Determination of volatile compounds in fresh and fermented *Nipa* sap (*Nypa fruticans*) using static headspace gas chromatography-mass spectrometry (GC-MS)

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Abstract

Nipa sap or air nira is a sweet natural beverage obtained from a type of palm tree, *Nypa fruticans*. It is readily and spontaneously fermented resulting in the development of alcoholic fermentation products. Objective of this study is to determine the volatile compounds (VOCs) responsible for the aroma in fresh and fermented *nipa* sap. The sap was left for natural fermentation at 30ºC for 63 days. VOCs of the sap were analysed using static headspace gas chromatography-mass spectrometry (GC-MS). Fresh nipa sap contained ethanol (83.43%), diacetyl (0.59%), and esters (15.97%). Fermented *nipa* sap contained alcohols (91.16 – 98.29%), esters (1.18 – 8.14%), acetoin (0.02 – 0.7%), diacetyl (0.04 – 0.06%), and acetic acid (0.13 – 0.68%). Concentration of ethanol in fresh *nipa* sap increased from 0.11% (v/v) to 6.63% (v/v) during the fermentation, and slightly decreased to 5.73% (v/v) at day 63. No higher alcohols were detected in the fresh *nipa* sap. Concentration of 1-propanol and 2-methylpropanol were constant throughout the fermentation with average of 0.004 to 0.006% (v/v) and 0.0001 to 0.0009% (v/v), respectively. 3-methylbutanol increased during the fermentation process. The highest concentration (0.001% v/v) was recorded at day 35. This study has shown differences in VOCs types between fresh and fermented *nipa* sap.

Introduction

Fresh palm sap, palm juice or air nira is a traditional beverage consumed by people in Malaysia. It has also been used in many Asian countries as sweetener in the form of palm sugar after going through heating process (Ho *et al*., 2007). Fermented palm sap on the other hand is usually called palm toddy, or simply palm wine and is widely consumed as a refreshing alcoholic beverage by people in some parts of Africa, Asia and South America (Jirovetz *et al*., 2001). Palm sap, which is sweet and translucent juice, is one of the important products from palm trees. Palm sap or palm toddy is a term commonly used by most of these countries. Special name is given according to the origin of the sap. Coconut sap or neera is sap obtained from coconut palm (Borse *et al*., 2007), while sap obtained from oil palm or Arenga palm is called palm sap or air nira (Ho *et al*., 2007).

In Malaysia, palm sap is usually obtained from various species of palm trees, including coconut tree (*Cocos nucifera*), nipa tree (*Nypa fruticans*), and Arenga tree (*Arenga pinnata*). These palms can be found in every part of Malaysia, especially in the middle and northern parts of Peninsular Malaysia. Fermented palm sap or palm toddy is whitish with ethanol content in the range of 5 to 8%, and pH at about 3.6 depending on fermentation stage at which the toddy is consumed (Iwuoha and Eke, 1996; Lasekan *et al*., 2007). Previous studies reported that the fermentation is dominated by yeasts (*Saccharomyces*, *Candida*, *Kloechera*) and lactic acid bacteria (Atputharajah *et al*., 1986; Chanthachum and Beuchat, 1997; Amoa-Awua *et al*., 2007).

Fermented palm sap has complex organic and inorganic compounds of which production depends on several factors, such as period of fermentation and microbial activity. In general, fermented palm sap contains low molecular mass carbonyls (C_1-C_6) as by-products of yeast fermentation, and alcoholic oxidation at various stages of fermentation (Anli *et al*., 2007). Volatile organic compounds impart aroma to fermented palm sap and have been subjected to many researches. However, the presence of some VOCs is undesirable as they are responsible for

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unpleasant organoleptic properties of alcoholic drinks (Vallejo-Córdoba *et al*., 2004). VOCs of fermented coconut sap and palm oil sap are largely dominated by alcoholic substances, such as ethanol, higher alcohols, acetic acid, esters, and acetoin (Uzochukwu, 1997; Jirovetz *et al*., 2001; Lasekan *et al*., 2007; Lasekan *et al*., 2009). Some of the VOCs in fermented palm sap resemble other alcoholic beverages, such as sake, spirits, mouro, and mescal, with major VOCs like propanol, 2-methylpropanol, 3-methylbutanol, 2-methylbutanol, acetaldehyde, acetic acid, ethyl acetate, and ethyl 2-hydroxypropanoate (Teramoto *et al*., 2000; Soufleros *et al*., 2004; León-Rodríguez *et al*., 2006).

