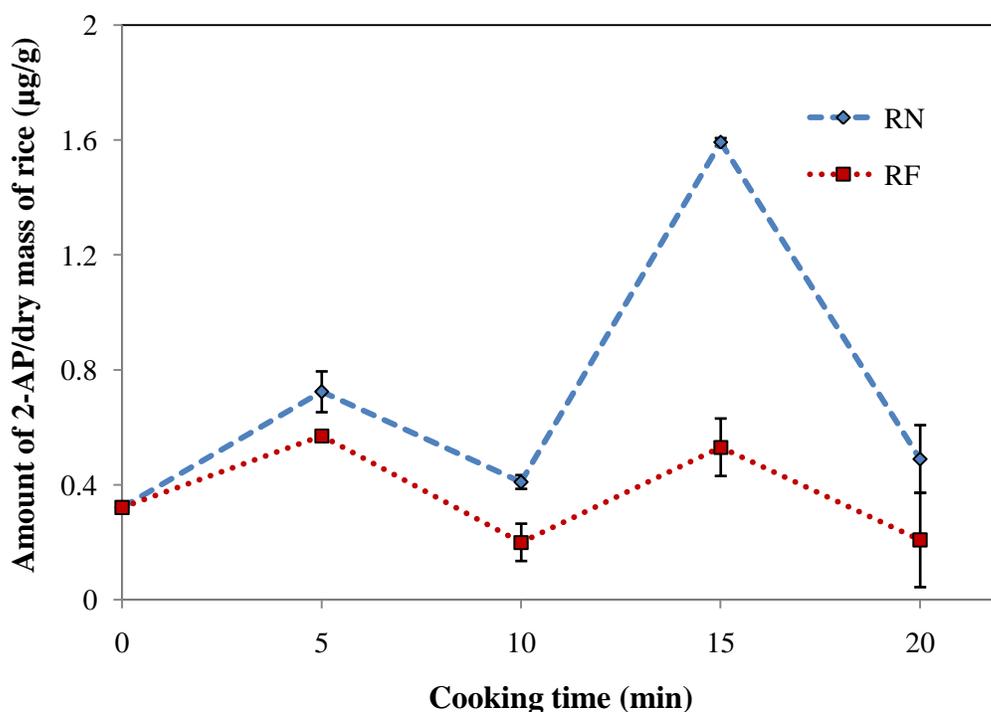


Figure 5.9: Effect of cooking time on the amount of 2-AP absorption by rice grains cooked with Pandan leaves in optimal water cooking at different collection points of rice sample. RN: rice sample is collected at 1 cm from Pandan leaves; RF: rice sample is collected at 7 cm from Pandan leaves.

Interestingly, a similar trend of 2-AP absorption by rice was observed from both collection points (Figure 5.9). As expected, the rice grains collected near (RN) to the tied Pandan leaves absorbed more 2-AP compared to those further away (RF) and the difference was obvious after 15 min of cooking time. These results can be explained by the mass transfer of 2-AP between the Pandan leaves and the rice grains during the cooking process. The distance between the rice and the Pandan leaves during cooking has a significant impact on the absorption of 2-AP by rice.

5.2.3.5 Amount of 2-AP in cooking water

Table 5.3 shows the changes of amount of 2-AP in optimal cooking water with time. The highest amount of 2-AP in water of 0.34 $\mu\text{g/g}$ was obtained at 15 min of cooking time. However, the amount of 2-AP in cooking water was observed to decrease after 20 min of cooking. When the water in the rice cooker was heated, more 2-AP molecules diffused from the Pandan leaves and were then transferred slowly to the water.

From 0 to 15 min of cooking, the amount of water seems enough to enable the 2-AP molecules to move out from the Pandan leaf cells. As the heating proceeded, less water remained in the rice cooker, and, therefore, less 2-AP was diffused from the Pandan leaves to the water at the end of 20 min of cooking. The results are also related to the experiment condition in which optimal water cooking was done in an open rice cooker thereby

resulting in evaporation–excess heating thus leads to the loss of the material from the vessel.

Table 5.3 : Amount of 2-AP in water during cooking of rice mixed with Pandan leaves under optimal water conditions at different cooking times.

Cooking time (min)	Temperature (°C)	Amount of 2-AP/cooking water (µg/g)
0	25.0 ± 0.5 ^a	0.00 ± 0.00 ^a
5	46.7 ± 0.8 ^b	0.18 ± 0.02 ^b
10	73.0 ± 1.3 ^c	0.28 ± 0.01 ^{bc}
15	91.5 ± 0.3 ^d	0.34 ± 0.12 ^{cd}
20	96.7 ± 0.6 ^e	0.17 ± 0.02 ^b

Values correspond to mean data, ± correspond to standard deviation (n=3). Means with different letters in the same column are significantly different ($P < 0.05$).

5.2.3.6 Effects of different distance between Pandan leaves and water sample on the amount of 2-AP in water

Similar to the rice, water samples were collected at two different collection points: 1 cm (WN) and 7 cm (WF) from the Pandan leaves in order to investigate the effect of different distances between the Pandan leaves and the water during cooking on the amount of 2-AP in the water. As indicated in Figure 5.10, a similar trend for the amount of 2-AP in the cooking water was obtained from these two collection points under optimal water conditions. The water sample collected near to the Pandan leaves was found to have a

higher amount of 2-AP during cooking compared to those collected further away from the Pandan leaves. The nearer the water to the Pandan leaves, the greater the 2-AP absorbed by the water.

