การเปรียบเทียบสารระเหยยากในน้ำมะนาวคั้นสดสี่พันธุ์โดยใช้สมบัติทางเคมีกายภาพและทางประสาทสัมผัส

นางสาวจุฑามาศ ก่อกิจพูนผล

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาเทคโนโลยีทางอาหาร ภาควิชาเทคโนโลยีทางอาหาร
คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2559

COMPARISON OF NON-VOLATILE COMPOUNDS IN FRESH-SQUEEZED JUICE FROM FOUR LIME CULTIVARS USING PHYSICOCHEMICAL AND SENSORY PROPERTIES

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A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Food Technology
Department of Food Technology
Faculty of Science
Chulalongkorn University
Academic Year 2016

Thesis Title	COMPARISON OF NON-VOLATILE COMPOUNDS IN FRESH-SQUEEZED JUICE FROM FOUR LIME CULTIVARS USING PHYSICOCHEMICAL AND SENSORY PROPERTIES
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จุฑามาศ ก่อกิจพูนผล : การเปรียบเทียบสารระเหยยากในน้ำมะนาวคั้นสดสี่พันธุ์โดยใช้สมบัติทางเคมีกายภาพและทางประสาทสัมผัส (COMPARISON OF NON-VOLATILE COMPOUNDS IN FRESH-SQUEEZED JUICE FROM FOUR LIME CULTIVARS USING PHYSICOCHEMICAL AND SENSORY PROPERTIES) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ดร. พนิตา งามเชื้อชิต, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: สุวิมล กีรติพิบูล, ปรีชา ภูวไพรศิริศาล, 73 หน้า.

มะนาว (*Citrus aurantifolia*) จัดอยู่ในกลุ่มผลไม้ตระกูลส้ม (Citrus) ซึ่งนับเป็นส่วนประกอบสำคัญชนิดหนึ่งในอาหารเนื่องจากลิ่นรสที่เป็นเอกลักษณ์ ทั้งนี้รสชาติหลักของผลไม้ตระกูลส้ม อันได้แก่ รสเปรี้ยวและรสหวาน เกิดจากสมดุลระหว่างกรดอินทรีย์และน้ำตาลซึ่งเป็นองค์ประกอบหลักของสารระเหยยากในผลไม้ตระกูลส้ม นอกจากนี้อาจมีรสขมเกิดขึ้นเนื่องจากสาร ลิโมนิน และ นารินจิน อนึ่งรสชาตินับเป็นหลักเกณฑ์หนึ่งที่สำคัญในการบ่งบอกคุณภาพ การยอมรับของผู้บริโภค งานวิจัยนี้จึงมีวัตถุประสงค์เพื่อเปรียบเทียบองค์ประกอบของสารระเหยยากในน้ำมะนาวคั้นสดสี่พันธุ์ ได้แก่ มะนาวพันธุ์แป้นรำไพ แป้นพวง แป้นพิจิตร(*Citrus aurantifolia* (Christm&Panz) Swingle) และตาฮิติ (*Citrus latifolia* Tanaka) โดยใช้คุณสมบัติทางเคมีกายภาพและการประเมินคุณภาพทางประสาทสัมผัส โดยจากการวิเคราะห์ปริมาณสารระเหยยาก ได้แก่ กรดอินทรีย์ (กรดซิตริก กรดมาลิก กรดแอสคอบิก และกรดซัคซินิก) ปริมาณน้ำตาล (กลูโคส ฟรุกโตส และ ซูโครส) ปริมาณสารให้รสขม (ลิโมนินและนารินจิน) ด้วยลิควิดโครมาโทกราฟฟี พบว่าในน้ำมะนาวคั้นสดกรดอินทรีย์ที่มีปริมาณมากที่สุด ได้แก่ กรดซิตริก ตามด้วย กรดมาลิก แอสคอบิกและซัคซินิก ตามลำดับ น้ำตาลฟรุ๊กโตสเป็นน้ำตาลที่มีปริมาณมากที่สุด ตามด้วยน้ำตาลซูโครสและกลูโคสมะนาวพันธุ์แป้นพวงและแป้นรำไพมีปริมาณกรดอินทรีย์และปริมาณกรดที่ไทเทรทได้ (Titratable acidity, TA) สูงที่สุด มะนาวพันธ์ตาฮิติพบปริมาณน้ำตาล สารที่ให้รสขม (ลิโมนิน) ปริมาณของแข็งที่ละลายน้ำได้ (Total soluble solid, TSS) และ TSS/TA สูงที่สุด มะนาวพันธุ์แป้นพิจิตรมีปริมาณกรดอินทรีย์ น้ำตาล สารให้รสขม (ลิโมนิน) TSS และ TSS/TA ต่ำที่สุด จาก Principal Component Analysis (PCA) และ Hierarchical Cluster Analysis (HCA) พบว่าสามารถแบ่งตัวอย่างน้ำมะนาวทั้งสี่พันธุ์ออกเป็น 3 กลุ่มตามความเหมือนของตัวอย่าง ได้แก่กลุ่มของมะนาวตาฮิติ กลุ่มของมะนาวแป้นพิจิตร และกลุ่มของมะนาวแป้นรำไพและแป้นพวง ทั้งนี้พบว่ามะนาวพันธุ์แป้นพวงและแป้นรำไพมีความสัมพันธ์กับปริมาณกรดอินทรีย์และรสเปรี้ยวที่สูงกว่ามะนาวพันธุ์อื่นๆ และมะนาวตาฮิติมีความสัมพันธ์กับปริมาณน้ำตาล สารให้รสขม TSS TSS/TA และรสหวานที่สูงกว่ามะนาวพันธุ์อื่นๆ ในขณะที่มะนาวแป้นพิจิตรมีความสัมพันธ์ผกผันกับปริมาณสารระเหยยาก คุณสมบัติทางเคมีกายภาพและรสชาติจากการประเมินทางประสาทสัมผัสเชิงพรรณนา จากการทดสอบการยอมรับของผู้บริโภคและกาทดสอบความชอบโดยวิธี Preference ranking test โดยนักเรียนการเรือน พบว่ามะนาวพันธุ์แป้นพวงได้รับคะแนนการยอมรับจากผู้บริโภคสูงที่สุด ในขณะที่มะนาวแป้นพิจิตรซึ่งเป็นพันธุ์ได้รับการยอมรับจากผู้บริโภคน้อยที่สุด

ภาควิชา เทคโนโลยีทางอาหาร	ลายมือชื่อนิสิต ....
สาขาวิชา เทคโนโลยีทางอาหาร	ลายมือชื่อ อ.ที่ปรึกษาหลัก ....
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	ลายมือชื่อ อ.ที่ปรึกษาร่วม ....

5871923023 : MAJOR FOOD TECHNOLOGY
KEYWORDS: LIME / CITRUS / NON-VOLATILE COMPOUNDS / CHEMOMETRICS / HPLC

JUTAMAS KORKITPOONPOL: COMPARISON OF NON-VOLATILE COMPOUNDS IN FRESH-SQUEEZED JUICE FROM FOUR LIME CULTIVARS USING PHYSICOCHEMICAL AND SENSORY PROPERTIES. ADVISOR: PANITA NGAMCHUACHIT, Ph.D., CO-ADVISOR: PROF. SUWIMON KEERATIPIBUL, Ph.D., ASSOC. PROF. PREECHA PHUWAPRAISIRISAN, Ph.D., 73 pp.

Lime (*Citrus aurantifolia*) is classed as citrus fruit. In various Thai dishes, lime juice is one of the important ingredients due to its unique flavor (aroma and taste). The uniqueness of taste in citrus is from the balance of organic acids and sugars. Organic acids and sugars contribute sourness and sweetness in citrus while limonin and naringin contribute bitterness in citrus. Taste is one of the important criteria of eating quality and consumer acceptance. This study aimed to compare the fresh-squeezed juice from four lime cultivars i.e., 'Pan Rumpai', 'Pan Puang', 'Pan Pichit' (*Citrus aurantifolia* (Christm&Panz) Swingle) and 'Tahiti' (*Citrus latifolia* Tanaka) using physicochemical properties and sensory evaluations. The analyses of non-volatile compounds i.e., organic acid (citric, malic, ascorbic, and succinic acids) contents, the sugar (sucrose, glucose, and fructose) contents, and the bitter compounds (limonin and naringin) were analyzed using liquid chromatography. Citric acid is the predominant acid in four lime cultivars, followed by malic, ascorbic, and succinic acids respectively. The predominant sugar is fructose followed by, sucrose and glucose. 'Pan Puang' and 'Pan Rumpai' had the highest organic acid contents and TA, while the highest sugar content, bitter compound (limonin) and TSS were found in 'Tahiti'. 'Pan Pichit' had the lowest organic acids, sugars, bitter compound (limonin), TSS and TSS/TA. From Principal component analysis (PCA) and Hierarchical cluster analysis (HCA), fresh-squeezed lime juice samples could be separated into 3 clusters based on the similarity of samples; a cluster of 'Tahiti', a cluster of 'Pan Pichit' and a cluster of 'Pan Puang and Pan Rumpai'. The higher organic acids content strongly associated with 'Pan Puang' and 'Pan Rumpai' cultivars than others. The higher sugar content, bitter compound, TSS, TSS/TA ratios and sweetness score were associated with 'Tahiti' lime than others, whereas 'Pan Pichit' cultivar negatively associated with the all non-volatile compound contents, physicochemical properties and sensory attributes from a descriptive analysis. From consumer acceptance test and preference ranking test performed by culinary students, 'Pan Puang' was the highest preference cultivar, while 'Pan Pichit' is the least preference cultivar.

Department:	Food Technology	Student's Signature	....
Field of Study:	Food Technology	Advisor's Signature	....
Academic Year:	2016	Co-Advisor's Signature	....
		Co-Advisor's Signature	....

ACKNOWLEDGEMENTS

I would first like to thank my thesis advisor Dr. Panita Ngamchuachit and co-advisor Prof. Suwimon Keeratipibul and Assoc. Prof. Dr. Preecha Phuwapraisirisan who guied, devoted, supported and encouraged me through my graduate education. My appreciation also extends to the thesis committee members, Asst. Prof. Dr. Varapha Kongpensook,Dr. Kannapon Lopetcharat and Dr. Sarn Settachaimongkon for their constructive comments and contribution to the improvement of my thesis.

The Scholarship from the Graduate School, Chulalongkorn University to commemorate 72nd Anniversary of his Majesty King Bhumibol Adulyadej is gratefully acknowledged and the 90th Anniversary Chulalongkorn University Fund (Ratchadaphiseksomphot Endowment Fund.)

I would like to sincerely acknowledge Mr. Channarong Puangsun, Provincial Agricultural Extension Office, who kindly supported lime samples and cooperated with other lime’s farm in Petchburi province for collecting lime samples and also Mr. Suparlerks Narubetkraisee, the owner of Wasa farm who supports the best Tahiti limes samples.

I would like to sincerely acknowledge Betagro Science Center Co., Ltd. and School of Culinary Arts, Suan Dusit University for cooperation in sensory evaluation part.

Next, I would like to thank to all staffs and graduated students at the department of Food Technology, Chulalongkorn University for their valuable help and suggestions.

Finally, I am sincerely grateful to my friends and family; mom, dad and my lovely sister for their supports and encouragement that gave to me when I faced the hardest time.

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CHAPTER I
INTRODUCTION

Lime is one of the citrus fruit families that has unique aromas and tastes which leads lime to be an important and favorable ingredient in various foods and beverages. The Thai lime's cultivar 'Pan' (*Citrus aurantifolia* (Christm&Panz) Swingle) includes 'Pan Rumpai', 'Pan Puang', and 'Pan Pichit'. 'Pan Rumpai' and 'Pan Puang' are popular in Thai market. 'Pan Pichit' would present in the market only in the dry season (March-April) when 'Pan Rumpai' and 'Pan Puang' cultivars were lacked. Tahiti (*Citrus latifolia* Tanaka), a seedless lime, is another famous cultivated variety in Thailand and other countries e.g. South Asia, South America that is widely used for beverage. Taste is one of the most important quality attributes that influence customer acceptance. The taste profile of each cultivar could represent their uniqueness or show a fingerprint of fruit to identify characteristics of each fruit or cultivar.

In addition, there are numerous studies focus on factors affecting flavor and chemical compositions of fruit that have been studied, for example, cultivars, environmental condition, geographical origin, seasons, fruit maturity etc. (Bai et al., 2016; Cheong, Liu, et al., 2012; Cheong, Zhu, et al., 2012; Zheng et al., 2009).

In Thailand, even though, there are numerous Thai lime cultivars, the information on chemical and sensory profile of taste compounds among lime cultivars remains unexplored.

In order to gain the taste profile, the correlation between chemical compositions and sensory profile of fruit should be determined. Liquid chromatography is widely used to identify and quantify non-volatile compounds. In addition, chemometrics is a class of statistical tool that aids researcher to correlate chemical measurements, consumer preference and sensory attributes of food (Marini, 2013). It is well-known and widely used in analytical chemistry and food science field. Giansante et al. (2003) mentioned that if taxonomy is a useful tool in characterization of the cultivar by morphological characteristics of plants and fruits, chemometrics is a useful tool in the characterization of the cultivar by chemical composition. The unsupervised pattern recognition techniques e.g. cluster analysis

(CA), principal component analysis (PCA) are carried out for a preliminary evaluation of the information content in the data metrics (Abad-García et al., 2012). Several previous studies focused on determination of volatile and non-volatile compounds in various citrus fruit such as Turkish cv. Dortyol orange juice (*Citrus sinensis* L. Osbeck), pomelo (*Citrus grandis* (L.) Osbeck), calamansi (*Citrus microcarpa*) (Cheong, Liu, et al., 2012; Cheong, Zhu, et al., 2012; H. Kelebek and Selli, 2011); however, correlation of sensory profile and chemical compositions has not been reported in some of these studies.

Hence, this study aimed to compare the non-volatile compositions, sensory profiles in fresh-squeezed juice from 4 lime cultivars (i.e., 'Pan Rumpai', 'Pan Puang', 'Pan Pichit' (*Citrus aurantifolia* (Christm&Panz) Swingle) and 'Tahiti' (*Citrus latifolia* Tanaka)) using high performance liquid chromatography (HPLC) and chemometrics.

CHAPTER 2
LITERATURE REVIEWS

2.1 Lime

Lime is a non-climacteric fruit (Paul et al., 2012). It is one of the citrus fruit families (Rutaceae) which originated in north-east of India and widely spread to Asia and other tropical regions. In the 13th century, lime has been known in Europe. Furthermore, it has spread to West Indies islands and has been widely cultivated in Mexico and Egypt (Pongsomboon, 2015). The lime fruit size ranges from very small to medium with round, obovate, or oblong in shape. Lime fruit has very small neck, a flat base, and a small nipple at the apex with a thick to very thin and papery peel and green to yellow in color. They are seedy to seedless. The peel surface is smooth and the flesh is tender, juicy, and yellowish-green (Ladaniya, 2008a).

In 2015, the cultivation areas for Thai lime were 168,000 m^3 (Office of Agriculture Economics, 2015). Most of Lime cultivated areas are located in the middle of Thailand including, Petchburi, Samutsakorn, Nakorn Phathom and Ratchaburi provinces. Thai lime 'Pan' cultivar (*Citrus aurantifolia* Swingle) is the most favorable in Thailand as it has the most acceptable taste and aroma, thin peel and high productivity; however, the availability of this cultivar is limited only in dry season during March to April which potentially cause high price of lime fruits.

2.1.1 Lime cultivars

Limes are categorized into 3 groups including, **Mexican lime** (*Citrus aurantifolia* Swingle), **Tahiti or Persian lime** (*Citrus latifolia* Tanaka), **Sweet lime** (*Citrus limetta*). 'Pan' cultivar (*Citrus aurantifolia* Swingle) from the Mexican lime group is the most cultivated Thai lime accounted for 74% of the total cultivation in Thailand. 'Pan Puang', 'Pan Rumpai' and 'Pan Pichit' are the most commercially available 'Pan' cultivars in Thai market. Apart from 'Pan' cultivars, 'Tahiti' cultivar, a seedless lime, from Persian lime group is also widely used in beverage industry. In this study, 'Pan Puang', 'Pan Rumpai', 'Pan Pichit' and 'Tahiti' limes were investigated (Table 2.1).

Table 2. 1 Lime cultivars grown in Thailand(Pongsomboon, 2015)

Cultivars	**Characteristics**
'Pan Rumpai' (*Citrus aurantifolia* (Christm&Panz) Swingle)	'Pan Rumpai' cv. grows with spreading tree shape. It has 7.3 cm in length and 4.3 cm in width dark green leaf with ovate shape and crenate leaf lamina margin. There are white flowers which budding more than once a year. The fruits are 4.5 cm in width and 3.8 cm in length with oblate shape and green skin. The skin is 1.8 mm thickness. There are 11.5 segments per fruit. The pulp and juice are light green color. There are 14.2 light brown ovoid seeds per fruit. The commercial maturity is 4 months.
'Pan Paung' (*Citrus aurantifolia* (Christm&Panz) Swingle)	'Pan Paung' cv. grows with spreading tree shape. It has 6.9 cm in length and 4.0 cm in width dark green leaf with ovate shape and crenate leaf lamina margin. There are white flowers which budding more than once a year. The fruits are 3.9 cm in width and 3.5 cm in length with oblate shape and green skin. The skin is 1.9 mm thickness. There are 9.6 segments per fruit. The pulp and juice is light green color. There are 12.5 brown ovate seeds per fruit. The commercial maturity is 4-5 months.

