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SCREENING AND IDENTIFICATION OF SPECIAL COCOA FLAVOR COMPOUND IN FRESH COCOA BEANS USING RAPID AND FAST MICROANALYSIS TECHNIQUES FOR MALAYSIAN RENAISSANCE COCOA

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ABSTRACT - *The analysis of pharmacological content in cocoa beans is important and crucial for quality control of chocolate products since cocoa flavor is one of the major components in the chocolate industry. Accurate microanalysis remains challenging, because it is still in development. Conventionally, this is achieved by combining multiple microanalysis techniques such as GCMS, HPLC and UV/Vis Spectrophotometry. Development of a simpler and quicker analytical method is anticipated. This study will focus on determination of several compound that contribute to special flavor such as fruity flavor (ethyl-3-methylbutanoate, ethyl-2-methylbutanoate and linalool), and flowery flavor (2-phenylethyl acetate and 2-phenylethanol) using fresh beans analysis and application of microanalysis with 10mg of samples. At the same time, we would like to share a new unique identification and qualification strategy that will discover and allow accurate identification and qualification of important compounds in fresh cocoa beans.*

Keywords: cocoa flavour, microanalysis technique, ethyl-3-methylbutanoate

INTRODUCTION

In traditional cocoa plant breeding, crosses are made between selected clones and seeds are planted. The resultant trees are evaluated for agronomic traits such as yield, precocity, and disease and insect resistance. One breeding cycle takes about 10 years and perhaps only a few trees are saved for the next cycle. After several breeding cycles, some clones are released to farmers. Almost 20 years of traditional breeding were required before Malaysian Cocoa Board (MCB) released its own MCB 1-9 clones. And much land, labor, and money were needed.

An unfortunate consequence of breeding primarily for agronomic traits is that flavor and other bean traits may suffer. This is fine if only ordinary low-value bulk beans are wanted. But Malaysia does not want such beans. We are not a Third World country desperately trying to employ as many people as possible at low wages. We are an emerging industrialized country with a well-paid workforce. That is why MCB is creating high value beans unlike any others on earth today – "Malaysian Renaissance Cocoa" (MRC). In addition, by processing our exclusive MRC beans, Malaysia can become the Gourmet Chocolate Capital of the world, not merely a supplier of beans, butter, and liquor.

In our program to create MRC, flavor and other bean traits are of paramount importance. They are selected for first. There are several important feature of MRC trees that sets them apart from ordinary trees now in production:

1. MRC trees are *populations* of trees, rather than individual clones. Our populations will be continuously improved, unlike clones, which are static.

2. Each of the various populations will be highly genetically diverse, but share an enhanced bean trait:

- Intense cocoa flavor
- High aroma notes (fruity or nutty or floral or spicy, etc.)
- High flavanols for health chocolate
- High caffeine for a stimulating beverage (to partially replace coffee)
- Low theobromine and caffeine for low bitterness
- High cocoa butter (the most valuable component of beans and the most expensive edible lipid)
- Other unique characteristics, such as high in compounds that relax people

To create MCR trees quickly and inexpensively, MCB has developed a single beans analysis method to select for trees that will bear beans with enhanced traits. We know of no other group anywhere performing such analyses. We are the pioneers.

Single bean analyses and selection is based on the realization that every cocoa bean ever produced – even from the same pod - has different trait potentials. Thus the few superior beans from an otherwise mediocre population of beans will produce trees that will on average produce superior beans.

Six properties of cocoa make single bean selection feasible:

- Cotyledons (nibs) are the source of chocolate and cocoa powder
- The vast bulk of the embryo is cotyledon
- The cotyledons are genetically identical to the germ
- Cocoa is highly genetically diverse
- Cocoa is highly heterozygous
- Individual embryos are large enough to perform the necessary chemical analyses easily (personal observation)
- Embryos can be cut in half without affecting germination (personal observation)

Chocolate flavor development involves two complex processes: fermentation and roasting. During fermentation storage protein degrades into amino acids and short oligopeptides. They can be reached with reducing sugar to produce a complex mixture of compounds during roasting. These compounds are the major contributors to chocolate flavor.

Besides components that contributed to chocolate flavor, seeds also contain ones that detract from chocolate flavor, notably condensed tannins and methylxanthines (theobromine and caffeine). Tannins reduce perceived chocolate flavor and cause astringency, and methylxanthines are bitter. Malaysian beans are discounted due to their low chocolate flavor. Producing is also currently low in Malaysia. Producing beans with novel and exceptional properties can revive and revolutionize the Malaysian cocoa industry.