Fermented nipa sap contains several particular VOCs which are responsible in giving crude and malty aroma of the sap as VOCs are generally important for the quality and aroma of alcoholic beverages. Fermented nipa sap is characterized by the increase of ethanol and acetic acids during fermentation. The information on volatile and flavour compounds from natural fermentation of nipa sap is required to determine the quality of the sap. It can also serve as an indicator for fresh palm sap industries in monitoring their freshness. VOCs information is also relevant to palm toddy industries and also the community to determine the optimal acceptable flavours. Therefore, the aim of this research was to determine the VOCs responsible for the aroma in fresh and fermented *nipa* sap.

Materials and Methods

Sample collection

Nipa sap was randomly collected from nipa trees in Kedah, Malaysia. The sap was obtained by cutting the stalk of matured nipa fruit and sap was collected four times daily to avoid spontaneous fermentation (Figure 3). The end cut of the stalk was wrapped with sterile plastic bags to maintain hygiene. A thin slice of the stalk was cut away every four hours to reduce accumulation of microorganisms. The collected sap was then pooled together and transferred into 250 mL of laboratory bottles and immediately kept in cooler box with dried ice at 0ºC in temperature. The samples were then taken and kept in a temperature of -20ºC at the laboratory for further analysis.

Fermentation of nypa sap

10 laboratory bottles containing 250 mL of nipa sap were fermented in an incubator for 63 days with 30ºC in temperature. Anaerobiosis and static fermentation were carried out. Fermentation liquid was collected every seven day, starting from day 0 until day 63 for analysis of VOCs.
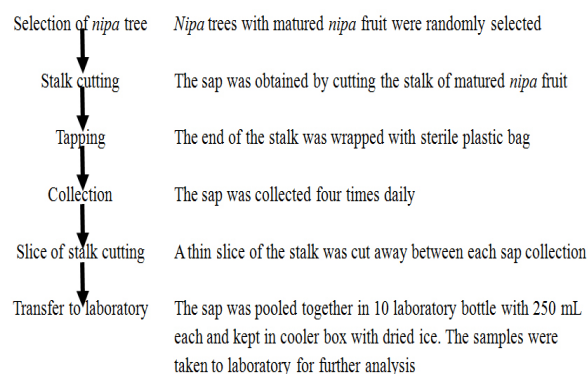

Figure 3. Flowchart indicating the process to obtain nipa sap

Reagents and materials

Ethanol and 1-propanol were purchased from Merck, Japan, while 2-methylpropanol and 3-methybutanol with purities of 99% were purchased from ACROS Organics, St. Louis, USA. As for internal standard of acetonitrile, it was purchased from Merck, Japan. Single and mixed standard stock solutions and their further dilutions were prepared in deionised water.

Headspace gas chromatography-mass spectrometry analysis

Volatile constituents of samples were analysed by static headspace gas chromatography-mass spectrometry (GC-MS). All VOCs of *nipa* sap were analyzed without any prior treatment by using the Agilent Technologies 7890A gas chromatography coupled with Agilent 5975C inert mass spectrometry detector (California, USA). The method used was according to Forney *et al*. (1991), with a slight modification. Only 0.001 mL of sample was injected into the capillary column. About of 3 mL of sample was put in the headspace vial for the analysis of VOCs besides ethanol; while only 0.1 mL of sample was put in the headspace vial to analyze ethanol. The analysis method was done separately due to the high content of ethanol in each sample compared to other VOCs. The separation was achieved using a J&W Scientific DB-WAX fused silica polar capillary column, 30 m × 0.25 mm in diameter, and 0.25 μm film thickness. GC oven temperature was set to 40ºC for 1 min, and later increased to 65ºC at 20ºC/min, and further increased to 220ºC at 50ºC/min, held at 220ºC for 1 min with a linear helium carrier flow of 1 mL/min. Mass spectra were obtained by electron ionization at 70 eV. The transfer line and ion source temperature was set to 200ºC and 230ºC, respectively. Mass range was set at 30-330 amu. For headspace configuration, oven, loop, and transfer line temperature were set to 70ºC, 80ºC, and 90ºC respectively. The samples were injected with 100:1 splitting for screening; 200:1 for

quantification of ethanol, and 50:1 for quantification of higher alcohols. Spectra were recorded by a Hewlett-Packard ChemStation. Identification of the compounds were made by matching against the Wiley/NBS library and by GC retention time against standards. The area of each peak was determined by ChemStation software (Agilent Technologies). Quantification of alcohols in the samples was carried out by adding acetonitrile as an internal standard. Twenty millilitres clear headspace vials (Agilent, California, USA) and 20 mm aluminium seal cap with PTFE/silicone septum (Agilent, California, USA) were used. The analyses were carried out in triplicates.

