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Factors Affecting the Formation of Alkylpyrazines during Roasting Treatment in Natural and Alkalinized Cocoa Powder

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The cocoa roasting process at different temperatures (at 125 and 135 °C for 3 min, plus 44 and 52 min, respectively, heating-up times) was evaluated by measuring the initial and final free amino acids distribution, flavor index, formol number, browning measurement, and alkylpyrazines content in 15 cocoa bean samples of different origins. These samples were also analyzed in manufactured cocoa powder. The effect of alkalinization of cocoa was studied. Results indicated that the final concentration and ratio of tetramethylpyrazine/trimethylpyrazine (TMP/TrMP) increased rapidly at higher roasting temperatures. The samples roasted with alkalies (pH between 7.20 and 7.92), such as sodium carbonate, or potassium plus air injected in the roaster during thermal treatment, exhibited a greater degree of brown color formation, but the amount of alkylpyrazines generated was adversely affected. The analysis of α-free amino acids at the end of the roasting process demonstrated the importance of the thermal treatment conditions and the pH values on nibs (cocoa bean cotyledons), liquor, or cocoa. Higher pH values led to a lower concentration of aroma and a higher presence of brown compounds.

KEYWORDS: Cocoa; alkylpyrazines; amino acids; flavor index; brown compounds; roasting; quality

INTRODUCTION

Amino acids and peptides are released from proteins enzymatically during the fermentation of cocoa beans (*1*). The overall α-free amino acid (FAA) content is increased by a factor of 3. In general, this increase is due to hydrophobic FAA (5−10 times). The maximum content is obtained around the fifth day of fermentation, showing that storage proteins are hydrolyzed by proteases and lead to the formation of >80 different oligopeptides and FAAs (*2*). The action of proteases can be coupled with polyphenol oxidase (PPO) during fermentation to produce additional products: [polyphenol −(PPO activity)→ *o*-quinone; *o*-quinone + amino acids → additional products]. "In vitro" experiments have shown the formation of additional brown products from quinones and amino acids as a result of PPO and protease activities (*3*). All of these modifications tend to reduce bitterness and astringency. Furthermore, the additional products are able to catalyze the oxidative deamination of free amino acids, without the intervention of PPO: [protein −(protease activity)→ amino acids; amino acids + $^1/_2$ O_2 → α-keto acid + $NH_3\uparrow$] (*4*). In the case of alkalinized roasted cocoa the oxidation is not enzymatic; it is oxidized by air injection. During its roasting a stream of air is injected into the roaster, provoking the deamination of the free amino acids. The principal chemistry of this reaction involves the competition between the Maillard reaction to generate aroma compounds and the oxidative deamination (*5*). Furthermore, the number of compounds detected depends on the amount of air and alkali incorporated in the roaster and the amounts of free amino acids that are involved. Alkalinization during roasting is one of several routes that may be taken by the manufacturer to modify the color of cocoa with different susceptibilities to develop *o*-quinones and Maillard chemistry, especially nonenzymatic brown compounds (*5, 6*). It is well-known that the pH is of great importance in secondary reactions involving *o*-quinones (*7*−*9*). These preliminary observations suggested that alkalinization played a prominent role not only in browning but also in wettability or dispersibility, flavor, and the nutritional and biological quality of cocoa. The chemistry of the alkali process provides a basis for the biochemical characterization and browning behavior of cocoa beans (*10*). Pigment cells are responsible for color, because they contain anthocyanins, alkaloids, and polyphenols (tannins, catechins, procyanidins, and anthocyanidins). Tannins such as flavone and flavan-3-ol are responsible for the different color formations found in alkalized products. In general, reactions of amino acids, peptides, polyphenolic compounds, and proteins with quinones cause deterioration of food during storage and industrial processing. The loss in digestibility and nutritional quality due to the destruction of essential amino acids is accompanied by the destruction of potential anticarcinogenic polyphenolic compounds—negatively affecting the quality of cocoa (*11*−*13*). Cocoa polyphenols exhibit antioxidant properties "in vitro" and "in vivo" (*14*−*16*). Thus, the production of