The objectives of this research were to determination of tannin level for different beans and screening of specialty flavor compounds in fresh beans by using microanalysis techniques

MATERIALS AND METHODS

Single bean from high flavor characteristic were analyses using several steps::

1. Crosses are made between trees that are already known to bear flavorful beans.
2. Each bean (embryo) is cut transversely without injuring the germ
3. Plant the portion with the germ (we know it will germinate and grow well.)
4. Analyze the upper nib (cotyledon) portion for compounds that influence bean traits:

- ♦ Flavan-3-ols (condensed tannins = procyanidins): astringent and reduce cocoa flavor precursors: want low for high cocoa flavor or want high for health chocolate
- ♦ Caffeine and theobromine: want low for low bitterness or want high caffeine for stimulating beverage
- ♦ Storage proteins (vicilin-class globulin): Generates flavor precursors - always want high
- ♦ Aroma note (nutty, fruity, floral) compounds: esters, alcohols, aldehydes, etc.

5. Seedlings from beans with the desired level of compound are saved, the others discarded.
6. Saved trees are given to breeders to select those that have acceptable agronomic traits.
7. The entire selection process is repeated with beans from the first improved population. And so on. Superior populations keep improving with each selection cycle.

Selection of trees for good agronomic traits is still done, but after beans are selected for desirable bean traits. Thus single bean analysis is a powerful pre-agronomic trait selection method.

In genetic terms, single bean analysis enriches populations of trees for alleles and their combinations that enhance bean traits.

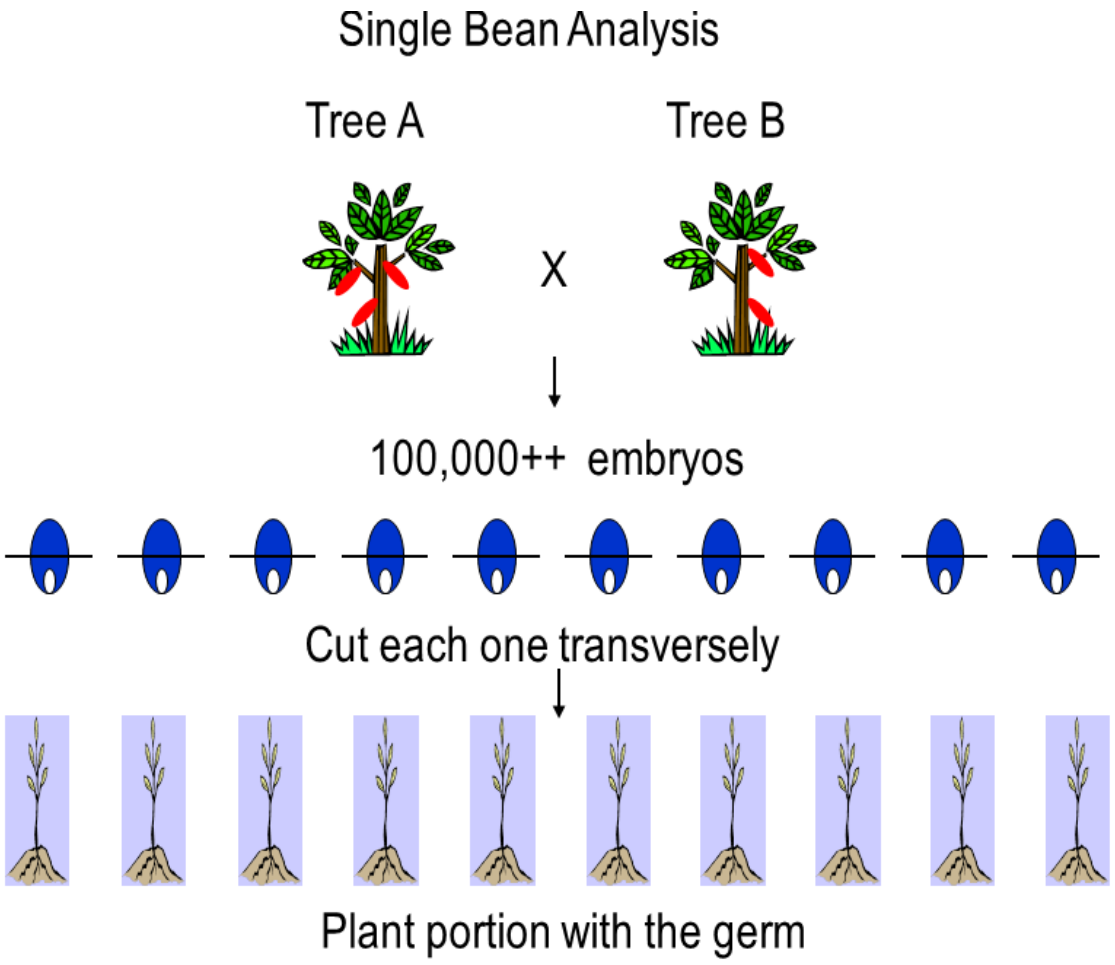
Single bean analyses must be:

1. Fast
2. Easy
3. Sensitive
4. Precise
5. Reproducible
6. Inexpensive (<RM20.00/sample)
7. (Able to be automated is a bonus)

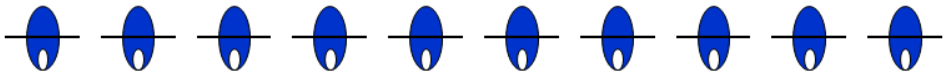
At this early stage in the selection process, we need to know the *relative* amounts of compounds in samples, not the absolute amounts. That is, single bean analysis is a selection tool.

