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# Role of carboxypeptidases to the free amino acid composition, methylpyrazine formation and sensory characteristic of under-fermented cocoa beans

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**Abstract:** The role of carboxypeptidases B (from porcine) and Y (from baker's yeast), applied to under-fermented cocoa beans, on the formation of cocoa-specific aroma precursors, the aroma and sensory quality after roasting was investigated. The application of carboxypeptidases in under-fermented cocoa beans was to overcome the slaty and purple beans with an excessive taste of their bitterness and astringency after roasting, and the lack of cocoa-specific aroma attribute. The 5% carboxypeptidases B and Y were applied separately on the dry-powdered under-fermented cocoa beans at several incubation periods (6, 12, 24, 48 h). The levels of free amino acids, especially hydrophobic amino acids as essential cocoa aroma precursors, were significantly higher in cocoa beans treated with carboxypeptidase B as compared to the control. This led to the higher levels of 2,5-dimethyl-, 2,3,5-trimethyl- and 2,3,5,6-tetramethylpyrazines found in the samples after roasting at 150ºC for 15 min. Therefore, carboxypeptidase B was more efficient for the formation of the cocoa-specific aroma compared to carboxypeptidase Y. However, both carboxypeptidase treatments had no significant effect ($p>0.05$) on flavor attributes of cocoa liquors made from the roasted cocoa beans, eventhough there is a significant correlation between the formation of hydrophobic free amino acids with cocoa-specific flavor attribute and between the methylpyrazines with the flavor attribute ($r2 = 0.91 - 0.99$).

**Keywords: Under-fermented cocoa beans, methylpyrazine, carboxypeptidase, sensory characteristic, cocoa, aroma, precursor, biodegradation**

## Introduction

Cocoa aroma or cocoa flavor is mainly developed after cocoa beans undergo fermentation and then followed by roasting (Rohan, 1964a,b). During fermentation, the microbial activities and indigenous enzymes from cocoa beans may induce chemical changes which result in the formation of brown pigments and the release of aroma precursors such as reducing sugars, amino acids and peptides. During the roasting of cocoa beans, Maillard reaction and Strecker degradation are occurred by the presence of these precursors to produce intermediate compounds such as pyrazines which directly contribute to cocoa aroma (Rohan, 1972; Ziegleder and Biehl, 1988), eventhough a small number of pyrazines has been detected by several researchers since the fermentation process of cocoa beans (Reineccius *et al.*, 1972b; Bauermeister, 1981; Barel *et al.*, 1985; Hashim and Chaveron, 1994; Jinap *et al.*, 1994). Di-, tri-, and tetramethylpyrazines are the most reported pyrazines in cocoa beans after roasting (Reineccius *et al.*, 1972b; Lopez and Quesnel, 1976; Bauermeister, 1981; Ziegleder and Biehl, 1988; Yusep *et al.*, 2002; Jinap *et al.*, 2008). Reineccius *et al.* (1972b) reported that the concentrations of pyrazines, especially tetramethylpyrazine, were determined by country origins, cocoa varieties, fermentation period, and degree of roasting.

The fermentation process affecting cocoa attributes has been unequivocally understood. It is known that cocoa flavor attributes, described as cocoa flavor intensity, bitterness, astringency and acidity, are influenced not only by the degree of bean fermentation but also the roasting process (Rohan, 1969). Rohan (1964a) divided the degree of fermentation into three categories, including unfermented, under-fermented with 1–3 days of fermentation, and fully fermented with 5 days of fermentation. Thus, under-fermented beans have a cocoa aroma intensity lower than that of fully fermented. Moreover, Shamsudin and Dimick (1986) and Jinap and Dimick (1990a, 1991) stated that under-fermented beans were extremely bitter and lack of cocoa flavor. However, the addition of

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carboxypeptidases as exogenous enzymes to the under-fermented cocoa beans may give a significant effect to the concentration of free amino acids and peptides. These aroma precursors could then directly contribute to the cocoa specific aroma formation during roasting of the cocoa beans.

To our knowledge, there are very few studies have addressed the sensory research of the cocoa beans. The investigations of Clapperton *et al.* (1993, 1994) on six varieties of cocoa beans grown in Sabah (Malaysia) revealed a genotypic effect on sensory attributes such as bitterness, astringency and cocoa flavor intensity. They also reported significant correlations of polyphenols content to astringency and cocoa flavor intensity, and alkaloids to bitterness intensity. Moreover, other studies by Lopez (1983) and Jinap and Dimick (1990a) pointed the significant effect of organic acids, mainly acetic and lactic acid, to the cocoa flavor and acidity. Therefore, the aroma and sensory characteristics of under-fermented cocoa beans treated with carboxypeptidases, after roasting, were investigated to know the role of carboxypeptidases on the cocoa flavor attributes. In this study, the carboxypeptidases were introduced to the defatted and dry-powdered under-fermented cocoa beans to have the effective effect of the enzymes.

## Materials and Methods

*Cocoa pod*

Cocoa pods were obtained from Golden Hope Plantation Berhad, Perak, Malaysia, and their clone was specified as the Prang Besar Clone (PBC) 140.

*Chemicals*

The chemicals and reagents used were of analytical grade or High Performance Liquid Chromatography (HPLC) and Gas Chromatography (GC) grades for HPLC and GC analysis.