Table 2.1 Lime cultivars grown in Thailand (Pongsomboon, 2015) (continued)

Cultivars	**Characteristics**
'Pan Pichit' (*Citrus aurantifolia* (Christm&Panz) Swingle)	'Pan Pichit' cv. grows with spreading tree shape. It has 8.6 cm in length and 4.5 cm in width dark green leaf with ovate shape and crenate leaf lamina margin. There are white flowers which budding more than once a year. The fruits are 5.12 cm in width and 4.71 cm in length with oblate shape and green skin. The skin is 2.3 mm thickness. There are 11.1 segments per fruit. The pulp is light green and juice is white color. There are 29.4 brown ovoid spheroid seeds per fruit. The commercial maturity is 4-5 months.
'Tahiti' (*Citrus latifolia* Tanaka)	'Tahiti' cv. grows with spreading tree shape. It has 9.05cm in length and 5.02 cm in width dark green leaf with ovate shape and crenate leaf lamina margin. There are white flowers which budding more than once a year. The fruits are 5.8 cm in width and 6.1 cm in length with Ellipsoid shape and yellow-green skin. The skin is 2.5 mm thickness. There are 10.4 segments per fruit. The pulp and juice are light green color. It is seedless. The commercial maturity is 4-5 months.

(a) (b)


(c) (d)


Figure 2.1 Four lime cultivars ‘Pan Rumpai” lime (*Citrus aurantifolia* (Christm&Panz) Swingle) (a), ‘Pan Puang’ lime (*Citrus aurantifolia* (Christm&Panz) Swingle) (b), ‘Pan Pichit’ lime (*Citrus aurantifolia* (Christm&Panz) Swingle), ‘Tahiti’ lime (*Citrus latifolia* Tanaka)

2.2 Non-volatile compounds and their taste contributions in citrus fruit

Biochemical compounds and secondary metabolites, such carbohydrates, organic acids, nitrogenous compounds, enzymes, lipids, waxes, phenols, flavonoids and limoniods, play a very important role in the physiology and metabolism of citrus plants and fruits. The uniqueness of taste in citrus is from the balance of organic acids and sugars. Organic acids and sugars contribute sourness and sweetness in citrus while limonin and naringin contribute bitterness in citrus fruits. The perception of sweetness is due to the presence of glucose, fructose and sucrose, whereas sourness is due to the presence of organic acids, particularly citric acid, Apart from organic acids and sugars, naringin and limonin are flavor constituents contributing bitterness (Cheong, Zhu, et al., 2012; Farnworth et al., 2001; Ladaniya, 2008b; Tietel et al., 2011).

2.2.1 Sugars

Free sugars in citrus juices are predominantly glucose, fructose, and sucrose (Table 2.1). Lime and lemon juices have a small quantity of sucrose, accounting for 0.7-0.8% in lime. Glucose and fructose are reducing monosaccharide. Sucrose is a non-reducing disaccharide in citrus. Lime and lemon juice has a trace amount of sucrose (Ladaniya, 2008b).

Table 2. 2 Sugar composition of citrus fruits(Ladaniya, 2008b)

Fruit	Glucose (%)	Fructose (%)	Maltose (%)	Sucrose (%)	Total
Sweet orange, Mosambi	4.05	4.55	Traces	<0.5	8.59
Mandarin, Nagpur	4.00	1.98	–	6.80	–
'Kagzi' lime (light green)	0.39	0.19	–	–	–
Dark green	0.37	0.13	–	–	–
Acid lime (full yellow)	0.61	0.23	–	–	–
Lemon	0.52	0.92	Traces	–	–
Grapefruit	2.97	3.08	<0.5	1.26	7.31

Swisher and Higby (1961); Veldhuis (1971); Hurst et al. (1979); Selvaraj and Edward Raja (2000); Ladaniya and Mahalle (2006).

In addition, It was found that the sweetness correlated with sugar content. The sweet taste is from the structure of sugar which forms hydrogen bonds with water. The mechanism of sweet taste reaction is the intermolecular hydrogen bonding between a sweet compound's saporous site unit and the taste bud receptor site according to AH-B theory (Shallenberger, 1963). Thus, the degree of association with water might be a factor conducting sweet taste (Belitz et al., 1979). The structure of glucose, fructose and sucrose were showed in Figure 2.2(a), Figure 2.2(b) and Figure 2.3, respectively.

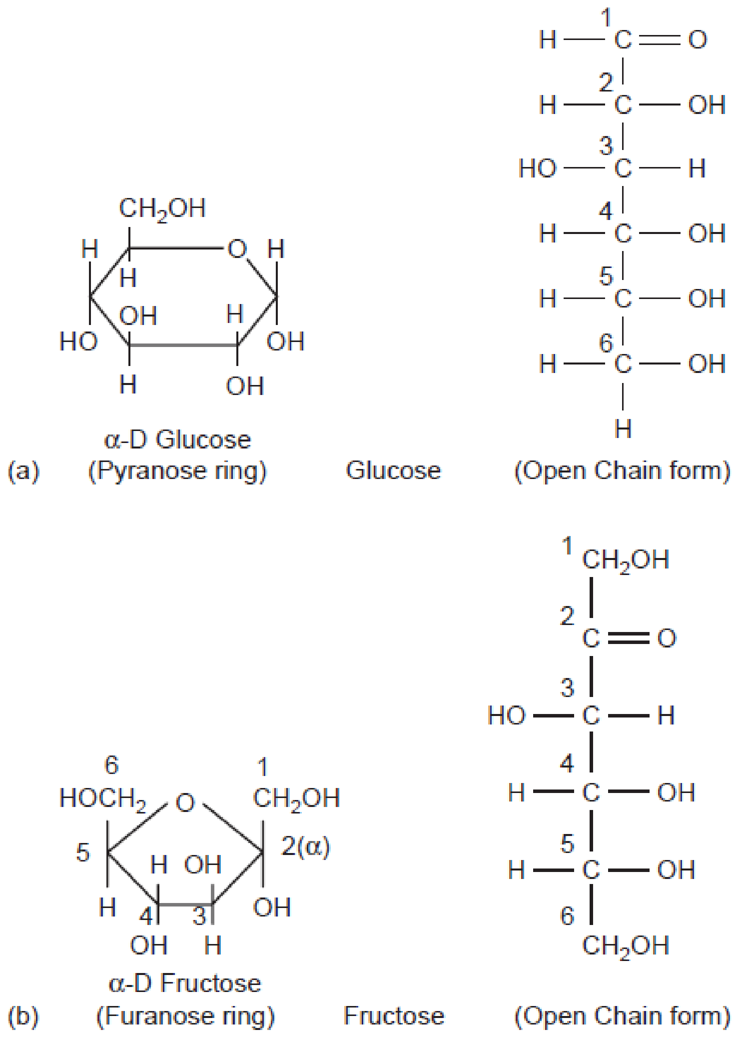


Figure 2. 2 Ring and chain structure of monosaccharide (Ladaniya, 2008b)

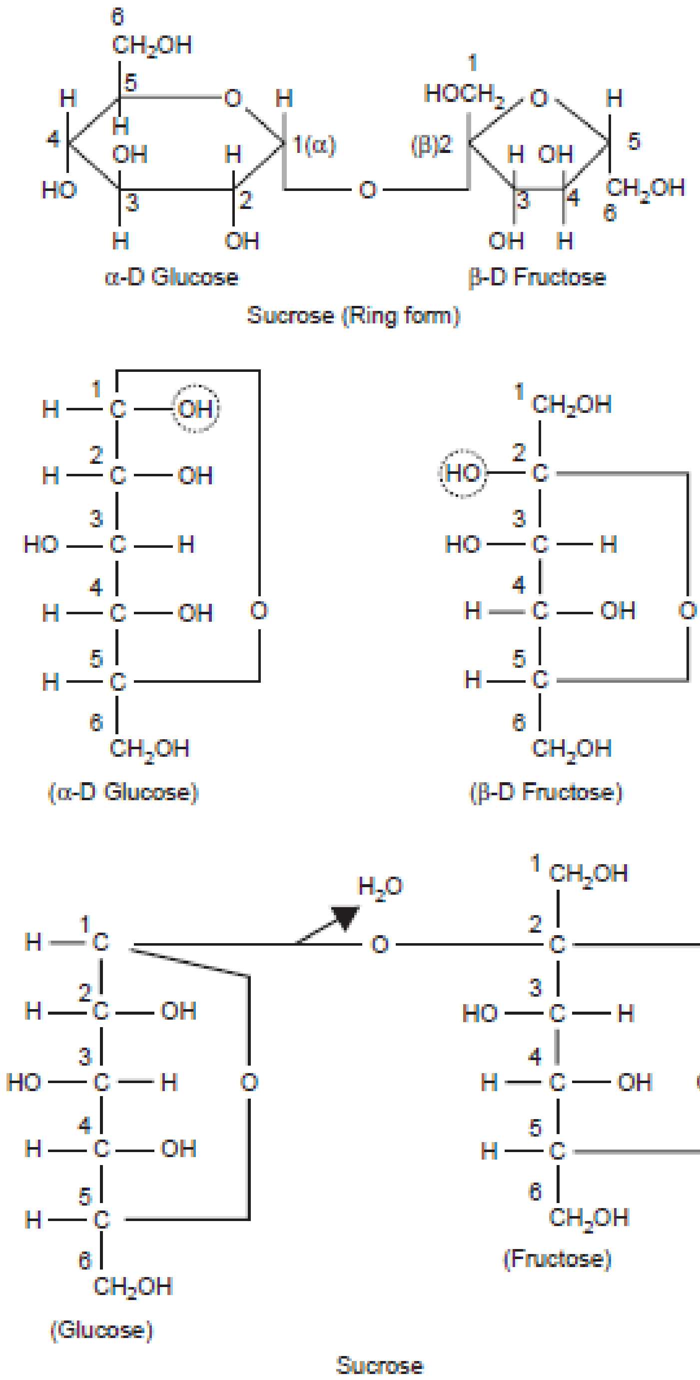


Figure 2. 3 Chain and ring structure of sucrose(Ladaniya, 2008b)

2.2.2 Organic acids

Organic acids are a main source of energy in plant cells. Organic acids are synthesized by different pathways (Figure 2.2). Citric and malic acids are synthesized from Krebs cycles (Famiani et al., 2015). Ascorbic acid is synthesized from imported sugars via GDP-mannose, GDP-L-galactose, L-galactose, and L-galactono-1,4-lactone, while tartaric acid is synthesized from ascorbic acid (Famiani et al., 2015; Smirnoff and Wheeler, 2000). Succinic acid is synthesized from the degradtion of citrate through GABA synthesis pathway (Etienne et al., 2013). Organic acids present in free form or in combined with salts, esters, or glycosides. The synthesis site of organic acids are located in juice vesicles of the fruit, thus most of organic acid is present in the endocarp of citrus fruit (Ladaniya, 2008b).

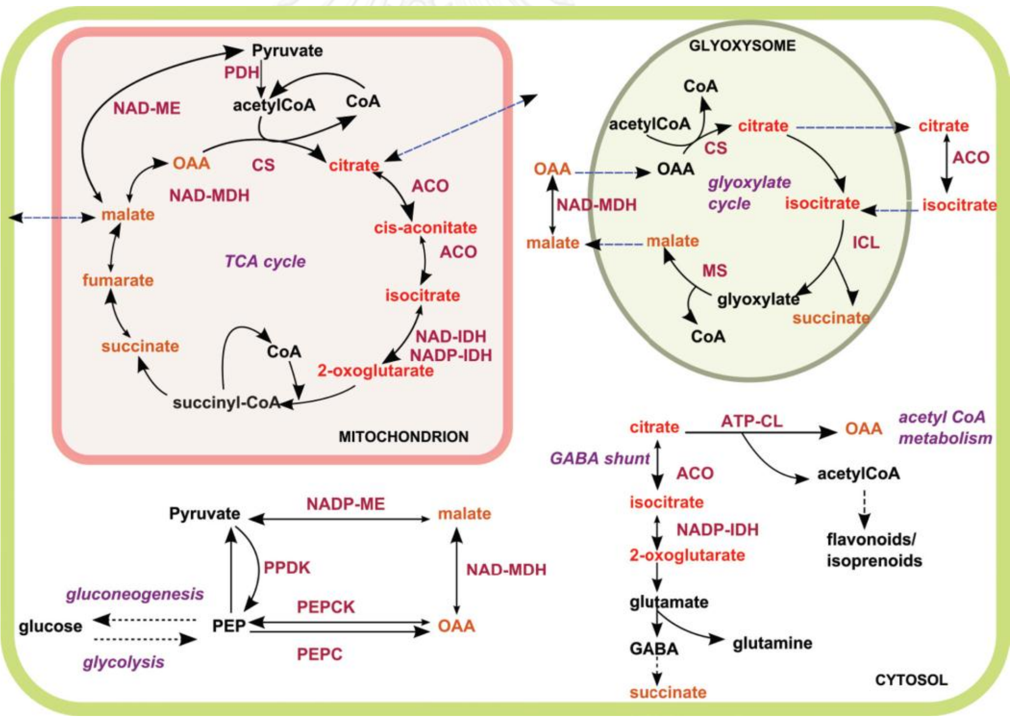


Figure 2. 4 Citrate and malate metabolic pathways in fruit mesocarp cells(Etienne et al., 2013)

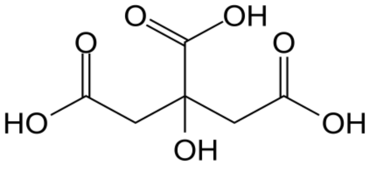
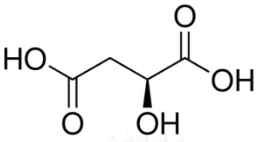
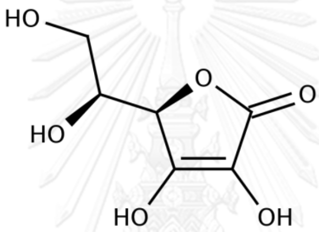
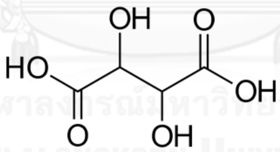
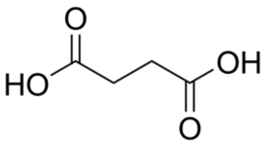
Citric acid is the major acid in citrus juice. The other acids in citrus juice are oxalic, malic, tartaric ascorbic, and succinic acids. Table 2.2 showed that species significantly impact on organic acids distribution of citrus fruit juices (Nour et al., 2010). Ladaniya (2008b) reported that lime juice mainly contains citric acid (5.56-6.60%), malic acid (0.46%) and succinic acid (0.01%).

Table 2. 3 Organic acid composition of citrus fruits(Nour et al., 2010)

Species	Oxalic (g/l)	Tartaric (g/l)	Malic (g/l)	Lactic (g/l)	Citric (g/l)	Ascorbic (g/l)
Sweet orange	0.109	0.336	1.516	1.857	13.918	0.636
Clementine	0.049	0.141	1.367	0.821	11.921	0.340
Mandarin orange	0.088	0.214	1.775	1.229	12.735	0.515
Pomelo	0.268	0.237	0.871	-	12.998	0.419
Lemon	0.094	0.073	1.465	1.545	73.936	0.718
Lime*	0.110	0.012	5.183	0.915	61.497	0.354
White grapefruit	0.117	0.169	0.089	0.641	23.053	0.580
Pink grapefruit	0.143	0.115	1.819	0.595	21.907	0.463

Organic acids are main contributor of sour taste in citrus fruit due to its hydrogen ion in carboxyl group. Richards (1900) reported that sour taste associated with hydrogen ion. In 1920, it was found that only the basis of hydrogen ions could not explain sour taste stimulation mechanism (R. Harvey, 1920). In addition, sour taste intensity is associated with the dissociation of organic acid which depends on the pKa values of the acids. It also has been reported that the acid having lower pKa (a higher capacity to dissociate) induces higher sour taste (Makhlouf, 1972). Sour taste intensity is directly related to the total molar concentration of organic acid species with one or more protonated carboxyl groups accumulate with the concentration of free hydrogen ions probably provides a basis for predicting sour taste (Da Conceicao Neta et al., 2007).

Table 2. 4 Structure and pKa of some organic acids commonly found in citrus fruits.

Organic acids	**Structure**	**pK_{a1}***	**pK_{a2}***	**pK_{a3}***
Citric acid		3.06	4.74	5.40
L-Malic acid		3.48	5.10	-
L-Ascorbic acid		4.10	11.79	-
Tartaric acid		3.07	4.39	-
Succinic acid		4.21	5.64	-

*(Rajković et al., 2007)

2.2.3 Bitter compounds

Bitter taste is an indicator of dietary toxic therefore detection thresholds of bitter taste are extremely low. The mechanism in the perception of bitter taste is still unclear, however many studies suggested that a common mechanism of bitter taste perception probably liked to G protein same in sweet taste (Belitz et al., 1979; Drewnowski and Gomez-Carneros, 2000). According to AH-B, it was found that the AH (hydrogen donor) to B (Lewis base) orbital distance was 1.5 Å, while it was 3.0 Å in sweetness. The bitter compounds can be found in many chemical classes, but it usually associated with alkaloids and glycosides which naturally present in plants (Belitz et al., 1979)

In citrus fruits, Limonoids (limonin) and flavonoids (naringin) are main contributor for bitterness (Ladaniya, 2008b; Maier and Beverly, 1968; Yusof et al., 1990).

2.2.3.1 Naringin

Naringin is a glycoside composed of aglycone naringenin and neohesperidose, a disaccharide moiety (Figure 2.3). The free form structure of naringenin has no bitterness, whereas naringein with disaccharides moiety, neohesperidose, is a bitterness contributor. Naringin is the principal flavonoids and bitter component in grapefruit, pomelo, sour orange and kumquat (Ladaniya, 2008b; Yusof et al., 1990). Yusof et al. (1990) determined naringin content in local Malaysian citrus fruits, including musk lime, Mexican lime, kaffir lime, pomelo and mandarin orange, by the high-performance liquid chromatographic method. The study showed that naringin could only be found in pomelo and kaffir lime. In addition, this study also reported the higher naringin content in the skin than in the juice and seeds. The detection threshold of naringin is 10-100 ppm (Ladaniya, 2008b).

