CHANGES IN THE POLYPHENOL CONTENT OF DIFFERENT CACAO GENOTYPES AND QUALITY OF COCOA BEANS HARVESTED AT TWO MATURITY STAGES

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ABSTRACT – *The polyphenol content of cocoa beans and the products derived from the beans varies between genotypes and pod harvesting maturity stages. The aim of the study was to compare the polyphenols compositions of three cocoa genotypes (QH 1003, MCBC 1 and KKM 22) and the sensory quality of the beans harvested at the mature and ripe stages. UPLC-QTOF analysis showed that major components found at the cacao peel extracts of the pods consisted of flavonoid and procyanidin which are commonly found in cacao beans. KKM 22 was found to contain lower epicatechin, procyanidin B2 and procyanidin C1 compared to QH 1003 and MCBC 1. Among the cacao genotypes, QH 1003 contained more cocoa flavour while KKM 22 tastes more bitterness, astringency and sour. Beans harvested at mature stage had 36.9% more acidity than beans harvested at ripe stage. The conducted research showcases the influence of different cocoa genotypes on the polyphenol content and the beans harvested at different maturity stages affect the sensory quality of the beans.*

***Keywords*:** maturity stage; polyphenol; cocoa genotype; sensory; peel extract; bean quality

INTRODUCTION

Quantification of secondary metabolites that control the external pigmentation can potentially serve as an augmenting criterion for determination of pod maturity among different cacao genotypes. These metabolites are also widely distributed in six major subgroups, including anthocyanidins, proanthocyanidins, chalcones, flavonols, flavandiols and flavones (Winkel-Shirley, 2006). Cacao consists of a large amount of polyphenols, which are large and heterogenous group of active secondary metabolites which provide color to pods to attract insects and pollinators, cell wall support materials and defence mechanisms under different environmental stress conditions (Hakkinen, 2000). Polyphenols in cacao include flavonoids that can be subdivided into anthocyanins and flavonols. Anthocyanins in the vacuole of epidermal and hypodermal cells of cacao pod determine the red colour of the pod appearance in several cacao genotypes. Despite the antioxidant role of anthocyanins in cacao, they are believed to protect the photosynthetic apparatus from high levels of light incidence (Merzlyak and Chivkunova, 2000). The colorless flavonols in the peel of cacao pod absorb ultraviolet (UV) radiation and protect the underlying tissues against UV-induced damage (Solovchenko and Schmitz-Eiberger, 2003).

The biosynthesis of these secondary metabolites within the pod during pod development can be affected by genetic differences, soil characteristics, environmental changes and cultural practices. The composition and concentration of these secondary metabolites may vary significantly depending on genotypes (Mattivi *et al*., 2006). Furthermore, latest research done by Joanna *et al*. (2015) found that polyphenols, which are the largest group compounds among natural antioxidants, can be very diverse even within one species of cacao

and affected by many factors, mainly pod maturity, climatic conditions during growth, time of harvest and storage period after harvest. To date, many research have been carried out on cacao beans as cacao contain polyphenols with properties that enable them to act as anticarcinogenic anti-inflammatory, antibacterial and antiallergenic compounds (Heim *et al*., 2002; Jalil and Ismail, 2008; Djoussé *et al*., 2011).

Cacao beans are processed into chocolates and other semi-finished products such as cacao liquor, cacao butter, cacao cake and raw cacao powder. Cacao flavour is one of the important criteria in the finished products and the precursors are triggered during fermentation and drying of cacao beans. The aroma precursor exits in cacao beans include free amino acids, peptides and reducing sugars that will eventually develop into specific aroma through Maillard reactions which involve in the sensory, flavour and color appeal of cacao (Bonvehi and Coll, 2002). The bean flavour on chocolate can only be developed during fermentation and thus unfermented beans do not develop any flavour during roasting and the beans become excessively astringent and bitter in taste (Puziah *et al*., 1998). During fermentation, mucilaginous pulp surrounding the beans are broken down and caused the death of cotyledon. Subsequently, it triggers the aroma precursors inside the beans and leads to reduction in bitterness and astringency, at the same time, flavour has been developed (Rodriguez-Campos *et al*., 2011). The quality of bean flavour during fermentation can be influenced by factors such as different types of cacao variety, disease, climatic, frequency of bean turning during fermentation, cacao batch size and quantity of the beans (Afoakwa, 2010). Hence, this study identified the polyphenol composition of different cocoa genotypes and revealed the quality of beans harvested at different maturity stages using sensory attributes.

