Kasetsart J. (Nat. Sci) 31 : 479 - 487 (1997)

Effect of Fermentation Time on Pyrazine Concentration of Thai Forasero Beans

Yusep Ikrawan[1], Siree Chaiseri[1], and Orawan Vungdeethum[2]

ABSTRACT

A study on the effect of fermentation time on pyrazine concentration of Thai Forastero cocoa beans was carried out. Samples were taken during 4, 5, 6, and 7 days of fermentation and then dried and roasted at 150 °C for 15 min. Fructose was the major sugar in cocoa beans followed by glucose and sucrose. Aspartic acid, glutamine, leucine, and arginine were the major amino acids in Thai cocoa beans. As fermentation progressed all sugars and amino acids decreased. Three major methyl pyrazines, *i.e.* dimethylpyrazine, trimethylpyrazine and tetramethylpyrazine were found in Thai cocoa beans after roasting. The concentrations of tetramethylpyrazine was the highest (89.95 μg/100g) in the samples that had been fermented for 5 days. Five-day fermented beans had the strongest cocoa aroma that agreed with the highest in pyrazine concentration of the sample. Sensory score was well correlated with the pyrazine concentration but was not strongly correlated with the flavor precursor concentrations. It is indicated that fermentation for 5 days is the most appropriate for Thai *Forastero* cocoa beans to produce the highest pyrazine concentration.

Key words : Forastero, cocoa, fermentation, pyrazines

INTRODUCTION

Cocoa flavor is developed after cocoa beans undergo fermentation and roasting. During fermentation, microbial activities and indigenous enzymes from cocoa beans induce chemical changes resulting in brown pigments and flavor precursors, mainly reducing sugars, peptides, and amino acids. During roasting, these compounds undergo Maillard reaction and Strecker degradation and produce intermediate compounds that contribute to cocoa flavor. Major compounds in cocoa flavor components are pyrazines. Reineccius *et al.* (1972) found that 0.2 % of free amino acids and reducing sugars reacted during the roasting process to produce 142 - 689 mg pyrazine/100 g beans. The concentrations of pyrazines, especially tetramethylpyrazine, are depended on degree of roasting, fermentation time, cocoa varieties, and country of the origins (Reineccius *et al.*, 1972). Fluctuations from samples of the same origins are relatively small (Ziegleder and Biehl, 1988).

Well fermented cocoa beans give higher pyrazine concentrations than the underfermented ones (Reineccius *et al.*, 1972). Well fermented and roasted Ghanaian beans contained 2.3-7.5 ppm tetramethylpyrazines, underfermented and insufficient roasting gave less than 1 ppm. Although

[1] Department of Food Science and Technology, Kasetsart University, Bangkok 10900, Thailand.
[2] Department of agriculture, Chemical Division, Ministry of Agiculture, Bangkok 10900, Thailand.

alkylpyrazines were formed through Maillard reaction, they were not strongly correlated with the amount of reducing sugar and free amino acid lost during cocoa bean roasting (Keeney, 1972).

The concentrations of pyrazine in roasted beans varies among cocoa varieties and growing areas (Maga and Sizer, 1973; and Reineccius *et al.*, 1972). Arriba beans contained 0.6-2.5 ppm, Trinidad 19 ppm, and Ghana 2.3-7.5 ppm.

Proper fermentation time to produce good flavored cocoa beans with high pyrazine concentration after being roasted, is unique for particular cocoa varieties and growing areas. It has been suggested that Forastero, which is the type that generally grows in Thailand, should be fermented for 5-7 days. Sensory evaluation showed little difference in preference between Thai cocoa beans that were naturally fermented for 4 and 6 days (Laempet, 1996).

There was no information on pyrazines concentrations in Thai cocoa beans. The proposes of this study are to investigate pyrazine concentrations and the proper fermentation time for Thai Forastero beans to produce high flavor compounds, *i.e.* pyrazines, during roasting.