Method validation

Validation of the method is an important process to determine the methodology suitability in achieving analytical data and should be performed to provide evidence that the method used is fit for the quantification process (ICH, 1996; Thompson, 2002; European Commission, 2010). Calibration curve, linearity, LOD, LOQ, accuracy (recovery), and precision were done in accordance with International Conference on Harmonization (ICH) guideline (1996).

Calibration curve

A series of standard alcohols with different concentrations were prepared by diluting the original solution as recommended by ICH (1996). Internal standard was added and ratio of peak area of alcohol of the internal standard was used as data variables for alcohols quantification in this study.

Linearity

Linearity of any analytical procedure is the ability to obtain directly proportional test results of variable data against the concentration of samples. Linearity was evaluated by using calibration curve to calculate coefficient of correlation (r^2) and intercept values.

Limit of detection (LOD) and the limit of quantification (LOQ)

According to ICH, LOD was defined as the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value, while LOQ was defined as the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. ICH with Q2R1 guideline also suggested that there were three different approaches in determining the LOD and LOQ; visual inspection, signal to noise S:N ratio, and variability of calibration curve slope, which involved

a statistical approach. Standard deviation (SD) of intercept from the calibration curve regression was used to determine the LOD and LOQ in this study. LOD and LOQ were calculated using equations A and B where s is the standard deviation of intercept, and is a slope estimated from the calibration curve of the analyte (ICH, 1996).

LOD = 3.3   *s/a*   (Equation A)
LOQ = 10   *s/a*   (Equation B)

Accuracy

The measuring system to detect errors was evaluateed by carrying out recovery assays in order to get the accurate method (ICH, 1996; Garrido-Frenich *et al*., 2006). Recovery test was carried out by spiking a known concentration of analyte into the sample (Quevauviller and Morabito, 2000).

Precision

According to ICH (1996), precision is measured under the same operating conditions of analytical method over a short interval of time, together with the same equipment. The precision test should be carried out in a minimum of six determinations and expressed in coefficient of variation, CV (%).

Statistical analysis

Statistical analysis was conducted using SAS 9.2 software, with three replications for each sample. The data were analysed by ANOVA. Comparison of means was done by Tukey's test.

Results and Discussion

Method validation

The calibration curve, linearity, LOD and LOQ of the method were summarized in Table 1. Linearity of the calibration curve for all alcohols ranged from 0.982 to 0.998. The precision and recoveries of alcohols were shown in Table 2. The precision, which was expressed in coefficient variation, was in the range of 1.03 to 8.54%. Percentage recoveries obtained from the samples were in the range of 92.26 to 104.55%.

Volatile compounds from gc-ms analysis

The VOCs identified in the samples were shown in Table 3, along with the percentage of peak area in the samples. A total of four VOCs in fresh nipa sap were identified as ethanol, diacetyl, ethyl acetate, and ethyl lactate. More VOCs were detected in the fermented nipa sap which largely consisted of four alcohols, three esters, diacetyl, acetoin, and acetic acid. Ethanol, esters, and acetoin were the highest

Table 1. Results obtained for calibration curve of alcohols

Alcohols	Linear calibration curve	Linearity (r^2)	LOD (% v/v)	LOQ (%v/v)
Ethanol	$y = 7.741x + 0.028$	0.998	0.029	0.087
	$y = 0.116x + 0.089$	0.996	1.650	5.000
1-propanol	$y = 146094x - 0.007$	0.998	0.002	0.007
2-methylpropanol	$y = 288169x + 0.243$	0.990	0.0003	0.001
3-methylbutanol	$y = 4259771x + 0.091$	0.982	0.0004	0.001

Table 2. Percentage recoveries and precision values for alcohols in *nipa* sap

Alcohols	Recovery (%)	Precision (CV%)
Ethanol	97.45±2.24	1.03±0.030
1-propanol	92.85±4.77	4.20±0.0002
2-methylpropanol	104.55±8.28	8.54±0.000
3-methylbutanol	92.26±6.26	6.20±0.0001