MATERIALS AND METHODS

Plant material

Three genotypes of cacao (QH 1003, KKM 22 and MCBC 1) were collected from the field plot (N 03°53.752' E 100°52.061') of Cocoa Research and Development Centre, Bagan Datuk, Perak. The field measurement was carried out between October and November of 2019. All three genotypes were selected from the same location, and were subjected to identical atmospheric conditions of light and temperature. Thirty-six trees were tagged and pods were bagged with green plastic bags once the pods emerged and was considered as day 1 (D1). Number of days of pod development was counted. One hundred and thirty-five healthy pods (three pods x three genotypes x five developmental stages x three replications = 135 samples) were selected for measurements as pods developed from one-month (D30) to five-month old (D150) after flower pollination. Fifteen mature pods of the three genotypes were harvested (three pods x three genotypes = 15 mature pods) when the pods reached maturity at five months (D150) and brought to laboratory for further analysis. Pods were cleaned with distilled water before measurements were taken.

Reagents and chemicals

Methanol, acetonitrile and formic acid (LC/ MS grade) were purchased from Fisher Scientific (Pittsburgh, PA, USA). Water was prepared using a Milli-Q system (Millipore Corp., Bedford, MA, USA). The standards of catechin, epicatechin, procyanidin B2, procyanidin C1 and quercetin were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Cyanidin 3-O-glalactoside chloride and cyanidin 3-O-arabinoside were purchased from ChemFaces (China). All other chemicals were of analytical grade.

Extraction of polyphenolic compounds

Mature cacao pods were harvested fresh from the field plot and transferred to the laboratory for further analysis. Pods were cleaned with distilled water before removing the peels with a peeler. Next, the peels were dried with a freeze-dryer (Labconco, United States) and the samples were sieved using a 1 mm sieve after the peels were ground into powder. 0.25 g of sieved powder was extracted in a glass tube with 5 ml of 80% aqueous methanol and the mixture was shaken for two minutes with a shaker (1500 ShaQer, United States) before being centrifuged at 12,000 rpm for 15 min at a temperature of 20 °C. The supernatant was then separated by a 0.2 µm nylon membrane, and then injected into a liquid chromatography; Acquity UPLC/Vion

IMS/QTOF-MS system (Waters Corporation, Milford, MA, USA).

Quantification of polyphenolic compounds
Separation of polyphenolic compounds was performed using an Acquity UPLC HSS T3 column featuring 1.8 µm high-strength silica (HSS) particles (100 mm x 2.1 mm) with the column temperature set at 40 °C. The mobile phase consists of solvent A (0.1% formic acid in water) and solvent B (acetonitrile and 0.1% formic acid) with the following gradient elution in Table 1. All solvents were LCMS graded. 1 µL of sample was injected into the UPLC system. The conditions for mass spectrometry were described (Table 2).

Table 1: Gradient for compound separation in the peel of cacao pods

Time (min)	Flow rate (mL min^{-1})	%A	%B	Curve
0.0	0.6	99	1	Initial
0.5	0.6	99	1	6
16.0	0.6	65	35	6
18.0	0.6	0	100	1
20.0	0.6	99	1	1

Table 2: Mass spectrometry conditions

MS conditions	Descriptions
Capillary voltage	1.5 kV
Source temperature	120 °C
Desolvation temperature	550 °C
Desolvation gas flow	800 L hr^{-1}
Collision energy	4 eV (low energy); 10 - 40 eV (high energy)
Acquisition mode	ESI negativity/ sensitivity
Experiment mode	High definition MS^e
Scan range	50 - 150 Da
Cone gas flow	50 L hr^{-1}
Lock mass	Leucine Enkephalin (LE), 50 pg μL^{-1} in 50/ 50 CAN : water + 0.1% formic acid
lock mass scan interval	0.5 min