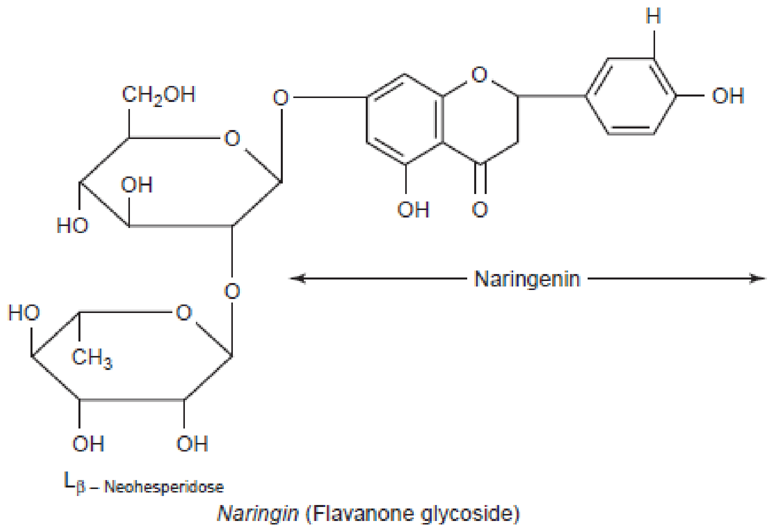


Figure 2. 5 Structure of naringin (Ladaniya, 2008b)

2.2.3.2 Limonin

Limonin has a basic tri-terpene structure. The precursor of limonin is limonin monolactone which is a non-bitter compound. It presents in albedo and endocarp tissues of fruits and stable at neutral pH (Ladaniya, 2008b). Limonin monolactone could convert to limonin by the enzyme limonin-D-ring lactone hydrolase which is accelerated by heat and acidic condition (pH<6.5) (Ladaniya, 2008b; Roy and Saraf, 2006; Siddiqui et al., 2013). The structure of lominin and its precursor are shows in Figure 2.4. Limonin has low amount in fresh juice and could gradually be developed during juice extraction, heat treatment and prolonged storage therefore, limonin is referred as delayed bitterness (Sandhu and Minhas, 2007; Siddiqui et al., 2013).. The highest content limonin was reported in seeds followed by, flavedo tissues, albedo tissues, segment wall and less in juice vesicle (Siddiqui et al., 2013). The threshold of limonin was reported at 4.0 mg/l in a simple matrix (sucrose and citric acid) (Dea et al., 2013)

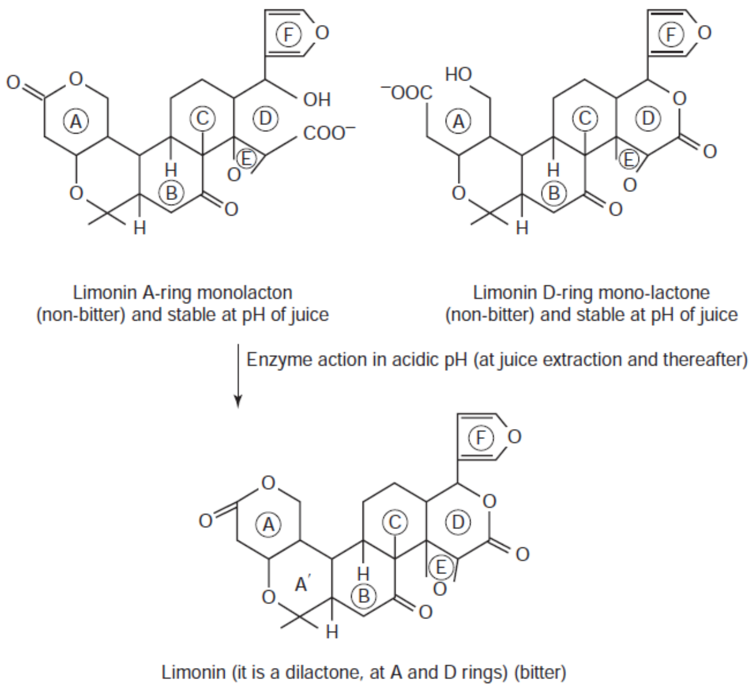


Figure 2. 6 Non-bitter mono-lactone and bitter dilactone structures of limonin(Ladaniya, 2008b)

2.3 High Performance Liquid Chromatography (HPLC)

High-performance liquid chromatography is an analytical technique used to separate, identify, and quantify each component in a mixture sample. It is carried on pumps to pass a pressurized liquid solvent (mobile phase) containing the sample mixture through a column filled with a solid adsorbent material (stationary phase). Each compound in the sample interacts differently with the adsorbent material causing different flow rates for different compounds, and then the compounds will be separated when they flow out for column to detector (Yashin and Yashin, 2012). Due to its simplicity and to the more suitable chromatographic conditions, HPLC separation is thought to be attractive for the fast and quantitative separation of the main organic acids, sugars, and flavonoids in fruit juices. Figure 2.5 shows a block diagram of HPLC system.

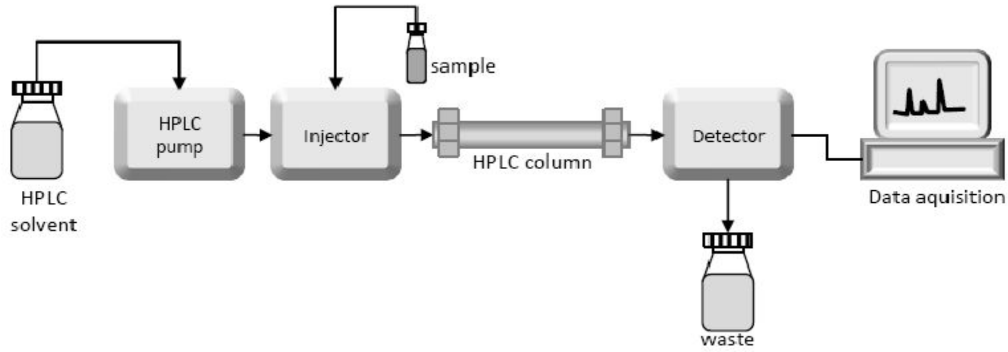


Figure 2. 7 Block diagram of HPLC system (source : http://laboratoryinfo.com/hplc/)

2.4 Standard addition method

Standard addition method is used for quantitative chemical analysis. It is well-known and widely used in analytical chemistry. Standard addition aims to eliminate the influence of matrix effects interference on the result that leading to be impossible to compare the analytical signal between samples and standards by traditional calibration method. This method provides the addition of a concentrated standard in to the samples with the assumption that an increased concentration of analytes would change the responses of analyzes in a linear relationship. By plotting a linear calibration curve between the responses (y-axis) and the amounts of standard added (x-axis), the unknown sample concentration can be determined from the absolute x-intercept values (Figure 2.6) (Andersen, 2017; Bader, 1980; Saxberg and Kowalski, 1979) which is calculated from following equation.

$$S_{sample} = k_A C_A \frac{V_0}{V_f} \tag{2.1}$$

$$S_{spike} = k_A \left(C_A \frac{V_0}{V_f} + C_{std} \frac{V_{std}}{V_f} \right) \tag{2.2}$$

$$S_{spike} = kAC_A\frac{V_0}{V_f} + kA\ C_{std}\frac{V_{std}}{V_f}$$

$$Y = \text{y-intercept} + (\text{slope})\ X$$

$$0 = kAC_A\frac{V_0}{V_f} + kA\ (\text{x-intercept})$$

(2.3)

Therefore,

$$\text{x-intercept} = -\frac{kAC_A\frac{V_0}{V_f}}{kA} = \frac{y-intercept}{slope} = -C_A\frac{V_0}{V_f}$$

Where,

S_{sample} = measured signal/response of sample without standard solution

S_{spike}= measured signal/response of sample with standard solution

C_A = concentration of analyte

C_{std} = concentration of standard stock solution

V_0 = volume of sample added

V_{std} = volume of standard stock solution added

V_f = final volume

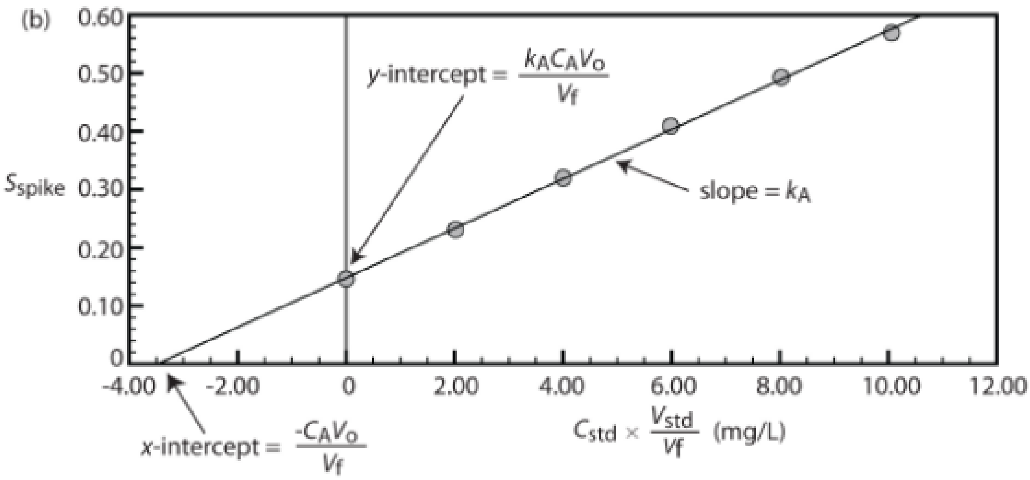


Figure 2. 8 Standard curve from standard addition method (D. Harvey, 2016)

2.5 Factors affecting non-volatile compounds and their sensory quality in citrus fruit

2.5.1 Cultivars or geographical origins

Cultivars or geographical origins are affected by genotypes and environmental conditions such as, amounts of sunshine, rainfall, etc. Chemical composition, physicochemical properties, and sensory profile of Malaysian pomelo juices from 2 cultivars including, *Citrus grandis* (L.) Osbeck PO51 (White pomelo) and *Citrus grandis* (L.) Osbeck PO52 (Pink pomelo) were investigated. Both pomelo juice were difference in the total soluble solid contents of both pomelo juices were different, white pomelo was characterized by mild acidity and a higher pH value, while pink pomelo was found to be higher in its organic acid content, of which, citric acid was the main organic acid. The white pomelo juice with higher sucrose content was highly correspond to sweeter taste, while the pink pomelo juice was rated higher in bitter and sourness (Cheong, Liu, et al., 2012).

Hasim Kelebek (2010) determined the organic acids, sugars, and phenolic composition of the grapefruit juice obtained from 4 cultivars, including 'Rio red', 'Star ruby', 'Ruby red' and 'Handerson'. The studied reported that the major sugar and organic acid were sucrose and citric acid, respectively. For the total sugar, the highest total sugars were found in 'Handerson' and the lowest one was found in 'Ruby red'. With regard to organic acids, the highest sum of organic acids was found in 'Star ruby' and the lowest in 'Rio red'. Naringin and narirutin were the most dominant flavones in grapefruit juices, The highest level of naringin was detected in 'Star Ruby', followed by 'Handerson', 'Rio red' ,and 'Ruby red'. Naringin is an important flavanone in grapefruit juices since it is known to be responsible for the bitter taste of grapefruit juices.

Cheong, Zhu, et al. (2012) studied on characterization of calamansi (*Citrus microcarpa*) juices from three countries (Malaysia, the Philippines and Vietnam). The profiles of physicochemical properties, volatiles and non-volatiles were investigated. For the fructose and glucose contents, calamansi juice from the Philippines had the highest concentration, followed by those from Vietnam and Malaysia. In contrast, the sucrose contents of calamansi juices were not statistically significant. Calamansi juice from the Philippines had the lowest amount of organic acids, notably citric acid, while those from Vietnam had the highest citric acid content. On the other hand, the Philippines calamansi juice had the highest amount of succinic acid (0.15%), and its concentration was about twice as high as those from Malaysia and Vietnam.

2.5.2 Harvest Maturity

Bai et al. (2016) investigated the major flavor chemicals, volatile (aroma), non-volatile (taste) of "Valencia" Orange Juice over the four-month commercial harvest seasons. The study showed that TA content decreased consistently over harvest seasons, resulting in steady increase of SSC/TA. Changes in TA and SSC/TA ratio were derived from some increase in SSC, but predominantly from a decrease in TA due to the decrease of citric acid, the dominant acid in citrus fruit. In mature orange juice sacs, both aconitase and citrate lyase activities were absent. Thus, decreasing the synthesis of oxaloacetate, the precursor of citrate, during maturation, which play a major role in the acid decline over the season. The bitter limonoids, limonin and nomilin generally decreased over the harvest season. Dilution and degradation during ripening cause a reduction in limonin levels. Individual sugars (sucrose, glucose and fructose) generally increased over the harvest seasons.

2.5.3 Extraction method

Baldwin et al. (2012) studied on changes in flavor and other quality parameters due to differences in the methods of juice extraction on the same batch fruit including, hand-squeezed juice (HSJ), fresh-commercial juice (FCJ), processed/pasteurized juice (PPJ). The 'Valencia' processed/ pasteurized juice contained 0.023% peel oil, while the FCJ juice contained 0.240% peel oil, and the HSJ contained a very low peel oil level of 0.003% total ascorbic acid was highest in FCJ but decreased after 4 days, while ascorbic acid in HSJ increased after 4 days. The HSJ had higher TA content which caused a lower SSC/TA ratio. SSC had no difference in all treatments. Although individual sugars varied, total sugar was lowest in HSJ on day 0 but highest by day 4, due to an increase in fructose. The HSJ had higher TA content because of higher citric acid content HSJ had lower malic acid content, although there was no difference in pH. ‘Valencia’, on the first day, HSJ was preferred; there were no differences for sweetness, sourness and mouthfeel, despite HSJ having higher TA and lower SSC/TA. HSJ was perceived as fresher. FCJ had more peel oil; and PPJ had more cooked flavor. FCJ had the most off-flavor like bitter, probably from the excess of peel oil.

Álvarez et al. (2012) investigated the effects of two commonly used juice extraction techniques on the chemical composition and functional properties of clementine juice. Two juice extraction method are Zumex squeezer (A) which, cutting the fruit through the middle and passing the halves between two rotating cylinders pressing, and Fresh’n Squeeze (B) which cutting the fruit in the center and pushing a strainer up inside the fruit, a mechanical hand presses the juice and pulp against this strainer, keeping the juice away from the strongly flavored peel oils in the exterior of the fruit. The study found that juice B had higher scores than juice A in some key descriptors such as fruit taste and fresh juice taste. At the same time, juice B had the lowest score for the following undesirable descriptors: bitterness, peel oil taste, green taste, spicy, and astringency. The essential oil of citrus peel contains high levels of limonene; therefore, high levels of peel oil could contribute to a bitter flavor.

2.5.4 Thermal processing and storage conditions

Siddiqui et al. (2013) studied on bitter compound of sweet orange juice. It was found that pasteurized juice become bitter in taste. Limonin content in pasteurized juice was significantly increased to a hundred times. Moreover, the limonin content reached 10.2 mg/l in 6 hours from an initial content of 0.15mg/l in freshly extracted juice. In addition, Farnworth et al. (2001) studied on the effect of thermal processing and storage conditions on the composition and physical properties of Mexican orange juice. They determined the impact of three alternative methods of processing and storing orange juice including, the unpasteurized frozen juice stored at -18°C (method A), the pasteurized frozen juice stored at -18°C (method B), and the pasteurized stored at 1°C (method C). Juice processed by method B exhibited significantly larger sucrose and fructose concentrations compared to methods A and C. Throughout storage, the individual sugar concentrations of the orange juices did not change, but total soluble solid significantly increased with time. Pasteurized orange juice samples in this study (methods B and C) exhibited higher total sugar content and the larger TSS compared to unpasteurized orange juice. G. Sadler et al. (1992) reported that sucrose concentrations in 'Valencia' orange juice decreased during storage at 4°C, apparently due to microbial contamination, with the smallest decrease in sucrose observed in unpasteurized orange juice. Malic acid content in the pasteurized orange juice (method A) samples was significantly lower than the unpasteurized orange Juices (methods B and C). Malic acid concentrations of orange juice increased significantly during storage. There was no change in citric acid concentration during storage, and method of processing did not affect juice citric acid level. The concentration of ascorbic acid was affected by both the processing method and storage time.

2.6 Sensory evaluations

Sensory evaluation is a scientific method used to evoke, analyze, measure and interpret reaction to characteristics of food products or materials since they are perceived through sensory system. Sensory evaluation includes techniques of measurement and evaluation which can be gain the accurate measurement of sensory response to foods or materials and minimizes the potential of other information influencing consumer perception (Lawless and Heymann, 2010; Stone et al., 2012a).

Sensory evaluations can be divided into three types according to their primary purpose i.e. discrimination tests, descriptive analysis and affecting test

2.6.1 Descriptive analysis

Descriptive analysis is the most comprehensive and informative sensory test. It provides quantitative descriptions of samples in term of the perceived sensory attributes. It is used to investigate a detailed specification of product's sensory attributes or compare the sensory differences among products. A descriptive analysis usually has 8 to 12 trained panelists. The panelists would be trained with reference standards to understand and agree on the meaning of the attributes used. A quantitative scale was used to specify intensity which provides the sensory profile to be able to statistically analyze.

There are several types of descriptive i.e. Flavor Profile®, Texture Analysis, Quantitative Descriptive Analysis®,

2.6.1.1 Flavor Profile®

Flavor Profile® is a technique which considers the overall flavor, individual detectable flavor, intensity, aftertastes, and overall impression. The trained panelists would identify reference standards and definitions for each descriptor use during training. The samples are used in the test as same as the consumer would be served. Panelists usually determine the amplitude before they focus on the individual flavor notes of the sample. The scale used in this method is a combination of number and symbols, thus preventing data analysis by statistical method. Hence, Flavor Profile® is classified as a qualitative descriptive technique.