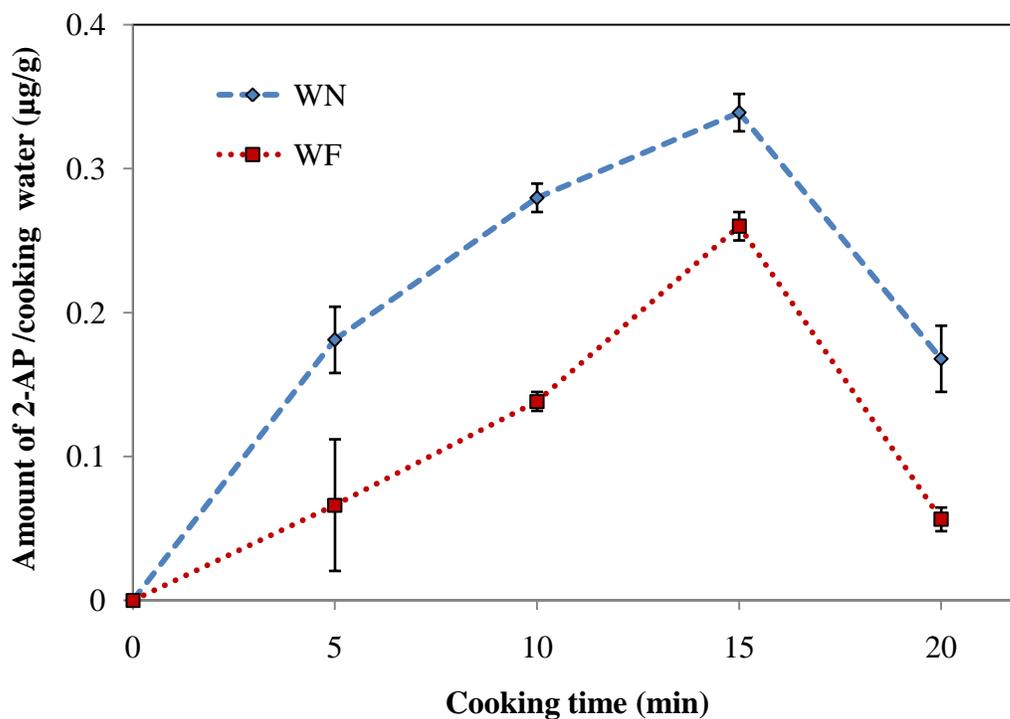


Figure 5.10: Effect of cooking time on the amount of 2-AP in water during the cooking of rice mixed with Pandan leaves in optimal water conditions at different collection points of water samples. WN: cooking water is collected 1 cm from Pandan leaves; WF: cooking water is collected 7 cm from Pandan leaves.

5.2.3.7 Release of 2-AP from Pandan leaves

In Figure 5.11, the percentage of 2-AP release from Pandan leaves in optimal water cooking was plotted as a function of cooking time. As expected, the release rate of 2-AP

from Pandan leaves decreased with cooking time. Depending on the presence of enough water and hydrothermal effects, 2-AP molecules from Pandan leaves were transferred to the vapour (air), water and rice during the cooking process. At 20 min of cooking, a 63% release of 2-AP from Pandan leaves was observed in optimal water. The release of 2-AP from Pandan leaves is significantly affected by temperature and time of cooking of rice as well as the condition of cooking. For example, at 20 min of cooking, the temperature of cooking was 97.6%. This temperature point was not strong enough to extract all the 2-AP from the Pandan leaves.

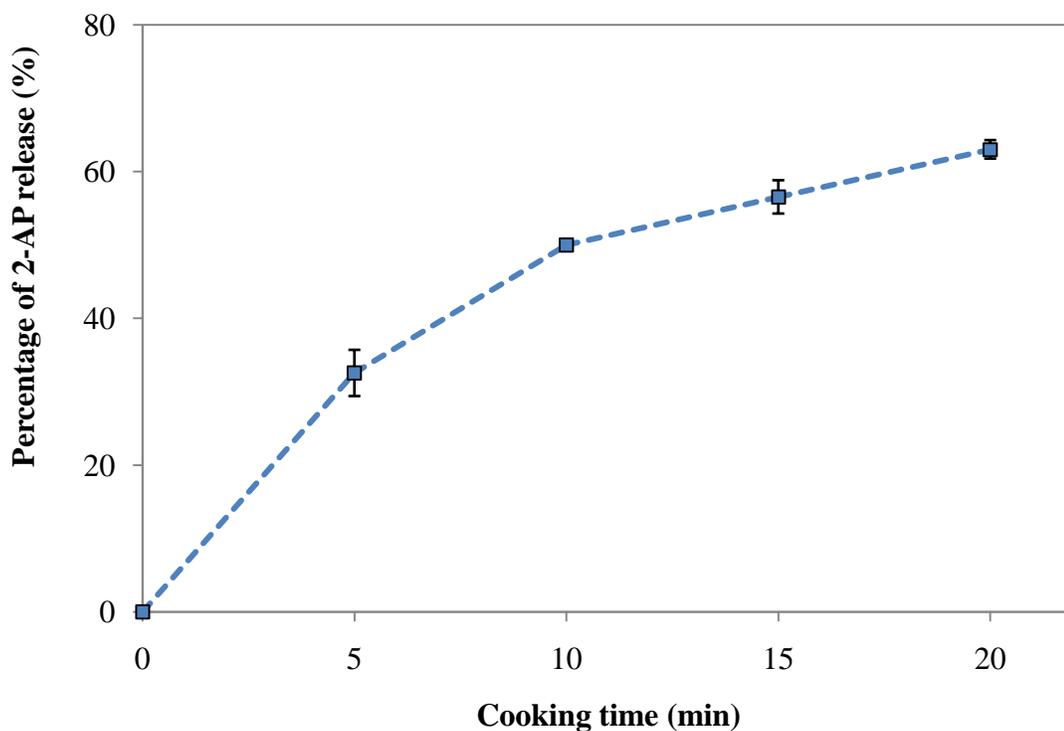


Figure 5.11: Effect of cooking time on the 2-AP release from Pandan leaves during the cooking of rice mixed with Pandan leaves in optimal water cooking conditions.