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antinutritional and additional products may further reduce the nutritional and biological value and, for instance, the quality of the final product (*17*). The amounts of Maillard reaction precursors (α-free amino acids and reducing sugars) have been determined in a few cocoa samples of different varieties and especially of different geographical origins (*18*). Nevertheless, these differences in the compounds cannot be attributed to a genotype effect alone, but result from the combined effect of the variety and the postharvest treatments (*19*). Typical flavor is dependent on the chemical precursors formed during fermentation coupled with the thermal reactions of compounds in the later stages of manufacturing cocoa. The Maillard reaction mainly involves the reaction of free amino groups of α-amino acids and reducing sugars. With aldoses, 1-amino-1-deoxyketoses (ketose-amino acids, Amadori compounds) are the first stable intermediates to be formed. Among these Maillard-type flavors, the heterocyclic compounds with desirable aromas and low odor thresholds make the most significant contribution. Heterocyclic compounds are very widely distributed in our nutrition, and principally among aroma compounds of cocoa, including furans, thiazoles, oxazoles, pyrroles, pyridines, and alkylpyrazines (*20*). The alkalized roasted cocoa has many fewermuch less volatile heterocyclic components than the natural roasted cocoa; pyrones and furaneol are destroyed (*21*). The nitrogen sources of these heterocyclic compounds come from amino acids; thus, the nature of the nitrogen source has a profound effect on both the kinds and amounts of flavors formed. Among the heterocycles, the most dominant are the alkylpyrazines, with ~80 derivatives, followed by furans and pyrroles, the concentrations of which are variable depending on the time and temperature of the thermal treatment. Ziegleder (*22*) has tried to use these compounds as indicators of the degree of cocoa roasting. The effects of different amino acids on the Maillard reaction have been widely studied. Proline and amino acids with hydrophobic side chains reacted more slowly than other amino acids; in contrast, amino acids with aliphatic hydroxy side chains reacted more rapidly (*23*).

Fewer aromatic compounds are generated from the oxidative deamination of the amino acids, due to the participation of polyphenolic compounds in the reaction that enhances the color, ultimately there is a loss of anticarcinogenic potential properties.

From the carbohydrate fraction, fructose is one of the most abundant reducing sugars in cocoa beans under alkaline conditions, a fact that is tremendously important here. Reducing sugars and the other flavor precursors developed during fermentation interact in the roasting process to produce the desired chocolate flavor. Roasting is the most important step for flavor development. In the analytical procedure to determine the flavor index (FI), part of the volatile cocoa components are collected by steam distillation. The components measured in this way are mainly alkylpyrazines. The variety and quantity of the alkylpyrazine formation depended on the reactivity and type of amino acid used, pH, and roasting procedure (*24*, *25*). Thus, the FI may be useful as a parameter of control during roasting, but the result of the roasting process can be interpreted only within strict limits of conditions, and some correlations may be useful to control the treatment in order to get a better product. Ziegleder (*22*) showed that a correlation can be established between the sensorially perceptible roasting degree of cocoa and selected pilot components of the methylpyrazine fraction.

Because of the complexity of the reactions, the purpose of our work was to examine the factors affecting the generation of alkylpyrazines during roasting treatment in natural and alkalinized cocoa powder.

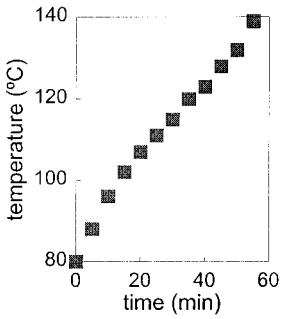

Figure 1. Thermal increase of the industrial roasting process.