2.6.1.2 Texture Analysis

Texture Profile was developed from Flavor Profile in order to specifically analyze texture characteristic of foods with regard to mechanical, geometrical, fat and moisture characteristics, including, the degree of each present and the order in which they appear from first bite through complete mastication (Brandt et al., 1963; Lawless and Heymann, 2010). A standardized terminology and rating scale are used to describe the texture characteristics. Definitions and order of appearance of the terms is consensus agreement of panelists. This method can provide direct comparison of results with known materials by using the reference product and also provide a relationship between the results and instrument measurements (Szczesniak et al., 1963).

2.6.1.3 Quantitative Descriptive Analysis®

Quantitative Descriptive Analysis® (QDA®) was developed from Flavor Profile and Texture Profile methods. Similar to Flavor Profile, panelists develop a set of vocabulary to describe differences among the samples. In addition, the reference standards and attribute definitions are decided by the panelists. The evaluating sequence of each attribute is decided during the training period. The panel leader is only a facilitator, direct discussion, supply materials and also help other panelists to sort out the attributes used in the test. Unlike Flavor Profile, QDA® samples are not necessary served to the panelists as same as the consumer. A 6-inch line scale with words generated by the panel is used. The QDA® data are gained as relative values not absolutes. The result from QDA® can be using statistically analysis such as multivariate analysis of variance, principal component analysis, cluster analysis (Lawless and Heymann, 2010; Stone et al., 2012b; Stone et al., 2008).

2.6.1.4 Sensory Spectrum®

Sensory Spectrum® is an expansion of descriptive analysis. In this method, panelists use a standardized lexicon of terms instead of creating a panel-specific vocabulary to describe sensory attributes of products. The numeric 15-point scales are used for intensity scales, so the data values are absolute. All panelists would be train to use the descriptor scales in the same way.

However, Flavor Profile®, QDA® and Sensory Spectrum® descriptive analysis can be adapted and apply to suite with different food products. Many previous studies developed descriptive sensory analysis for evaluating the sensory profile of their own product. Descriptive sensory analysis also frequently used to evaluate sensory profile of fruit juice. Carbonell et al. (2007) evaluated sensory profile of fresh and processed mandarin and orange juices using descriptive analysis with 29 descriptors by 11 panelists. From the study of Luckow and Delahunty (2004), a descriptive sensory analysis was used to examined the sensory impact of functional ingredients, e.g. probiotic, prebiotic, vitamins and minerals, on the aroma and taste of orange fruit juices. Four added functional ingredients orange juices and seven conventional orange fruit juices were evaluated by ten trained panelists with 37 sensory attributes

2.6.2 Affecting testing/ Consumer sensory testing

Affective testing is a screening task which can identify which product is preferred by consumers or find consumer acceptance on the product based on its sensory characteristics. The result from affective testing can combine with other sensory analyses, product formulation to investigate the optimal design of food products for consumers. The panelists do not need to be trained, but they should be selected from the product's target group (Lawless and Heymann, 2010; Stone et al., 2012c). There are two main methods to approaches affecting testing or consumer sensory testing including,

2.6.2.1 Preference test

Preference test is used to compare among two products or several products which one or two or more is preferred by consumers. There are several types of preference testing including,

- Simple Paired preference testing
- Non-forced Preference
- Replicated Preference tests
- Replicated Non-forced Preference
- Other Related Method e.g. Ranking, Best-Worst Scaling, Rated Degree of Preference

2.6.2.1.1 Preference ranking test

In this test, products would be ranked by consumers from most liking to least liking and panelists would be forced to make choices, resulting in no tied ranks happens. The result from preference ranking test is usually analyzed by using Friedman's test. If the result is significance, the least significant ranked different (LSRD) values will calculated to find out which samples are significantly preferred to the others(Lawless and Heymann, 2010; Luckow and Delahunty, 2004). Luckow and Delahunty (2004) used preference ranking test in their study to investigate the effect of adding functional ingredients in orange juice on consumer acceptance.

2.6.2.2 Acceptance test

Acceptance test provides the degree of acceptability of food products rating on an acceptance scale, thus the data could be gain in absolute score of liking. In contrast to preference test, acceptance test does not need alternative samples to compare. The test can be done using a single product. The 9-point hedonic scale is commonly used in foods, beverages or non-food product to quantify acceptability in consumer sensory test since it is very simple and easy to implement. It has been reported that the hedonic scale is reliable and high stability (Pimentel et al., 2016). The samples are served to the panelist one at a time or all samples can be placed on one tray with the three-digit code, then the panelists were asked to indicate their liking to the sample in each topic in the questionnaire. Furthermore, there are other acceptance scales can be used e.g. line scales, magnitude scale, labeled magnitude scales etc.

2.7 Chemometrics

Nowadays, with high technology and powerful instruments, e.g. Gas chromatography (GC), High performance liquid chromatography (HPLC), spectroscopic techniques, scientists can obtain a lot of data from each sample analyzed. Also, in food products, the samples can be described by a number of chemical and physical parameters such as rheological properties, color, pH, textural properties, sensory profile, etc. To handle and analyze a huge amount of data and various variables, Chemometrics becomes a powerful tool to solve such problem. The large set of variables and data obtained from the experiments are described by data vectors (Oliveri and Forina, 2012).

Chemometrics is the science of relating measurements made on a chemical system or process to the state of the system via application of mathematical or statistical methods (International Chemometrics Society: ICS). In addition, chemometrics also could build the bridge between consumer preferences, sensory attributes and molecular profiling of food (Bertacchini et al., 2013). Multivariate data analysis is a tool of chemometrics that aim to find the correlations between samples and variables (Kumar et al., 2014). Due to their difference in magnitude and scale, a proper pre-processing data analysis is required to be the first step. Then, to find out the similarities or differences among samples based on the data set, a pattern recognition method is perform which includes the unsupervised pattern recognition method and the supervised pattern recognition method. (dos Santos et al., 2013)

2.7.1 Unsupervised pattern recognition techniques

Unsupervised pattern techniques aim to visualize the relations between samples and variables. They do not need prior knowledge about the data (Roggo et al., 2007). The unsupervised pattern recognition techniques can be performed a preliminary evaluation of the information content in the data matrices by using Cluster analysis (CA) and Principal component analysis (PCA) (Abad-García et al., 2012).

2.7.1.1 Hierarchical Cluster Analysis (HCA)

Hierarchical Cluster analysis is a part of cluster analysis. It highlights the existence of similarity or dissimilarity and natural groupings among samples inside the data set by evaluating the distance of data matric between samples. The distances between samples can be calculated by different method. The result is performed by dendogram which shows the cluster of sample and the distances (dos Santos et al., 2013; Roggo et al., 2007). Hierarchical clustering is divided in two types :

- *Agglomerative clustering*

Agglomerative clustering is a “bottom up” approach. Initially, each object represents its own cluster. Then, a selected pair of clusters with the smallest intergroup dissimilarity is merged into a single cluster. A grouping would produce one less cluster at the next level. (Hastie et al., 2009; Rokach and Maimon, 2005)

- *Divisive clustering*

Divisive clustering is a “top down” approach. Initially, all objects represent in a same cluster. Then, the cluster is divided into the two new sub-clusters which produce the largest between-group dissimilarity in the next level. Divisive clustering focused on partition the data into a relatively small number of clusters. (Hastie et al., 2009; Rokach and Maimon, 2005)

2.7.1.2 Principal component analysis (PCA)

Principal component analysis is a factor analysis which reduces the number of variables presenting in n-dimensional data set into smaller number of dimensional data structure which still retain the maximum amount of variability present in the data by creating a set of orthogonal axes (the linear combination of the original variables) in order to provide a better visualization of data structure. The linear combination of the principal components is based on the data correlation matrix (the standardized data) or the data covariance matrix (the unstandardized data). The correlation matrix is useful when the variables were measured on widely largely different scales which can affect the result. On the other hand, the covariance matrix is used when the data are measured ion the same scale. The row of a data matric correspond to samples or objects, called scored, and the column correspond to variables or factors, called loadings (dos Santos et al., 2013; Kumar et al., 2014; Lawless and Heymann, 2010) .Generally, the first principal component (PC1) represents the maximum possible amount of variance among the samples direction, while the second principal component (PC2) describes the remaining points. Practically, two or three PCs can be describes and represented the objects (score plot), the original variables (loading plot), or both objects and variables (biplot) (Oliveri and Forina, 2012).

In sensory evaluation, there are a lot of sensory attributes or samples which lead to be difficult to explain the results or the relationship among samples. PCA simplifies and describes interrelationships among the descriptors or sensory attributes (dependent variables) and among the samples (objects). Ngamchuachit et al. (2015) determined influence of cultivar and ripeness stage of fresh-cut mango by measuring physico-chemical and sensory quality. PCA was used to illustrate the difference in fresh-cut mango samples using sensory attributes from descriptive sensory analysis as variables in the covariance matrix. In addition, PCA is also widely used for the differentiation or classification of food products or fruits due to geographical origin or chemical profile. Cheong, Liu, et al.

(2012) compared the chemical profile and sensory profile of pomelo juice from two cultivars (*Citrus grandis* (L.) Osbeck PO 51 and PO 52). PCA was used based on the correlation matrix to determine the difference in volatile and non-volatile compound of two pomelo cultivars.

2.7.2 Supervised pattern recognition techniques

In unsupervised pattern recognition techniques, they just show the correlation between variables from the data as presented. In contrast with supervised pattern recognition techniques, they perform the regression model which proposes to predict a qualitative or quantitative property of sample. The reliability of the model in prediction should be evaluated before using the model in practice by validation. To validate the model, the available samples are divided into two subsets including, a training set which is used for calculating the model and an evaluation set which is used for evaluating the reliability of the model used (Giansante et al., 2003; Oliveri and Forina, 2012).

2.7.2.1 Partial Least Squares regression (PLS)

Partial Least Square regression (PLS) is one of supervised quantitative modeling which performs regression defining mathematical relationships between variables. PLS determines a relationship between dependent variables or target values (Y) and independent variables or input matrix (X). It finds the components in the input variables describe the maximal relevant variation in the input variables which have a maximal correlation with the target values in Y. Therefore, PLS regression concurrently accounts the latent variables in X which will predict the latent variables in Y and also maximizes the covariance between matrices X and Y. (Berrueta et al., 2007; Oliveri and Forina, 2012; Westad et al., 2013). PLS is a useful tool explaining and predicting the relationship between sensory properties and chemical compositions in food. There are several previous studies using PLS to find the correlation of sensory attributes and chemical components in fruit. Xi et al. (2016) determined the correlation of flavor compounds and sensory attribute of apricot fruit using PLS regression model to find the key characteristic flavor factors contributing to consumer acceptance during fruit development and ripening. Tiitinen et al. (2005) used PLS to explain the relationship between the sensory properties and chemical compounds of juice from seven sea buckthorn (*Hippophaë rhamnoides* L.) varieties.

CHAPTER III
METHODOLOGY

3.1 Materials and instruments

3.1.1 Plant material

- Lime cv. 'Pan Rumpai' (*Citrus aurantifolia* (Christm & Panz) Swingle)
- Lime cv. 'Pan Puang' (*Citrus aurantifolia* (Christm & Panz) Swingle)
- Lime cv. 'Pan Pichit' (*Citrus aurantifolia* (Christm & Panz) Swingle x *Citrus latifolia* Tanaka)
- Lime cv. 'Tahiti' (*Citrus latifolia* Tanaka)

3.1.2 Chemicals

- Acetonitrile, HPLC grade (RCL Labscan, Thailand).
- Citric acid monohydrate (Fisher scientific, United States)
- D-(-) fructose (Sigma-aldrich, USA)
- D-(+) glucose (Supelco, USA)
- Deionized water
- L-(-)-malic acid, $C_4H_6O_5$ (Sigma-aldrich, USA)
- L-ascorbic acid (Fisher scientific, United States)
- Limonin (Sigma-aldrich, Germany)
- Naringin (Sigma-aldrich, Isarael)
- Succinic acid, C4H6O4 (Fisher scientific, United States).
- Sucrose (Supelco, USA)
- Sulphuric acid 98% (QREC chemical, Thailand)
- Sodium hydroxide

3.1.3 Instruments

- 1290 Infinity II UHPLC system (Agilent Technologies, Germany)
- Alliance Waters 2690 HPLC system equipped with Waters 410 differential refractometer detector (Waters Corporation, United States)
- Centrifuge (Hermle Z36HK, HERMLE Labortechnik GmbH, Germany)
- Ceramic knife
- Cheese cloth
- Colorimeter (Minolta CR-300, Tokyo, Japan)
- Hand refractometer (Atago® master M, Japan)
- Micropipet (Pipet-Lite XLS, Rainin®, Mettler Toledo, Thailand)
- Nylon syringe filter 0.22 µm, 25 mm (CNW technologies®, Shinghai)
- pH meter (CyberScan® pH 1000 meter, Eutech instruments, Netherland)
- Rezex ROA Organic acid column (300mm×7.8 mm) (Phenomenex®, United States)
- Water bath (One 7, Memmert, Germany)
- Whatman® no.1 filter paper
- Zorbax Eclipse Plus C18 Rapid Resolution HD (2.1 x 50mm, 1.8 um) (Agilent technologies, Germany)
- Zorbax Eclipse Plus C18 rapid resolution HD column

3.2 Methods

3.2.1 Sample preparation

Lime samples were harvested at a commercial maturity on February 2017 (5 months after flowering for limes cv. ‘Pan Rumpai’, ‘Pan Puang’ and ‘Pan Pichit’ and 4 months after flowering for limes cv. ‘Tahiti’). ‘Tahiti’ lime was harvested from Wasa farm in Nakhonnayok province. ‘Pan Rumpai’, ‘Pan Puang’ and ‘Pan Pichit’ were harvested from the Mr. Channarong Puangsun’s farm in Petchburi province. All lime samples were transported in an air-conditioned car. On the same day, limes were visually sorted to discard damaged and defective fruits, and then washed with clean water and stored at 6°C until analyses. To obtain lime juice for each sample replicate, lime juice was prepared from10 fruits by hand-squeezing. The removal of the peel must be done using a ceramic knife in order to avoid contamination from the components in the flavedo and albedo.

All fresh-squeezed lime juice samples were taken for physicochemical properties measurement and non-volatile compound analysis.

3.2.2 Physicochemical properties measurement

Physicochemical properties including, CIE L*a*b*, pH, titratable acidity (TA), and total soluble solids content (TSS) were investigated. CIE L*a*b* values of lime juice were measured by Colorimeter (Minolta, model CR-300 series, Japan). The pH was measured with a pH meter (CyberScan pH 1000 meter, Eutech instruments, Netherland). Titratable acidity was carried out by titrating 5 g of lime juice with 0.1 M NaOH. Titratable acidity was expressed as citric acid (AOAC, 1999). Total soluble solid content was determined in °Brix with a refractometer (Atago, Tokyo, Japan). All experiments have been done in triplicate.

3.2.3 Non-volatile compounds analysis

The 1290 Infinity II UHPLC system (Agilent Technologies, United States) was used for organic acid (citric, malic, ascorbic, and succinic acids and bitter compound (limonin and naringin) analyses. Alliance Waters 2690 system equipped with Waters 410 differential refractometer detector (Waters Corporation, United States) was used for sugar (glucose, fructose, and sucrose) analyses.

3.2.3.1 Influence of storage time and temperature on non-volatile profile of fresh-squeezed Thai lime juice

The experiments have been done in triplicate. For each replicate, lime juice sample was prepared from 10 lime fruits. The 25 ml of fresh-squeezed lime juice ('Pan Rumpai' cultivar) was kept in 6 closed cap amber glass bottles stored at 4°C (3 bottles) and 35°C (3 bottles). One bottle from each storage temperature (4°C and 35°C) was taken periodically at 6, 10 and 24 hours for non-volatile analysis. Then, each lime juice sample was centrifuged at 7000 g for 10 minutes and was filtered through Whatman® no.1 filter paper. The supernatants were kept at -20°C until analyzed. All samples were filtered through 0.22 µm nylon filter before HPLC injection. HPLC conditions are shown in Table 3.1.

3.2.3.2 Analysis of non-volatile compounds in fresh-squeezed juice from four lime cultivars

The experiments have been done in triplicate. For each replicate of each cultivar, lime juice sample was prepared from 10 lime fruits for each replicate. Then, each fresh-squeezed lime juice sample was centrifuged at 7000 g for 10 minutes and was filtered through Whatman® no.1 filter paper. The supernatant was kept at -20°C for HPLC analysis. All samples were filtered through 0.22 µm nylon filter before HPLC injection. HPLC conditions are shown in Table 3.2.

Table 3. 1 HPLC conditions for analysis of Influence of storage time and temperature on non-volatile profile of fresh-squeezed Thai lime juice

Targets	Column	Conditions				
		Mobile phase	**Flow rate (ml/min)**	**Injection volumn (µl)**	**Column Temp.(°C)**	**Detector**
Organic acids Citric acid Malic acid Succinic acid Ascorbic acid	Phenomenex Rezex ROA-Organic acids 300x7.80 mm	0.005 N Sulfuric acid	0.5	10	40	DAD UV 210 nm
Sugar Glucose Fructose Sucrose	Thermo scientific APS-2 Hypersil column 150 x 4.6mm, 3um	80% Acetonitrile	1	10	40	Differential refractometer detector
Bitter compounds Limonin Naringin	Allech Econosil (C18) 5U 250x4.6mm	60% Acetronitrile	0.5	10	40	DAD UV 220 nm

Table 3. 2 HPLC conditions for Analysis of non-volatile compounds in fresh-squeezed juice from four lime cultivars

Targets	Column	Conditions				
		Mobile phase	**Flow rate (ml/min)**	**Injection volumn (µl)**	**Column Temp.(°C)**	**Detector**
Organic acids Citric acid Malic acid Succinic acid Ascorbic acid	Phenomenex Rezex ROA-Organic acids 300x7.80 mm	0.005 N Sulphuric acid	0.5	10	40	DAD UV 210 nm
Sugar Glucose Fructose Sucrose	Phenomenex Rezex ROA-Organic acids 300x7.80 mm	0.005 N Sulphuric acid	0.5	10	40	Differential refractometer detector
Bitter compounds Limonin Naringin	Zorbax Eclipse Plus C18 Rapid Resolution HD 2.1x 50mm, 1.8 um	A : Deionized water B : Acetronitrile 15% - 50% B : 0-3 min 50% -100% B : 3-6 min 100% B : 6-9 min	0.25	5	25	DAD UV 220 nm

3.2.4 Sensory evaluations

3.2.4.1 Descriptive analysis

Descriptive analysis was performed by the 8 trained panelists (2 males and 6 females, aged 30-55 years old) from Betagro science center. They had been trained for 10-12 hours using the following steps below.