*Preparation of under-fermented cocoa beans*

The ripe cocoa pods were collected, split with a knife, and then the beans were extracted manually. The placenta was removed prior subjected to fermentation. The beans (45 kg) were fermented in a wooden box of size 0.50 m × 0.50 m × 0.40 m, giving a depth of 25 cm and the bean mass being turned every 24 h. To produce under-fermented beans, the bean mass was fermented for 3 days (instead of 6 days for fully fermented beans). After 3 days, the beans were dried under conventional sun drying until the moisture content of the beans reduced to c.a. 7%. The dried beans were then stored at −20 ºC until further analysis.

*Preparation of dry cocoa powder*

After removing the shells, the dry cotyledons of the beans were crushed using mortal and pestle, and then divided into portions of 10 g. Each portion was defatted by Soxhlet extraction using 250 ml of petroleum ether for 8 h. The defatted cotyledon was then ground into powder using a blender (Braun ZK 100, Kronberg, Germany).

*Preparation of incubated cocoa powder with 5% of carboxypeptidases B or Y*

The fat-free dry cocoa powder (containing polyhydroxyphenol) was suspended in 100 mL of 0.2 M McIlvaine buffer (0.2 M $Na_2HPO_4$ adjusted to pH 5.8 by addition of citric acid). Carboxypeptidase B from porcine (200 units $mg^{-1}$ protein) (Sigma Chemical Co. St. Louis, USA) at the concentration of 5% were added to the suspension and incubated at 45°C in a Orbital Shaker Incubator (YIH-DER Interments Co. Ltd, Taiwan) for 6, 12, 24, and 48 h, respectively. After incubation, the suspensions were lyophilized and stored at −20°C until further analysis. Digestions with 5% of carboxypeptidase Y from baker's yeast (12 units $mg^{-1}$) (Fluka Chemie, Switzerland) were performed using the same procedure above. The preparation of under-fermented cocoa beans, treated with carboxypeptidases, was done in triplicate.

*Roasting*

Fifty grams of each incubated cocoa powder were placed in glass petri dish uncovered and roasted in an oven (Memmert Model UM 400, Schwabach, Germany) at 150°C for 15 min. After roasting, the samples were stored for further analysis. Further analyses were performed for the triplicate samples.

*Determination of free amino acids by HPLC*
*Sample preparation*

The extraction of the free amino acid was performed as described by Kirchhoff *et al.* (1989) and Biehl *et al.* (1993). The incubated dry cocoa powder sample (0.7 g) was mixed with 1.4 g of polyvinylpyrolidone (PVPP) and 15 ml of distilled water by stirring for 1 h in the cold room (0 to 5°C). The pH of the mixture was adjusted to 2.5 with acetic acid glacial. After the addition of 5 ml of 2.5 $\mu$M α-amino butyric acid (AABA) as an internal standard, the mixture was made up to 25 ml distilled water. Protein was separated by adding 4 ml acetone per ml incubation suspension. After 30 min standing at 3 °C, the precipitate was separated by centrifugation (Kubota 2100, Kubota Corp, Tokyo, Japan), and discarded. The supernatant was used for free amino

acids analysis using HPLC.

*Derivatisation of amino acids*

Derivatisation of free amino acids was carried out with phenylisothiocyanate (PITC) (Sigma Chemical Co., St. Louis, USA) following the method of Heinrikson and Meredith (1984), and Bidlingmeyer *et al.* (1984).

*Determination of free amino acids*

The free amino acid derivatives were separated and analyzed by reversed phase HPLC equipped with a UV detector following PICO TAG method of Waters. The UV detection was performed at 254 nm. The column was a 3.9 x 300 mm Waters Pico-Tag free amino acid (Waters Associates, Milford, MA, USA). Eluant A (50mM sodium acetate buffer at pH 5.7) and eluant B (Acetonitrile:Water = 1:1) were used as mobile phase. Gradient conditions were (1) 0.5min, 100% (A) : 0% (B), (2) 5 min, 75% (A) : 25% (B), (3) 6 min, 52% (A) : 48% (B), (4) 0.5 min, 0% (A) :100% (B), (5) 14 min, constant 100 % (A) :0% (B).

*Determination of methylpyrazines*

Methylpyrazines were extracted using Lickens Nickerson's Simultaneous Distillation Extraction (SDE) by the method of Jinap *et al.* (1994) and Puziah *et al.* (1998). The determination of methylpyrazines was carried out using Hewlett-Packard II 5890 Gas Chromatography (Hewlett-Packard, Wilmington, USA) and a flame ionization detector (FID) (Hewlett-Packard, Wilmington, USA). The 2,5-dimethyl-, 2,3,5-trimethyl-, and 2,3,5,6-tetramethylpyrazine (Aldrich Chemical Co., Inc., Milwaukee, USA) were used as standards. A solution of 0.1% of 4-picoline was used as an internal standard. The capillary column was Carbowax 20M (50 m, 0.32 mm, 0.30 μm) (Hewlett-Packard, Wilmington, USA). Injection mode was split/splitless with split ratio of 25:1. The column temperature was programmed from 60°C to 180°C at 5°C $min^{-1}$ increment. Helium was used as a carrier gas at a flow rate of 1.5 mL $min^{-1}$. Both detector and injector temperatures were set at 200°C.