MATERIALS AND METHODS

Thai Forastero cocoa beans were used in this study. The fermentation process of cocoa beans was conducted at Chumphon Horticultural Research Center, Sawee, Chumphon, Thailand. The experiments in this study were done in duplicate.

Sample preparation

Forastero cocoa pods were harvested when the color changes from green to yellow. The pods were collected and then broke by knives. The beans were extracted by hand and fermented in two 0.84 m (L) × 0.4 m (W) × 0.4 m (D) wooden boxes. Each box contained approximately 80 kg cocoa beans (25 cm depth) and covered with jute bags. The beans were turned twice at 24 and 48 hours. Three kilograms of cocoa beans was taken from each box at 4, 5, 6 and 7 days of fermentation. Temperatures and pHs of the beans mass were measured during fermentation using a thermometer and a pH meter (pH- vision 6071, Jenco Electronics, Taipei, Taiwan).

Cocoa bean samples were sun dried until the moisture content was reduced to 6 - 7 %. The dried beans were kept in sealed plastic bags and stored at room temperature for further analyses.

Two hundred and fifty grams of the dried beans were roasted in an oven (Memmert Model UM 400, Schwabach, Germany) at 150°C for 15 min. The samples were analyzed reducing sugar and amino acid concentrations before and after roasting. Pyrazine concentrations were determined only after roasting.

Methods of analyses

During fermentation cocoa beans were determined for acidity (as % acetic acid) and pH. The dried samples, roasted and unroasted, were separately shelled and ground in a Waring laboratory blender (Waring Co., New York, USA) prior to chemical analyses. Reducing sugar and total amino acid concentrations were determined before and after roasting. Pyrazines, that mainly form through roasting, were determined after roasting.

Determination of reducing sugars

Cocoa powder sample was mixed with 50 ml petroleum ether in a 100 ml centrifuge tube to extract lipids. The mixture was then centrifuged in a Hettich Universal Centrifuge 7200, Tuttlinger, Germany) for 15 min at 1,800 x g. The supernatant was discarded. The extraction was repeated. The residue was added with 100 g water. The defatted sample was placed in a 85 - 90°C waterbath

(Memmert, Schwanbach, Germany) for 25 min. The sample was cooled to room temperature and centrifuged for 10 min at 2,000 x g. The clear supernatant was filtered through a 0.45mm MF-Millipore membrane using a Swinney syringe filter (Millipore Corp., Bedford, MA).

The determination of reducing sugars was carried out by High Performance Liquid Chromatography (HPLC). The system equipped with Waters 600 Controller Pump, Waters 717 plus Autosampler, and Waters 410 Differential Refractometer (Waters Associates, Milford, MA, USA).

Sample solution was filled in a thirty-microliter injection loop and injected into a 5 µm, 200 mm × 4.6 mm id APS Hypersil (NH_2) column (Hewlett-Packard, Wilmington, USA). The mobile phase, acetonitrile:water 80:20 (J.T. Baker, Phillipsburg, NJ, USA), was pumped at 1.0 ml/min. Standard mixture of sucrose, fructose, and glucose (Sigma Chemical Co., St. Louis, USA) was used.

Determination of amino acids

Amino acids of cocoa beans before and after roasting were determined. Cocoa powder weighed 0.1 g was mixed with 10 ml of 6 M HCl and heated at 110°C for 24 h in a sealed glass tube. The hydrolyzed solution was cooled and neutralized with 7.5 N sodium hydroxide. HCl was removed by addition of distilled water and repeated evaporation in a rotary evaporator. The volume of the sample was adjusted to 1 ml with boric acid buffer solution and filtered through a 0.22 µm Nylon 66 membranes (Alltech, Deerfield, IL, USA). Amino acids were determined using Amino Acid Analyzer (Pharmacia LKB-Alpha plus, Uppsala, Sweden). A standard mixture of amino acids (Pierce Chemical Co., Rockford, Illinois, USA) were used.