Each value is mean of triplicate ± standard deviation
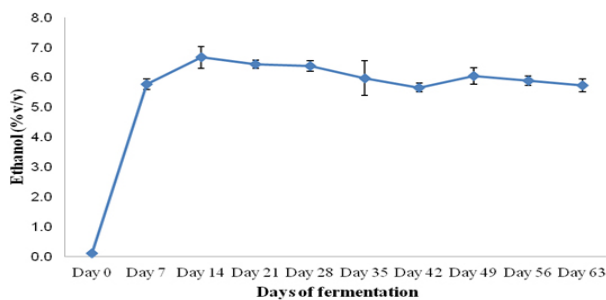
Figure 1. Changes of ethanol content in fresh and fermented *nipa* sap during 63 days of fermentation at 30ºC
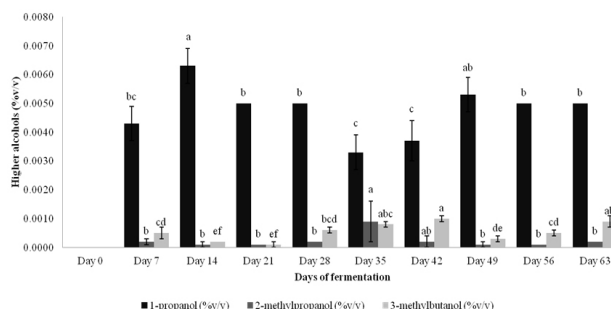
Figure 2. Changes of higher alcohols content in fresh and fermented *nipa* sap during 63 days of fermentation at 30ºC

VOCs found in the samples. According to previous studies, (Vallejo-Córdoba *et al*., 2004; León-Rogríguez *et al*., 2006) ethyl esters, mainly ethyl acetate and alcohols were responsible for the flavour of alcoholic beverages. The presence of minor VOCs in palm sap was also important as they were able to produce unique aroma and characteristic of alcoholic beverages (León-Rogríguez *et al*., 2006). All the identified VOCs in the samples were resulted by-products of yeast metabolisms (Uzochukwu *et al*., 1994a; Uzochukwu *et al*., 1994b; Uzochukwu *et al*., 1999).

The number and amounts of VOCs isolated from fresh and fermented nipa sap were method dependent. According to Lasekan *et al*. (2007), abundant of VOCs could be collected when analyzed using static headspace. Therefore, there were only few VOCs collected in this study as compared to more VOCs when using other methods (Jirovetz *et al*., 2001; Ho *et al*., 2007).

Alcohols

Ethanol identified from fresh and fermented nipa sap was the major VOC with range of 83.43 to 98.29%. The concentrations of alcohols were determined by using standard calibration curve and quantified using the peak area ratio of compound to internal standard. There were significant changes of ethanol ($P<0.05$) from fresh and fermented nipa sap. Ethanol content throughout the fermentation process increased drastically from 0.11% during the initial stage of fermentation to 6.66% (v/v), before slowly decreasing to 5.73% (v/v) by day 63 (Figure 1). The detection of ethanol at day 0 or in a fresh *nipa* sap was due to the naturally-present yeast in tapping sap. Consequently, the sap tends to spontaneously become fermented even while still in the tapping process. Therefore, interval hours between sap tapping should be decreased to get lower ethanol content in fresh *nipa* sap. The decrease in ethanol to some extend was explained by Ingledew (1999), saying that the production of other by-products such as higher alcohols, as well as the growth of yeast cells, might direct some glycolytic intermediates to the corresponding metabolic pathway.

Higher alcohols

There were no higher alcohols detected in fresh *nipa* sap. Production of higher alcohols (Figure 2) was observed starting from day 7 of fermentation onwards. The concentration for 1-propanol and 2-methylpropanol were steady at the range of 0.004 to 0.006% (v/v) and 0.0001 to 0.0009% (v/v), respectively throughout the fermentation process. The detection of higher alcohols in fermented nipa sap was due to the metabolisms of yeast *Saccharomyces cerevisiae* through two metabolic pathways; amino acids such as isoleucine and leucine (Boulton *et al*., 1996) and glycolysis (Peterson *et al*. 2004). Hammond (1993), also suggested that sugary sap was converted to higher alcohols by yeasts via pyruvate pathway. The level of higher alcohols was influenced by nature of the nipa sap and fermentation condition. 3-methylbutanol increased during the fermentation with highest concentration of 0.0010% (v/v) at day 35, while the lowest was 0.0001% (v/v) at day 21. The increasing amount of 3-methylbutanol gave alcoholic and malty aroma that contributed to the unpleasant aroma of fermented palm sap (Lasekan *et al*., 2007). Higher alcohols are the group responsible for the aroma and essential character of alcoholic