*Samples preparation for sensory evaluation*

The incubated cocoa powder (25 g) from under-fermented cocoa beans treated either with carboxypeptidase B or carboxypeptidase Y was mixed with deodorised cocoa butter (KL Kepong Sdn. Bhd.), at a ratio of 1:0.5 (w/w). The mixture was filled into petri dishes as thin layers (2-3mm in height) and roasted for 15 min in an oven at 150°C. The samples were ground with mortal and pestle to obtain cocoa liquor. The liquor was kept in air tight jars at 10°C until used for sensory evaluation.

*Training of sensory panel*

Twelve panelists from the Department of Food Science, University of Putra Malaysia were invited for screening test. In an initial meeting, they were asked to taste and identify four different solutions: caffeine (0.05% and 0.1%); tannic acid (0.05% and 0.1%); citric acid (0.1%); and sucrose (1%) for basic recognition test, as described by Watts *et al.* (1989). They were also asked to perform a triangle test (using the 1 citric acid solution and 2 caffeine solutions). In the last session, they were requested to rank 4 different caffeine solutions according to bitterness intensity (0.025%, 0.05%, 0.1% and 0.2%). Based on the results of their sensory sensitivity, 9 panelists were selected for further training. In the training session, panelists were first introduced to bitterness (caffeine at 0.05% and 0.1%), astringency (tannic acid at 0.05% and 0.1%), acidity (citric acid at 0.1% and 0.2%) and cocoa flavor of Ghanaian cocoa liquor from Malaysia and Ghana. They were then trained to taste several cocoa liquor from the unfermented beans, under-fermented beans and well-fermented beans.

*Sensory analysis*

Cocoa liquor was heated to 40°C for 5 min and brought to room temperature prior to sensory evaluation. A maximum of four samples were evaluated in each session. Five sensory attributes were evaluated using a scale from none (0 point) to high (10 points) intensity. The sensory attributes evaluated were bitterness (basic taste, quickly perceived on the back of the palate and the throat), astringency (substances causing a contraction of mouth tissues), acidity (basic taste on the tongue, especially the sides, near the back, associated with acids), cocoa flavor (flavor of well-fermented cocoa beans) and off-flavor (undesirable cocoa flavor). Cocoa liquor from Ghana was used as the reference in all evaluations.

*Data analysis*

The sensory and analytical data (in triplicate) were analyzed by two-ways analysis of variance followed by Duncan's multiple range test using the Statistical Analysis System (1985) (SAS Institute Inc., Cary, New York) to examine whether there were significant differences between treatments ($p<0.05$). The sensory as well as analytical data were also analyzed further by multiple linear regression using the same Statistical Analysis System (1985) to evaluate whether there was a relationship existed

between the free amino acids, methylpyrazines and cocoa flavor attributes.

## Results and Discussion

*Characteristic of free amino acids in under-fermented cocoa beans treated with carboxypeptidases*

Free amino acids and oligopeptides are known as key precursors for cocoa-specific aroma and flavor formed during roasting (Rohan, 1972; Ziegleder and Biehl, 1988). They are produced by proteolysis of the vicilin-like globulin (Jinap *et al.*, 2008; Kratzer *et al.*, 2009). This particular storage protein consists of three subunits with molecular weights of 47, 31 and 19 kDa reported by Jinap *et al.* (2008), or 47, 31 and 15 kDa reported by Kratzer *et al*. (2009). Under-fermented cocoa beans treated with 5% of carboxypeptidase B had significantly ($P<0.05$) higher concentrations of free amino acids than those treated with carboxypeptidase Y (Tables 1 and 2). The results with carboxypeptidase B were also significantly higher than those without carboxypeptidase as reported by Yusep *et al.* (2002). Total free amino acids in samples treated with carboxypeptidase B were observed at a range of 46.55–61.19 g/kg (Table 1), whereas those treated with carboxypeptidase Y contained 30.76–42.59 g/kg (Table 2). Without carboxypeptidase, the total free amino acids were ranged at 34.38–50.42 g/kg (Yusep *et al.*, 2002). Among the three amino acid groups shown in Tables 1 and 2, hydrophobic amino acids were the dominant amino acids present in the samples. These results had the similar profile of free amino acids as those treated with 10% of the respective carboxypeptidase B or Y as reported by Yusep *et al.* (2002). However the amounts of free amino acids in samples treated with 5% of the enzymes were lower. This could give a lower effect to the formation of cocoa-specific aroma.

After roasting, the concentrations of all amino acids were reduced. The acidic, hydrophobic and other free amino acid contents has significantly decreased ($p<0.05$) in the samples after roasting at 150ºC for 15 min. The concentration of total free amino acids after roasting was decreased by more than 88% to 93% in the samples treated by 5% of carboxypeptidase B at incubation periods of 6 to 48 h. The reduction of free amino acid concentrations after roasting was also observed in the untreated samples (Yusep *et al.*, 2002). The free amino acids decreased by 63 to 73% at the range of incubation period.

The data in Table 1 shows that the total free amino acid concentrations decreased from 46.55 to 3.30 g/kg at 6 h, 54.56 to 4.78 g/kg at 12 h, 59.95 to 4.70 g/kg at 24 h and 61.19 to 6.66 g/kg at 48 h incubation period. The concentrations of total acidic, hydrophobic and other free amino acids in the samples treated with 5% of carboxypeptidase B, after roasting, decreased by 84 to 97%, 85 to 96% and 90 to 94%, respectively, at different incubation periods (6 to 48 h). If compared to the results reported by Yusep *et al.* (2002), their amounts after roasting, between samples treated with 5% and 10% of carboxypeptidase B, were relatively the same.