Determination of pyrazines

Pyrazines were extracted by volatile oil distillation. One hundred grams of cocoa powder was mixed with 200 ml distilled water and distilled for 1 hr. The distillate was collected and mixed with 20 ml *n*-pentane. The mixture was added with anhydrous sodium sulfate (Sigma Chemical Co., St. Louis, USA) and set aside for 2 hr to absorb moisture. The distillate was later dried using nitrogen stream and adjusted volume to 1 ml before the analysis.

The determination of pyrazines was carried out on a Hewlett-Packard II plus 5890 Gas Chromatograph (Hewlett-Packard, Wilmington, USA). 2,5-dimethylpyrazine, 2,3,5-trimethylpyrazine, and 2,3,5,6-tetramethylpyrazine (Aldrich Chemical Co., Inc., Milwaukee, USA) were used as standards. The capillary column was a 25 m × 0.32 mm × 0.25 µm Carbowax 20M (Hewlett-Packard, Wilmington, USA). The detector was a flame ionization detector (FID). The column temperature was programmed to increase from 60°C to 180°C at 5°C/min. The carrier gas was helium at 1.5 ml/min. Both detector and injector temperatures were set at 200°C.

Sensory evaluation

Cocoa powder samples were prepared from roasted cocoa beans as mentioned earlier. The sample and icing sugar were mixed at the ratio of 1:1 and passed through 20-mesh screen. Cocoa beans from Ghana was used as a reference sample. Ten trained panelists were evaluated cocoa flavor of the samples in comparison to the reference. The scores were reported as 1=extremely weaker, 2=much weaker, 3=moderately weaker, 4=slightly weaker, 5=no difference between sample and the reference, 6=slightly stronger, 7=moderately stronger, 8=much stronger, and 9=extremely stronger.

RESULTS AND DISCUSSION

Changes in temperatures and pHs in cocoa beans mass during fermentation

Temperatures of cocoa beans mass during fermentation are presented in Figure 1. During the early stage of fermentation the temperatures had increased to as high as 45°C on the third days and gradually declined to 31°C on the seventh day. The maximum temperature indicated that the bean death could occur after 3 days of fermentation. The raise of the temperature associates with an enhanced aeration by turning of cocoa mass that promoted the growth of acetic acid bacteria (Jinap and Danker, 1992).

The changes of pH in pulp and cotyledon of cocoa beans during fermentation are presented in Figure 2. The pH of the pulp increased after the second day of fermentation. It was because citric acid in the pulp was utilized by microorganisms. Lactic and acetic acids later produced by lactic and acetic acid bacteria were less dissociated when

Table 1 Influence of fermentation time on sugar concentration (mg/100g) in Thai cocoa nibs before and after roasting at 150°C, 15 min.

Sugar		Fermentation time (days)			
		4	5	6	7
Fructose	before roasting	6.51 $^{a1/}$	5.23 ^{c}	3.01 ^{e}	2.20 ^{g}
	after roasting	5.73 ^{b}	4.41 ^{d}	2.55 ^{f}	1.87 ^{h}
Glucose	before roasting	2.87 ^{a}	1.64 ^{b}	0.55 ^{c}	nd ^{d}
	after roasting	2.12 ^{b}	1.79 ^{b}	0.44 ^{cd}	nd ^{d}
Sucrose	before roasting	1.80 ^{a}	1.86 ^{a}	0.88 ^{b}	nd ^{c}
	after roasting	1.70 ^{a}	1.66 ^{bc}	nd ^{c}	nd ^{c}

1/ Means within the same sugar rows with the same following letter are not significantly different by Duncan's Multiple Range Test at the 5% level.