MATERIALS AND METHODS

Samples. The samples of natural and alkalinized cocoa powder (10−12 or 20−22% cocoa butter) were obtained from processed [fermented, dried, and cut into nibs (cotyledons)] cocoa beans of different origins (Ghana, Ivory Coast, Nigeria, Madagascar, and Ecuador). The nibs were roasted in batches of 3 tons. The roaster consisted of a rotating cylinder (5 m long and 2 m diameter) (Barth, Germany) the envelope of which was heated by the combustion gas burner, in three basic steps: (1) cocoa nibs were mixed with water in natural cocoa or with 20 L of an aqueous solution of potassium carbonate (9.5−16.8 kg of Na_2CO_3/100 L of H_2O) in 100 kg of cocoa bean, plus incorporated air (225 m^3/h for 30 min) in the case of alkalinized cocoa, and heated to 85 °C by steam injection into the roaster; (2) progressive heating took place until the selected temperature (125 or 135 °C) was reached; and (3) the selected temperatures were maintained for 3 min in the roaster (total roasting process times of 47 and 55 min). A thermal sonde permitted measurement of the internal temperature at the moment of deduction. The thermal increase of the industrial roasting process is shown in **Figure 1**. The 15 samples used in the study came from batches of unroasted cocoa (no. 1−15) and cocoa roasted at 125 and 135 °C in natural acid (pH 5.60−5.90) and alkaline (pH 7.20−7.92) extracts. In the latter case, the degree of brown color is dependent on the balance of pH and the incorporation of air in the roaster. Samples were preserved at room temperature and analyzed as soon they arrived at the laboratory.

Reagents and Reference Standards. Solvents were of analytical (Panreac, Barcelona, Spain), GC, and HPLC (Merck, Darmstadt, Germany) grades. Ultrapure water (Milli-Q, Millipore Corp., Bedford, MA) was prepared for chromatographic use. Sugars and amino acids were obtained from Sigma Chemical Co. (St. Louis, MO) and Merck.

Physicochemical Analyses. *Free Sugars.* The sugars were isolated from 3 g of pulverized cocoa with 40 mL of water/methanol (80:20) in an ultrasonic bath, precipitating the polyphenols with 1 mL of Carrez I solution [3.6 g of potassium hexacyanoferrate(II) trihydrate [$K_4Fe(CN)_6 \cdot 3H_2O$] dissolved in water and diluted to 100 mL] and 1 mL of Carrez II solution [7.2 g of zinc sulfate heptahydrate ($ZnSO_4 \cdot 7H_2O$) dissolved in water and diluted to 100 mL]. The sugars were silylated prior to gas chromatography according to the method of Serra Bonvehí and Bosch Callís (*26*). Individual sugars were identified by comparison with reference compounds.

Free Amino Acids. Free amino acids were extracted from 1.5−2 g of pulverized cocoa with 40 mL of distilled water. The samples were shaken for 1 h and centrifuged at 10000 rpm (12062*g*) for 15 min at 4 °C. The supernatant was poured into a 50 mL volumetric flask and made up to volume with distilled water. A two-step derivatization technique was developed. In the first step the primary amino acids are derivatized using a mixture of *o*-phthalaldehyde (OPA) and 3-mercaptopropionic acid (MPA) (*1*). The reaction was rapid and complete within 60 s. In the second step the secondary amino acids were derivatized using 9-fluorenylmethylchloroformate (FMOC). The amino acids were separated by reversed phase HPLC. A diode array detector was used for detection and identification of the primary amino acid derivatives at a detection wavelength of 338 nm and at 262 nm for the detection of secondary amino acid derivatives. A Hypersil ODS 5 (240 × 4.6