If a compound in embryos is not vital for survival (e.g. secondary metabolite), can be measured precisely, and embryos are genetically diverse, it's amount in beans can be increased or decreased. Procyanidins, aroma note compounds, methylxanthines (theobromine and caffeine), and - to a large extent – the vicilin-class globulin cocoa flavor/aroma precursor protein meet these criteria.

FIRST STEP SINGLE BEANS ANALYSIS

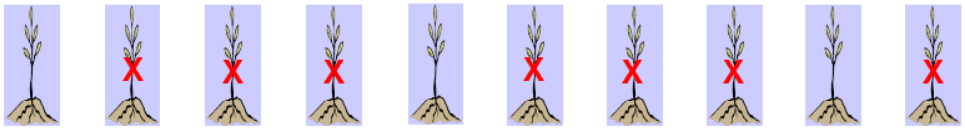


SECOND STEP SINGLE BEANS ANALYSIS



Analyze

- Flavan-3-ols (Procyanidins): Astringent and reduce cocoa flavor precursors
  - Want low for high cocoa flavor
  - Want high for health chocolate
- Caffeine and theobromine: Bitter
  - Want low for low bitterness
  - Want high caffeine for stimulating beverage
- Storage proteins: Generates flavor precursors - always want high
- Aroma note (nutty, fruity, floral) compounds



Flavanols	−	−	+	−	+	−	+	−	+	+
Caffeine/Theobro	−	−	+	−	+	+	−	+	−	+
Storage proteins	+	−	−	+	+	−	−	+	+	−
Aroma notes	−	−	+	−	+	−	−	−	+	−
Good mol markers (Agronomic traits)	+	−	−	−	+	+	−	−	+	−

Sample preparation and tannin analysis

Cocoa samples were taken from Tenom and stored one day after harvest from the trees. 20 cocoa seeds were used along the experiment. Every single bean was peeling from the pod (separated from the pod). After that, every single bean was moved from their mucilage by using wooden waste and leaves it for a while to make sure it dry. Then, 10mg from the single beans was weighed and put into methanol. There are 3 replications used for every sample. After that, 10mg of the sample was crushed by small mortar and mixed with glass powder. After that, the sample was centrifuge 5000rpm at 5min. Supernatant of the sample was taken out and filled-up in new tubes and analysed.

Vanillin-HCL Assay (Yamiko et al., 1998)

Proanthocyanidins in the sample solutions of 5 GSEs, 4 health foods, and 2 grape seed oils were determined by the vanillin-HCL assay described by Sun et al., (1998). To 1 ml of CT (catechin) solution (0-300ug/ml in methanol) or test solution (150-250ug/ml polyphenol in methanol) in a test tube, 2.5ml in methanol (control) or 1% vanillin solution in methanol (sample) and 2.5ml of 9 mol/L HCL in methanol was added. The reaction mixture was incubated for 20 min at 30^0C and the absorbance at 500nm was measured by using UV Spectrometer (UV Lambda 35, Perkin Elmer).

The following A_0, A_b, A_c, A_s was measured for each standard and sample.

A_0 = Absorbance at 500nm of the control of o mg CT (1ml methanol + 2.5ml methanol + 2.5ml 9 mol/L HCL.

A_b = Sample of 0 mg CT (1ml methanol + 2.5ml 1% vanillin solution + 2.5ml 9 mol/L HCL.

A_c = control (1 ml CT (20-300ug/ml) or test solution + 2.5ml methanol + 2.5ml 9 mol/L HCL.

A_s = sample (1 ml CT (20-300ug/ml) or test solution + 2.5 ml 1% vanillin 2.5ml 9 mol/L HCL.

A was calculated as follows for each standard and sample solution:

$A = (A_s - A_b) - (A_c - A_0)$

A calibration curve was prepared using A for the CT solution using the above calculation. Total flavan-3-ol in each test was calculated from the calibration curve.

The Vanillin-HCL assay was performed 3-5 times for each sample.

RESULTS AND DISCUSSIONS

Single bean analyses and selection are based on the realization that every cocoa bean produced and the same pod, has different trait potentials. Nine hundred twenty five cocoa seeds cut transversely. The upper portions were used for analysis while the lower portions were planted in the nursery. Only 792 plants survived. 223 cocoa plants were ready to transfer to the field. And there are 569 cocoa plants that were successfully transferred to the field. Currently, 605 samples were analyzed using HPLC for methylxanthine (theobromine and caffeine).