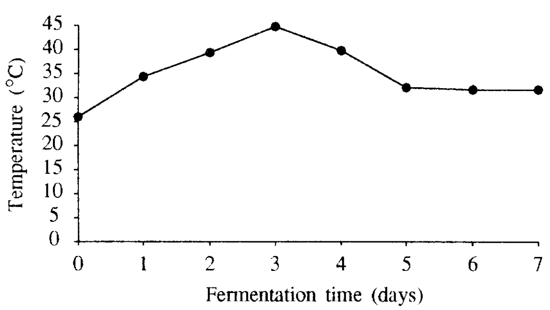

Figure 1 Temperatures of cocoa bean mass during fermentation.

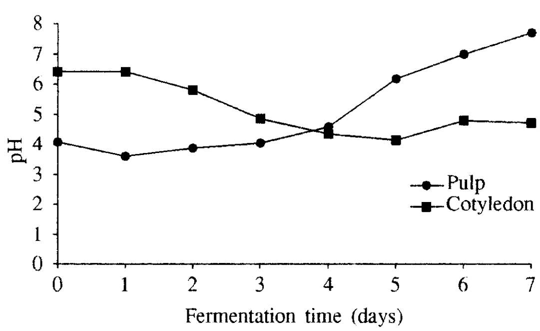

Figure 2 Changes in pHs of the pulp and the cotyledon of cocoa beans during fermentation.

compared to citric acid. Furthermore, turning of cocoa bean mass and draining of liquidified pulp provided more oxygen contact to the bean and caused the oxidation of lactic and acetic acids. As a result, the pH of the pulp continued to rise as fermentation proceeded.

The pH of the cotyledon, in contrast, decreased from 6.39 in unfermented beans to 4.13 after 5 days of fermentation. The decrease in cotyledon pH during fermentation was caused by the diffusion of acids, mainly acetic acid, from the pulp into the cotyledon. As a result, the pulp became less acidic and the cotyledon was more acidic. Acetic acid, provided an acidic environment which was the optimum for enzymatic reactions in the cotyledon. The acid is partially responsible for the death of beans that initiated the diffusion of substrates and enzymes to migrate across cell wall before enzyme activities could occur (Biehl *et al.*, 1977, and Dimick and Hoskin, 1981). After five days, the pH of the cotyledon started to increase due to the degradation of proteinaceous compounds to ammonia by bacteria.

Changes in sugar concentrations

The amount of reducing sugars in cocoa beans before roasting is important to cocoa flavor compounds that will form during roasting.

Fructose was the major sugar in cocoa beans followed by glucose and sucrose (Table 1). As

Table 2 Influence of fermentation time on total amino acid concentration (mg/100g) in Thai cocoa nibs before roasting.

Amino acid	Fermentation time (days)			
	4	5	6	7
Aspartic acid	24.0	20.2	17.6	19.7
Threonine	19.0	7.5	5.6	7.5
Serine	24.6	10.0	9.0	9.2
Glutamic acid	20.5	31.3	25.5	21.5
Proline	16.1	8.4	6.3	8.3
Glycine	20.9	7.9	6.8	8.0
Alanine	20.1	7.7	7.0	7.8
Cystine	5.3	2.2	1.5	2.2
Valine	27.2	9.0	9.4	5.4
Methionine	4.7	1.1	1.2	1.1
Isoleucine	16.0	5.5	2.5	2.0
Leucine	29.2	10.9	9.1	8.0
Tyrosine	18.3	7.4	7.3	6.3
Phenylalanine	18.1	7.9	7.4	6.8
Histidine	8.7	3.2	2.1	1.9
Lysine	24.6	9.0	6.1	7.0
Arginine	33.3	13.5	8.5	8.3
Total	330.6	162.7	132.9	131

fermentation progressed from the fourth to the seventh day, fructose decreased from 6.36 to 2.06 mg/100g. Glucose concentrations continuously reduced from 2.48 mg/100g in the fourth day of fermentation until it was not detected after 7 days. Low concentration or the absence of glucose resulting from long fermentation time could impair cocoa flavor.