5.2.4 Effect of different headspace analysis on absorption of 2-AP by rice

Two different headspace analyses were used in order to determine the optimum absorption of 2-AP by rice during cooking under excess water conditions. The cooking procedure of excess water was carried out as previously described in section 3.2.3.1. The sample of cooked rice filled in the headspace vial was incubated using a water bath at 100°C for 30 minutes or at 80°C for 15 min, as clearly mentioned in section 3.2.9 prior to GC-FID analysis. The results were then compared for each cooking time, as shown in Figure 5.12.

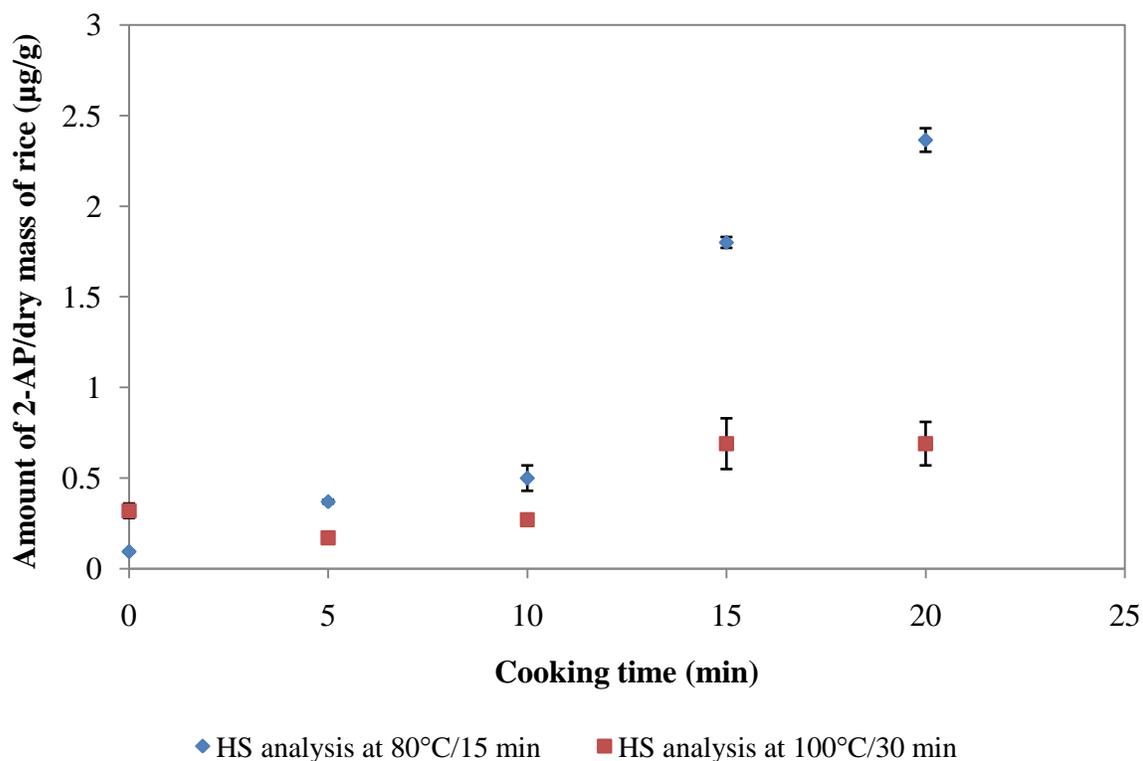


Figure 5.12: Effect of different equilibrium state of headspace (HS) analyses on the amount of 2-AP absorption by rice under excess water conditions.

As one can see in Figure 5.12, the trend of 2-AP absorption by rice grains was similar for both headspace procedures. However, incubation with a water bath at 80°C for 15 min promotes the optimum equilibrium state of headspace, which leads to a higher concentration of 2-AP in the headspace above the cooked rice when compared to incubation at 100°C for 30 min. At the early stage of cooking, the temperature and time of the headspace analysis procedure slightly changes the absorption of 2-AP by cooked rice. However, there a significant increase in the 2-AP amount of cooked rice was obtained after 15 and 20 min of cooking, especially by the procedure of 80°C for 15 min of headspace analysis. These results are in good agreement with Wakte *et al.* (2010) who found that a reduction in the 2-AP peak area of Pandan leaves was observed with an increase in temperature above 80°C using headspace solid phase microextraction. Therefore, the headspace procedure using incubation of 80°C for 15 min was used for further analysis on 2-AP absorption by cooked rice during storage time for both excess and optimal water conditions. These are discussed in detail in Chapter 6.

5.2.5 Effect of prolonged cooking time on the absorption of 2-AP by rice

According to the results achieved in section 5.2.3.1, the cooking of rice under optimal water conditions for 20 min is not enough to obtain fully cooked rice. Therefore, in this section, the effects of prolonging the cooking time to 40 min on the absorption of 2-AP under optimal water conditions using a rice cooker without a lid were investigated. The concentration of 2-AP was analysed by headspace analysis using 80°C for 15 min prior to

GC-FID analysis. The changes of 2-AP absorption by cooked rice during cooking are shown in Figure 5.13.