1) Developing and selecting the taste attributes,

   The panelists tasted the fresh-squeezed lime juice samples and tried to describe the received taste. Then, they discussed and selected the suitable attributes (standardized vocabularies describing the sensory differences among the samples) for lime juice samples. All panelists should understand and agree on the definitions of all used attributes.

2) Defining the reference standards,

   After the development of the attributes, the concentration and score of the reference standards would be set to define the attributes used in the test.

3) Pre-testing

   The simulated testing with the real samples was set for training the panelists. The panelists practiced on rating the lime juice samples in each attribute on intensity line scale until they could give the consistency scores among the panelists. (The definition of attributes and the concentration of reference standards used shows in Table 4.1)

4) Testing with the fresh-squeezed lime juice samples

   Fresh-squeezed lime juice samples from four lime cultivars were prepared in the same method as the physicochemical analysis. For each cultivar, lime juice samples were prepared in triplicate. Each replicate was freshly prepared from 10 limes. Fresh lime juice stored at 4°C until served to the panelists. Ten gram of fresh-squeezed lime juice was contained in the white plastic cup with the cover lid (Figure 3.1). The panelists were served a set of three-digit coded tasting cups covered with lids at 20°C with randomized block design (RBD). The panelists tasted the samples using a coffee stir spoon (Figure 3.2) for one serving. The intensity was scored on 0-15 unstructured line scale from low intensity to high intensity. The panelists were asked to clean their mouths with drinking water and biscuits and they were forced to take a 10 minutes break between samples.

Figure 3. 1 The tasting cup used in descriptive analysis

Figure 3. 2 Coffee stir spoon used in descriptive analysis

3.4.2 Consumer acceptance test

3.4.2.1 Preference ranking test

Preference ranking test was performed by 60 students from School of Culinary Arts, Suan Dusit University (males and females, age 18-22 years old). The present study focus on the sensory evaluation of four lime cultivars in cooking used purpose thus, the culinary students were chosen to be panelists. Ten grams of fresh-squeezed lime juice samples from four lime cultivars were served in the white plastic cup with the three-digit code and cover lid to the panelists. The samples were served at room temperature (25°C) with randomized block design (RBD). The panelists were asked to rank the lime juice samples from the most to the least preferred using number 1-4 (1 = most preferred, 4 = least preferred) based on cooking used purpose. The panelists were asked to clean their mouths with drinking water and they were asked to take a 30 seconds break between samples. Then, the data were analyzed by Friedman test (Equation 3.1) and the least significant ranked different (LSRD) (Equation 3.2)

$$\chi^2 = \left\{ \frac{12}{[K(J)(J+1)]} \left[\sum_{j=1}^{J} T_j^2 \right] \right\} - 3K(J+1) \tag{3.1}$$

Where

J = number of samples

K = number of panelists

Tj = rank total, and degrees of freedom for $\chi 2$ = (J–1)

$$LSRD = t\sqrt{\frac{JK(J+1)}{6}} \tag{3.2}$$

Where

J = number of samples

K= number of panelists

t = the critical t-value at a = 0.05 and degrees of freedom = 1

3.4.2.2 Acceptance test (9-point hedonic scale)

Acceptance test was performed by 117 culinary students (39 males and 78 females, aged 18-22 years old) from School of Culinary Arts, Suan Dusit University with 9-point hedonic scale. Ten grams of fresh-squeezed lime juice samples from four lime cultivars were served in the three-digit coded plastic cup covered with lids. The samples were served at room temperature (25°C) with randomized block design (RBD). The panelists would mark the accepting score of aroma, color, taste and overall liking from 1-9 points (1= dislike extremely, 2=dislike very much, 3= dislike moderately, 4 = dislike slightly, 5= neither like nor dislike, 6= like slightly, 7= like moderately, 8= like very much and 9= like extremely). The panelists were asked to clean their mouths with drinking water and they were asked to take a 30 seconds break between samples.

3.5 Statistical analysis

The statistical analyses were carried out using XLSTAT-software version 2017 (Addinsoft, Paris, France). Physicochemical properties, non-volatile compounds, and sensory evaluations of fresh-squeezed juice from four lime cultivars were analyzed using the one-way analysis of variance (ANOVA). Fisher's least square difference (LSD) was used for the multiple comparisons of the mean values with 95% confidence level. The difference in sensory intensities among four lime cultivars from the descriptive analysis was analyzed using multivariate analysis of variance (MANOVA) and Duncan's multiple-range tests were used for the multiple comparisons of the mean values with 95% confidence level. Further, multivariate analysis was carried out using Principal Component Analysis (PCA) based on the correlation matrix, Hierarchical Cluster Analysis (HCA) based on similarity with Ward's method. The relationship between the taste attributes and chemical data matrices was analyzed with Partial Least-Square regression (PLS) using jack-knifing.

CHAPTER IV
RESULTS AND DISSCUSSION

4.1 Influence of storage time and temperature on non-volatile profile of fresh-squeezed lime juice

To optimize the proper sample preparation for fresh-squeezed lime juice, the effect of storage time and temperature on the non-volatile profile of fresh-squeezed lime juice were investigated. Lime cv. 'Pan Rumpai' was selected to be a representative of lime in this study due to the chef's recommendation and market survey. 'Pan Rumpai' lime is the most favorable used in Thai kitchen because of its unique aroma, thin peel, and juicy pulp. The result of this study showed that citric acid (82.33 g/l) was the predominant acid in lime juice, followed by malic acid (3.49 g/l), ascorbic acid (0.73 g/l), and succinic acids (0.56 g/l), respectively. There was a trace of sugars (glucose 0.56 g/l, fructose 0.60 g/l, and sucrose 0.47 g/l) and limonin (0.24g/l) in fresh-squeezed lime juice.

During storage, there was no change in malic acid content. Citric and succinic acids contents in lime juice stored at 35°C had no change throughout storage for 24 hours. However, lime juice stored at 4°C was significantly decreased when stored for 24 hours (Figure 4.1). Ascorbic acid content of lime juice stored at 4 and 35°C significantly decreased throughout storage (Figure 4.1). In agreement with previous studies (Igual et al., 2010; Kaanane et al., 1988), most of the organic acid content (citric, malic and succinic acids) remained constant, then decreased after 24 hours while ascorbic acid content significantly decreased during storage. The decrease of ascorbic acid during storage is due to the oxidation reaction in aerobic condition which causes ascorbic acid degraded to dehydroascorbic acid (Kimoto et al., 1993; Yuan and Chen, 1998). Moreover, ascorbic acid could also be degraded to furfural under anaerobic condition through several steps. Furfural may react with amino acid causing a non-enzymatic browning. The ascorbic acid degradation depends on various factors such as oxygen, heat, light, pH, storage temperature and time (Burdurlu et al., 2006; Yuan and Chen, 1998). In addition, the reduction rate of ascorbic acid content in lime juice stored at 35°C was higher than lime juice stored at 4°C. This occurrence was potentially explained by the previous studies that reported the decrease of ascorbic acid content coincides with the increase of temperature and acidic conditions (Burdurlu et al., 2006; Yuan and Chen, 1998).

The sugar content results of lime juice samples showed that fructose and glucose contents of ‘Pan Rumpai’ lime juice significantly increased in 24 hours stored lime juice (Figure 4.1). The increase of these reducing sugars and the decrease of sucrose content in lime juice is due to the acid hydrolysis of sucrose which could release glucose and fructose. The acid hydrolysis approximately occurs at pH 2.5 (Echeverria, 1990) and high storage temperature also promotes more sucrose hydrolysis (Kaanane et al., 1988).

Limonin content was not significantly different between treatments. In agreement with previous studies (Chareonkit and Jirapakku, 2011; Pareek et al., 2011; Siddiqui et al., 2013), limonin content tended to increase with longer storage time and higher storage temperature (Figure 4.1). Limonin usually presents low amount in fresh lime juice, however, it gradually increases during processing (juice extraction, heat treatment) and prolonged storage due to the conversion of limonin monolactone to limonin. Under the acidic environment of lime juice ($pH<6.5$), the enzyme could accelerate the conversion of limonin monolactone to limonin (Roy and Saraf, 2006; Sandhu and Minhas, 2007; Siddiqui et al., 2013). The bitterness of limonin could be reduced by immediately removing the peel of citrus fruit, a rich source of limonin precursor, after extraction or avoiding any heat treatments.

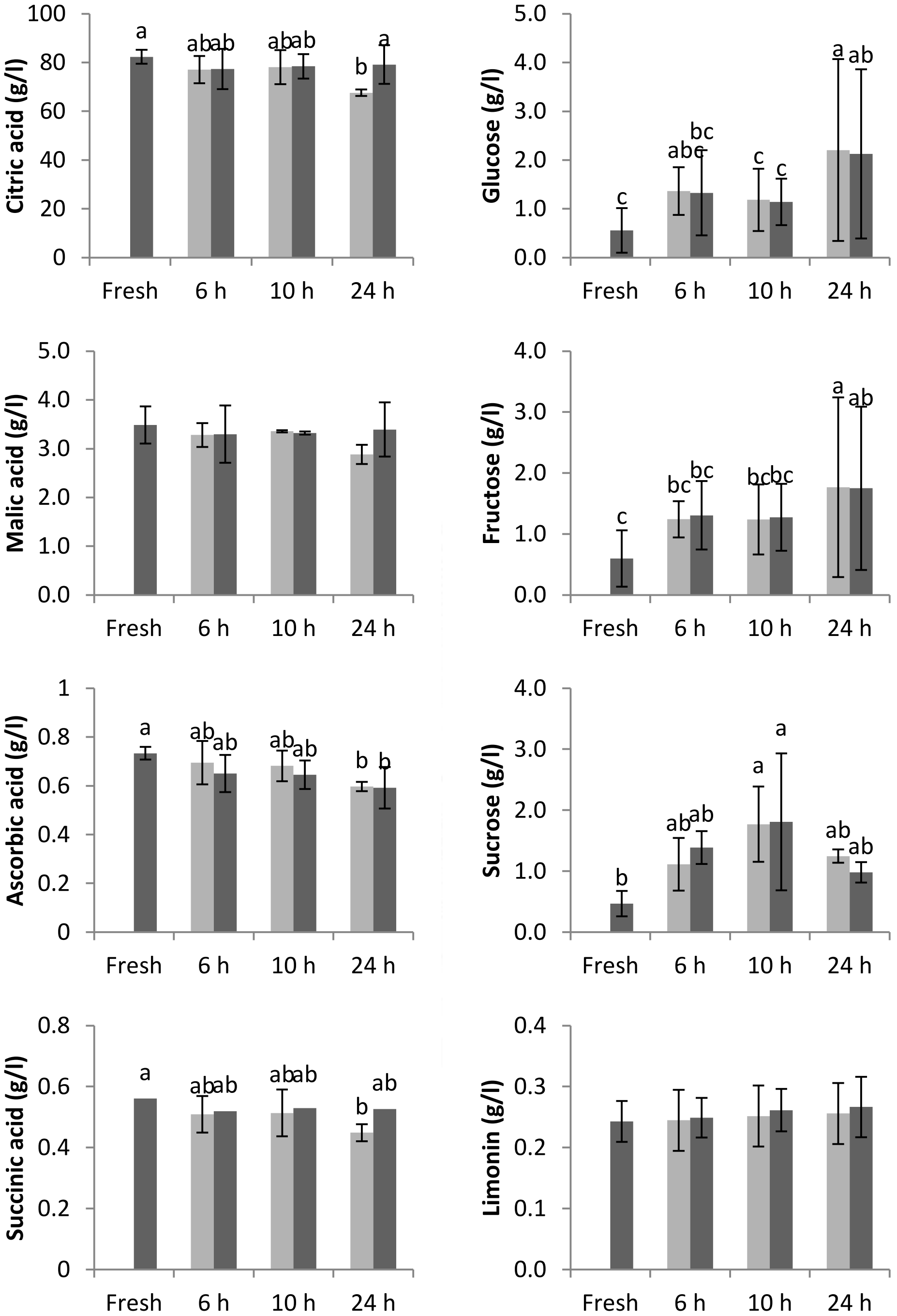


Figure 4. 1 Means of organic acids, sugar, and limonin content of lime juice (Citrus aurantifolia (Christm&Panz) Swingle) cv. 'Pan Rumpai' during storage at 4°C(■) and 35°C(■) for 6, 10, and 24 hours. Vertical bars with different letters are significantly different at $\alpha = 0.05$

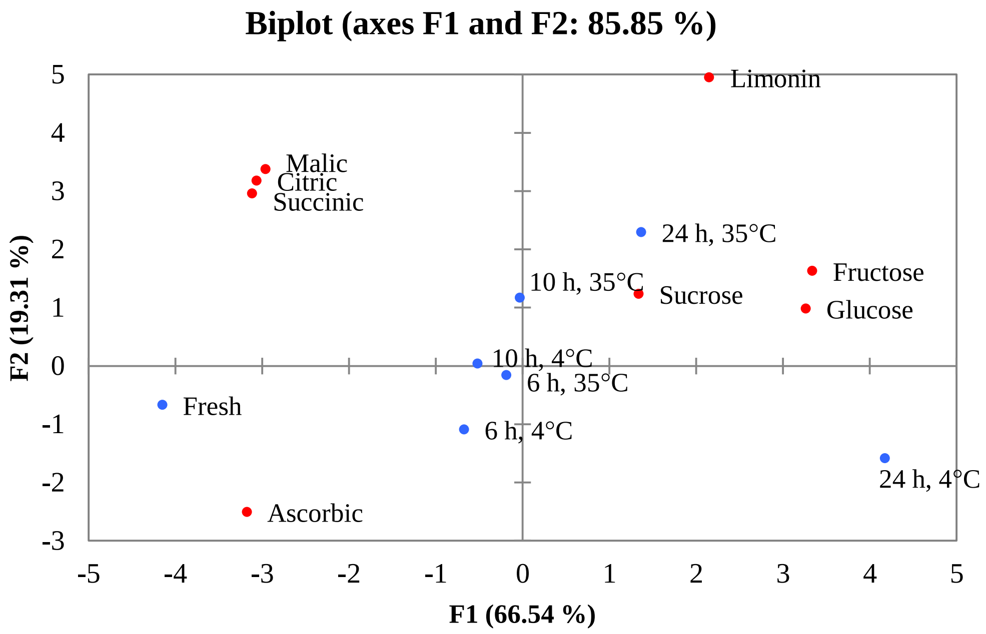


Figure 4. 2 Principal component analysis (PCA) of non-volatiles profile of Thai lime juice (*Citrus aurantifolia* (Christm&Panz) Swingle) cv. 'Pan Rumpai' , stored at 4°C and 35°C for 6, 10, and 24 hours.

Principal component analysis result (Figure 4.2) shows the first two principal components (PCs) accounted for 85.85% of the total variance. The 8 variables were considered as analytical data in PCA in the correlation matrix PC1 explained 66.54% of the total variance among four lime cultivars which was related to fructose, glucose and was negatively associated with citric, malic, ascorbic and succinic acids PC2 explained 19.31% of the total variance which was related to limonin. Fresh Thai lime juice had a strong positive relation with higher in organic acids and lower in sugar content, whereas lime juice stored at 4°C for 24 hours associated with higher in sugar content and lower in organic acids content. The longer stored lime juice (24 hours) at 35°C was strongly correlated with higher limonin content. Thus, higher in sugar contents and lower in ascorbic content could imply the decrease of taste stability and quality of lime juice.

4.2 Comparison of non-volatile compounds of fresh squeezed juice from four lime cultivars

4.2.1 Physicochemical properties of four lime cultivars

In this study, physicochemical properties; titratable acidity (TA), total soluble solid content (TSS), TSS/TA ratio, pH and color measurement were investigated. All of these physicochemical measurements are the preliminary methods used to evaluate quality of fruit and vegetable. TSS has been commonly used to be a representative of dry substance content of solution mainly sucrose (Magwaza and Opara, 2015). Generally, TSS/TA ratio is an index of fruit ripeness because the accumulation of sugars and the loss of acidity are involved in the maturation of citrus (Barros et al., 2012; Ladaniya, 2008b). In addition, TSS and TSS/TA ratio could indicate the sweetness in many fruits which could relate with sensory quality and consumer acceptability (Magwaza and Opara, 2015).