Table 2 shows that after roasting the total free amino acids decreased by more than 58 to 85% in the samples treated with 5% of carboxypeptidase Y at different incubation periods, 6 to 48 h. Their concentrations decreased from 30.76 to 11.49 g/kg at 6 h, 37.71 to 15.90 g/kg at 12 h, 52.03 to 15.74 g/kg at 24 h and 42.59 to 11.80 g/kg at 48 h. The concentration of acidic, hydrophobic, and other free amino acids in the samples treated with 5% of carboxypeptidase Y, after roasting, respectively decreased by 70 to 96%, 50 to 67% and 39 to 80% at 6 to 48 h of incubation periods.

Free amino acids were mostly reduced during roasting because of their reaction with carbohydrates to form cocoa-specific aroma, particularly alkylpyrazines (Brunetto *et al.*, 2009). In this case, they act as cocoa aroma precursors in Maillard reactions and Strecker degradation occurred during roasting (Pinto and Chichester, 1966; Mohr *et al.*, 1976; Lopez and Quesnel, 1976; Ziegleder and Sandmeier, 1982). The involvement of free amino acids in Maillard reaction to form the cocoa-specific aroma compounds, had also been reported by many researchers (Rohan, 1972; Reineccius *et al.*, 1972a; Maga and Sizer, 1973; Dimick and Hoskin, 1981; Maga, 1982; Hoskin and Dimick, 1984; Barel *et al.*, 1985; Humbert and Sandra, 1987; Puziah *et al.*, 1998; Yusep *et al.*, 2002; Jinap *et al.*, 2008; Kratzel *et al.*, 2009).

*Effect of carboxypeptidases on methylpyrazines*

The essential cocoa-specific aroma, methylpyrazines, are derived from the precursors, free amino acids or oligopeptides (Jinap *et al.*, 2008; Brunetto *et al.*, 2009; Kratzer *et al.*, 2009), mainly during roasting, eventhough a small number of methylpyrazines are known to be generated during the fermentation of cocoa beans (Jinap *et al*., 2008). The roasting process in this study was conducted at the temperature of 150 °C to have the desired coloration (Krysiak, 2006). The changes in concentrations of methylpyrazines (2,5-dimethyl-, 2,3,5-trimethyl- and 2,3,5,6-tetramethylpyrazines) during roasting are shown in Tables 3 and 4. Samples treated with 5% of carboxypeptidase B or Y showed significant increase

**Table 1.** Changes in free amino acids concentrations (g/kg) of under-fermented cocoa beans treated with 5% of carboxypeptidase B before and after roasting during incubation period

| Free amino acids | | Before roasting | | | | After roasting | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Incubation period (h) | | | | | | | |
| | | 6 | 12 | 24 | 48 | 6 | 12 | 24 | 48 |
| | | g/kg* | | | | | | | |
| Acidic | | | | | | | | | |
| | Asp | 0.53e | 3.48cd | 2.58d | 3.41d | 0.70(+32)† | 0.83(-76) | 0.54(-79) | 0.24(-93) |
| | Glu | 2.72cd | 2.98c | 3.03c | 5.72a | 0.12(-95) | ND(-100) | ND(-100) | 0.05(-99) |
| | Ser | 1.91c | 1.67c | 1.62c | 0.42d | ND(-100) | ND(-100) | ND(-100) | ND(-100) |
| | His | ND | ND | ND | ND | ND | ND | ND | ND |
| | | 5.16 | 8.13 | 7.23 | 9.55 | 0.82(-84) | 0.83(-90) | 0.54(-93) | 0.29(-97) |
| Hydrophobic | | | | | | | | | |
| | Ala | 7.09a | 5.04b | 2.68cd | 3.09c | 0.17(-98) | 0.08(-99) | 0.03(-0.99) | 0.40(-87) |
| | Tyr | 5.32d | 5.21d | 10.14b | 16.13a | 0.05(-99) | 0.23(-96) | 0.25(-98) | 0.64(-96) |
| | Val | 2.28c | 4.71a | 1.01de | 1.75cd | 0.64(-72) | 0.93(-80) | 1.38(+37) | 1.16(-34) |
| | Iso | 1.65def | 1.62def | 3.41b | 0.32h | 0.10(-94) | 0.15(-91) | 0.13(-96) | 0.24(-25) |
| | Leu | 7.04a | 7.25a | 6.45ab | 2.78f | 0.12(-98) | 0.36(-95) | 0.25(-96) | 0.56(-80) |
| | Phe | 1.71fg | 2.03ef | 4.85bc | 2.58e | 0.05(-97) | 0.55(-73) | 0.76(-84) | 0.92(-64) |
| | | 25.09 | 25.77 | 28.54 | 26.65 | 1.13(-96) | 2.30(-91) | 2.80(-90) | 3.92(-85) |
| Others | | | | | | | | | |
| | Gly | ND | ND | ND | ND | ND | ND | ND | ND |
| | Arg | 1.33e | 2.36d | 2.90c | 4.47a | ND(-100) | ND(-100) | ND(-100) | ND(-100) |
| | Thre | 0.74d | 1.32abc | 1.22abc | 1.60bcd | ND(-100) | 0.92(-30) | 0.82(-33) | 1.84(+15) |
| | Pro | 4.03d | 4.54d | 4.54d | 7.59b | 0.73(-82) | ND(-100) | ND(-100) | ND(-100) |
| | Met | 4.92a | 3.24b | 2.86b | 0.97f | ND(-100) | 0.17(-0.95) | 0.11(-96) | 0.06(-94) |
| | Cys | 2.43ef | 3.92c | 7.49a | 7.36ab | 0.41(-83) | 0.35(-91) | 0.32(-96) | 0.24(-97) |
| | Lys | 2.85de | 5.28a | 5.17a | 3.00cd | 0.21(-93) | 0.21(-96) | 0.11(-98) | 0.31(-90) |
| | | 16.30 | 20.66 | 24.18 | 24.99 | 1.35(-92) | 1.65(-92) | 1.36(-94) | 2.45(-90) |
| Total | | 46.55 | 54.56 | 59.95 | 61.19 | 3.30(-93) | 4.78(-91) | 4.70(-92) | 6.66(-89) |