Sensory analysis (Quantitative descriptive analysis – QDA)
A total of 1200 g of fermented dried beans were prepared from three cacao genotypes (QH 1003, KKM 22 and MCBC 1) at two maturity stages (mature and ripe) for sensory analysis. Liquor from each samples were extracted according to methods proposed by Federation of Cocoa Commerce (FCC, 2012) with slight modifications. First, dried beans were roasted in a forced airflow-drying oven for 15 min at 150°C. Roasted beans were then left to cool to approximately 50 °C before shells were removed and broken into nibs. Next, 100 g of roasted nibs from each sample was ground using a mill (IKA, Germany) until a smooth cacao paste was obtained. Sensory analysis was carried out by nine trained panelist at Cocoa Innovative and Technology Centre (CITC), Nilai, Malaysia. The QDA contains scale 0 to 10. Scale 0 indicates the absence or minimum intensity while scale 10 indicates maximum intensity. Five flavour attributes, include cocoa, bitter, astringent, acid/ sour and fruity/ floral/ bouquet were evaluated.

Statistical analysis

The experimental design was a 3 x 5 factorial arrangement in randomized complete block design (RCBD) of three cacao genotypes applied to five stages of pod development (months after emergence of pod from pollinated flower) with three replications. Data analysis was performed using Statistical Analysis System (SAS Institute, 2002). Multiple mean comparisons were analyzed using Least Significant Difference (LSD). Results were further computed in graphs to study the trend of each parameter during pod development and were displayed as means ± standard error using Microsoft Excel (Microsoft Corporation, 2003).

RESULTS AND DISCUSSION

Qualitative polyphenol compounds in mature cacao pods

Comparable values in FLAV index were found among genotypes (Tee *et al*., 2019). This finding is in agreement with the destructive UPLC-QTOF analysis of cacao peel extracts which also showed similar compounds of flavonols in QH 1003, KKM 22 and MCBC 1. The gradient for compound separation in the peel of cacao pods is given in Table 1. In the peel of the cacao pod, different classes of polyphenol compounds were characterized: flavonols (quercetin-3-arabinoside, rutin) and procyanidin (catechin, epicatechin, procyanidin B2, procyanidin C1). Despite polyphenols, alkaloids (caffeine and theobromine) were also found in the peel extracts (Figure 1 and 2).

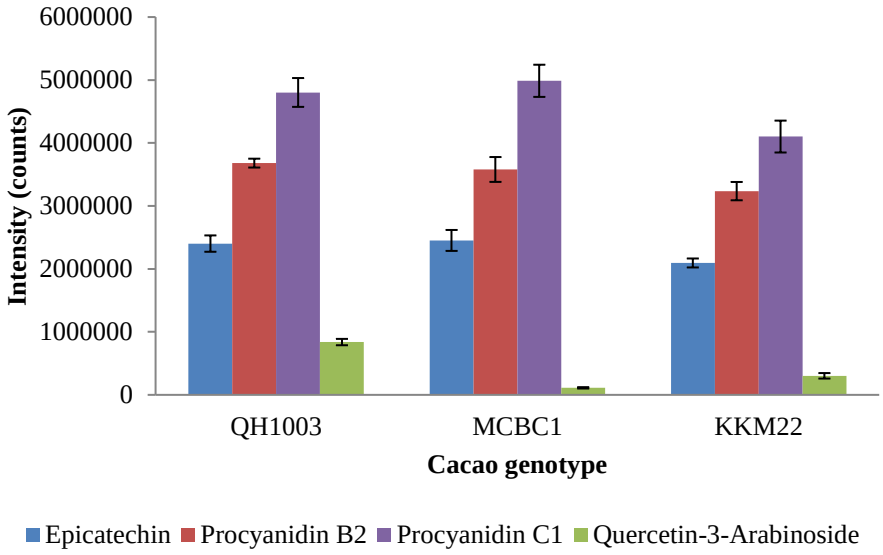

Figure 1: Major compounds found in the peel extracts of cacao genotypes with UPLC-QTOF analysis (mean ± S.D)