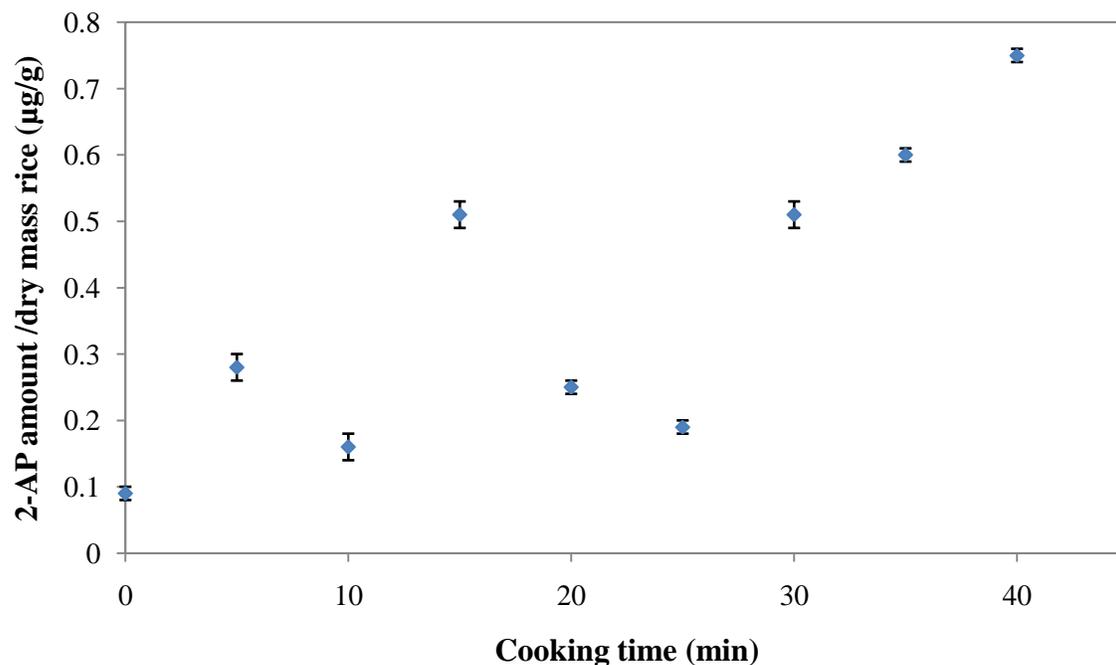


Figure 5.13: Changes of 2-AP concentration during prolonged cooking time under optimal water conditions. The 2-AP concentration was quantified using static headspace analysis (80°C for 15 min) and GC-FID analysis.

The absorption of 2-AP did not increase smoothly with time until after 20 min of cooking. However, a similar trend of 2-AP absorption, determined either by static headspace of 100°C and 30 min or 80°C for 15 min, was obtained. Interestingly, from 30 to 40 min, a sharp increase of 2-AP absorption was observed, as shown in Figure 5.13, with the highest absorption of 0.75 µg/g of 2-AP per dry mass of rice being observed at 40 min of cooking. This concentration value was 8 times higher when compared to uncooked rice. At this

cooking time, the whole rice grains were observed as fully cooked rice in which there no or little water remained on top of the rice as the water was completely absorbed by the rice grains.

Chapter 6

Changes of 2-acetyl-1-pyrroline absorption during storage of cooked rice

6.1 Introduction

A study was undertaken to quantify the 2-AP compound during the storage of cooked rice. As a continuation of the work performed in Chapter 5, long grain white rice was cooked in excess and optimal water conditions. In this chapter, excess and optimal water cooking were conducted for 20 min and 40 min, respectively, in order to obtain fully cooked rice. Previous studies focus more on raw rice including the effect of packaging (Tulyathan *et al.*, 2008) and changes of aroma during storage (Sirisoontaralak and Noomhorm, 2006). Zhou *et al.* (2007) investigated the effect of storage on the changes of rice properties including

residual cooking water and cooked rice grains texture while Yu *et al.* (2009) studied the impact of amylose content on starch retrogradation and the texture of milled rice during storage.

The knowledge of aroma changes occur in cooked rice during storage is important as normal practice; rice is not consumed immediately after cooking. In addition, the aroma is one of sensory attributes may influence the consumption of cooked rice. However, little work has been done on the aroma of cooked rice during storage. Therefore, the aim of this work was to investigate the changes of 2-AP absorption during the storage of cooked rice under different cooking conditions. The temperature and moisture content of cooked rice, which also affects the 2-AP absorption, were determined.

6.2 Results and Discussion

The effect of the different cooking conditions (i.e., amount of water and Pandan leaves) on the phenomena that occur during the storage of rice was investigated. Cooking practices similar to those occurring in typical household conditions were considered.

6.2.1 Effect of quantity of Pandan leaves added during cooking on the absorption of 2-AP during storage of cooked rice

In order to investigate the effect of the quantity of fresh Pandan leaves used during the cooking of long grain white rice on the absorption of 2-AP during storage, rice, with 5 g and 10 g of Pandan leaves, were cooked separately under optimal water cooking conditions for 40 min using an electric rice cooker without a lid. The samples of cooked rice were analysed at intervals of 15 min until 60 min of storage. The storage process was conducted at 24.0 ± 1.0 °C in which cooked rice and Pandan leaves remained in the cooker.

Table 6.1 clearly shows that the 2-AP amount of stored cooked rice decreased with time for both quantities of Pandan leaves during cooking. As an uncovered cooker was used in this experiment, prolonging the storage time would prolong the exposure of cooked rice to the air. These conditions may lead to more 2-AP molecules from rice grains moving to the vapour phase as well as water molecules.

As previously described in Chapter 5, 5 g of Pandan leaves was mixed with rice during cooking. The same procedure for cooking was undertaken in this study. When the temperature reached 100°C, after 40 min of cooking, 0.75 µg/g of 2-AP per dry mass of rice was obtained (Figure 5.13). However, after the cooked rice was stored at 24.0 ± 1.0 °C for 15 min, the concentration of 2-AP decreased sharply to 0.18 µg/g of dry mass of rice

(Table 6.1). This result suggested that a decrease in temperature from 100°C to 80.5°C significantly influenced 2-AP amount in cooked rice.

Table 6.1: Amount of 2-AP during storage of rice cooked with different quantity of Pandan leaves under optimal water conditions.

