

METABOLITE PROFILING OF CORE OIL PALM TRUNK (COPT) SAP: THE EFFECTS OF DIFFERENT STORAGE DURATIONS, CONDITIONS AND TEMPERATURES

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ABSTRACT

In Malaysia, core oil palm trunk (COPT) is one of the biomass that has been left underutilised due to its low properties. Nonetheless, metabolites contained in COPT sap may provide an alternative natural resources for bio-chemicals and pharmaceuticals. Metabolites such as sugars are easily affected by analytical factors during storage. In this study, the changes of metabolite contents in COPT sap stored at different storage durations, conditions and temperatures were observed by using gas chromatography-mass spectrometry (GC-MS) based metabolomics approach. The changes of metabolite contents, particularly sugars and organic acids were analysed using univariate and multivariate statistical analysis. The separation trends observed in the principal component analysis (PCA) score plot was greatly influenced by storage temperatures. However, two-way analysis of variance (ANOVA) revealed that the majority of significant metabolites ($P < 0.05$) was strongly influenced by storage durations. The metabolite increased significantly when COPT was stored as raw rather than sap. The highest sugar concentrations in COPT were found at 10°C for one month (R-10-1M). Furthermore, the organic acids increased significantly when stored at 4°C for one month (R-4-1M). The results indicated different storage durations, conditions and temperatures led to variation in the COPT sap metabolite content.

Keywords: GC-MS, metabolomics, principal component analysis, free sugars, organic acid.

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INTRODUCTION

Palm oil is a highly valuable commodity with values reaching RM 2780 t⁻¹ of crude palm oil, RM 2886 t⁻¹ for palm olein and RM 2711 t⁻¹ for palm kernel oil (MPOC, 2017). According to the Malaysian Palm Oil Board (MPOB), oil palm are replanted every 25

years, which contributed up to 8.2 million tonnes of oil palm trunk (OPT) per year (MPOB, 2016). OPT is one of the most underutilised biomass from the palm oil industry, which usually would be chopped and left to decompose naturally at the plantation (Abdullah and Sulaiman, 2013). As a strategy to promote sustainability, the usage of OPT into valuable materials such as compressed wood (Sulaiman *et al.*, 2012), bioethanol (Yamada *et al.*, 2010), lactic acid (Chooklin *et al.*, 2011), 5-hydroxymethyl furfural (Mostapha *et al.*, 2016) and methyl levuinate (Jahar *et al.*, 2017) are of great interest. Furthermore, the utilisation of OPT into such products will encourage a proper oil palm waste management as well as making it profitable to the industry. This will open

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up more opportunities and benefits, particularly to rural regions and supports the usage of the oil palm biomass for a sustainable future.

Metabolomics is the global analysis approach to study metabolites in a biological system. The approach relies heavily on analytical tools and multivariate data analysis to obtain and process metabolite profile. Gas chromatography-mass spectrometry (GC-MS) is an important analytical tool in metabolomics and often used to detect key small molecules such as sugars, amino acids and organic acids (Warth *et al.*, 2015). GC-MS is relatively economical, easy to use and offer good sensitivity in detecting metabolites. Metabolites are usually known as small molecules that have important and various functions in a biological process. Metabolites are highly diverse, easily affected by environmental perturbation and their changes are correlated to a specific phenotypic characteristic (Field and Lake, 2011). Therefore, metabolite analysis via metabolomics can generate important information to comprehend phenotype of a biological system in response to the ever-changing surroundings.

Previous research showed that several metabolomics studies have been conducted on various parts of oil palm including leaves (Vargas *et al.*, 2016), fruits (Neoh *et al.*, 2013) and mesocarp (Bourgis *et al.*, 2011; Teh *et al.*, 2013). To our knowledge, there is no comprehensive metabolomics studies done on OPT sap, particularly on OPT sap stored at different conditions. Ahmad *et al.* (2010) reported that OPT contains roughly 23.3% lignin, 72.12% holocellulose and 2.3% ash. The sap from OPT is rich with free sugars such as glucose (25.29 g litre⁻¹), fructose (6.28 g litre⁻¹) and sucrose (2.44 g litre⁻¹) (Shamsir *et al.*, 2017). Particularly, these free sugars increased as they are stored at 30-60 days after logging (Yamada *et al.*, 2010). Additionally, organic acids such as citric acid (380.6 µg g⁻¹), malic acid (371.8 µg g⁻¹) and maleic acid (119.1 µg g⁻¹) were dominantly found in sap (Kosugi *et al.*, 2010).