In the present study, the result showed (Table 4.1) that TSS, TA, and TSS/TA ratios of four Thai lime cultivars were not different between ‘Pan Rumpai’ and ‘Pan Puang’, but they were significantly different from ‘Tahiti’ and ‘Pan Pichit’. ‘Tahiti’ had the highest TSS (8.83±0.15) and TSS/TA ratio (1.45±0.03) while the lowest TSS (6.87±0.06) and TSS/TA ratio (1.08±0.01) was found in ‘Pan Pichit’. The highest TA was found in ‘Pan Puang’ (7.05±0.03%) while ‘Tahiti’ had the lowest titratable acidity (6.11±0.03%). In addition, ‘Tahiti’ had the lowest pH while ‘Pan Rumpai’ and ‘Pan Puang’ had the highest pH. Barros et al. (2012) studied the antioxidant capacity, phenolic compounds, vitamin C and minerals contents of five citrus species grown in Brazil. The study showed a significant difference in physicochemical properties among citrus species. The TA, TSS and TSS/TA of ‘Tahiti’ lime in the previous study were slightly different from the present study. They reported that ‘Tahiti’ lime had lower TA (4.73%) and higher TSS (2 ºBrix) and TSS/TA ratio (2.2).This is probably due to the difference in geographical origin of Tahiti lime.Furthermore Barros et al. (2012) also suggested that the TSS/TA ratio is an important parameter used to characterize the good eating quality of citrus fruits.

Table 4. 1 Physicochemical properties of four lime cultivars

	Cultivars			
	Pan Rumpai	Pan Puang	Pan Pichit	Tahiti
TA (%w/w)	6.72±0.35 a	7.05±0.03 a	6.37±0.03 b	6.11±0.03 b
TSS (°Brix)	7.80±0.10 b	8.17±0.12 b	6.87±0.06 c	8.83±0.15 a
TSS/TA	1.19±0.05 b	1.16±0.02 b	1.08±0.01 c	1.45±0.03 a
pH	2.34±0.02ab	2.37±0.01 a	2.32±0.02 b	2.27±0.02 c
Color				
L*	32.58±0.10 b	34.62±0.40 a	30.99±0.11 c	30.90±0.39 c
a*	-0.65±0.19 b	-1.25±0.32 c	0.85±0.02 a	-0.51±0.10 b
b*	-1.60±0.08 b	-0.51±0.67 a	-3.02±0.09 c	-1.41±0.25 b

[a,b,c] Different letters in the same column indicate statistical differences at $p \leq 0.05$

The color measurement of lime juice from four lime cultivars was measured in the CIE L*a*b* system which L* is represented as the darkness/lightness (0/100). a* is represented as green/red (-a*/+a*), b* is represented as blue/yellow (-b*/+b*) (Lawless and Heymann, 2010). From table 4.1, the highest L* value or lighter in color was found in 'Pan Puang' followed by 'Pan Rumpai', 'Pan Pichit' and 'Tahiti'. 'Tahiti' had the lowest a* value, indicating greener color followed by 'Pan Puang' and 'Pan Rumpai', and 'Pan Pichit'. The highest b* value found in 'Pan Puang', indicating more yellowness followed by 'Pan Rumpai', 'Tahiti', and 'Pan Pichit'.

4.2.2 Analysis of non-volatile compounds in fresh-squeezed juice from four lime cultivars using HPLC

The result of organic acid compositions of fresh-squeezed juice from four lime cultivars (Table 4.2) showed that four lime cultivars had the same main organic acid composition but with different amounts. Citric acid is the predominant acid of the lime juices (61.0-76.10 g/l), followed by malic acid (2.55-5.32 g/l), succinic acid (0.25-0.45 g/l), and ascorbic acids (0.22-0.39 g/l), respectively. In agreement with previous studies, citric and malic acids are main organic acids in citrus juice with a trace amount of other organic acids such as ascorbic, succinic, tartaric acids (Cunha et al., 2002; Ladaniya, 2008b; Nour et al., 2010). The malic acid content of lime juice was significantly higher than other citrus juice while lactic acid of lime juice was lower than other citrus juice (Nour et al., 2010). The organic acid content of lime juice (*Citrus aurantifolia*) reported in our study was slightly different from the study of Nour et al. (2010). They reported that lime juice consist of citric acid (61.50 g/l), malic acid (5.18 g/l), lactic acid (0.915 g/l), ascorbic acid (0.354 g/l), tartaric acid (0.012 g/l), and oxalic acid (0.11 g/l). In contrast with our current study that lactic, tartaric and oxalic acids were not detected. The highest citric acid, malic acid and succinic acid content were found in 'Pan Puang'. 'Pan Puang' and 'Pan Rumpai' had the highest ascorbic acid content (0.39 g/l). 'Pan Pichit' had the lowest level in all organic acid contents (Table 4.2). The difference in organic acids contents of fruit juice among different cultivars has been reported in many studies (Bordonaba and Terry, 2008; Cheong, Liu, et al., 2012; Hasim Kelebek, 2010; Muñoz-Robredo et al., 2011; Tiitinen et al., 2005). The difference in organic acids content is due to the variation of genotype of each cultivar, resulting in the difference in metabolism and producing different organic acid contents (Etienne et al., 2013; Famiani et al., 2015; Kader, 2008).

Table 4. 2 Organic acid compositions of four lime cultivars

Cultivars	**Citric acid (g/l)**	**Malic acid (g/l)**	**Ascorbic acid (g/l)**	**Succinic acid (g/l)**
Pan Puang	76.10±5.33 a	5.32±0.05 a	0.39±0.04 a	0.45±0.01 a
Pan Rumpai	73.74±5.90 a	4.27±0.40 b	0.39±0.03 a	0.39±0.04 ab
Tahiti	64.13±2.61 b	4.69±0.35 ab	0.33±0.01 b	0.35±0.05 b
Pan Pichit	60.60±5.03 b	2.55±0.59 c	0.22±0.01 c	0.25±0.04 c

^{a,b,c} Different letters in the same column indicate statistical differences at $p \leq 0.05$

Additionally, it can be noticed that Tahiti showed the least TA values (Table 4.1), even it did not show the least amount of organic acid contents (Table 4.2). The reason is probably came from the difference in sensitivity and selectivity methods used to analyze TA and organic acids content. Titratable acidity is the sum of acids that can be titrated by strong base standard solution which are the total available hydrogen ions (the dissociated acid) in lime juice, while organic acid contents analysis using HPLC measured all the organic acids anions in lime juice (both dissociated and undissociated acids) (R. Boulton, 1980; Rajković et al., 2007; G. D. Sadler and Murphy, 2010). In addition, other cations, such as sodium (Na+), potasium (K+) presenting in juice can interfere the titration causing discrepant TA values (R. Boulton, 1980; R. B. Boulton et al., 2013).

The present study showed that four lime cultivars had low sugar content (Table 4.3). Fructose is predominant sugar in lime followed by sucrose and glucose except in ‘Tahiti’ which had glucose content higher than sucrose content. In contrast reported in ‘Mandarin’ and ‘Valencia’ orange, sucrose was the major sugar and the ratio of sucrose, glucose, and fructose was 2:1:1 (Ladaniya, 2008b). ‘Tahiti’ had the highest level of sucrose, glucose and fructose (4.36, 9.89 and11.60 g/l, respectively) followed by, ‘Pan Puang’ and ‘Pan Rumpai’ while the lowest sugar content was found in ‘Pan Pichit’. ‘Pan Puang’ and ‘Pan Rumpai’ showed no difference in sugar contents. The sugar content in lime juice was highest in ‘Tahiti’ and the lowest values in ‘Pan Pichit’.

Table 4. 3 Sugar compositions of fresh-squeezed juice four lime cultivars

Cultivars	**Sucrose (g/l)**	**Glucose (g/l)**	**Fructose (g/l)**
Pan Puang	2.33±0.30 b	1.74±0.43 b	6.10±0.98 b
Pan Rumpai	1.83±0.20 b	1.32±0.45 b	5.00±0.43 bc
Tahiti	4.36±0.90 a	9.89±1.70 a	11.60±1.79 a
Pan Pichit	0.57±0.28 c	0.40±0.07 b	2.62±1.60 c

[a,b,c] Different letters in the same column indicate statistical differences at $p \leq 0.05$

Limonin, a bitter compound, was significantly different among four lime cultivars (Table 4.4). The highest limonin content was found in 'Tahiti' (25.95 ppm) while 'Pan Pichit' had the lowest limonin content (9.75 ppm). 'Pan Puang' and 'Pan Rumpai' showed no difference in limonin content and had an intermediate amount among the four lime cultivars. Naringin was found in a trace amount among lime juice samples from four lime cultivars. There was no difference in naringin content between four lime cultivars while Yusof et al. (1990) have reported that naringin was not detected in Mexican lime.

Table 4. 4 Limonin and naringin content of fresh-squeezed juice four lime cultivars

Cultivars	**Limonin (ppm)**	**Naringin (ppm)**
Pan Puang	17.27±2.53 b	1.52 ±1.10 a
Pan Rumpai	18.40±2.55 b	1.96±0.82 a
Tahiti	25.95±2.24 a	2.31±1.36 a
Pan Pichit	9.75± 2.54 c	0.55±0.12 a

[a,b,c] Different letters in the same column indicate statistical differences at $p \leq 0.05$

4.2.3 Sensory evaluations of fresh-squeezed juice from four lime cultivars

4.2.3.1 Descriptive sensory analysis

A descriptive sensory analysis was performed by 8 trained panelists. The taste attributes used in the test were sourness, sweetness, and bitterness. From training session, three taste attributes, which are sourness, sweetness, bitterness, were selected to be used in the test. Citric acid, sucrose, and caffeine were used as reference standard for sourness, sweetness, and bitterness, respectively. The definition of attributes and the concentration of reference standards used shows in Table 4.5.

Table 4. 5 Definition of attributes and concentration of reference standards used

No	Attribute	Definition	Reference	Intensity
Taste : Taste the sample for one coffee stir spoon and evaluate the intensity of taste (low-high)				
1	Sourness	*Definition:* The level of sourness when tongue was stimulated with citric acid	Citric acid 1.5%	8
			Citric acid 2.5%	13
2	Sweetness	*Definition:* The level of sweetness when tongue was stimulated with sucrose	Sucrose 0.5%	0.5
			Sucrose 1%	1
3	Bitterness	*Definition:* The level of bitterness when tongue was stimulated with caffeine	Caffeine 0.05%	2
			Caffeine 0.08%	5

The result shows that the sweetness was significantly different among four limes cultivars (Table 4.6). There was no significant difference in sourness and bitterness among four lime cultivars. The sweetness score of 'Tahiti' was significantly higher than that of 'Pan Rumpai', 'Pan Puang' and 'Pan Pichit' which was coincided with chemical and physicochemical results that Tahiti had the highest sugar contents, TSS and TSS/TA ratios. However, the result showed very low scores in all lime cultivars (0.50-0.98). This is due to the strong sourness taste of lime juice which caused the panelists difficult to detect the sweetness. Thus, descriptive sensory analysis could not suitably use for evaluating sweetness and bitterness of lime juice in this study. The other sensory method, such as discrimination test, should be taken into consideration. And the sweetness should not be a good indicating the difference among four lime cultivars.

Table 4. 6 Mean sensory scores of fresh-squeezed lime juices from four lime cultivars

Cultivars	**Descriptors**		
	Sourness	Sweetness*	Bitterness
Pan Puang	11.43± 0.60	0.54+0.14 b	0.56±0.16
Pan Rumpai	11.50±0.44	0.50+0.00 b	0.70±0.25
Tahiti	10.75±0.33	0.98+0.10a	0.50±0.00
Pan Pichit	10.74±0.25	0.50+0.00 b	0.63±0.22

^{a,b} Different letters in the same column indicate statistical differences at $p \leq 0.05$

4.2.3.2 Preference ranking test

Preference ranking test was performed by 60 students from School of Culinary Arts, Suan Dusit University. The culinary students were chosen for this study because of their culinary skill and their experience on using lime for cooking purpose. They were asked to rank lime juice from four lime cultivars from the most preferred to the least preferred for cooking. From Friedman test, the calculated chi-square value from ranking preference data (14.97) was higher than the chi-square value from the table (7.81) (table B.1), thus indicating that there was a significant difference between fresh-squeezed lime juices from four lime cultivars ($p \leq 0.05$). Then, the least significant ranked difference (LSRD) was used to determine which lime samples differed in preference from one another by comparison of rank total separation. The rank total and significant group of four lime cultivars are showed in Table 4.7. 'Pan Pichit' was the least preference comparing with 'Pan Puang', 'Pan Rumpai' and 'Tahiti' while there was no difference in preference among 'Pan Puang', 'Pan Rumpai' and 'Taihiti'.

Table 4. 7 Rank total and significant group of four lime cultivars ($p \leq 0.05$)

	Pan Puang	**Pan Rumpai**	**Tahiti**	**Pan Pichit**
Rank total	134	136	143	186
Significance group	A	A	A	B

4.2.3.3 Acceptance test

Consumer acceptance test was performed by 117 students from School of Culinary Arts, Suan Dusit University with 9-point hedonic scale. The result is showed in Figure 4.3. The significant difference in consumer acceptance was found among four lime cultivars. 'Pan Puang' had the highest scores in aroma (6.9), color (6.6), tastes (6.2) and overall liking (6.5) followed by 'Pan Rumpai', 'Tahiti' and 'Pan Pichit', respectively. It can be noticed that 'Pan Puang' which had the highest sourness score from descriptive sensory analysis (Table 4.6) showed the most preference and acceptance from chef students both in preference ranking test and acceptance test. The culinary students were asked to choose the most preferred lime juice, they mainly selected the lime samples based on the unique aroma and sourness to the dish. From Figure 4.3, 'Pan Puang' showed the highest scored in aroma which was coincided with Suwannaprom (2016) who reported that 'Pan Puang' had the highest intensity of citrus, lemon, green, juicy, peely, and floral aromas in descriptive analysis test.

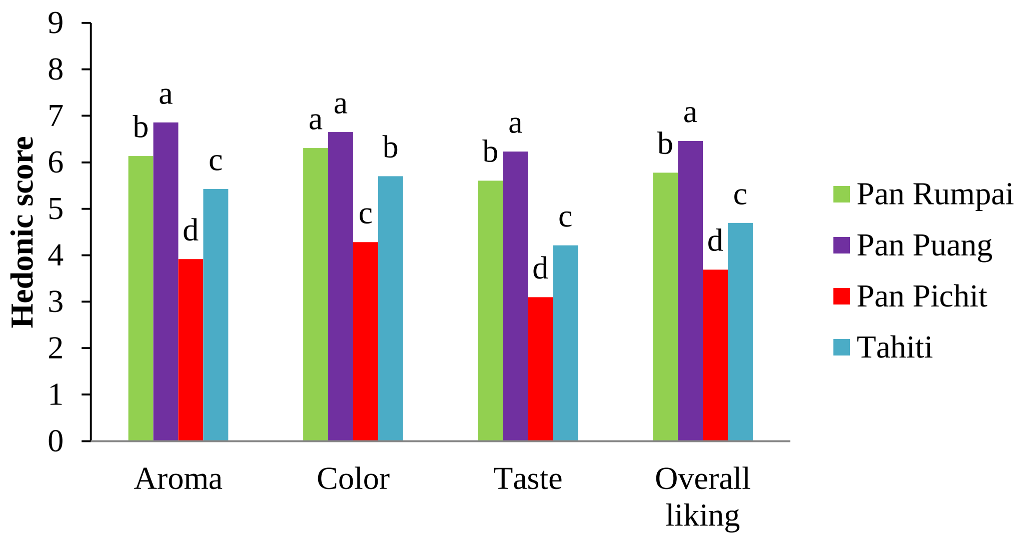


Figure 4. 3 Means of consumer acceptance scores. Vertical bars with different letters are significantly different at $\alpha = 0.05$

4.2.4 Comparison of non-volatile compounds in fresh-squeezed juice from four lime cultivars using chemometrics

4.2.4.1 Unsupervised pattern recognition technique

Hierarchical Cluster Analysis (HCA) is one of the unsupervised data analysis methods. Figure 4.4 shows the dendrogram obtained from clustering the different lime cultivars, according to 9 variables of non-volatile compounds by HCA using Ward's method. The lime juice samples were divided into 3 clusters based on similarity. 'Pan Puang' is the most similar to 'Pan Rumpai' followed by 'Pan Pichit' and 'Tahiti'. Almost samples classified into the same cluster were same cultivars including, a cluster of 'Pan Pichit' and a cluster of 'Tahiti', whereas 'Pan Puang' and 'Pan Rumpai' were classified into the same cluster since they showed no difference in non-volatile contents. Figure 4.5 shows the dendrogram and heat map of four lime cultivars which illustrates the difference in organic acid and sugar contents among cluster. The more green color presented the lower concentration and the more red color presented the higher concentration of compounds.

The 16 variables consisting of 9 non-volatile compounds, 4 physicochemical and 3 sensory attributes were considered as analytical data in PCA in the correlation matrix. The first two principal components (PCs) accounted for 79.36% of the total variance (Figure 4.8). PC1 explained 49.82% of the total variance among four lime cultivars (Figure 4.6a) which was related to TSS, limonin, sucrose, fructose, glucose, sweetness, and TSS/TA and was negatively associated with succinic acid, TA and pH. PC2 explained 29.55% of the total variance which was related to citric acid, malic acid, ascorbic acid, sourness and TA. In agreement with HCA result, the score plot (Figure 4.6b) could be discriminated into 3 groups. 'Pan Puang' and 'Pan Rumpai' associated with the higher organic acid, TA, pH, and sourness score while the higher sugar content, bitter compound, TSS, TSS/TA ratios and sweetness score were associated with 'Tahiti' lime. On the other hand, 'Pan Pichit' was found negatively correlated with all variables.

The classifications of lime cultivars in these three clusters were influenced by the difference in concentration of the non-volatile compound, physicochemical properties, and sensory profile. Numerous studies suggested that the variation of genotypes in different cultivars of fruits influences metabolic pathway of fruits which lead to the difference in chemical profile and sensory properties (Abad-García et al., 2012; Bordonaba and Terry, 2008; Crespo et al., 2010; Etienne et al., 2013; Kader, 2008; Zheng et al., 2009).