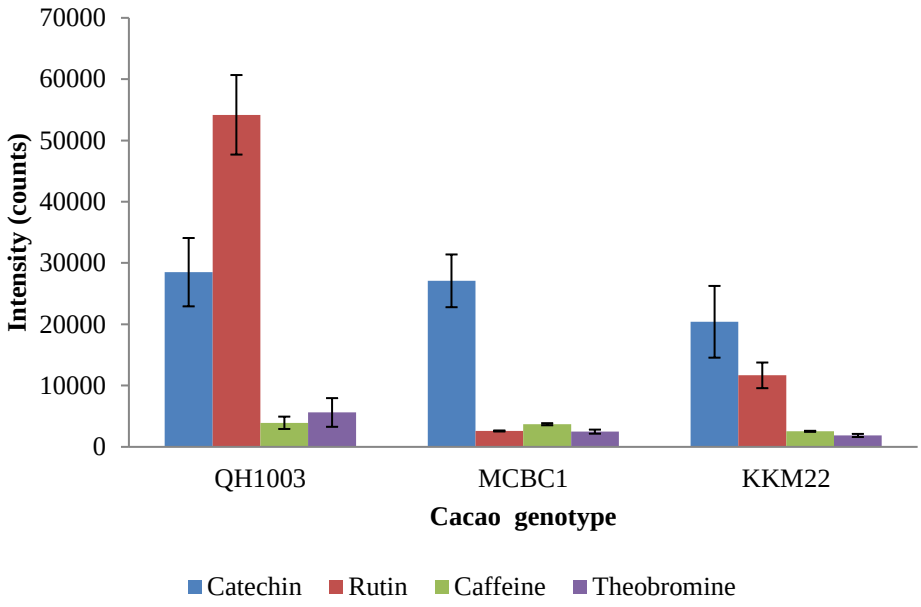

Figure 2: Traces of compounds found in the peel extracts of cacao genotypes with UPLC-QTOF analysis (mean ± S.D)

Analysis showed the highest compounds found in peel extracts of the three genotypes were procyanidin C1, followed by procyanidin B2, epicatechin and quercetin-3-arabinoside (Figure 1). Traces such as catechin, rutin, caffeine and theobromine were also observed in the peel extracts (Figure 2). These results are in agreement with a recent study by Aprotosoaie *et al*. (2016) who reported 58% polyphenols, followed by 37% flavanols (epicatechin and catechin) and 4% anthocyanins in cacao beans.

Flavonols in cacao are central to the development of bean quality because they bind with amino acids and peptides to generate bean aroma during fermentation. During chocolate processing, flavonols are reported to contribute bitter flavour and astringent properties (Stark *et al*., 2005). Prior to harvest, flavonols play important roles in the growing seeds as defence compounds as they protect the seeds against oxidative damage (Scarpari *et al*., 2005; Ndoumou *et al*., 1996). So, it is not surprising that flavonols such as quercetin-3-arabinoside and rutin exist in the cacao pods with similar functions. In this study, QH 1003 ccumulated the largest amount of quercetin-3-arabinoside and rutin compared to other cacao genotypes (Figure 1 and 2). Pinelli *et al*. (2013) reported that the relationship between FLAV index and total peak areas of flavonols demonstrated a strong linear regression (R^2 = 0.83) in kiwifruits.

Procyanidins exist naturally in plant-based foods and beverages such as apples, red wine, sorghum, tea, blueberry and cranberry (Degenhardt *et al*., 2001; Kelm *et al*., 2002; Guyot *et al*., 2001; Peng *et al*., 2001). Procyanidins in cacao are members of the proanthocyanidin (condensed tannins) class of flavonoids which exhibit potential antioxidant properties (Sanbongi *et al*., 1998; Rein *et al*., 2000). Chocolate has been recently categorized as food high in procyanidins and has become a leading model used to develop techniques to analyze procyanidins (Hammerstone *et al*., 1999; Adamson *et al*., 1999; Wollgast *et al*., 2001). In this study, matured cacao genotypes showed high abundance of procyanidin C1 and procyanidin B2 in the peel extracts (Figure 1).