In this study, GC-MS-based metabolomics analysis was conducted to identify and quantify the metabolite profiles of core oil palm trunk (COPT) sap. The aim of the study is to investigate the effects of storage conditions (sap and raw), temperatures (4°C and 10°C) and durations (one week and one month) of COPT sap. The univariate and multivariate statistical analysis (MVA) were then used to evaluate the relationship between these storage parameters and their influence on the metabolite contents.

MATERIALS AND METHODS

Plant Materials and Chemicals

The middle (height position) parts of COPT, approximately 29 years old oil palm (*Elaeis*

guineensis), a *Tenera* variety (hybrid between the *Dura* and *Pisifera*) were obtained from Leong Brothers Earthworks Construction, Kluang, Johor, Malaysia (N1°43'39.3", E103°41'38.4"). Chloroform (99.8%), ribitol (99%), pyridine (99.8%), methoxyamine hydrochloride (MeOX) (98%) and *N*-Methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA) (99.5%) were purchased from Sigma-Aldrich, USA. The HPLC-grade methanol (99.9%) was purchased from Merck, USA. All chemicals were used without further purification.

Samples Preparation

The COPT was stored in two conditions as sap and raw under different storage durations and temperatures (*Appendix 1*). Briefly, sap was extracted from COPT by using a laboratory press scale machine. The collected COPT sap was then stored at 4°C (S-4) and 10°C (S-10) for one week (1W) and one month (1M). After storage durations completed, the COPT sap was then subjected to freeze drying for derivatisation and GC-MS analysis. These samples were later named as S-4-1W, S-10-1W, S-4-1M and S-10-1M. On the other hand, raw COPT was obtained and wrapped with paper and stored at 4°C (R-4) and 10°C (R-10) for one week (1W) and one month (1M). Sap from the stored raw COPT was then extracted by using the laboratory press scale machine and subjected to freeze drying prior to derivatisation and GC-MS analysis. These samples were later named as R-4-1W, R-10-1W, R-4-1M and R-10-1M. The fresh COPT sap was used as a control (C-0) sample.

Total Metabolite Extraction

Approximately 20 mg of freeze dried COPT sap samples were extracted by using 1.0 ml mixture of chloroform:methanol:water (1:1:1). The liquid mixtures were then shaken for 30 s by using a vortex mixer and rotated for 20 min at 200 rpm. The extracted samples were stored at -80°C overnight. Then, the samples were thawed and centrifuged at 4°C, 10 000 rpm for 10 min to separate the two resulting fractions. The top fraction was transferred into a new 1.5 ml microcentrifuge tube and ribitol (20 µl, 0.18 mg ml⁻¹) was added as an internal standard (Neoh *et al.*, 2013). The extracts were then dried using nitrogen gas to remove the excess solvent.

Derivatisation Procedure

Derivatisation method was carried out as described by Ruitz-Matute *et al.* (2011) with minor modifications. Briefly, 40.0 µl pyridine containing MeOX was added to the dried extract samples. The mixture was shaken for 30 s by using a vortex mixer

and then it was incubated. Later, 40 μl of MSTFA was added into the mixture and re-shaken for 30 s and re-incubated. The reactions were incubated at 40°C for 90 min. The samples were then analysed by using GC-MS.