Storage time (min)	Amount of 2-AP/dry mass of rice ($\mu\text{g/g}$)	
	Pandan leaves (5 g)	Pandan leaves (10 g)
15	0.18 ± 0.02^c	0.33 ± 0.01^d
30	0.08 ± 0.02^b	0.24 ± 0.01^{bc}
45	0.08 ± 0.01^{ab}	0.21 ± 0.02^b
60	0.06 ± 0.01^a	0.17 ± 0.02^a

Values correspond to mean data, \pm correspond to standard deviation (n=3). Means with different letters in the same column are significantly different ($P < 0.05$).

As expected, storing rice cooked with a larger quantity of Pandan leaves results in a larger amount of 2-AP. As one can see in Table 6.1, the concentration of 2-AP in rice cooked with 10 g of Pandan leaves is more than twice as high compared to that cooked with 5 g of Pandan. These results reveal that the amount of Pandan leaves added during cooking significantly contributes to the absorption of 2-AP during the storage of cooked rice. Therefore, in the following studies, 10 g of Pandan leaves was used.

Figure 6.1 shows the changes of 2-AP concentration in Pandan leaves during the storage of cooked rice. Each value represents the mean of 2-AP concentration in stored cooked rice per 1 g Pandan leaves used during cooking. Similar to Figure 6.1, the changes of 2-AP concentration decreased smoothly with storage time.

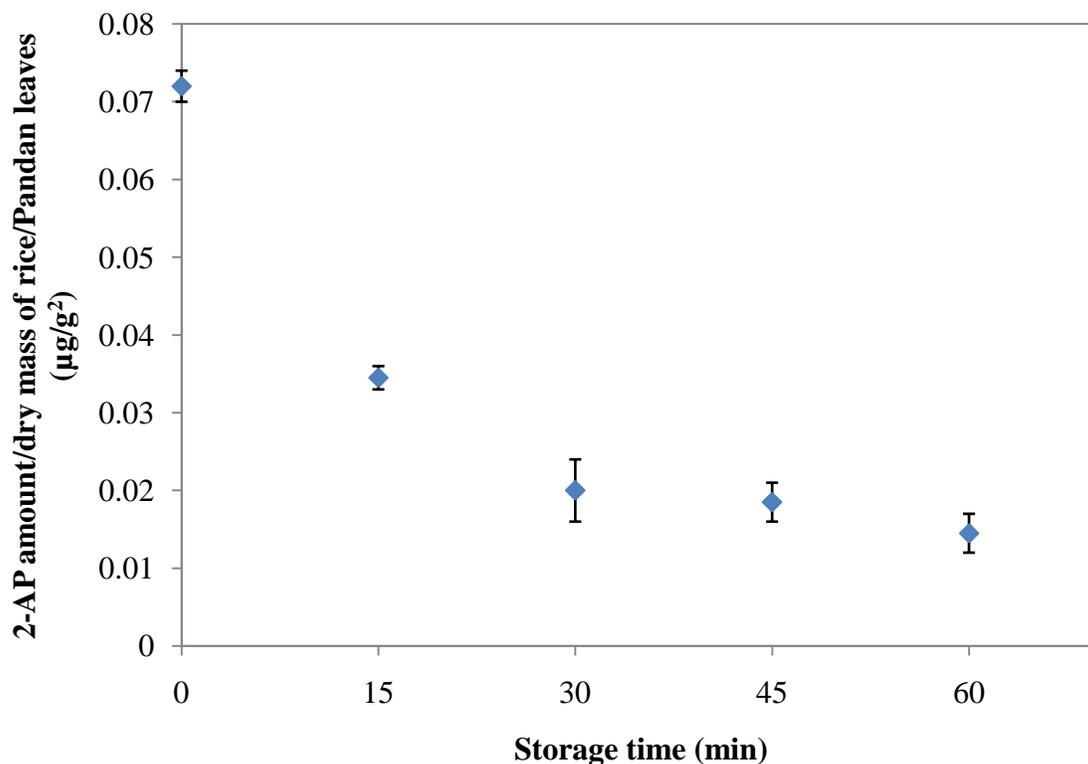


Figure 6.1: Changes in 2-AP concentration per 1 g Pandan leaves during storage of rice under optimal water conditions.

The amount of Pandan leaves plays an important role in the absorption of 2-AP. Even though different amounts of Pandan leaves (i.e., 5 g and 10 g) were used in this study, the level of 2-AP amount per dry mass of rice per 1 g of Pandan leaves at each time of storage

was observed for the same value due to experimental conditions (i.e., amount of water, amount of rice, rice cooker, time and temperature used) being kept constant.

6.2.2 Changes of temperature, moisture content and amount of 2-AP of excess water cooked rice at different storage times

As previously mentioned in Chapter 3, rice with fresh Pandan leaves was cooked under excess water conditions in a covered aluminium cylinder. After cooking for 20 min, boiling water in the cylinder was removed while cooked rice was placed in a covered plastic container together with tied Pandan leaves. The temperature, moisture content and amount of 2-AP of cooked rice were determined at different storage times, as shown in Table 6.2.

Table 6.2: Changes of temperature, moisture content and amount of 2-AP of excess water cooked rice at different storage times.

Storage time (min)	Temperature (°C)	Moisture content (%)	Amount of 2-AP/dry mass of rice (µg/g)
0	100.0 ± 0.6 ^c	73.5 ± 2.2 ^a	0.69 ± 0.03 ^d
15	30.3 ± 0.9 ^b	72.0 ± 1.4 ^a	0.52 ± 0.02 ^c
30	25.0 ± 0.4 ^a	71.5 ± 0.4 ^a	0.32 ± 0.04 ^b
45	23.7 ± 0.4 ^a	71.9 ± 0.4 ^a	0.18 ± 0.06 ^a
60	22.7 ± 0.6 ^a	72.2 ± 0.2 ^a	0.36 ± 0.01 ^b

Values correspond to mean data, ± correspond to standard deviation (n=3). Means with different letters in the same column are significantly different ($P < 0.05$).