*Triplicate data, ND (not detected) †(-) and (+): % decreasing and increasing from that of initial unroasted samples

**Table 2.** Concentration of free amino acids (g/kg) of under-fermented cocoa beans treated with 5% of carboxypeptidase Y before and after roasting during incubation period

| Free amino acids | | Before roasting | | | | After roasting | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Incubation period (h) | | | | | | | |
| | | 6 | 12 | 24 | 48 | 6 | 12 | 24 | 48 |
| | | g/kg* | | | | | | | |
| Acidic | | | | | | | | | |
| | Asp | ND | ND | 7.33a | 5.12b | 0.72(+72)† | 0.35(+35) | 0.16(-98) | 0.57(-89) |
| | Glu | 2.67cd | 1.67e | ND | ND | 0.70(-74) | 0.15(-91) | 0.14(+14) | 0.66(+66) |
| | Ser | 2.76b | 1.45c | 5.73a | ND | 0.23(-92) | 0.22(-85) | 0.24(-96) | 0.31(+31) |
| | His | ND | ND | ND | ND | ND | ND | ND | ND |
| | | 5.43 | 3.12 | 13.06 | 5.12 | 1.65(-70) | 0.72(-77) | 0.54(-96) | 1.54(-70) |
| Hydrophobic | | | | | | | | | |
| | Ala | 4.69b | 4.44b | 2.01d | 2.42cd | 0.47(-90) | 0.83(-81) | 0.75(-63) | 0.68(-72) |
| | Tyr | 5.13d | 5.03d | 7.73c | 7.64c | 0.98(-81) | 0.94(-81) | 0.95(-88) | 0.99(-87) |
| | Val | 1.50de | 3.02b | 0.99e | 1.57de | 2.49(+66) | 6.30(+109) | 5.19(+424) | 2.24(+43) |
| | Iso | 1.26efg | 1.18fg | 1.97de | 0.77gh | 0.71(-44) | 0.49(-58) | 0.48(-76) | 0.62(-20) |
| | Leu | 5.81bc | 6.92a | 4.23de | 3.46ef | 1.64(-72) | 1.03(-85) | 1.33(-69) | 1.38(-60) |
| | Phe | 1.56fg | 1.25g | 4.56c | 1.47fg | 0.28(-82) | 1.11(-11) | 1.19(-74) | 0.38(-74) |
| | | 19.95 | 21.84 | 21.49 | 17.33 | 6.57(-67) | 10.70(-50) | 9.89(-54) | 6.29(-64) |
| Others | | | | | | | | | |
| | Gly | ND | ND | ND | ND | ND | ND | ND | ND |
| | Arg | ND | 1.09e | 1.41e | 3.30b | ND | ND(-100) | ND | ND(-100) |
| | Thre | ND | 1.26abc | 0.91cd | 1.44ab | ND | ND(-100) | 0.46(-50) | 0.26(-82) |
| | Pro | 3.48d | 3.46d | 3.83d | 6.41c | 1.60(-54) | 3.12(-10) | 3.87(+1) | 2.50(-61) |
| | Met | 1.60cde | 1.96c | 1.07f | ND | 0.53(-67) | 0.22(-89) | 0.14(-87) | 0.22(+22) |
| | Cys | ND | 2.87de | 7.16ab | 6.55b | 0.99(+99) | 0.16(-94) | 0.15(-98) | 0.86(-87) |
| | Lys | 0.30g | 2.11f | 3.10cd | 2.44ef | 0.15(-50) | 0.98(-54) | 0.69(-78) | 0.13(-95) |
| | | 5.38 | 12.75 | 17.48 | 20.14 | 3.27(-39) | 4.48(-66) | 5.31(-70) | 3.97(-80) |
| Total | | 30.76 | 37.71 | 52.03 | 42.59 | 11.49(-63) | 15.90(-58) | 15.74(-70) | 11.80(-72) |

*Triplicate data, ND (not detected)
†(-) and (+): % decreasing and increasing from initial unroasted samples

in all three methylpyrazines ($p<0.05$) after roasting at 150 °C for 15 min. This is in accordance with the results found by Reineccius *et al.* (1972b) that the pyrazines were generated rapidly and linearly during the first 30 min of roasting at 150°C. The significant increase of methylpyrazine concentrations may correlate to the significant decrease of free amino acid concentrations, especially hydrophobic amino acid concentrations.