GC-MS Analysis

The operation parameter of GC-MS analysis was optimised as described by Azizan *et al.* (2015) with minor modifications. A Perkin Elmer Clarus 600 GC-MS with auto sampler (quadrupole mass selective detector) on an electron ionisation (EI) operated at 70 eV was used for the analysis. Samples (1 μl) were injected into the Elite-5MS capillary column (30 m \times 0.25 mm i.d. \times 0.25 μm thickness) in a split mode (50:1) with an injection temperature at 250°C and ion source temperature at 300°C. The helium gas flow was constantly set at 1 ml min^{-1} with oven temperature set from 70°C to 300°C. The full scan mode was acquired at mass range of m/z 45-600.

Data Processing

Data analysis was carried out by using Turbo Mass software (Perkin Elmer, USA). Metabolites were identified by comparing MS spectra with the National Institute of Standards and Technology (NIST) library with cut off probability of ≥ 700 . Heat map was constructed by using MetaboAnalyst 3.0 software (Xia *et al.*, 2016). All metabolites were normalised by the internal standard ribitol (100 ppm) and subjected to log₁₀ transformation. Principal component analysis (PCA) was performed by exporting the normalised data into Soft Independent Modeling of Class Analogy (SIMCA)-P (version 11.0, Unimetrics AB, Umea, Sweden) to identify metabolites that were differentiated in the sample treatments. The metabolites were classified using Pubchem database (Kim *et al.*, 2016). Subsequently, two-way analysis of variance (ANOVA) using SPSS version 23 was performed to determine the significantly different metabolites towards different storage parameters. $P < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Effects of Storage Conditions, Durations and Temperatures on COPT Metabolites

GC-MS and MVA were used to determine the metabolite changes in the COPT sap in response to different storage conditions, durations and temperatures. In total, 44 metabolites were identified and their relative concentrations were determined. By comparison, sugars and organic acids had the highest levels of relative concentration among the

other identified metabolites (Table 1). The trends in metabolite concentrations have variously been attributed to environmental and analytical factors (Maier *et al.*, 2010; Heuberger *et al.*, 2014).

Table 1 shows the relative concentration of sugars such as glucose, glucopyranose, fructose, xylose, galactofuranose, galactose, mannose, arabinose and mannopyranose were identified in the samples. Sugars are important to OPT as they provide nutrient substrates and important roles as primary messengers in signal transduction (Rolland *et al.*, 2002). The significant increment of those sugar concentrations were obviously depicted as the sap was stored for 1W and 1M, particularly in raw condition. From the findings, the highest concentrations of fructose (78.11 \pm 3.23 mg ml^{-1}), glucose (219.45 \pm 3.74 mg ml^{-1}) and galactose (77.12 \pm 2.98 mg ml^{-1}) were obtained in R-4-1W, R-10-1M and R-4-1W samples respectively. While in the sap storage conditions, the highest fructose (12.99 \pm 0.85 mg ml^{-1}), glucose (200.61 \pm 1.26 mg ml^{-1}) and galactose (6.76 \pm 1.12 mg ml^{-1}) were determined in S-4-1M, S-10-1W and S-4-1W.

Hamid *et al.* (2010) found similar trend of sugar contents increment as the storage time increased due to the different species, cultivation and storage conditions. The increment of sugars in COPT sap may be attributed to plant carbohydrate storage which was instantly metabolised into sugars upon stresses (Lamade *et al.*, 2016). Subsequently, the activation of plant carbohydrate metabolism resulted in free sugars accumulation as response to stress (Prawitwong *et al.*, 2012; Hamid, 2016). Despite that, tautomerisation was also involved during storage as influenced by amino acid (Liu *et al.*, 2014; Varanasi *et al.*, 2016) such as serine, alanine, glutamic acid and aspartic acid (Kosugi *et al.*, 2010).

Organic acids found in stored COPT include acetic acid, malic acid, oleic acid, lactic acid and ribonic acid. The organic acid levels were highly found at 4°C compared to 10°C. Metabolites such as malic acid and ribonic acid were two organic acids found in C-0 and similarly found in S-4 and R-4. Similar levels were observed from 8.88 \pm 0.13 to 8.25 \pm 0.22 mg ml^{-1} for malic acid and from 0.35 \pm 0.03 to 0.47 \pm 0.05 mg ml^{-1} for ribonic acid, particularly in S-4-1 m. This is probably due to the lower temperatures which may well preserve the organic acids.