As one can see, the temperature of cooked rice gradually decreased with storage time. The temperature significantly ($P<0.05$) decreased from 100°C (at 0 min of storage) to 30.3°C within 15 min of storage. This was mainly due to transferring the cooked rice from the aluminium cylinder to the plastic container. Overall, cooked rice reaches ambient temperature within 30-45 min of storage. The mechanisms of cooling are due to natural convection.

No significant differences were noted for the moisture content during storage of cooked rice. It is interesting to note that in contrast to the temperature reduction, the moisture content remains the same. One of the possible reasons is that cooked rice was kept in a covered container during storage. Therefore, the moisture evaporating from cooked rice was not removed from the container, and, thus, was condensed and eventually reabsorbed by the grains. As a result, the texture of aged cooked rice was observed to remain soft and was less sticky compared to the long grain white rice with high amylose content used in this study.

The amount of 2-AP of excess water cooked rice was significantly ($P<0.05$) different among storage times. One can see that there is a significant decrease of 2-AP concentration from the early stage to 45 minutes of storage. This phenomenon is related to the evaporation of water molecules to the headspace above the cooked rice stored in a covered cooker. Therefore, within 45 min of storage, less water was present in the whole grains. As a result, less 2-AP molecules were absorbed by cooked rice. This result suggests

that the interaction of 2-AP and rice starch molecules is promoted by the presence of adequate moisture content in the rice grains.

Interestingly, a small increase of 2-AP concentration in stored cooked rice was obtained at the end of 60 min of storage. This result may be attributed to the presence of enough moisture in the rice grains. At a low temperature of $22.7\pm 0.6^{\circ}\text{C}$ (60 min of storage) some of the evaporated vapour as well as the 2-AP molecules from Pandan leaves were condensed and reabsorbed by the rice grains. In addition, more amylose remained in the rice grain, as it is difficult to leach out from starch granules at low temperature. These conditions may help the interaction between 2-AP and rice starch through the hydrogen bond. Cooked rice, which has more than 72% of moisture content, absorbs more 2-AP molecules. This result is in good agreement with the results obtained during cooking under the excess water conditions (Chapter 5).

6.2.3 Changes of temperature, moisture content and amount of 2-AP of optimal water cooked rice at different storage times

In this storage study, optimal water cooking was conducted in two different conditions in which rice cooked with fresh Pandan leaves using a covered and an open rice cooker, for 40 min to get fully cooked rice. Cooked rice and tied Pandan leaves were left in the cooker during the storage time. The power supply of the rice cooker was switched off prior to the

storage process. The cooked rice and tied Pandan leaves still remained in the cooker for an additional one-hour. Samples of the top layer of cooked rice were obtained at 15, 30, 45 and 60 min of storage.

The temperature of optimal water cooked rice using both a covered and an open rice cooker, was measured during storage and the results are shown in Table 6.3 and Table 6.4, respectively. Cooked rice and tied Pandan leaves were left in the cooker during the storage time and the power supply was switched off immediately after 40 minutes of cooking. As expected, for both cooking conditions, the temperature of cooked rice smoothly decreased as the storage time decreased. The condition of storage significantly affected the temperature of stored cooked rice.

Table 6.3: Changes of temperature, moisture content and amount of 2-AP of cooked rice at different storage times. Rice was cooked using a covered rice cooker.

Storage time (min)	Temperature (°C)	Moisture content (%)	Amount of 2-AP/dry mass of rice (µg/g)
0	98.5 ± 0.4 ^e	76.1 ± 0.4 ^b	1.35 ± 0.02 ^d
15	91.5 ± 0.8 ^d	71.1 ± 0.7 ^a	1.05 ± 0.04 ^c
30	79.5 ± 1.8 ^c	71.1 ± 0.1 ^a	1.04 ± 0.03 ^c
45	68.3 ± 0.7 ^b	71.0 ± 0.1 ^a	0.77 ± 0.05 ^{ab}
60	59.8 ± 1.2 ^a	71.4 ± 0.3 ^a	0.62 ± 0.05 ^a

Values correspond to mean data, ± correspond to standard deviation (n=3). Means with different letters in the same column are significantly different ($P < 0.05$).

Table 6.4: Changes of temperature, moisture content and amount of 2-AP of cooked rice at different storage times. Rice was cooked using an open rice cooker.

Storage time (min)	Temperature (°C)	Moisture content (%)	Amount of 2-AP/dry mass of rice (µg/g)
0	96.5 ± 3.3 ^e	35.2 ± 0.9 ^b	0.52 ± 0.05 ^d
15	80.5 ± 1.0 ^d	28.8 ± 1.4 ^a	0.33 ± 0.06 ^c
30	63.0 ± 0.5 ^c	29.2 ± 0.3 ^a	0.32 ± 0.03 ^c
45	50.8 ± 0.3 ^b	29.7 ± 0.3 ^a	0.21 ± 0.04 ^{ab}
60	42.7 ± 0.4 ^a	28.5 ± 0.2 ^a	0.13 ± 0.02 ^a

Values correspond to mean data, ± correspond to standard deviation (n=3). Means with different letters in the same column are significantly different ($P<0.05$).

The temperature of stored rice cooked using a covered rice cooker appeared to be higher by 10-20°C when compared to the temperature of cooked rice stored in an open rice cooker, except for the temperature at 0 min of storage. One possible reason is that the cooker with the cover used as storage container may help to retain rice heat even though the cooker is not tightly covered. In addition, the heating element and metal pot used may control the circulation of heat. Therefore, less heat moved out from the cooker. In contrast, cooked rice stored in an open cooker was cooled by the surrounding air. As a result, cooked rice stored in a covered cooker has a higher temperature. This study also revealed that 40-60°C temperature of optimal water cooked rice can be reduced after storage in a rice cooker at room temperature for one hour.