The results from the present study showed that samples treated with 5% of carboxypeptidase B had a significant increase in concentrations of 2,5-dimethyl, 2,3,5-trimethyl- and 2,3,5,6-tetramethylpyrazines after roasting (Table 3). The 2,5-dimethyl-pyrazines concentration after roasting increased by 233%, 1635%, 4540% and 3323% at 6, 12, 24 and 48h, respectively, whereas, the 2,3,5-trimethyl- and 2,3,5,6-tetramethylpyrazine concentrations increased by 673%, 3143%, 7156%, 13755% and 86%, 1129%, 2505%, 1301% at 6, 12, 24 and 48 h, respectively, compared to those before roasting. These results had the similar pattern of methylpyrazines as those treated with 10% of carboxypeptidase B (Yusep *et al.*, 2002). However the amounts of methylpyrazines of samples treated with 5% of carboxypeptidase B were much lower. In untreated samples reported by Yusep *et al.* (2002), the highest concentration of 2,5-dimethyl, 2,3,5-trimethyl- and 2,3,5,6-tetramethylpyrazine after roasting was also reached at 48 h of incubation (406.83, 406.62 and 656.58 µg/100g, respectively).

The concentration of methylpyrazines after roasting in samples treated with 5% of carboxypeptidase Y also significantly increased (Table 4). Concentration of 2,5-dimethylpyrazines after roasting increased by 377%, 1701%, 2115% and 2946% at 6, 12, 24 and 48 h of incubation, respectively, whereas, the concentrations of 2,3,5-trimethyl- and 2,3,5,6- tetramethylpyrazines showed an increase by 1980%, 4425%, 5367%, 6181%; and 113%, 602%, 237%, 246% at 6, 12, 24 and 48 h of incubation, respectively, compared to those before roasting. These results were almost similar to those treated with 10% of carboxypeptidase Y (Yusep *et al.*, 2002). However the concentrations of methylpyrazines treated with carboxypeptidase Y, either at 5% or 10%, were significantly lower than those without enzyme treatment, eventhough the free amino acid concentrations as their precursors were relatively higher than those without enzyme treatment.

According to Reineccius *et al.* (1972b) the concentration of pyrazines formed during roasting depend upon the geographical origin of the cocoa beans and type of fermentation process. In addition, the aroma potential of raw cocoa beans has been found to be strongly dependent on the degree and the period course of cocoa beans fermentation, which considerably affects the proteolysis of seed proteins (Biehl *et al.*, 1982, 1985; Yusep *et al.*, 2002; Jinap *et al.*, 2008). Pyrazines are formed due to the presence of free amino acids, peptides and reducing sugars which are generated during fermentation. Koehler *et al.* (1969) stated that in a complex natural product such as cocoa beans, some chemical and physical variables influence the accumulation rate of the pyrazines during roasting. Our findings indicated that the treatment with 5% of carboxypeptidase B did have a significant influence ($p<0.05$) on cocoa-specific aroma, especially methylpyrazines formed during roasting.

*Sensory characteristic of under-fermented cocoa beans treated with carboxypeptidases*

The sensory score of under-fermented cocoa bean samples untreated and treated with 5% of carboxypeptidase B or carboxypeptidase Y at different periods of incubation (6, 12, 24 and 48 h), for bitterness, astringency, acidity, cocoa flavour and off-flavour are summarized in Table 5. Samples treated with 5% of carboxypeptidase B for 48 h of incubation significantly had the highest score for bitterness ($p<0.05$). Whereas, for 6 and 12 h of incubation, the samples treated with 5% of either carboxypeptidase B or carboxypeptidase Y were significantly scored less bitter ($p<0.05$). Bitterness in cocoa is mainly due to the caffeine and theobromine, which naturally occur in cocoa beans. Bitterness can also be caused by diketopiperazines-theobromine complex which is formed during roasting, and the presence of purines in the beans (Pickenhagen *et al.*, 1975).

Treatment with 5% of carboxypeptidases did not significantly influence astringency scores ($p>0.05$). This means that there was no significant difference ($p>0.05$) in astringency between samples untreated and treated with 5% of carboxypeptidases at all incubation periods. Thus, it indicates that both incubation and carboxypeptidases treatment did not reduce the astringent taste in liquor obtained from under-fermented cocoa beans. However, all samples were significantly more astringent ($p<0.05$) when compared with reference (Ghanaian cocoa). The presence of astringent taste in under-fermented cocoa beans might be caused by polyphenol and flavonoids content in the cocoa beans (Biehl *et al.*, 1985; Misnawi *et al.*, 2004). Among the polyphenolic and flavonoid compounds of cocoa, tannins (hydrolysable and condensed), flavan-3-ol group and anthocyanins are responsible for the astringent taste, and affect

**Table 3**. Concentration of methylpyrazines of under-fermented cocoa beans untreated and treated with 5% of carboxypeptidase B before and after roasting as influenced by incubation period

| Methylpyrazines* | Before roasting | | | | After roasting | | | |
|---|---|---|---|---|---|---|---|---|
| | Incubation period (h) | | | | | | | |
| | 6 | 12 | 24 | 48 | 6 | 12 | 24 | 48 |
| | µg/100g | | | | | | | |
| 2,5 - DMP | 7.30a | 7.28a | 7.87a | 7.24a | 24.31(+233)† | 126.32(+1635) | 365.19(+4540) | 247.84(+3323) |
| 2,3,5 - TrMP | 6.43e | 6.51e | 7.23e | 4.76f | 49.68(+673) | 211.10(+3143) | 524.61(+7156) | 659.49(+13755) |
| 2,3,5,6 - TMP | 44.07bc | 36.27de | 23.87f | 39.92cd | 81.78(+86) | 445.90(+1129) | 621.78(+2505) | 559.27(+1301) |
| Total | 57.80 | 50.06 | 38.97 | 51.92 | 155.77(+169) | 783.32(+1465) | 1511.58(+3779) | 1470.03(+2731) |