Sucrose concentrations were constant at 1.80-1.86 mg/100g during 4-5 days of fermentation but then decreased and was absent after 7 days.

Roasting conditions affect the rate of Maillard reactions through which reducing sugars are consumed. In this study, roasting condition for Thai cocoa beans was controlled at 150°C for 15 min. Our preliminary study showed that roasting at lower temperatures than 150°C could not develop full cocoa flavor in our samples unless the beans were roasted longer than 60 min. Roasting at 150 °C longer than 15 min gave the beans burnt characteristic.

Concentrations of reducing sugars in roasted cocoa nibs are presented in Table 1. Comparison of reducing sugar concentrations before and after roasting showed that fructose was the major sugar lost during roasting due to its presence at high concentration. Surprisingly, glucose showed a loss through roasting only in the 4-day fermented sample. The roasting condition in this study was sufficient to initiate Maillard reaction that should reduce the amount of glucose. However, there was no difference between glucose concentrations before and after roasting in the 5-6- day fermented samples. The reason could be the hydrolysis of sucrose during roasting. From Table 1, sucrose concentration remained the same during roasting

Table 3 Influence of fermentation time on total amino acid concentration (mg/100g) in Thai cocoa nibs after roasting (150°C, 15 min).

Amino acid	Fermentation time (days)			
	4	5	6	7
Aspartic acid	19.7	17.0	14.0	13.5
Threonine	6.6	5.0	5.3	5.1
Serine	8.7	8.2	7.0	6.5
Glutamic acid	28.1	21.8	22.0	21.8
Proline	3.2	6.6	5.5	5.6
Glycine	7.5	5.4	5.7	5.6
Alanine	6.6	6.0	5.8	5.6
Cystine	1.7	1.2	1.5	-
Valine	8.5	6.7	5.4	6.8
Methionine	0.9	1.3	-	-
Isoleucine	5.3	4.1	4.5	4.4
Leucine	10.7	7.5	8.0	7.8
Tyrosine	6.6	5.0	5.3	5.0
Phenylalanine	8.0	5.2	5.7	5.2
Histidine	3.1	2.2	2.4	2.3
Lysine	8.7	6.1	7.1	6.6
Arginine	14.4	6.1	6.5	6.4
Total	148.3	112.4	111.7	108.2

Table 4 Influence of fermentation time on pyrazine concentration (mg/100g) in Thai cocoa nibs after roasting at 150°C, 15 min.

Pyrazine	Fermentation time (days)			
	4	5	6	7
Dimethylpyrazine	6.42 $^{a1/}$	3.31 ^{ab}	4.50 ^{ab}	0.93 ^{b}
Trimethylpyrazine	21.95 ^{ab}	31.56 ^{a}	19.97 ^{b}	7.70 ^{c}
Tetramethylpyrazine	51.81 ^{b}	89.95 ^{a}	38.36 ^{bc}	11.47 ^{c}

1/ Means within the same row with the same following letter are not significantly different by Duncan's Multiple Range Test at the 5% level.

of the 4-day fermented sample but decreased in the samples that showed consistant in glucose concentration. Fructose seemed to have less impact. It is probrably because fructose can react at a faster rate in Maillard reaction than glucose. The reason why this phenomena occured in low moisture environment and was observed only in the 5- and 6- day but not on the 4-day sample is not well understood and needs further investigation.

Changes in amino acid concentration

Compositions of total amino acids, including free, peptide-bound, and protein-bound amino acids, in dried cocoa beans are presented in Table 2. Major amino acids in Thai Forastero were aspartic acid, glutamine, leucine, and arginine.

The concentrations of total amino acids in Thai Forastero beans continuously declined from 4 to 7 day of fermentation. It was possibly caused by microbial metabolism that degraded proteins, peptides, and amino acids to other nitrogen-containing compounds.