Six hundred thirty six samples were analysed using GC-MS to detect several compounds that contribute to special aromatic flavors (nutty, fruity and floral). This information will be used as an indicator for cocoa plants selection. It was found that only flowery compounds can be detected from all cocoa samples. From all selection, there are 20 plants selected from GCMS analysis.. Out of them, 4 died, and 16 plants still survived and will be added from time to time. At this early stage in the selection process, we need to know the *relative* amounts of compounds in samples, not the absolute amounts.

Results obtained from figure 1, it was shown that there were a total 20 samples listed from samples 1 to 20. The entire sample was calculated by conversion to 10mg because all the samples were targeted to be uniform 10mg of every single bean. There are three samples which showed the highest levels of the tannin content, with a high catechin content with absorbance below 0.5 (500nm). The high tannin samples are sample no. 8, 14, and 16. And the rest of the samples had shown lower levels of the tannin content. Sample no 1-7, 9-13, 15, 17-20 shown lower tannin levels.

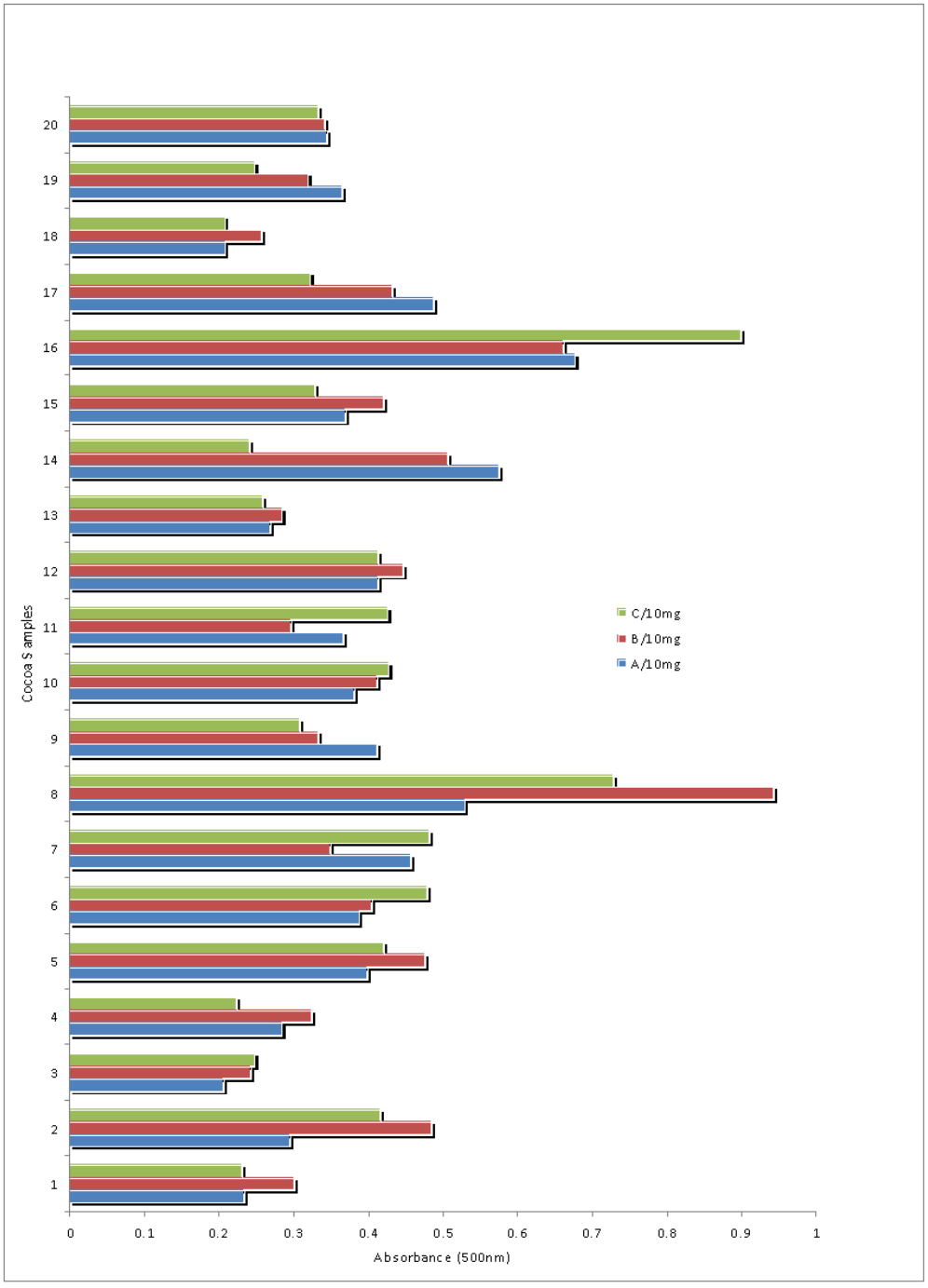


Figure 1: Tannin levels for 20 cocoa beans samples (3 replications for each samples)

It was shown that every single cocoa bean has tannin and can be separated into low and high levels of the tannin. This is due to different types of clones and their genetic variations between the

clones. In addition, information of the color in the cocoa beans also contributed for levels of tannins in every single bean.