However, more organic acids were found with decreasing levels observed as storage temperature increased to 10°C indicating the effects of higher storage temperatures on the total content of organic acids. Generally, organic acids are among the fundamental metabolites which are important for plants because they are considered as transitory or stored forms of fixed carbon. Organic acid can either be converted into carbohydrates thus their decrement may contribute to the accumulation of sugars in COPT. Results suggest that organic acids

TABLE 1. RELATIVE SUGAR AND ORGANIC ACIDS CONCENTRATION IN THE STORED CORE OIL PALM TRUNK (COPT) SAP (mg ml⁻¹)

			C-0	4°C		10°C	
				1W	1M	1W	1M
Sugars	Fructose	Sap	12.31±1.49	10.97±1.72	12.99±0.85	12.37±2.16	8.30±0.69
		Raw	-	78.11±3.23	15.79±0.78	19.53±1.78	71.71±3.41
	Glucose	Sap	44.87±1.99	41.57±1.52	42.33±1.93	200.61±1.26	78.53±2.15
		Raw	-	37.00±1.77	63.00±2.17	113.63±1.66	219.45±3.74
	Galactose	Sap	1.07±0.07	6.76±1.12	0.09±0.02	0.14±0.01	0.45±0.17
		Raw	-	77.12±2.98	47.44±2.65	0.68±0.03	2.79±0.55
	Glucopyranose	Sap	13.92±0.90	7.48±1.13	0.10±0.06	3.41±0.07	5.05±0.34
		Raw	-	4.12±2.83	4.30±0.55	9.04±0.06	1.05±0.01
	Xylose	Sap	4.93±0.92	4.03±0.36	3.84±0.22	4.20±0.22	5.22±0.38
		Raw	-	18.08±1.23	4.30±0.21	4.55±0.86	20.89±0.18
	Ribose	Sap	0.12±0.04	0.10±0.04	0.30±0.06	0.23±0.09	0.14±0.01
		Raw	-	0.50±0.04	0.20±0.06	nd	1.29±0.01
	Ribopyranose	Sap	nd	2.83±0.15	nd	nd	1.85±0.12
		Raw	-	4.50±1.19	0.30±0.01	nd	nd
	Mannose	Sap	0.48±0.18	0.27±0.13	0.49±0.14	0.06±0.01	0.11±0.01
		Raw	-	0.40±0.02	1.30±0.05	0.15±0.02	0.16±0.01
	Galactofuranose	Sap	1.90±0.63	1.26±0.23	1.62±0.22	nd	nd
		Raw	-	2.20±0.61	1.60±0.21	nd	nd
	Arabinose	Sap	nd	nd	nd	nd	nd
		Raw	-	nd	nd	0.05±0.01	0.07±0.02
Mannopyranose	Sap	nd	nd	nd	nd	nd	
	Raw	-	nd	nd	0.26±0.14	nd	
Organic acids	Acetic Acid	Sap	nd	nd	nd	0.12±0.05	0.32±0.01
		Raw	nd	nd	nd	0.09±0.00	
	Malic Acid	Sap	8.88±0.13	7.14±0.10	8.25±0.22	1.03±0.08	nd
		Raw	-	24.17±0.51	10.96±0.52	1.76±0.00	2.54±0.62
	Oleic Acid	Sap	nd	nd	nd	nd	0.16±0.04
		Raw	-	nd	nd	0.18±0.00	nd
	Lactic Acid	Sap	nd	nd	nd	nd	nd
		Raw	-	nd	nd	nd	0.086±0.00
	Ribonic Acid	Sap	0.35±0.03	0.34±0.02	0.47±0.05	nd	nd
		Raw	-	0.60±0.43	0.41±0.02	nd	nd

Note: nd - not detected. 1W - one week. 1M - one month.

were higher and well preserved when they are stored at 4°C compared to 10°C.