*2,5- DMP ( 2,5 - dimethylpyrazine)
2,3,5- TrMP ( 2,3,5- trimethylpyrazine)
2,3,5,6- TMP (2,3,5,6-tetramethylpyrazine)
†(-) and (+) : % decrease and increase from initial under-fermented-incubated cocoa powder before roasting

**Table 4**. Concentration of methylpyrazines of under-fermented cocoa beans untreated and treated with 5% of carboxypeptidase Y before and after roasting as influenced by incubation period

| Methylpyrazines* | Before roasting | | | | After roasting | | | |
|---|---|---|---|---|---|---|---|---|
| | Incubation period (h) | | | | | | | |
| | 6 | 12 | 24 | 48 | 6 | 12 | 24 | 48 |
| | µg/100g | | | | | | | |
| 2,5 - DMP | 6.95a | 7.28a | 6.71a | 6.91a | 33.17(+377)† | 131.10(+1701) | 148.60(+2115) | 210.50(+2946) |
| 2,3,5 - TrMP | 3.55fg | 3.19fg | 3.03g | 3.26fg | 73.78(+1980) | 144.34(+4425) | 165.64(+5367) | 204.76(+6181) |
| 2,3,5,6 - TMP | 35.40de | 34.03e | 34.37e | 33.57e | 75.57(+113) | 238.87(+602) | 115.88(+237) | 116.19(+246) |
| Total | 45.90 | 44.50 | 44.11 | 43.74 | 182.52(+298) | 514.31(+1056) | 430.12(+875) | 531.45(+1115) |

*2,5- DMP (2,5-dimethylpyrazine)
2,3,5- TrMP (2,3,5-trimethylpyrazine)
2,3,5,6- TMP (2,3,5,6-tetramethylpyrazine)
†(-) and (+) : % decrease and increase from initial under-fermented-incubated cocoa powder before roasting

**Table 5.** Sensory scores for bitterness, astringency, acidity, cocoa flavour and off-flavor of under-fermented cocoa beans treated with 5% of carboxypeptidases

| | Incubation (h) | Bitterness | Astringency | Acidity | Cocoa flavor | Off-flavor |
|---|---|---|---|---|---|---|
| WCP* | 6 | 5.1$^{abc}$† | 5.4$^{abc}$ | 6.4$^{a}$ | 4.0$^{abc}$ | 4.6$^{c}$ |
| | 12 | 5.2$^{abc}$ | 5.5$^{ab}$ | 5.7$^{abc}$ | 4.6$^{a}$ | 5.7$^{ab}$ |
| | 24 | 4.4$^{c}$ | 5.8$^{ab}$ | 5.5$^{abc}$ | 3.1$^{abc}$ | 6.0$^{ab}$ |
| | 48 | 4.8$^{bc}$ | 6.1$^{ab}$ | 4.5$^{bcd}$ | 3.3$^{abc}$ | 5.4$^{abc}$ |
| CPB | 6 | 4.4$^{c}$ | 4.7$^{abc}$ | 4.3$^{cd}$ | 4.2$^{ab}$ | 5.5$^{abc}$ |
| | 12 | 4.4$^{c}$ | 4.9$^{abc}$ | 4.3$^{cd}$ | 3.6$^{abc}$ | 5.2$^{abc}$ |
| | 24 | 6.5$^{ab}$ | 6.3$^{a}$ | 4.7$^{abcd}$ | 3.2$^{abc}$ | 5.6$^{abc}$ |
| | 48 | 6.8$^{a}$ | 6.6$^{a}$ | 6.3$^{ab}$ | 2.6$^{bc}$ | 6.1$^{a}$ |
| CPY | 6 | 4.0$^{c}$ | 3.6$^{c}$ | 3.0$^{d}$ | 2.3$^{c}$ | 5.4$^{abc}$ |
| | 12 | 4.3$^{c}$ | 4.3$^{bc}$ | 4.0$^{cd}$ | 2.8$^{bc}$ | 5.1$^{bc}$ |
| | 24 | 4.8$^{bc}$ | 4.8$^{abc}$ | 5.1$^{abc}$ | 3.2$^{abc}$ | 5.7$^{ab}$ |
| | 48 | 4.7$^{bc}$ | 5.7$^{ab}$ | 6.2$^{ab}$ | 3.2$^{abc}$ | 5.1$^{abc}$ |
| Ref. | | 2.5 | 3.0 | 3.4 | 7.0 | 2.6 |

*Mean values followed by the same letters within the column are not significantly different ($p>0.05$)
†WCP (the samples without carboxypeptidase)
CPB (the samples treated with 5% of carboxypeptidase B)
CPY (the samples treated with 5% of carboxypeptidase Y)
Ref. (reference) (Ghanaian cocoa)

stability and digestibility of cocoa (Biehl, 1985). Reduction in astringency could increase the cocoa flavor (Misnawi *et al.*, 2004).