The result of this analysis shows the absorbance of the samples that were analyzed in a day. This result shows that there are variabilities of the flavan-3-ol level in each bean that were analyzed. The variability of flavon-3-ol concentrations in each sample tells us that each bean in the same pod does not necessarily have the same level of flavon-3-ol concentration in each sample. There are reasons that lead to the variability of flavon-3-ol concentration in each sample. During the preparation of the beans, we observed that the size of each bean is different from each other, thus the weight of each bean is different too. We are also observed that the intensity of the colour in each bean is also different. These factors may affect the level of flavon-3-ol concentration in each bean. A darker bean may have high level of flavon-3-ol than a less dark bean.

Table 2 The absorbance and concentration of the samples

Number of beans	Beans ID	Absorbance at 500nm	Concentration
1	4617	0.4744	94.88
2	4618	0.3842	76.84
3	4619	0.5208	104.16
4	4701	0.7593	151.86
5	4702	0.9515	190.30
6	4703	0.8335	166.70
7	4704	0.5381	107.62
8	4705	0.6978	139.56
9	4707	0.6489	129.78
10	4708	0.4269	85.38
11	4710	0.5760	115.20
12	4711	0.6630	132.60
13	4713	0.4513	90.26
14	4714	0.6450	129.00
15	4715	0.6814	136.28
16	4716	0.6310	126.20
17	4717	0.5993	119.86
18	4719	0.5785	115.70
19	4720	0.5695	113.90
20	4721	0.4813	96.26
21	4724	0.6333	126.66
22	4726	0.2777	55.54
23	4727	0.4467	89.34