Table 3. Volatile compounds identified in fresh and fermented nipa sap by GC-MS analysis

Volatile compounds	Area (%)									
	Day 0	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42	Day 49	Day 56	Day 63
Alcohol										
Ethanol	83.433±0.833[g]	98.289±0.017[a]	96.850±0.026[b]	96.469±0.066[b]	94.322±0.071[c]	94.246±0.309[c]	93.391±0.114[d]	92.081±0.091[e]	90.782±0.109[f]	91.408±0.025[ef]
1-propanol	n.d.[e]	0.207±0.013[c]	0.291±0.007[a]	0.268±0.008[b]	0.258±0.004[b]	0.180±0.004[d]	0.190±0.011[cd]	0.270±0.004[ab]	0.251±0.010[b]	0.265±0.009[b]
2-methylpropanol	n.d.[d]	0.037±0.008[b]	0.018±0.002[c]	0.018±0.003[c]	0.038±0.004[b]	0.041±0.006[b]	0.057±0.002[a]	0.014±0.004[c]	0.034±0.007[b]	0.033±0.002[b]
3-methylbutanol	n.d.[d]	0.098±0.006[b]	0.031±0.002[c]	0.033±0.009[c]	0.093±0.009[b]	0.104±0.005[b]	0.331±0.008[a]	0.054±0.003[c]	0.090±0.006[b]	0.129±0.005[b]
Total(%)	83.433±0.833[g]	98.631±0.010[a]	97.190±0.025[b]	96.788±0.050[b]	94.711±0.079[c]	94.571±0.310[c]	93.969±0.108[d]	92.419±0.089[e]	91.157±0.096[f]	91.835±0.024[ef]
Acetoin										
Total(%)	n.d.[c]	n.d.[c]	n.d.[c]	n.d.[c]	0.023±0.002[bc]	0.034±0.001[b]	0.017±0.003[bc]	0.020±0.001[bc]	0.025±0.002[b]	0.700±0.025[a]
Acetic acid										
Total(%)	n.d.[e]	0.129±0.014[de]	0.248±0.035[cd]	0.187±0.020[cd]	0.303±0.021[c]	0.472±0.053[b]	0.471±0.100[b]	0.590±0.085[ab]	0.677±0.033[a]	0.540±0.023[ab]
Diacetyl										
Total(%)	0.594±0.454[a]	0.061±0.012[b]	0.044±0.006[b]	0.050±0.006[b]	n.d.[b]	n.d.[b]	n.d.[b]	n.d.[b]	n.d.[b]	n.d.[b]
Ester										
Ethylacetate	12.933±0.639[a]	1.125±0.019[f]	2.395±0.010[e]	2.757±0.029[e]	4.803±0.102[d]	4.342±0.199[d]	4.897±0.153[d]	5.922±0.122[c]	7.546±0.138[b]	6.481±0.050[c]
Ethyllactate	3.040±1.361[a]	0.022±0.001[b]	0.052±0.002[b]	0.086±0.014[b]	0.129±0.013[b]	0.225±0.020[b]	0.527±0.032[b]	0.627±0.117[b]	0.508±0.052[b]	0.362±0.009[b]
Formic acid, ethyl ester	n.d.[f]	0.032±0.004[ef]	0.072±0.005[de]	0.132±0.006[c]	0.031±0.002[ef]	0.355±0.056[b]	0.118±0.009[cd]	0.423±0.009[a]	0.087±0.005[cd]	0.080±0.012[cde]
Total(%)	15.973±0.787[a]	1.179±0.022[g]	2.519±0.014[f]	2.975±0.036[f]	4.963±0.171[e]	4.922±0.264[e]	5.542±0.132[d]	6.972±0.113[c]	8.141±0.112[b]	6.923±0.046[c]

drinks (Ferreira *et al*., 1999; Silva and Malcata 1999). Lasekan *et al*. (2007), also reported that the important higher alcohols of fermented palm sap were n-propanol, 2-/3-methylbutanol, 3-methylthio-1-propanol, and 2-phenylethanol.