One can see that during storage, no significant differences were noted for the moisture content of cooked rice from both conditions. These results suggested that storage at $24.0\pm 1.0^{\circ}\text{C}$ did not affect the moisture content of cooked rice stored for 60 min. However, the moisture content of stored optimal water cooked rice in a covered rice cooker was more than 2.5 times higher (71%) when compared to those obtained by rice stored in an open cooker (28%). When rice is cooked with a limited amount of water and the lid is not closed, moisture can escape into the atmosphere; a significant amount of water is not absorbed by the rice but evaporates. This results in rice with significantly lower moisture content when compared to storage with either a closed lid or in excess water. These results revealed that cooking and storage of cooked rice in a covered rice cooker is practically efficient as the cooked rice obtained had a better physical appearance compared to that stored in an open rice cooker.

Surprisingly, very low moisture content was obtained from the top layer of the rice stored in an open cooker. When using an electric rice cooker, the heat was transferred to the top of the cooker (Das *et al.*, 2006). This process leads to the evaporation of water to the air. Most of the cooked rice on the top surface was observed to become harder in texture when compared to that located in the middle or bottom layer of the cooker. Therefore, cooked rice taken from the top layer of an open rice cooker is less suitable for eating. This is due to evaporation from the surface; an open lid would result in lower relative humidity in the vapour phase.

Table 6.3 and Table 6.4 also show the changes on the 2-AP amount during the storage of cooked rice. After 15 min of storage, the concentration of 2-AP in the rice cooked in the covered rice cooker was found to be 1.05 µg/g of the dry mass of rice (Table 6.3). A significantly smaller amount (0.33 µg/g of dry mass) was found in the rice cooked in an open cooker (Table 6.4). The 2-AP contents in cooked rice were significantly ($P<0.05$) decreased during storage. One can see that the 2-AP content of rice stored in an open rice cooker continues to decrease. Storage conditions involving a low temperature and using an open cooker may promote the 2-AP molecules moving to the vapour phase as well as water. This result is in good agreement with the low partition coefficient of 2-AP obtained in water/air system at 25°C, as previously discussed in section 5.2.1.

Prolonging the storage time to 60 min appeared to decrease the 2-AP absorbed by optimal water cooked rice in both conditions by up to 25%. Interestingly, a similar pattern of 2-AP absorption appeared for both conditions. The less the amount of water presents in stored cooked rice results in less interaction between the 2-AP and the rice starch. Thus, a decrease in 2-AP absorption by cooked rice was obtained.

Chapter 7

Conclusions and Further work

7.1 Introduction

As mentioned in the previous chapters, this work was divided into three main experimental topics including extraction of 2-AP from Pandan leaves by using SC-CO₂ and solvent extraction (Chapter 4), absorption of 2-AP during cooking of rice with Pandan leaves (Chapter 5) and changes of 2-AP absorption during the storage of cooked rice (Chapter 6). The work undertaken promotes fundamental knowledge concerning the phenomena that occur during cooking and storage, and, in general, in enhancing the flavour of non-aromatic cooked rice.

7.2 Conclusions

7.2.1 Extraction of 2-AP from Pandan leaves

In this study, the effect of the particle size of Pandan leaves, either cut into small pieces or ground, followed by drying treatment including oven and freeze drying to the total yield and concentration of 2-AP from Pandan leaf extracts were investigated. Pandan leaves were extracted by using SC-CO₂ and solvent (hexane) extraction. 2-AP was identified by gas chromatography-mass spectrometry and was quantified using a gas chromatography-flame ionization detector. The results from treated samples were then compared to a sample of fresh Pandan leaves.

The total yield and 2-AP concentration of Pandan leaf extracts were highly affected by differences in particle size and drying pre-treatment. The number of epidermal cells was disrupted by the grinding and drying processes, which can affect the papillae structure; a storage site of 2-AP molecules in Pandan leaves. In spite of the higher yield and 2-AP concentration achieved by Soxhlet extraction using hexane as a solvent when compared to SC-CO₂, the extracts obtained by supercritical extraction were pure, without contaminants due to the high selectivity nature of SC-CO₂. The amount of 2-AP in the extracts of pre-treated Pandan leaves was in the range of 0.04 ± 0.01 to 0.45 ± 0.01 ppm. The grinding and freeze-drying method was revealed as the best pre-treatment for the supercritical extraction method.

7.2.2 Absorption of 2-AP during cooking of rice mixed with Pandan leaves

The results obtained in Chapter 4 revealed that Pandan leaves are a suitable source of 2-AP, and, therefore, Pandan leaves can be used in cooking of rice in order to enhance the flavour of cooked rice, particularly in increasing the concentration of 2-AP. According to these results, the absorption of 2-AP, typically by rice grains during the cooking of rice mixed with fresh Pandan leaves, has been studied, as previously mentioned in Chapter 5.

As a preliminary study, the partition coefficient of 2-AP in the water/air system was investigated using the phase ratio variation (PRV) method. It was concluded from the results that the PRV method is suitable for the accurate determination of water/air partition coefficients for a wide variety of chemical compounds including 2-AP. An increase in phase ratios increased the reciprocal chromatographic peak area of 2-AP. In addition, increasing the liquid volume of Pandan leaf solution in the headspace vial led to an increase in the GC peak area of 2-AP. The partition coefficient of 2-AP between water and air at 25°C was obtained at 0.909. This result indicates that the 2-AP molecules partition more into air than into water at this specific temperature.

Non-aromatic long grain white rice was mixed with tied Pandan leaves and was then cooked under excess and optimal water conditions for 20 min. The excess water cooking was carried out using an aluminium cylinder covered with aluminium foil while the optimal

water was conducted using an open electric rice cooker. In both conditions, the moisture content of rice grains was smoothly increased with time. After 20 min of cooking, moisture contents of 73% and 61% were obtained from the rice grains cooking under excess and optimal water, respectively. In contrast to the excess water, the duration of 20 min was not enough to obtain fully cooked rice under optimal water conditions.