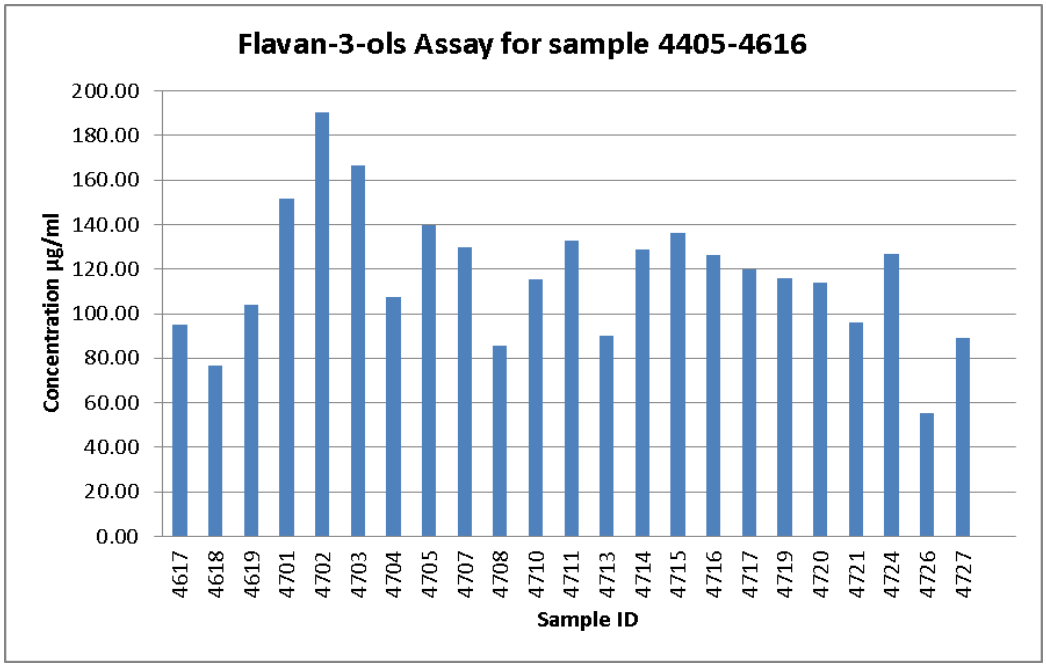


Figure 2 Concentration of samples at 500 nm

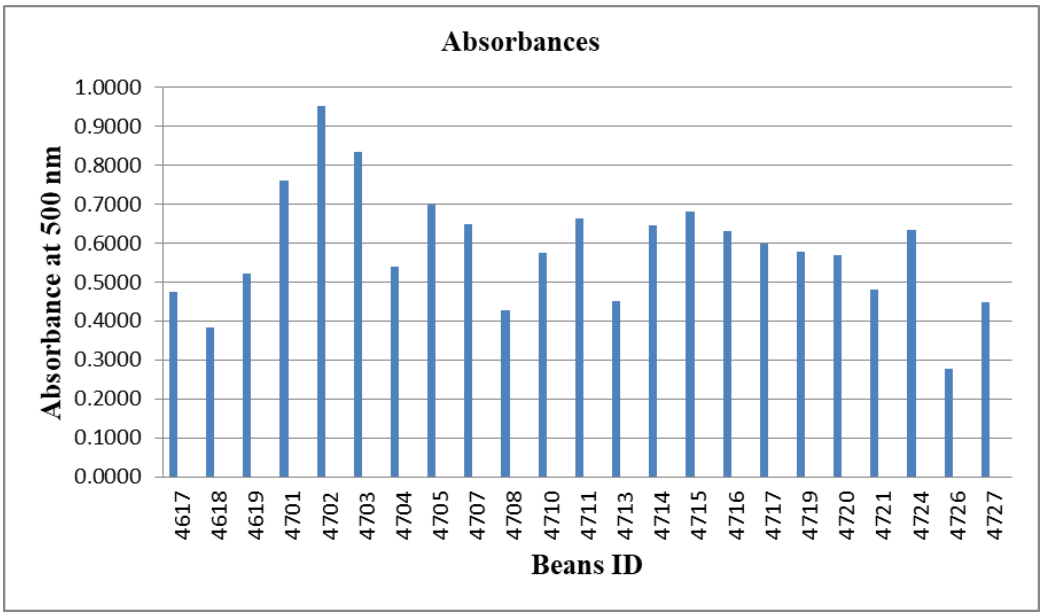


Figure 3 Absorbance of samples at 500nm

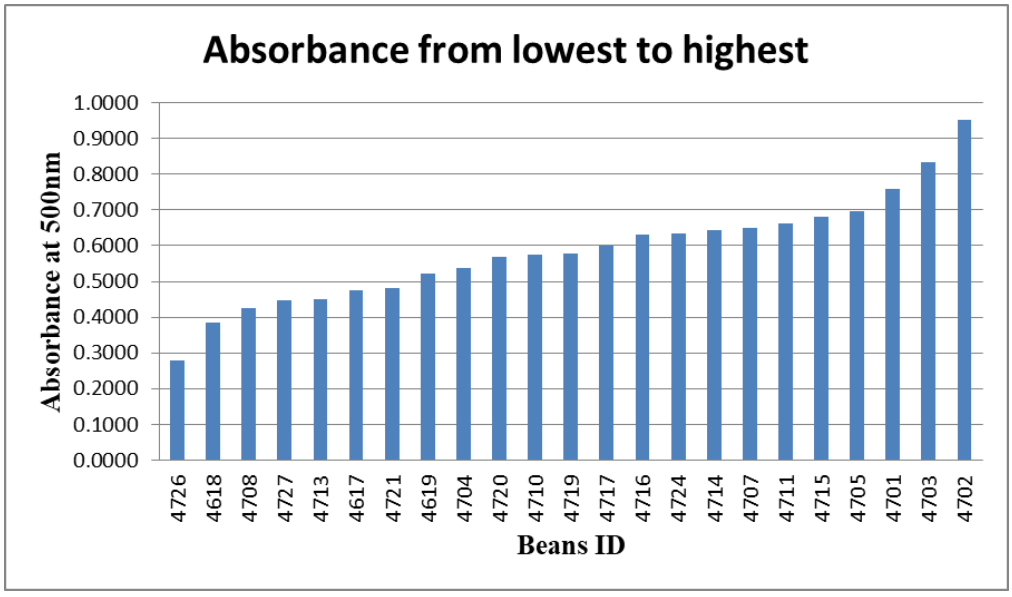


Figure 4 The absorbance from lowest to highest at 500nm

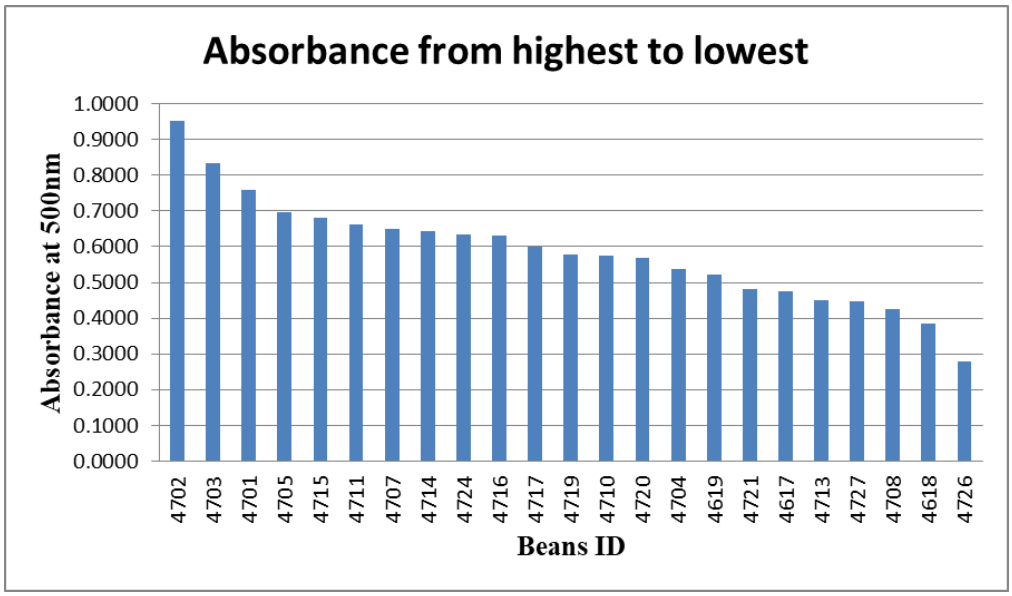


Figure 5 The absorbance from highest to lowest at 500nm

The purpose of this tannin assay was to analyze the level of flavon-3-ol in each bean in every cocoa pod. The type of cocoa pod may be selected from different clones that had been successfully pollinated. As the analysis was done, the result will be assorted and a number beans will be selected with the highest flavon-3-ol concentration which is considered as the health promoting cocoa beans. A number of beans also will be selected from the lowest flavon-3-ol concentration which is considered as high flavor cocoa beans. This process will continue from year to year to find the higher and the lowest level of flavon-3-ol concentration in cocoa beans. This analysis allows us to control the production of cocoa beans with high flavon-3-ol content to promote good health as we consume the cocoa product. This also allows us to control the flavor of cocoa products in the market as we produce cocoa beans with low flavon-3-ol content.