Ester

Ester was the second highest VOC in fermentation of nipa sap. A total of two and three esters were found in fresh and fermented *nipa* sap, respectively. Among these, ethyl acetate and ethyl lactate were the highest VOCs identified in all samples. Ethyl acetate which gives the fruity flavour (Cortes *et al*., 2005; Lasekan *et al*., 2007; Dragone *et al*., 2009) was present in all interval day of fermentation. The percentage of peak area of ethyl acetate was highest in fresh nipa sap (12.93%) and dropped to 1.13% during the initial stage of fermentation. The percentage however, increased gradually to the range of 2.40 to 7.55% at later stages.

The increase of ethyl acetate indicated long term storage of raw material and acetic acid spoilage (Mingorance-Cazorla *et al*., 2003; Apostolopoulou *et al*., 2005). Continuous oxidation of ethanol to acetic acid and the esterification of acid also contributed to the accumulation of ethyl acetate (Cole and Noble, 1997). Ethyl lactate, which is produced mainly by lactic acid bacteria (Apostolopoulou *et al*., 2005) was

observed to be the highest in fresh nipa sap (3.04%) and dropped during the initial stage of fermentation, and then increased to the range of 0.02 to 0.63%. Formic acid was not detected in fresh nipa sap, but the percentage of peak area increased from 0.03% to the range of 0.07 to 0.36% throughout the fermentation process.

According to Peterson *et al*. (2004), esters production has been investigated along with higher alcohols. Esters were expected to be present as the ethanol content increased during fermentation, and the build up of harsh and astringency aroma from fermented *nipa* sap. This was agreed by Borse *et al*. (2007), who reported that the harsh note of fermented palm sap could be due to the increased amount of acids, and along with produced ethanol and esters.

Diacetyl and acetoin

Diacetyl was detected in fresh *nipa* sap (0.59%) at the early stage of fermentation. The percentage decreased throughout the fermentation to 0.05 and 0.04% at day 14 and day 21, respectively. No diacetyl was detected in further fermentation. Interestingly, acetoin was detected only after 21 days of fermentation, with the percentage of 0.02 and keep on increasing until 0.7% at day 63. Drastic changes of acetoin content in later stage of fermentation could be due to the high availability of glucose.

According to Brandolini *et al*. (1995), acetoin is a natural by-product of fermentation and its determination assists in the evaluation of complete and proper fermentation. Acetoin, with buttery odour is one of the normal products of alcoholic beverages, which originated from yeasts during fermentation (Romano and Suzzi, 1996). Lasekan *et al*. (2007), reported a concentration of 664 mg/L of acetoin in fermented palm sap, which is higher than wine (80 mg/L). The acetoin production was significantly influenced by substrate, with larger amount being formed when the substrate is glucose (Deiana *et al*., 1990). According to Taminaidu *et al*. (2011), sucrose available in nipa sap was hydrolyzed to glucose and fructose to be further converted to ethanol. Therefore, it was expected that higher glucose concentration in the later stage of fermentation had influenced the increasing of acetoin production. The accumulation of acetoin in fermented nipa sap affects the formation of higher alcohols, especially the formation of 3-methylbutanol (Romano and Suzzi, 1996), which contributes to the malty odour (Lasekan *et al*., 2007).

Acetic acid

Low percentage of acetic acid was found at all intervals time of fermented *nipa* sap, but not in a fresh sample. The percentage of acetic acid increased with time as a result of ethanol oxidation. The highest percentage was detected at day 56 (0.68%) while the lowest was at day 7 (0.13%). According to Amoa-Awua *et al*. (2007), the detection of acetic acid in fermented nipa sap was indicated by the existence of acetic acid bacteria such as *Acetobacter* species. Some studies reported that lactic acid bacteria were also responsible in acetic acid production through heterofermentation (Adams and Moss, 1995; Tesfaye *et al*., 2002).

Conclusions

Natural fermentation of fresh nipa sap will lead to changes in aroma and taste, due to the highly fermentable nature of the sap. There were differences in the VOCs identified and their percentage when comparing fresh and fermented *nipa* sap. Generally, fresh nipa sap, which has sweet and fruit-like odour, does not contain any higher alcohols and acetic acid. Fermented *nipa* sap contained high amount of ethanol, as well as higher alcohols, esters, diacetyl, and acetoin. Further study with a large number of samples and from various sources should be carried out in order to increase the precision of the study.

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