Separation Pattern Analysis using PCA Score Plot and Loading Scatter Plot

In order to evaluate the metabolite profiles of stored COPT sap, PCA was performed to further determine the response of different storage durations, conditions and temperatures. Interestingly, the separation patterns found in PCA score plot (*Figure 1a*) was greatest driven by storage temperatures rather by storage conditions and durations. Specifically, samples stored at 4°C (S-4-1W, S-4-1M, R-4-1W and R-4-1M) were closely clustered whereas samples stored at 10°C (S-10-1W, S-10-1M, R-10-1W and R-10-1M) were further separated along positive axis of principal component 1 (PC1).

Subsequently, loading scatter plot was performed to further identify metabolites with major contribution towards the separation pattern found

in the PCA score plot. The loading plot in *Figure 1b* suggested that discrimination of samples were contributed by a number of metabolites including glucose, arabinofuranose, galactopyranose and sulfurous acid. The dissipation trends of samples stored at 10°C along positive PC1 were contributed by metabolites of mannose and ribose for R-10-1W, glucopyranose for R-10-1M, xylose for S-10-1W and propanoic acid for S-10-1M.

Differences in the relative concentration of metabolites are shown in the two-way hierarchical cluster analysis (HCA) and heat map (*Figure 2*). As shown in *Figure 2*, the heat map displays relative increase and/or decrease of metabolite levels. HCA dendrogram shows metabolite clustering between different storage conditions, durations and temperatures. Specifically, definitive differences in the relative concentration of metabolites can be observed between 4°C and 10°C. The dendrogram also indicates two major clusters of metabolites. The upper cluster shows relatively high levels of metabolites, particularly glucose, fructose, ribose,