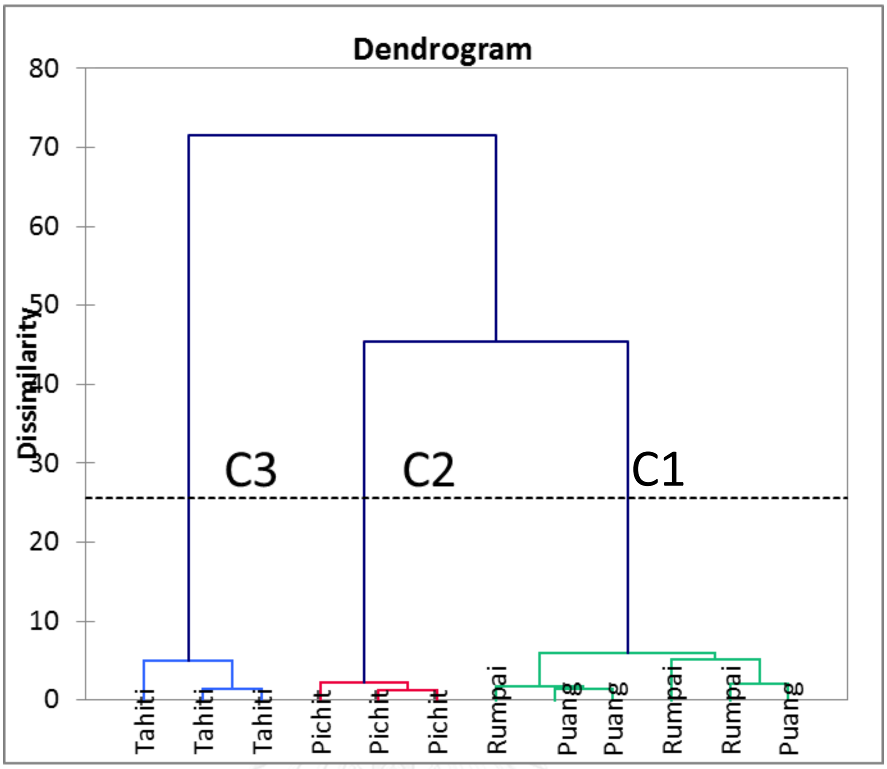


Figure 4. 4 Dendrogram obtained by Hirerachical cluster analysis (HCA) of fresh-squeezed juice from four lime cultivars

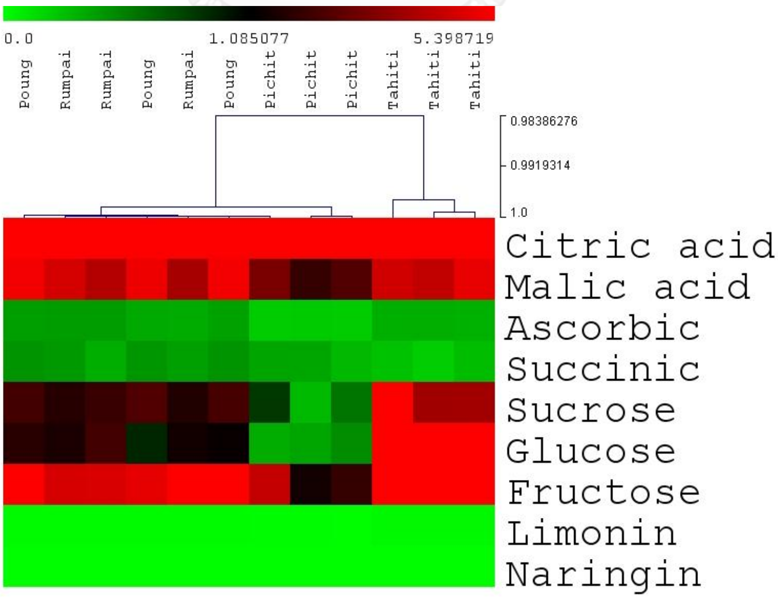


Figure 4. 5 The dendrogram and heat map of fresh-squeezed juice from four lime cultivars

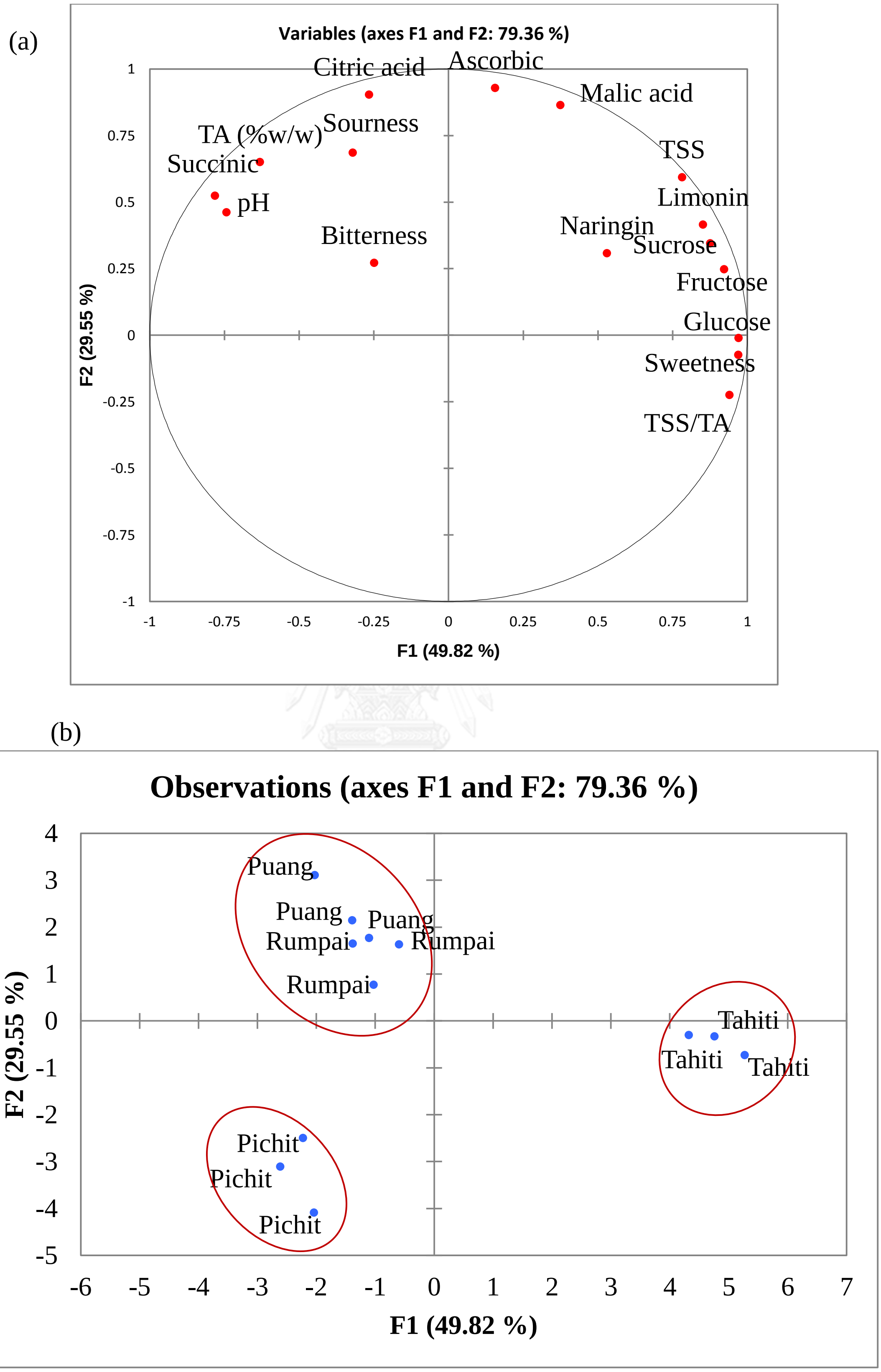


Figure 4. 6 Loading plots of non-volatile compounds, physicochemical and sensory profile (a) and score plot of lime's cultivars

4.2.4.2 Supervised pattern recognition technique

Partial least square (PLS) regression analysis which is a part of supervised pattern recognition technique was employed to correlate non-volatile compounds data with sensory attributes. Table 4.8 shows the standardized coefficient and R^2 for regression. The 9 non-volatile compounds variables (X-matrix) were used to predict each sensory attributes (sweetness, sourness and bitterness) or Y-matrix. The high R^2 value was found in sweetness (0.894), thus the PLS regression model could well explain the correlation of sweetness and non-volatile compounds. Glucose, fructose and sucrose had the three highest standardized coefficients therefore sweetness was mainly influence by glucose content followed by fructose and sucrose content, respectively (Table 4.8 and Figure 4.7a and b). Sourness was mainly effected by citric acid content followed by ascorbic, malic, succinic acid, respectively (Table 4.8 and Figure 4.8 a and b); however the correlation of sourness and non-volatile compounds could not be well explained by the PLS regression model (R^2= 0.526) as good as sweetness. On the other hand, bitterness could not be elucidated by the PLS regression model due to the very low R^2 (0.159) (Table 4.8 and Figure 4.9 a and b).

CHAPTER V
CONCLUSION AND SUGGESTIONS

5.1 Conclusion

From the study of the influence of storage time and temperature on nonvolatile profile of fresh-squeezed lime juice, organic acids, sugars, and bitter compound contents showed no change throughout 10 hours of storage of lime juice at 4°C and 35°C. Lime juice stored at 4°C has more stability of taste compounds than lime juice stored at 35°C. Ascorbic acid significantly decreased during 24 hours of storage due to the oxidation/reduction and intermolecular rearrangement reaction. Fructose and glucose contents of Thai lime juice significantly increased in 24 hours stored lime juice due to the acid hydrolysis of sucrose.

From the study of non-volatile compounds of fresh-squeezed lime juice from four lime cultivars using HPLC found that four lime cultivars consisted of largely organic acid contents (citric, malic, ascorbic and succinic acids) and a trace of sugar (glucose, fructose, and sucrose) and bitter compounds (limonin and naringin). Citric acid was the predominant non-volatile compound in lime juice (6.06-7.61%). The highest organic acids content and TA value were found in 'Pan Puang' and 'Pan Rumpai'. 'Tahiti' showed the highest content sugars and TSS content, and TSS/TA ratios, whereas 'Pan Pichit had the least amount of organic acids, sugar content, TSS and TSS/TA. According to descriptive sensory analysis, only sweetness showed the significant difference among four lime cultivars. In agreement with the physicochemical results, 'Tahiti' showed the highest intensity of sweetness. However, due to the very low sweetness intensity of lime juice perceived by panelists, a descriptive sensory analysis would not be a suitable method to evaluate, and sweetness could not indicate the difference among four lime cultivars. From acceptance test and preference ranking test, 'Pan Pichit' was the least preferred by culinary students, while 'Pan Puang' showed the highest acceptance scores. 'Pan Puang' which had the highest sourness intensities, organic acid contents, and unique aromas was the most preferred by culinary students. HCA and PCA have shown that fresh-squeezed lime juice from four lime cultivars was divided into three groups. 'Pan

Puang' and 'Pan Rumpai' were classed in the same cluster which was positively associated with the higher organic acid content, TA, sourness, and bitterness. 'Tahiti was' positively associated with sugar content, TSS, TSS/TA, and sweetness while 'Pan Pichit' was negatively associated with all variables. PLS regression used to elucidate the correlation between sensory attribute and non-volatile compounds which found only sweetness could be well explained by PLS the regression that sweetness in lime juice was mainly influenced by sugar content.

In conclusion, the lime cultivars could be characterized with physicochemical properties and sensory profile. In addition, the variation of genotype among lime cultivars influences the non-volatile compounds which probably affecting on taste.

5.2 Suggestions

In this study, non-volatile compounds analyzed was chose to be the representative of the taste including, organic acid, sugars, and bitter compounds. To efficiently characterize lime cultivar from chemical composition, the whole profile of non-volatile compounds, for example, amino acids and other phenolic compounds using liquid chromatography-mass spectrometry (LC-MS) should be taken into consideration. In addition, a descriptive sensory analysis was not proper to evaluate the lime juice sample due to the strong sour taste, thus other sensory methods such as discrimination test should be taken into consideration.

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APPENDIX

APPENDIX A
ADDITIONAL DETAIL OF STTANDARD METHOD

A.1 Total titratable acidity (AOAC, 1999)

Chemicals

1. phenolphthalein (1%): Dissolve 1 g of phenolphthalein in 100 ml 50% isopropanol
2. Sodium hydroxide solution (0.100 N): Dissolve 4.0 g of NaOH in 1 liters of distilled water

Method

1. Measure 5 g lime juice sample into 250 ml glass flasks
2. Add 95 ml of distilled water and mix.
3. Add 0.3 ml of phenolphthalein solution and mix thoroughly.
4. Titrate with 0.1 N NaOH solution until solution shows a faintest discernible pink color persisting for 30 seconds.
5. The total titratable acidity is expressed as anhydrous citric acid on a weight basis and calculated by the following equation.

$$\%Acid(w/w) = \frac{\left(\frac{ml\ Titrant}{1000\ ml}\right)(N\ Titrant)\left(\frac{64.04\ g\ Citric\ acid}{1\ mole OH^{-}}\right)}{Sample\ Weight(g)} \times 100$$

APPENDIX B
ADDITIONAL DATA

Table B. 1 Chi-square distribution table

Percentage Points of the Chi-Square Distribution

Degrees of Freedom	Probability of a larger value of x^2								
	0.99	**0.95**	**0.90**	**0.75**	**0.50**	**0.25**	**0.10**	**0.05**	**0.01**
1	0.000	0.004	0.016	0.102	0.455	1.32	2.71	3.84	6.63
2	0.020	0.103	0.211	0.575	1.386	2.77	4.61	5.99	9.21
3	0.115	0.352	0.584	1.212	2.366	4.11	6.25	7.81	11.34
4	0.297	0.711	1.064	1.923	3.357	5.39	7.78	9.49	13.28
5	0.554	1.145	1.610	2.675	4.351	6.63	9.24	11.07	15.09
6	0.872	1.635	2.204	3.455	5.348	7.84	10.64	12.59	16.81
7	1.239	2.167	2.833	4.255	6.346	9.04	12.02	14.07	18.48
8	1.647	2.733	3.490	5.071	7.344	10.22	13.36	15.51	20.09
9	2.088	3.325	4.168	5.899	8.343	11.39	14.68	16.92	21.67
10	2.558	3.940	4.865	6.737	9.342	12.55	15.99	18.31	23.21
11	3.053	4.575	5.578	7.584	10.341	13.70	17.28	19.68	24.72
12	3.571	5.226	6.304	8.438	11.340	14.85	18.55	21.03	26.22
13	4.107	5.892	7.042	9.299	12.340	15.98	19.81	22.36	27.69
14	4.660	6.571	7.790	10.165	13.339	17.12	21.06	23.68	29.14
15	5.229	7.261	8.547	11.037	14.339	18.25	22.31	25.00	30.58
16	5.812	7.962	9.312	11.912	15.338	19.37	23.54	26.30	32.00
17	6.408	8.672	10.085	12.792	16.338	20.49	24.77	27.59	33.41
18	7.015	9.390	10.865	13.675	17.338	21.60	25.99	28.87	34.80
19	7.633	10.117	11.651	14.562	18.338	22.72	27.20	30.14	36.19
20	8.260	10.851	12.443	15.452	19.337	23.83	28.41	31.41	37.57
22	9.542	12.338	14.041	17.240	21.337	26.04	30.81	33.92	40.29
24	10.856	13.848	15.659	19.037	23.337	28.24	33.20	36.42	42.98
26	12.198	15.379	17.292	20.843	25.336	30.43	35.56	38.89	45.64
28	13.565	16.928	18.939	22.657	27.336	32.62	37.92	41.34	48.28
30	14.953	18.493	20.599	24.478	29.336	34.80	40.26	43.77	50.89
40	22.164	26.509	29.051	33.660	39.335	45.62	51.80	55.76	63.69
50	27.707	34.764	37.689	42.942	49.335	56.33	63.17	67.50	76.15
60	37.485	43.188	46.459	52.294	59.335	66.98	74.40	79.08	88.38

Source: http://bitesizebio.com/25166/statistics-for-biologists-chi-square-test-and-its-use-in-biology/