The compositions of total amino acids in cocoa beans after roasting is shown in Table 3. During roasting, free amino acids and peptides underwent Maillard reaction and Amadori rearrangement. Major decrease in total amino acid concentrations during roasting was observed in the 4-day fermented sample that lost 55.1 % of the original amount. Most individual amino acids were lost more than 55 % of their original concentrations except for aspartic acid and glutamic acid. Roasting of samples from 5-, 6-, and 7-day fermentation lost less amino acids, *i.e.*, 30.9 %, 16.0 %, and 17.4 % of their original concentrations, respectively.

Researchers found different losses of individual amino acids during roasting. Seiki (1973) noticed the sharp decrease of leucine, glutamic acid, phenylalanine, and tyrosine during roasting.

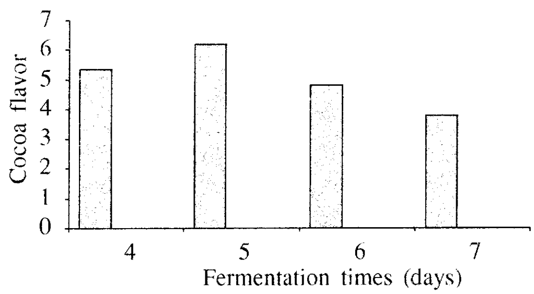

Figure 3 Effect of fermentation time on cocoa flavor score (1=extremely weaker, 2=much weaker, 3=moderately weaker, 4=slightly weaker, 5=no difference between sample and the reference, 6=slightly stronger, 7=moderately stronger, 8=much stronger, and 9=extremely stronger).

Pinto and Chichester (1966) reported a sharp decrease in glutamic acid in comparison to the other amino acids. Hoskin and Dimick (1984) stated that four amino acids, *i.e.*, leucine, threonine, glutamine, and valine had been shown a correlation with chocolate aroma produced during roasting. The differences can be caused by the differences in cocoa varieties and roasting conditions.

In our study, loss of individual amino acids during roasting seemed to be influenced by fermentation time. In Thai cocoa beans that had been fermented for 4 days, roasting caused most losses in arginine, leucine, valine, lysine, and serine (15.9-18.9 mg/100g beans). Roasting of cocoa beans that had been fermented for 5 days caused major destruction in arginine and glutamic acid.

Pyrazine concentration

Pyrazines are among the most important flavor compounds in cocoa produced through Maillard reaction during roasting. Three major methyl pyrazines, *i.e.* dimethylpyrazine, trimethylpyrazine and tetramethylpyrazine were found in Thai cocoa beans after roasting (Table 4). The principle compounds were trimethyl- and tetramethyl-pyrazines. Under the same roasting condition, dimethylpyrazine was consistant in 4- to 6- day fermented samples but lower in the sample that had been fermented for longer period.

The concentrations of tetramethylpyrazine was the highest (89.95 μg/100g) in the samples that had been fermented for 5 days. The tetramethylpyrazine concentrations were much lower in the samples that had longer fermentation time. The results indicated that the influence of fermentation time on these pyrazines were particularly sensible. Their concentrations increased from the fourth to the fifth day of fermentation and then decreased ($p < 0.05$). The decrease in pyrazine concentrations from 5 to 7 days of fermentation was correlated with the decrease in reducing sugar and amino acid concentrations in the beans before roasting.

On contrary, 4-day fermented sample that contained the highest reducing sugars and amino acids but had lower tetramethylpyrazine concentration than the 5-day fermented sample. Tetramethylpyrazine could form from metabolism of microorganisms, especially, *Bacillus subtilis* during fermentation (Ostovar and Keeney, 1973). The concentration of tetramethylpyrazine produced by microorganism usually was not higher than 20 μg/ 100 g beans. The difference in pyrazine concentrations between 4- and 5- day fermented samples was greater than 38 mg/ 100 g which could not be contributed alone by the microorganism. It was also possible that acid content of the beans might influence the rate of Maillard reaction which preferred basic environment. Cocoa beans that had been fermented for 4 days had lower pH, hence, less suitable condition for Maillard reaction during roasting. Greater loss of amino acids during roasting of the 4-day than the 5-day fermented sample indicated a possibility that proteinaceous compounds may undergo other chemical reactions besides the reaction with reducing sugars.