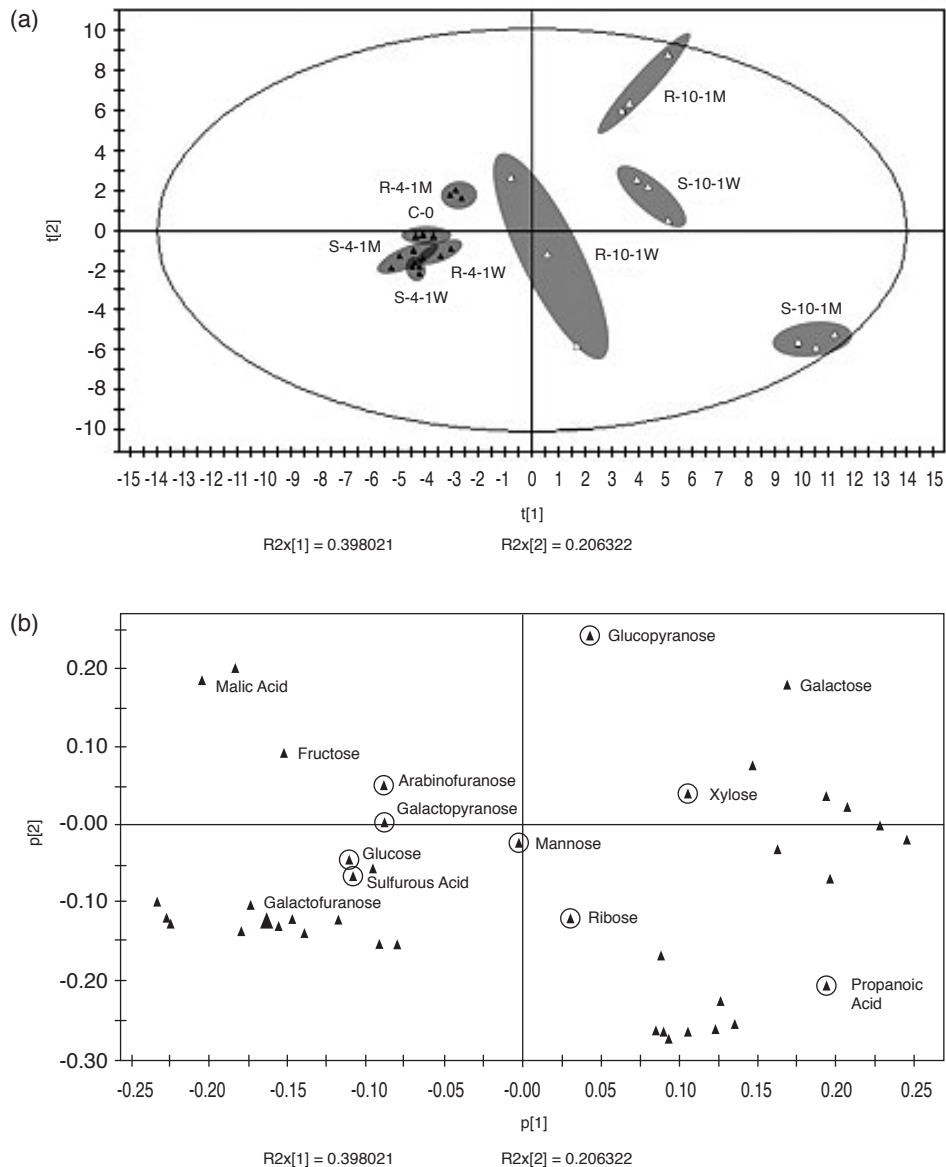


Figure 1. The general clustering patterns of stored core oil palm trunk (COPT) sap metabolites using principle component analysis (PCA): (a) PCA score plot of gas chromatography-mass spectrometry (GC-MS) based metabolites in COPT ($R^2=0.59$; $Q^2=0.367$), (b) PCA loading plot of GC-MS-based metabolites in stored COPT sap ($R^2=0.59$). The triangles labelled with circles were the metabolites that contributed towards the separation in PCA score plot.

mannose and organic acids of malic acid, lactic acid together with other sugar acids during the storage at 10°C. Meanwhile, organic acids (such as acetic acid and oleic acid) and sugars (such as galactose and xylose) dominated the lower cluster of dendrogram during the storage at 4°C.

Main and Interaction Effects Analysis of Different Storage Conditions, Durations and Temperatures Using Two-way ANOVA

The two-way ANOVA was used to determine the relationship and effect of the analytical factors on the sugars and organic acids that displayed significant difference (Table 2). The P -value showed that the majority of

the metabolites were affected significantly by the storage durations. Sugars such as fructose, glucose and galactose showed higher significant differences ($P<0.001$) under different storage durations. Malic, acetic and ribonic acids were affected significantly by storage durations and temperatures. Results indicated that sugars were mainly affected by the COPT storage conditions (sap or raw) and temperatures (Table 2). Organic acids were largely influenced by the COPT storage conditions and different storage durations.