APPENDIX C

CHROMATOGRAM OF NON-VOLATILE COMPOUNDS

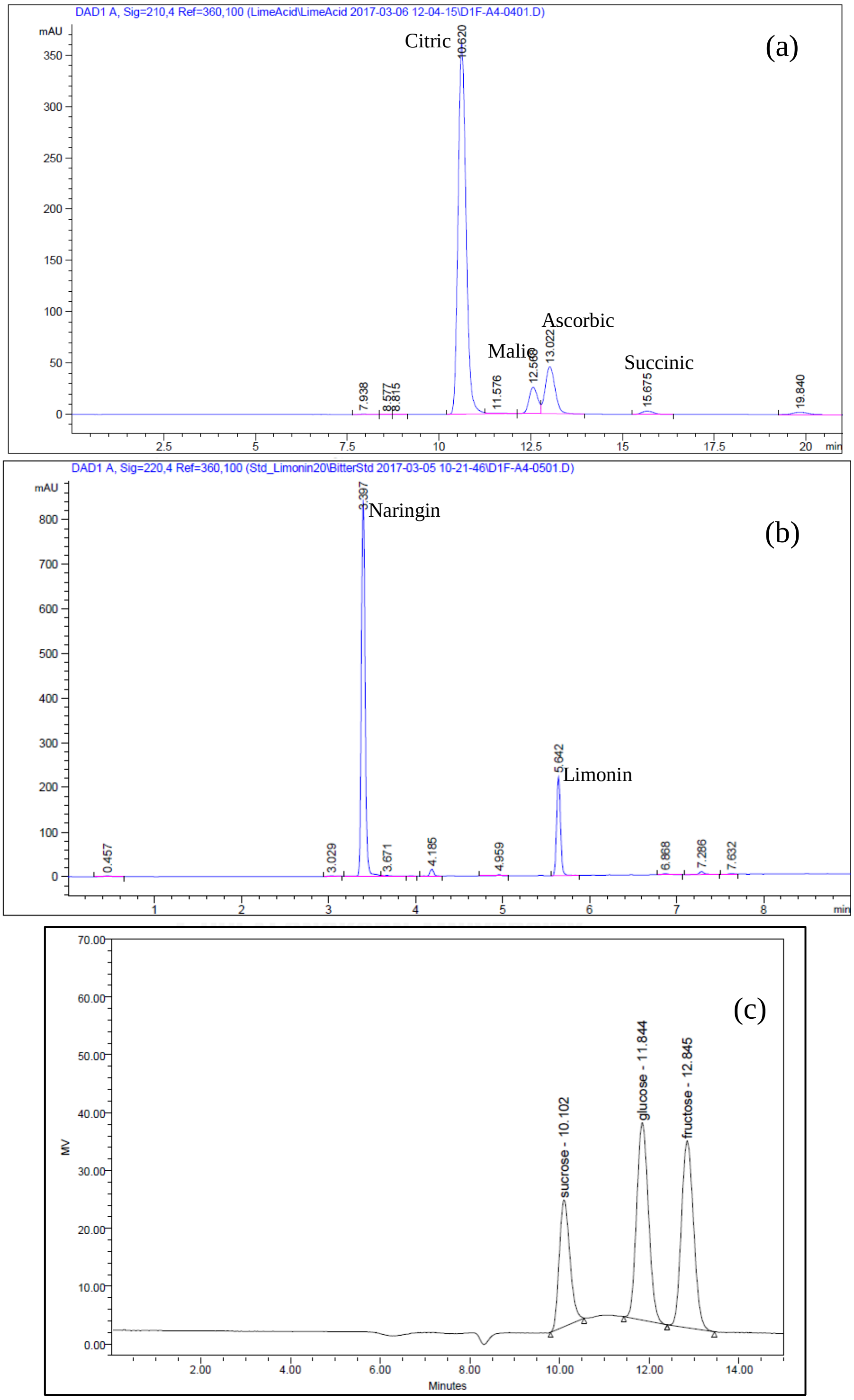

Figure C. 1 Chromatogram of bitter compound (a), organic acid (b) and sugar (c) standards

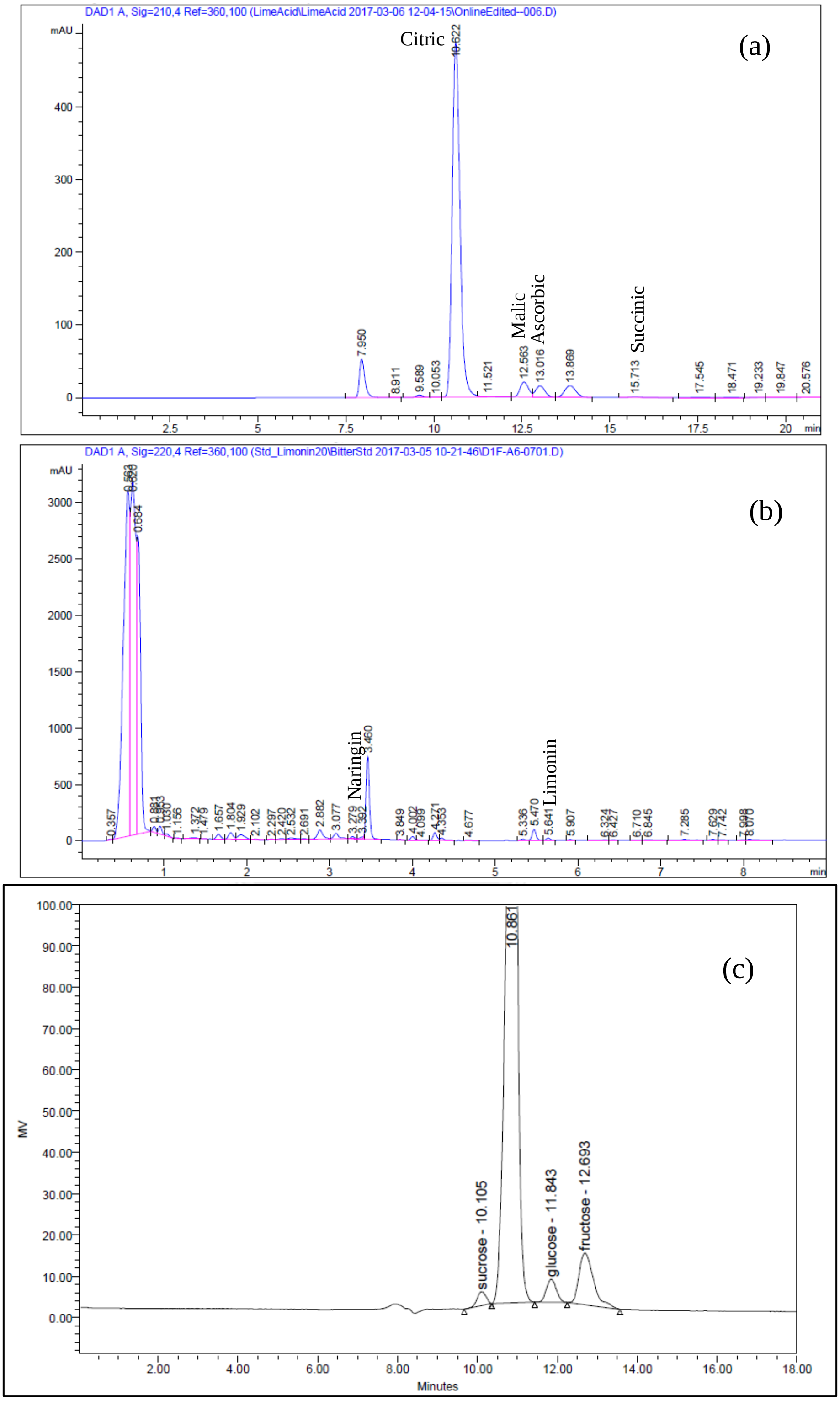

Figure C. 2 Chromatogram of organic acid (a), bitter compound (b) and sugar (c) of 'Pan Rumpai'

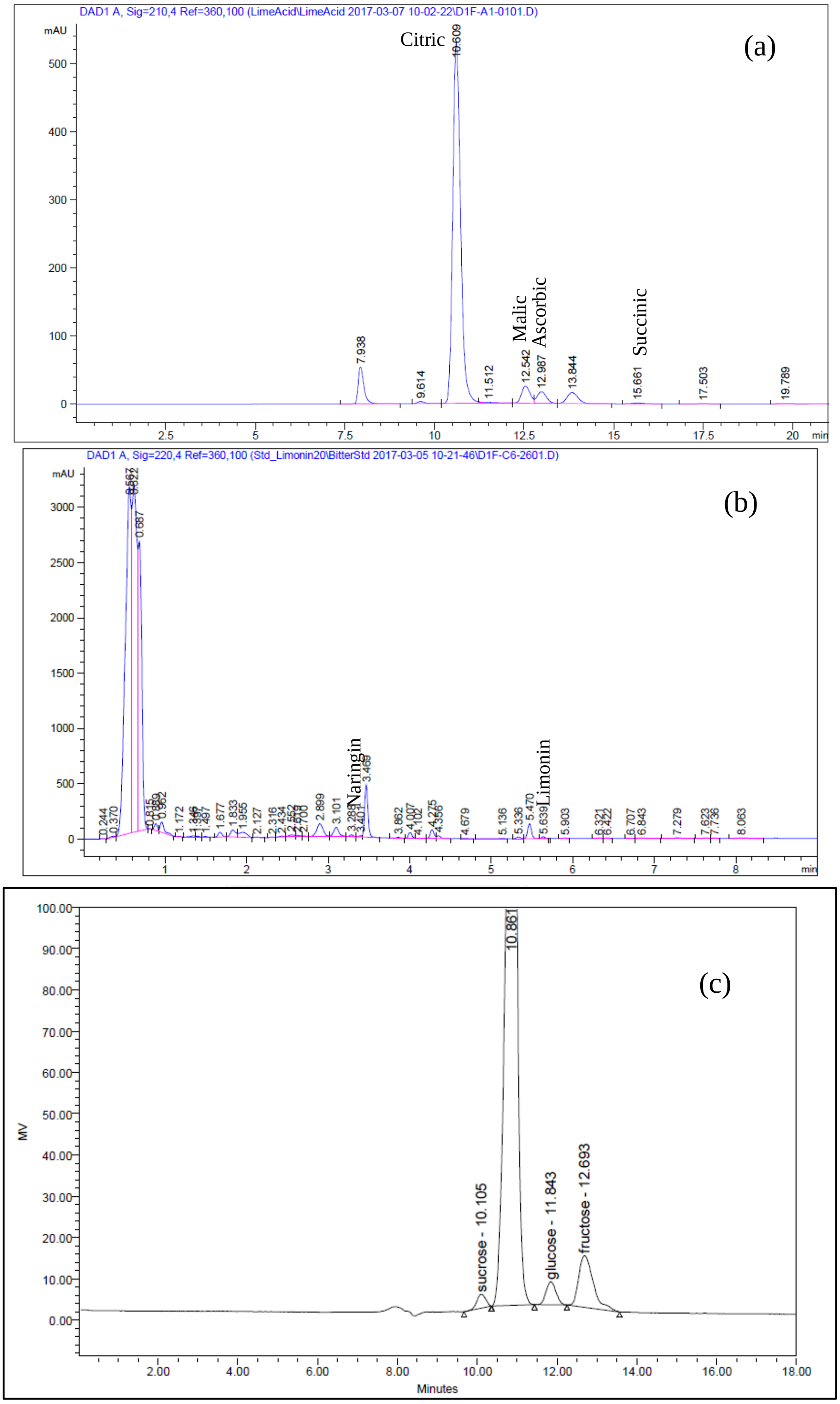

Figure C. 3 Chromatogram of organic acid (a), bitter compound (b) and sugar (c) of 'Pan Puang'

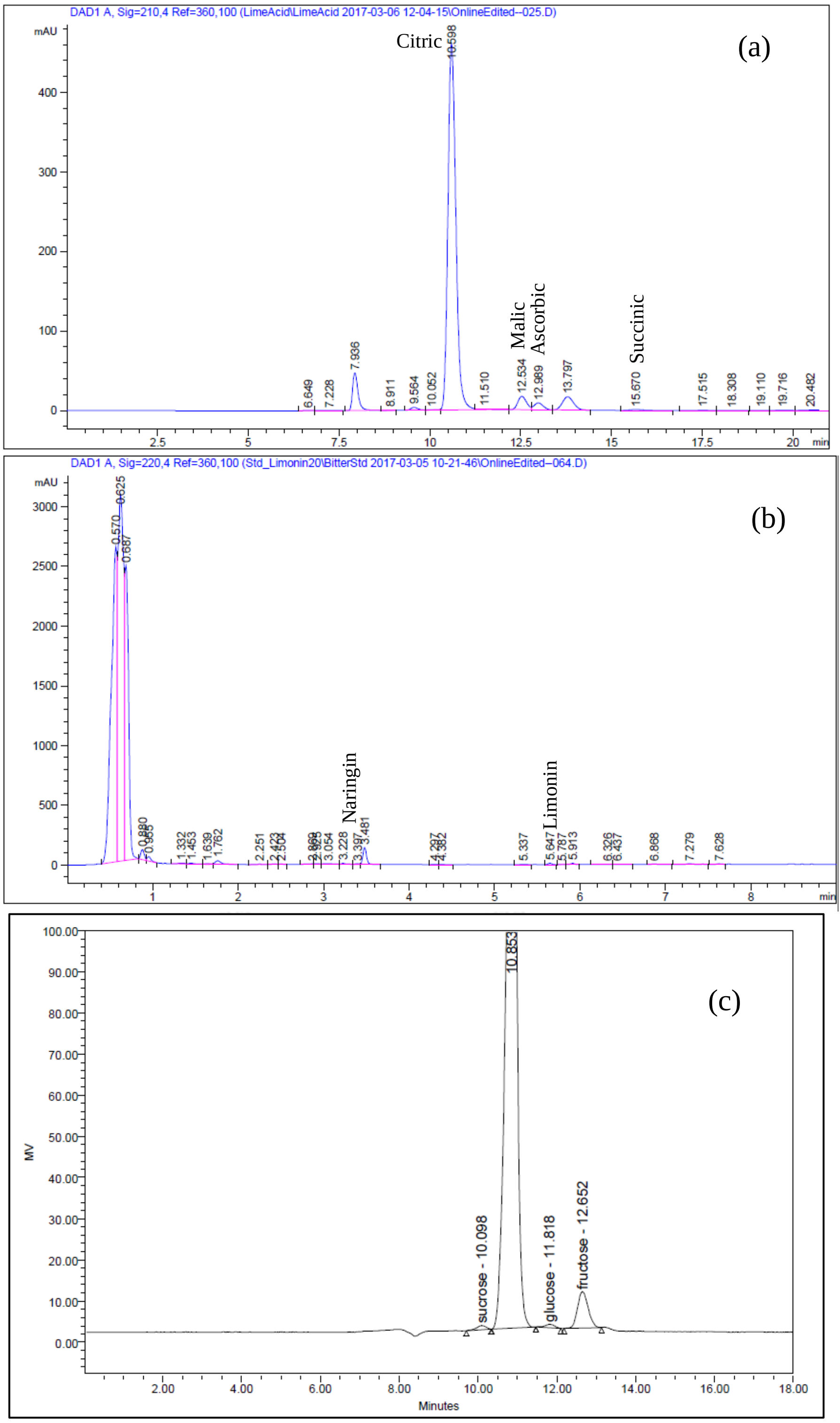

Figure C. 4 Chromatogram of organic acid (a), bitter compound (b) and sugar (c) of 'Pan Pichit'

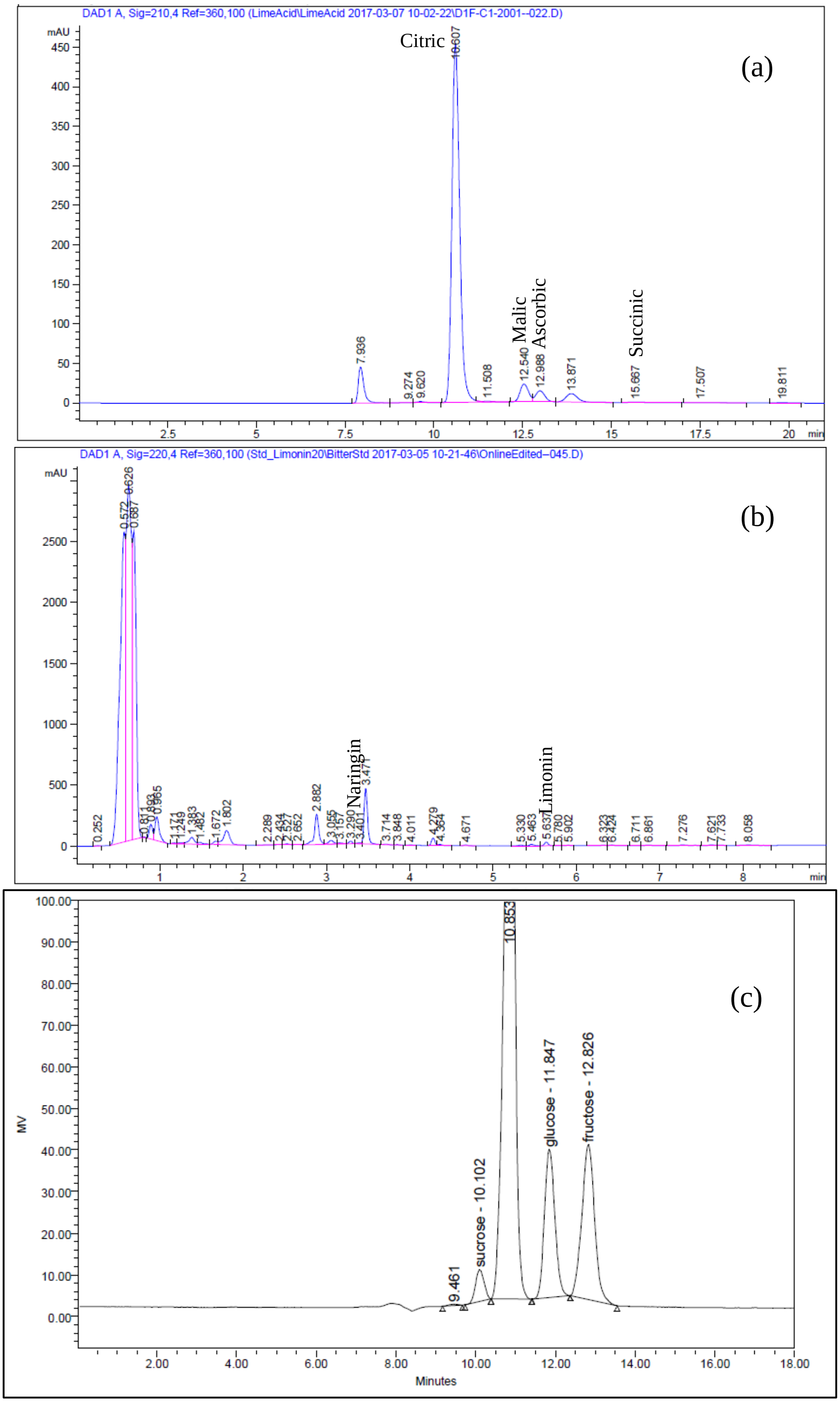

Figure C. 5 Chromatogram of organic acid (a), bitter compound (b) and sugar (c) of 'Tahiti'

VITA

Jutamas Korkitpoonpol was born on November 1st, 1991 in Bangkok, Thailand.In 2009, she finished her high school from St. Joseph Bangna school. In 2010, She entered Chulalongkorn University, Bangkok where she received her Bachelor of Science in Food Technology in 2013.

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