Acidity could be detected in this study as one of the cocoa flavor attributes. This sensory characteristic may come from acetic and lactic acids contained in cocoa beans (Jinap *et al.*, 1994; Jinap and Dimick, 1990a, 1990b; Gálvez *et al.*, 2007). Some researchers suggested that acetic acid was an important acid because it was present in high concentration and tasted more acid than other acids (Rohan and Stewart, 1966; Biehl, 1967; Lopez, 1983; Jinap and Dimick, 1990a; Jinap and Dimick, 1991), eventhough other researchers believed that lactic acid was more responsible for the acid flavor because of the lack of volatility (Jinap *et al.*, 1995). In general, the samples treated with 5% of carboxypeptidases did not show a significant difference ($p>0.05$) in acidity scores compared to untreated samples. However, at 48 h of incubation, samples treated with 5% of carboxypeptidases B and Y had significant high acidity scores (6.3 and 6.2, respectively). This result indicates that the acid concentrations in the treated samples were relatively high.

In term of cocoa flavor, generally there was no significant difference ($p>0.05$) between the untreated and treated samples. The results also indicate that both carboxypeptidase treatment and incubation period did not significantly influence cocoa flavor. This was probably because of a weak development of cocoa flavor after 2 days (48 h) of fermentation. The fact that all samples treated with 5% of carboxypeptidases were high in off-flavor compared to the reference, results in the reduction of the intensity of cocoa flavor in the treated samples. This result confirms the same results of Shamsudin and Dimick (1986) and Jinap and Dimick (1990a, 1991) who stated that under-fermented beans were extremely bitter and lack of cocoa flavor.

*The relationship between cocoa flavor, free amino acids and methylpyrazines*

According to the discussion above, methylpyrazines might be related to the intensity of cocoa flavor, whereas hydrophobic amino acids might be the most potential precursors of methylpyrazines formation. Therefore, the correlation between hydrophobic free amino acids, methylpyrazines content and cocoa flavor was evaluated. Table 6 shows the correlation between hydrophobic free amino acids, methylpyrazines content and cocoa flavor in treated samples. The hydrophobic free amino acids were found to be well correlated with the formation of methylpyrazines in under-fermented cocoa beans either treated with carboxypeptidase B or carboxypeptidase Y. In those samples, high correlations were obtained between hydrophobic free amino acids and 2,5-dimethyl-, 2,3,5-trimethyl- and 2,3,5,6-tetramethylpyrazines ($r^2$ = 0.92, 0.95, and 0.94, respectively). This results revealed that hydrophobic free amino acids were the amino acid group which directly contributed to the cocoa

**Table 6.** Correlation coefficients ($r^2$) between hydrophobic free amino acids, cocoa flavor and methylpyrazines in under-fermented cocoa beans treated with 5% of carboxypeptidases B and Y

| Samples* | | Correlation coefficient (r2) | | |
|---|---|---|---|---|
| | | 2,5-DMP | 2,3,5-TrMP | 2,3,5,6-TMP |
| CPB | | | | |
| | Hydrophobic | 0.53 | 0.91 | 0.73 |
| | Cocoa flavor | 0.75 | 0.94 | 0.99 |
| CPY | | | | |
| | Hydrophobic | 0.92 | 0.95 | 0.94 |
| | Cocoa flavor | 0.98 | 0.99 | 0.42 |

*CPB (with carboxypeptidase B from pancreas porcine )
CPY (with carboxypeptidase Y from baker's yeast )

flavor formation. During roasting, the amino acids reacted with reducing sugars to produce cocoa flavor (Kirchhoff *et al.*, 1989). Therefore, it could be seen that after roasting the methypyrazines increased (Tables 3 and 4) while the hydrophobic free amino acids decreased (Tables 1 and 2). Lopez and Quesnel (1976), Ziegleder and Biehl (1988), Yusep *et al.* (2002) and Jinap *et al.* (2008) stated that free amino acids were cocoa specific aroma precursors, which act as substrates for Maillard reactions to form methylpyrazines (Eichner *et al.*, 1994).

The correlation coefficients between cocoa flavor attribute and methylpyrazines in under-fermented cocoa beans are also shown in Table 6. In the sample treated with 5% of carboxypeptidase B, strong correlation was found between cocoa flavor attribute and 2,3,5- trimethylpyrazine ($r^2$ = 0.94), and that with 2,3,5,6-tetramethypyrazine ($r^2$ = 0.99). On the other hand, in the sample treated with 5% of carboxypeptidase Y, strong correlation was found between cocoa flavour attribute and 2,5-dimethylpyrazine ($r^2$ = 0.98), as well as with 2,3,5-trimethylpyrazine ($r^2$ = 0.99). These results indicate that there is a significant correlation between cocoa flavor attribute and the presence of methylpyrazines in the samples treated by both carboxypeptidases.

## Conclusions

The 5% of carboxypeptidase B or Y concentration was applied to compare the previous research using 10% of enzymes concentration at the same incubation periods (6, 12, 24, 48 h) of cocoa beans fermentation. Recent study gave a comparable result of 2,5,-dimethyl-, 2,3,5-trimethyl- and 2,3,5,6-tetramethylpyrazine levels found in the samples after roasting at 150ºC for 15 min. The cocoa specific aroma components were dominantly present in cocoa beans treated with porcine carboxypeptidase. They were generated from hydrophobic amino acid precursors during roasting. This is proved by a high correlation value between the precursors and cocoa-specific aroma attribute. This study could give a benefit to the cocoa bean producers and chocolate manufacturers to enhance their product quality.

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