Figure 6 shows the plotted catechin standard calibration curve was assorted from lowest to highest. We had determined the concentration of flavan-3-ols in each sample. From the curve, it shows the result of flavan-3-ol content which is that the bean ID 4702 has the highest content of flavan-3-ol while the bean ID 4726 has the lowest. The highest content flavon-3-ol is considered as the health promoting cocoa beans while the lowest is considered as the high flavor cocoa beans.

Table 2 Concentration and absorbance of catechin standard in Vanillin/H2SO4 at 500 nm

µg /ml	Absorbance at 500nm
7.8125	0.0261
15.625	0.0551
31.25	0.1364
62.5	0.3392
125	0.6505
250	1.2496
500	2.2476
1000	3.4216

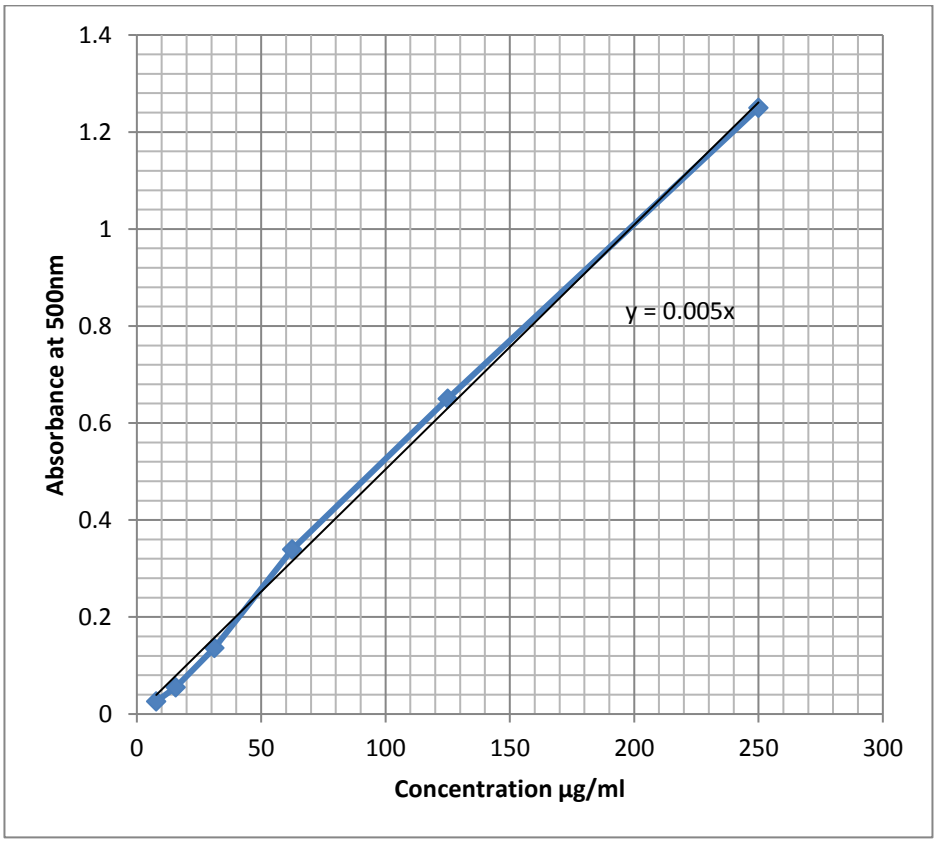


Figure 6 Catechin standard curve in Vanillin/H2SO4 at 500 nm.

In this analysis, the reagent used in the reaction of flavon-3-ol with the vanillin had been charged with H_2SO_4 are used instead of HCl. This is because H_2SO_4 is more stable than HCl. The stability of H_2SO_4 and HCl was determined from the previous analysis. In the previous analysis, the reaction of 35% HCl was compared to the 99% H_2SO_4. The result shows a better stability when H_2SO_4 was used in the analysis.