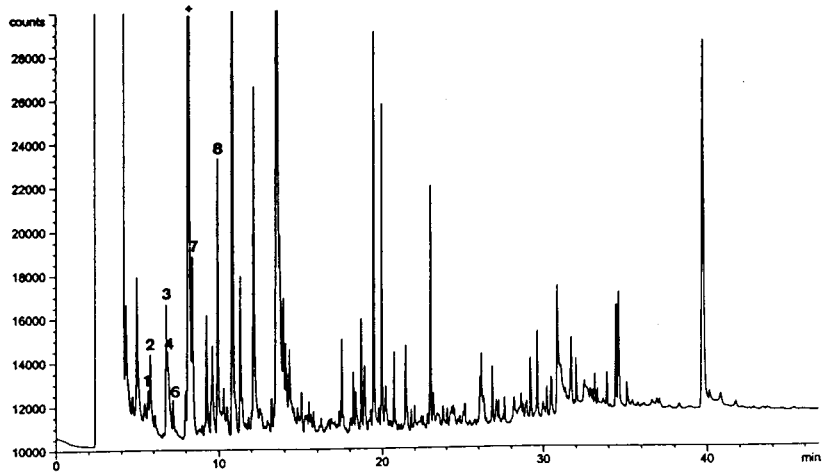

Figure 2. GC-FID alkylpyrazine profiles in natural processed cocoa powder: (1) pyrazine; (2) 2-methylpyrazine; (3) 2,5-dimethylpyrazine; (4) 2,6-dimethylpyrazine; (5) 2-ethylpyrazine; (6) 2,3-dimethylpyrazine; (+) internal standard; (7) 2,3,5-trimethylpyrazine; (8) 2,3,5,6-tetramethylpyrazine.

mm) column and a Hypersil ODS 10 (20 × 4.6 mm) precolumn were used. Samples of 20 μL were used for analysis. The reagents and sample were mixed automatically in the injection system of the chromatograph. The components were then injected immediately, eliminating problems such as the instability of the derivatives and human errors that can occur during manual sample preparation. With UV−visible absorbance detection a detection limit of 5 pmol/μL was achieved. Individual amino acids were identified by comparison with standard (1 nmol/μL).

Formol Number (FN). The FN was determined using a modification of the method described by Biehl et al. (*27*). The sample (5 g) of pulverized cocoa was homogenized in 40 mL of water in an ultrasonic bath. The extract was adjusted to pH 8.40 with 0.1 M NaOH, followed by the addition of 10 mL of a 35% formalin solution (pH 8.40); the liberated acidity was determined by titration to pH 8.40 and expressed as α-amino nitrogen. Because of its chemical structure, proline contributes only ∼7% of the FN.

Browning Measurements. These compounds were extracted twice by shaking 0.5 g of pulverized cocoa into test tubes equipped with Teflon-lined screw caps with 50 mL of water for 5 min in an ultrasonic bath and for 10 min in a laboratory shaker at 150−200 rpm. The capped tubes were then centrifuged for a period of 15 min in a centrifuge at 10 °C and 10000 rpm. The supernatant was filtered, and aliquots were filtered with a 0.45 μm nylon filter (Millipore Millex-HN) and then removed; the absorbance was determined at 420 nm using a modification of the method described by Izzo and Ho (*5*).

Steam Distillation. A suspension of 20 g of pulverized cocoa in 100 mL of distilled water was steam distilled. The condenser was maintained at 0 °C, and 150 mL of this fractional distillation was collected during 45 min. The steam volatile compounds in the condensate were well mixed (*28*).

Flavor Index (FI). The solution obtained in steam distillation was filtered and the optical density measured in a 10 mm quartz cell using a spectrophotometer. The extinction value obtained in the UV spectra (210−290 nm) at 278 nm (maximum absorbance) was multiplied by the collected final volume, determining the value of the FI (*29*).

Simultaneous Steam Distillation−Extraction (SDE). The distillate solution obtained in steam distillation was placed in flask A of the distillator Likens−Nickerson, and 10 g of NaCl was added. In flask B, 60 mL of a mixture of pentane.diethyl ether (2:1, v/v) was introduced. Boiling chips were added to flasks A and B. Flask B was heated in a oil bath at 55 °C and flask A in a balloon heater. The vapors were condensed by means of a coldfinger maintained at −5 °C by a cryostat. The SDE procedure was carried out under a 2 mL/min nitrogen flow. After 100 min of extraction, ∼55 mL of solvent, containing the aroma compounds, was collected. The extract was dried over 5 g of anhydrous Na_2SO_4 and concentrated to ∼0.50−1 mL on a Vigreux column (50 × 1 cm).