Headspace analysis using 100°C for 30 min was used to measure the concentration of 2-AP in cooked rice. Gas chromatography analysis revealed that 2-AP is transferred during cooking. It was observed that the absorption of 2-AP by rice grains did not increase smoothly with time, particularly under optimal water conditions. At 10 min of cooking, a sharp decrease of 2-AP absorption was observed. This unexpected result indicated that the phenomena that occur during cooking are quite complex. A possible explanation of these phenomena could be linked to starch gelatinization and the effect it has on the absorption of 2-AP by rice grains. This work quantified the potential of Pandan leaves as a flavouring agent in order to enhance the aromatic quality of non-aromatic cooked rice, especially under excess water conditions with two times higher 2-AP concentration when compared to those obtained in raw grains.

7.2.3 Changes of 2-AP absorption during storage of cooked rice

The absorption of 2-AP during the storage of cooked rice was investigated after rice was cooked with fresh Pandan leaves under excess and optimal water conditions for 20 and 40 min, respectively. Similar to the cooking procedure discussed in chapter 5, excess water cooking was conducted using an aluminium cylinder covered with aluminium foil. After cooking was completed, the water remaining in the cylinder was removed while the cooked rice and tied Pandan leaves were placed in a covered plastic container. In the optimal water condition, rice was cooked using an electric rice cooker either with or without a lid prior to the storage process. After 40 min cooking, the rice together with the Pandan leaves remained in the cooker. For both excess and optimal water of cooked rice, samples that were stored at $24.0 \pm 1.0^\circ\text{C}$ were taken every 15, 30, 45 and 60 min of storage for further analysis.

The preliminary study shows that the quantity of Pandan leaves added during the cooking of rice has a positive correlation with the 2-AP concentration of rice grains. The cooking as well as the storage conditions significantly contributed to the concentration of 2-AP, temperature and moisture content of stored cooked rice. In both excess and optimal water conditions, the temperature of cooked rice decreased with storage time while the moisture content of the rice remained constant. The absorption of 2-AP during the storage of the excess water cooked rice continuously decreased until 45 min but then slightly increased

after a further storage of 15 min, while the 2-AP concentration of stored optimal water rice cooked using a cooker either with or without a lid decreased smoothly overtime.

This study revealed that a covered rice cooker proved to be an efficient tool for cooking rice under the optimal water condition with the best duration being 40 min. The keeping of fresh Pandan leaves together with cooked rice during storage in a covered container shows a great impact of the absorption of 2-AP. These results also suggested that cooked rice should be consumed at an early stage of storage (within 15 min) in order to obtain great flavour and texture profiles.

7.3 Further work

According to the findings of this work, some further works would be interesting to undertake as follows:

- As this work only focussed on the 2-AP compound; other volatile compounds which also contribute to the flavour of rice, typically from different hydrophobicity-partition coefficients, would be interesting to investigate since the flavour of cooked rice is attributed to the combination of aromatic compounds.

- 2-AP from Pandan leaves was extracted by SC-CO₂ and solvent extraction. However, the optimization of supercritical extraction parameters, such as solubility, pressure, CO₂ flow rate, temperature and extraction time, which promotes the optimum extracts and 2-AP concentration from Pandan leaves, was not investigated in detail. This may result in finding better quality extracts with high concentrations of 2-AP.
- The changes that take place during the cooking and storage of rice significantly influence the absorption of 2-AP, particularly in cooked rice. Therefore, changes in the physicochemical properties of rice, such as water uptake, swelling, volume expansion, amylose and amylopectin content as well as amylose leaching during cooking and storage would be interesting to investigate followed by a study on the correlation of the results obtained with the absorption of 2-AP.
- A study on the absorption of 2-AP during cooking and storage of rice could be expanded using different cultivars of non-aromatic rice and cooked with Pandan leaves. The results might be compared with the absorption of 2-AP by cooked aromatic rice or even with cooked non-aromatic rice without the addition of Pandan leaves. The results could help to obtain a better understanding concerning how the differences in each cultivar of rice grain influences the cooked rice flavour as well as the role of Pandan leaves on the flavour enhancement of rice.

- Attempts have been carried out on the cooking of rice under excess and optimal water conditions by the boiling method using an aluminium cylinder and electric rice cooker, respectively. It may be possible to compare the absorption of 2-AP using other rice cooking methods – microwave, steaming or even using other domestic cooking appliances, such as a pressure cooker. Besides the cooking method, storage conditions at different temperatures and times may also help to better understand the absorption of 2-AP, particularly how it is affected by the retrogradation process of rice starch.
- Detailed studies on the interactions of flavour compounds with starch using different techniques, such as wide angle X-ray diffraction, Nuclear Magnetic Resonance (NMR), Fourier Transform Infrared (FTIR) spectroscopy, Differential Scanning Calorimetry (DSC) as well as the quantification of aroma compound by Gas Chromatography-Mass Spectrometry (GC-MS) analysis may lead to a comprehensive understanding, particularly concerning the structure, aroma composition and behaviour of starch-flavour inclusion complexes.
- As previously mentioned, it is easy to lose the 2-AP molecules during processing, particularly those involving heating treatment. In addition, the depletion of 2-AP may also occur due to exposure to light and the oxidation process. Therefore, the encapsulation of 2-AP molecules may be the best solution to protect this valuable volatile compound in food matrixes, which would be interesting to study.

- Further investigation concerning the phenomena that happen in the mouth is suggested for a better understanding concerning the release of aroma during the eating of cooked rice. Accordingly, using a model of a mouth, simulation of eating and parameters that influence the release of aroma, such as composition and volume of saliva, mastication and addition of amylase enzyme would be interesting to investigate. To study these phenomena in depth, a combination of real time measurement of flavour release in the mouth using Atmospheric Pressure Chemical Ionization Mass Spectrometry and sensory analysis are strongly recommended.

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