The main reason for the stability of the reaction was the concentration of the reagent used. A less concentration reagent have more water compared to more concentration reagent. At the same normality, H_2SO_4 is a better catalyst than HCl. This difference was distributed due to the different water content of the two acids. At the same normality, concentrated HCl contains more water than H_2SO_4.

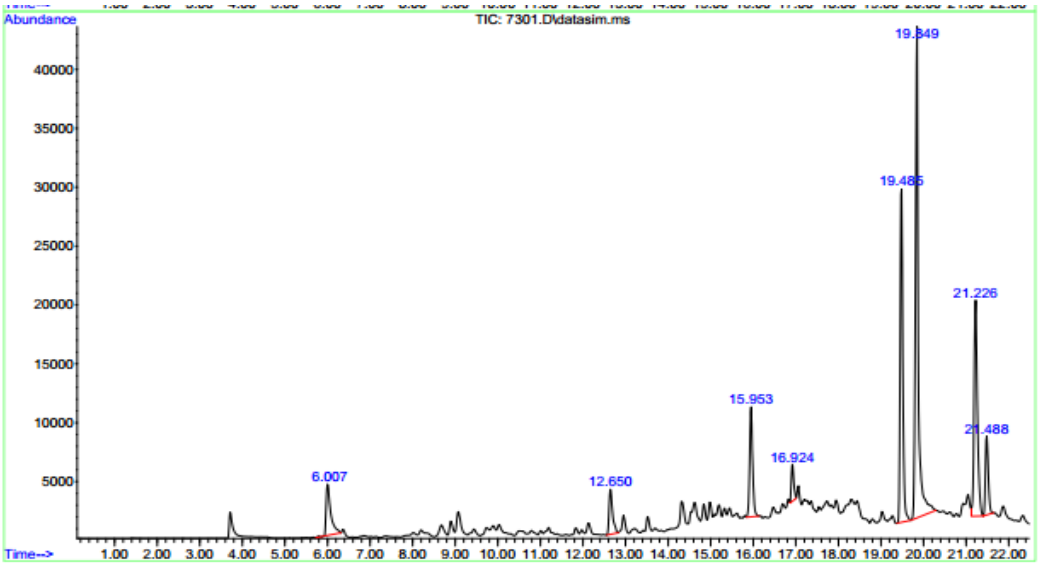


Figure 7: Detection of aromatic flavors (nutty, fruity, flowery) in cocoa beans using Gas Chromatographic Mass Spectrometry

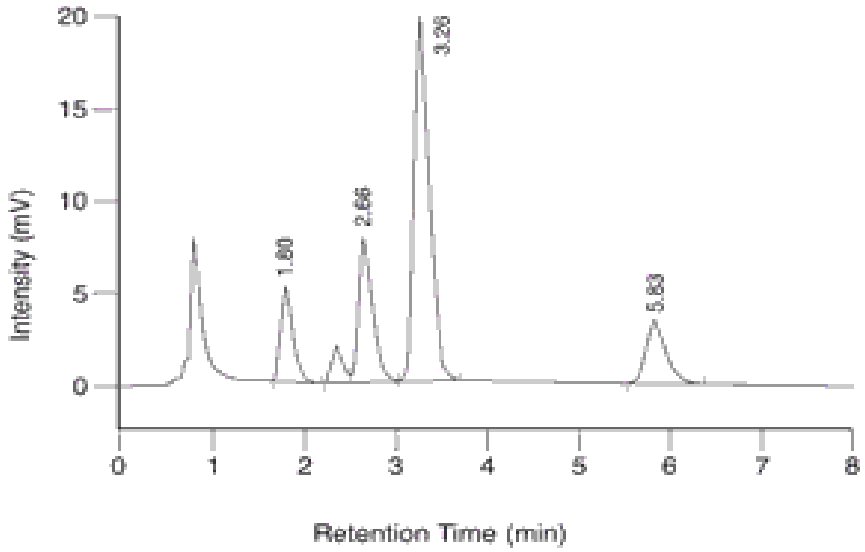


Figure 8: Detection of methylxanthine (theobromine and caffeine). using High Performance Liquid Chromatographic.

CONCLUSION

It was shown that analysis of special flavour compounds using GC-MS and tannin levels on cocoa beans by observation of the color intensity. This experiment was successfully found that with light color a high level of tannin and blackish color of the beans showed low tannin levels. This experiment will be applied in a thousand cocoa beans analysis for the improvement of the fast, cheap and accurate techniques for microanalysis of individual's beans.

As a conclusion, the analysis shows that each cocoa bean has different flavour compounds and different flavon-3-ol concentration. Within this analysis, we can produce the grade A of cocoa bean that has been chosen by level of flavan-3-ol concentration, because levels of flavon-3-ol are the main factor that influence the flavor of cocoa bean.

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