Miscellaneous Metabolites in COPT Sap

A number of interesting metabolites were also detected in COPT sap with relatively low

TABLE 2. MAIN AND INTERACTION EFFECTS OF ANALYTICAL FACTORS IN STORED CORE OIL PALM TRUNK (COPT) SAP METABOLITES

Metabolites	Sugars										Organic acids					
	Fructose	Galactose	Glucose	Ribose	Xylose	Ribo- pyranose	Gluco- pyranose	Mannose	Galacto- furanoside	Arabino- furanose	Manno- pyranose	Acetic acid	Lactic acid	Malic acid	Oleic acid	Ribonic acid
Storage durations (1W/1M)	0.000 ^c	0.004 ^b	0.000 ^c	ns	0.011 ^b	0.011 ^b	0.000 ^c	ns	0.007 ^b	ns	0.000 ^c	0.000 ^c	ns	0.000 ^c	ns	0.000 ^c
Storage temperatures (4°C/10°C)	0.002 ^b	ns	0.000 ^c	ns	ns	ns	ns	ns	ns	0.009 ^b	0.000 ^c	ns	ns	0.001 ^c	ns	0.000 ^c
Storage conditions (sap/raw)	0.002 ^b	ns	0.011 ^b	0.015 ^a	ns	ns	ns	0.029 ^a	ns	0.001 ^c	ns	0.000 ^c	ns	0.008 ^b	ns	0.022 ^c
Storage temperatures x storage durations	0.033 ^b	ns	0.000 ^c	ns	ns	ns	ns	ns	0.033 ^a	ns	0.000 ^c	ns	ns	0.003 ^b	ns	0.000 ^c
Storage temperatures x storage conditions	0.000 ^c	ns	0.012 ^b	0.004 ^b	ns	ns	ns	0.025 ^a	ns	0.002 ^b	0.000 ^c	0.000 ^c	ns	0.006 ^b	ns	0.001 ^c
Storage durations x storage conditions	ns	0.046 ^a	ns	ns	ns	ns	0.001 ^c	ns	0.027 ^a	0.01b	0.000 ^c	ns	ns	0.000 ^c	0.001 ^c	0.000 ^c
Storage durations x conditions x temperatures	0.000 ^c	ns	ns	ns	ns	ns	0.000 ^c	ns	ns	ns	0.006 ^b	ns	ns	0.000 ^c	0.005 ^b	0.001 ^c