Traces of alkaloids such as theobromine and caffeine were also found in the peel extracts of cacao pods (Figure 1). Caffeine and theobromine belong to the chemical group known as methyxanthine alkaloids and raw cacao beans contain about 4% alkaloids (Kadow *et al*., 2013). Theobromine (3,7-dimethylxanthine) is considered to be one of the major alkaloids of cacao constituting about 2-3% while caffeine (1,3,7-trimethylxanthine) is found only in small amounts of around 0.2% (Franco *et al*., 2013).

According to Anita-Sari *et al*. (2016) anthocyanin content in the cacao plant can be identified in flowers, leaves, seeds and fruits. Anthocyanins can be found in the cell palisade and the mesophyll cork. The synthesis of anthocyanins occurs during plant growth and when plant responds to abiotic stress (Woodall and Stewart, 1998; Close *et al*., 2000). Anthocyanins are pigmented phenolic compounds expressed in red and blue (Lee and Kevin, 2002) as well as purple color (Close and Christoper, 2003). Previous research has shown the presence of two major pigmented anthocyanins, 3-β-D-galactosidyl cyanidin and 3-α-L-arabinosidyl cyanidin, in seed extracts of cacao (Niemenak *et al*., 2006). In this study, the intense red pod color of KKM 22 and MCBC 1, which gave high a ANTH index is not well explained with the destructive analysis in peel extracts as no compounds of cyanidin was observed (data not shown). This might be due to the factors involved in the expression of anthocyanins during analysis as it is known that anthocyanin can be easily modified due to pH, co-pigmentation with proteins and other flavonols (Cakirer *et al*., 2010). The anthocyanin will be colorless under mild acidic condition between pH 3 and 6 (Tanaka *et al*., 2008). Despite cyanidin, there might be other anthocyanin pigments such as pelargonidin derivatives that produce red and purple color, delphinidin, petunidin and malvidin that exist in the peel extracts but were not analyzed in this work.

To date, there is no study that has documented polyphenol compounds in the peel of cacao pods. Based on results from this study, we hypothesize that the compounds found in the peel of cacao pods might be related to the fresh beans inside the pod as studies have shown that the main polyphenol compounds contained in fresh cacao beans are epicatechin followed by catechin, which were also found in the peel of cacao pods (Jalil and Ismail, 2008).

Sensory analysis of pods harvested at two maturity stages

From the study, there were significant differences observed between the interaction of three cacao genotypes and maturity stages in flavour attributes of cocoa and acid (sour) (Table 3). Among the cacao genotypes, QH 1003 obtained more cocoa flavour while KKM 22 tastes more bitterness, astringency and sour. Beans harvested at the mature stage (4 months after pollination) had significantly higher acidity and taste more sour while ripe beans showed higher flavours in cocoa, bitter and unique flavours (fruity, floral and bouquet) (Table 3).

Table 3: Sensory analysis of cacao genotypes harvested at two maturity stages (mature and ripe)

Source	Cocoa	Bitter	Astringency	Acidic/sour	Fruity/ floral/ bouquet
Cacao genotypes (G)					
QH 1003	5.15 a	3.44 b	3.71 b	1.96 c	0.13 a
KKM 22	3.78 c	3.77 a	4.08 a	3.94 a	0.05 a
MCBC 1	4.40 b	3.48 b	3.82 b	2.90 b	0.09 a
Maturity stages (M)					
Mature	4.28 b	3.48 b	3.93 a	3.59 a	0.02 b
Ripe	4.60 a	3.64 a	3.81 a	2.28 b	0.16 a
Interaction					
G*M	*	n.s	n.s	**	n.s

Means followed by the same letter in the same column are not significantly different by DMRT at $P \leq 0.05$.
NS, non-significant difference at $P > 0.05$. Significant difference at $*P \leq 0.05$ or $**P \leq 0.01$.