Sensory Evaluation

Sensory evaluation was performed by trained panelists. Mixture of icing sugar and cocoa powder was used in the test. Cocoa beans from Ghana was selected as a reference because of its neutral cocoa flavor and low acid note that could mask cocoa aroma.

The results of sensory evaluation on cocoa are shown in Figure 3. The panelists could differentiate cocoa aroma from the beans that had different fermentation times. Cocoa powder prepared from 5-day fermented beans had the strongest cocoa aroma that agreed with the highest in pyrazine concentration of the sample. The flavor score of the 4- day fermented sample was not different ($p < 0.05$) from that of Ghanaian beans. Cocoa powder made from 6- and 7- day fermented samples had weak cocoa flavor due to their low pyrazine concentrations.

It should be noted that the sensory score was well correlated with the pyrazine concentration but was not strongly correlated with the flavor precursor concentrations. This can be observed from the data of 4- and 5- day fermented samples.

From this study, it is indicated that fermentation for 5 days is the most appropriate for Thai Forastero cocoa beans to produce the highest pyrazine concentration. Longer fermentation times produce cocoa beans that have weak cocoa aroma due to low total amino acid and the absence of glucose, thus, produce low pyrazines.

ACKNOWLEDGEMENTS

The authors wish to thank Chumphon Horticultural Research Center at Sawee, Chumphon, Thailand, and their staff for their kindly support in cocoa samples and fermentation facilities.

LITERATURE CITED

Biehl, B., U. Passern, and D. Passern. 1977. Subcellular structures in fermenting cacao beans-effect of aeration and temperature during seed and fragment incubation. J. Sci. Food Agric. 28 : 41 - 45.

Dimick, P.S. and J.M. Hoskin. 1981. Chemico-physical aspects of chocolate processing : A review. Can. Inst. Food Sci. Technol. J. 4 : 269.

Hoskin, J.C. and P.S. Dimick. 1984. Role of non-enzymatic browning during theprocessing of chocolate: A review. Process Biochemistry 19 : 92 - 104.

Jinap, S. and P.S. Dimick. 1990. Acidic characteristic of fermented and dried cocoa beans from different countries of origin. J. Food Sci. 55 : 547 - 550.

Jinap, S. and R.M. Danker. 1992. Effect of clone and temperature on acetic acid diffusion and death of cocoa beans. ASEAN Food Journal 8 : 139 - 144.

Keeney, P.G. 1972. Various interactions in chocolate flavor. J. Am. Oil Chem. Soc. 49 : 567.

Laempet, B. 1996. Study on the quality of fermented cocoa beans inoculated with mixed culture. M.S. Thesis. Kasetsart University. Bangkok, Thailand.

Maga, J.A. and C.E. Sizer. 1973. Pyrazines in Food. : A review. J. Agric. Food Chem. 21 : 22-25.

Ostovar, K. and P.G. Keeney. 1973. Isolation and characterization of microorganism involved in fermentation of Trinidad's cocoa beans. J. Food Sci. 38 : 661.

Pinto, A. and C.O. Chichester. 1966. Changes in the content of free amino acids during roasting of cocoa beans. J. Food Sci. 31 : 726.

Reineccius, G.A., P.G. Keeney, and W. Weisberger. 1972. Affecting the concentration of pyrazines in cocoa beans. J. Agr. Food Chem. 20 : 202.

Ziegleder, V.G. and B. Biehl. 1988. Analysis of cocoa flavor component and flavour precursors, pp. 321 - 390. *In* H.J. Lickens and J.F. Jackson (eds) Modern Methods of Plants Analysis. Vol. 8. Analysis of Non-alcoholic Beverages. Springer - Verlag, Berlin.