Note: ns - not significant; ^aP <0.05; ^bP <0.01; ^cP <0.001. 1W - one week. 1M - one month.

concentrations. These metabolites include compounds from the class of fatty acids (hexadecanoic acid and heptadecanoic acid), carboxylic acid (benzoic acid) and plant lipid (sitosterol, campesterol, stigmasterol and glycerol) which are generally found in plants as shown in *Figure 2*. In particular, lower lipids such as fatty acid was previously found in the OPT sap (1-5 wt%) as compared to other parts of oil palm such as mesocarp (20-60 wt%) (Bourgis *et al.*, 2011; Teh *et al.*, 2013).

CONCLUSION

The dominant metabolites found in the COPT sap were sugars and organic acids. Highest sugars and organic acids concentration were found in R-10-1M sample. The results suggested that the sugar levels are highly influenced by all parameters; storage temperatures (4°C or 10°C) durations (1W/1M) and storage conditions (sap or raw). However, the P-value showed that the majority of the metabolites

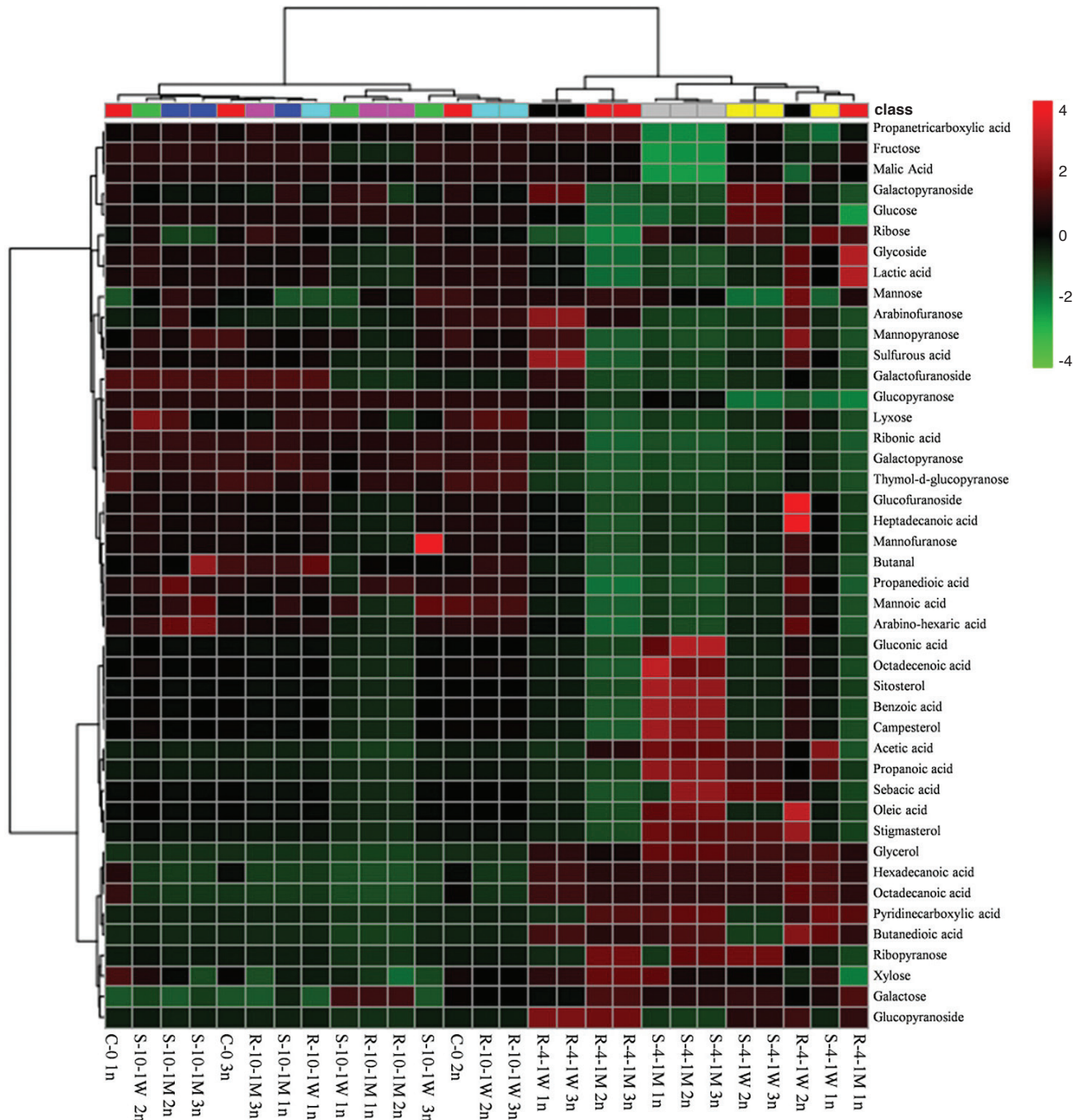


Figure 2. The heat map and hierarchal cluster dendrogram of stored core oil palm trunk (COPT) sap metabolites using hierarchical component analysis (HCA).

were affected significantly by the storage durations. Sugars such as fructose, glucose and galactose showed higher significant differences ($P < 0.001$) under different storage durations.

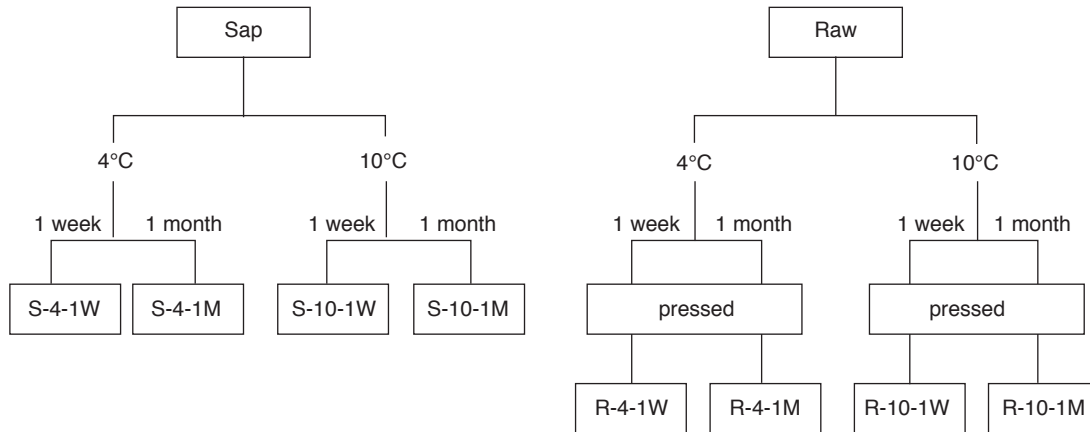
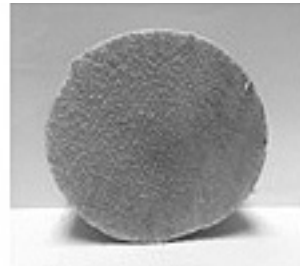
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Work flow of samples preparation at different storage conditions (sap/raw), storage temperatures (4°C/10°C) and storage durations (1 week/1 month).