In this study, QH 1003 contained similar attributes for mature and ripe beans in cocoa, bitter, astringent and unique flavours of fruity, floral or bouquet, except acidity. Beans harvested at mature stage had 36.9% more acidity than beans harvested at ripe stage (Figure 3).

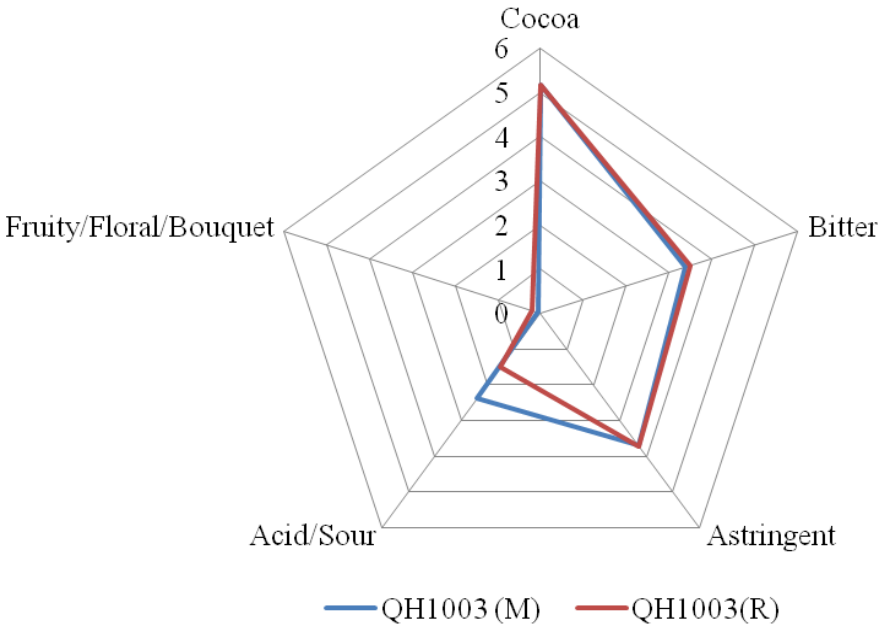

Figure 3: Flavour attributes of QH 1003 harvested at two maturity stages (M: Mature; R: Ripe)

As for KKM 22, it showed significant difference in flavour attributes of bitter and acid for mature and ripe beans (data not shown). Ripe beans had 8.9% higher bitterness compared to mature beans while mature beans tasted more sourness as it contained 61.8% higher acidity compared to ripe beans (Figure 4).

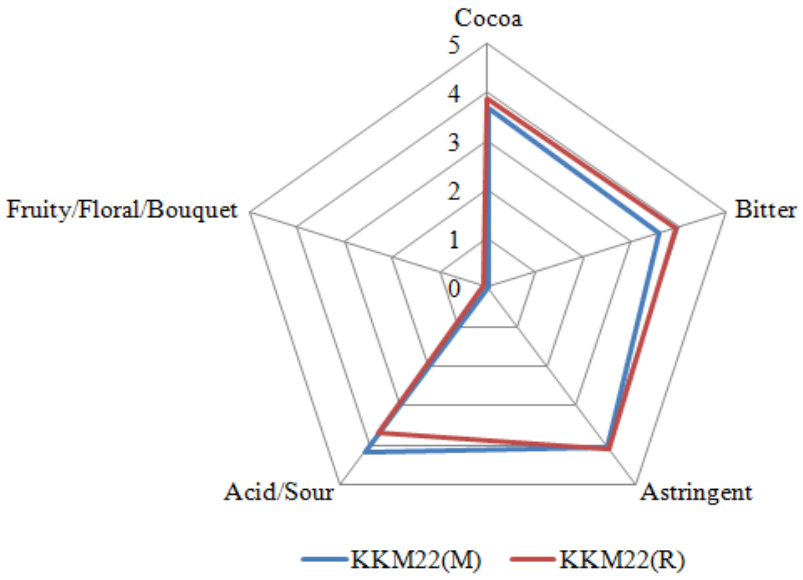

Figure 4: Flavour attributes of KKM 22 harvested at two maturity stages (M: Mature; R: Ripe)

For MCBC 1, ripe beans contained 16.7% higher cocoa and unique flavours of fruity/ floral/ bouquet only tasted in ripe beans. Like other cacao genotypes, mature beans of MCBC 1 had 61.8% more acidity than beans harvested at ripe stage (Figure 5).

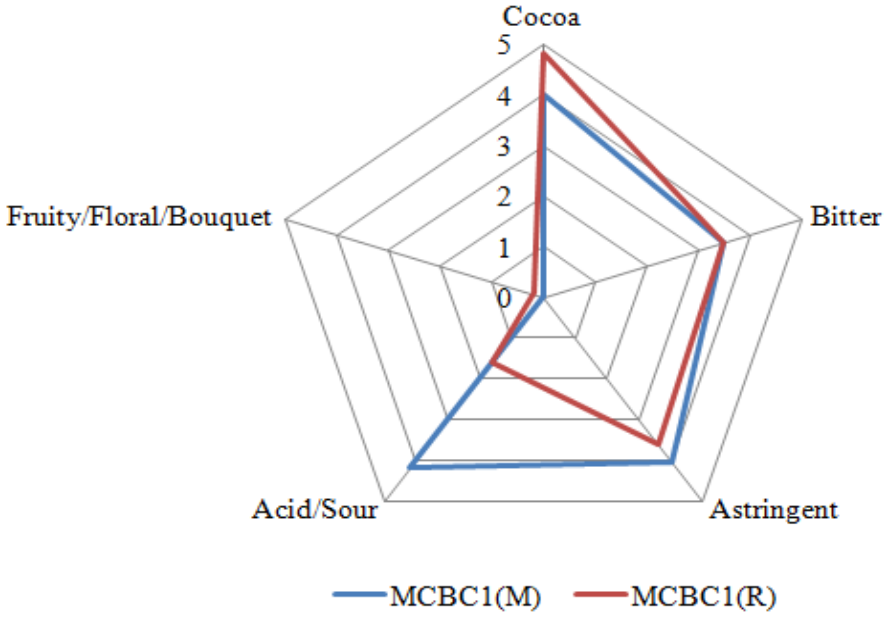

Figure 5: Flavour attributes of MCBC 1 harvested at two maturity stages (M: Mature; R: Ripe)

Overall, beans harvested at mature stage did not show much significant difference in flavour especially cocoa flavour. However, beans harvested at mature stage had higher acidity and acidic beans are characterized by high concentrations of acetic and lactic acids (Jinap and Dimick, 1990). The acidity can be detrimental to the chocolate flavour if present in large amounts. Mature beans with slightly higher acidity can be solved using an alternative method using pod storage. After harvesting, beans are allowed to keep in the pods for pre-conditioning of pulp in order to reduce acidification during subsequent cocoa fermentation. Pod storage helps to reduce the pulp volume per seed and reduction in pulp volume enhances mass aeration, thus increases the ratio of respiration to ethanol fermentation. Ethanol is then oxidized to acetic acid. Consequently, acidification of seeds during formation of flavour precursors is strongly reduced. Hence, pod storage provides stored beans in the pod with an anaerobic condition where initial stage of fermentation is initiated in the pod. Under these conditions, the pH value does not reduced below 5.0 and thus, drastic flavour and acid degradation are unnecessary at the end of fermentation (Meyer *et al.*, 1989). Pod storage of four days at the field can be done and beans are extracted to allow normal fermentation for a day using a fermentation box. After a day, fermented beans are dried and kept for sale.

CONCLUSIONS

Presence of polyphenols at the pod surface absorbs light according to their concentration in the visible spectral range which can be measured quantitatively from their fluorescent light emitted. The composition of polyphenols in three cacao genotypes were defined and beans harvested at different maturity stages showed interesting result in sensory attributes. Further research can be carried out to relate the influence of polyphenols in contributing to the flavour of the cocoa.

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