Instrumentation. *Spectrophotometric Equipment.* A Hitachi model UV-2000 double-beam spectrophotometer with 1 cm quartz absorption cells was used for all spectrophotometric measurements.

HPLC Equipment. HPLC-UV was carried out on an HPLC system consisting of a Waters 1090 with a 996 photodiode array detector (DAD) and a 712 WISP Rheodyne valve loop injector fitted onto a 20 μL loop (Waters Chromatography Division, Milford, MA).

High-Resolution Gas Chromatography (HRGC)−Flame Ionization Detection (FID) Analytical Conditions. GC was performed on a Hewlett-Packard model 5860 gas chromatograph equipped with an HP model 7673 automatic sampler, and a flame ionization detector (FID) with Agilent ChemStation Plus (all from Hewlett-Packard, Boise, ID). The volatiles were transferred onto a 50 m fused silica column (0.32 mm i.d., 0.2 μm film thickness) coated with OV-351 (Supelco, Inc., Bellefonte, PA). GC conditions were as follows: 2 μL splitless injection (1.20 s value delay); oven programmed from 60 to 220 °C at a rate of 5 °C/min; 250 °C injector temperature; 300 °C detector temperature; helium as carrier gas at a flow of 1.8−2 mL/min (**Figure 2**).

Gas Chromatography−Mass Spectrometry (GC-MS) Conditions. Identification and confirmation of aromatic compounds were achieved by GC-MS. Chromatographic conditions were the same as those used for the FID. The column was directly connected to an HP G1530A quadruple mass spectrometer. Electron impact mass spectra were recorded at 70 eV (filament current, 300 mA; electron multipler range, 2500; scan rate, 4 s^{-1}; *m*/*z* range, 40−300). Spectral recording throughout elution was automatically performed with the HP 59970C MS Chemstation analytical workstation. Identification was done on the basis of peak enrichment by co-injection with authentic standard compounds and by comparison with the NBS/EPA/NIH mass spectra library. Identification of the volatile compounds was based on the comparison of mass spectra of unknown compounds against library data for the GC-MS and comparison of experimental and theoretical Kovats indices (IK). Identification was considered to be tentative when it was based on only mass spectra data.

Calibration Factors. Solutions 0.1% (w/v) in methylene chloride of the alkylpyrazines were prepared: pyrazine; 2,5-dimethylpyrazine (DMP); 2,3,5-trimethylpyrazine (TrMP); 2,3,5,6-tetramethylpyrazine (TMP); and 4-ethylpyridine (internal standard). By adding 0.50 mL of each solution in a volumetric flask of 50 mL and diluting, 10 mg/kg solutions were obtained. HRGC was performed.

Calibration for Quantitative Analysis. Relative response coefficients for the various volatile constituents were determined by adding standard amounts (four points, in duplicates) of pure compounds to a cocoa sample prior to analysis. Retention times and areas of selected peaks, including the internal standard, were used for statistical analysis.

Statistical Analysis. The results and samples were subjected to analysis of variance and Duncan's multiple-range test, carried out using Statgraphics Statistical package, version 6.0 (*30*). Group differences were considered to be statistically significant at a level of $P \leq 0.05$. Analyses were carried out in duplicate.

Table 1. Free Amino Acid Content (Milligrams per 100 g) from Unroasted and Roasted Cocoa Bean (125 and 135 °C for 3 min)

	sample																
amino acid	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	*x*	SD
asparagine																	
unroasted	29	29	32	33	22	25	27	43	33	34	28	21	33	46	28	30.87	6.81
roasted (125 °C for 3 min)	13	14	12	17	13	15	13	13	13	16	12	18	16	15	18	14.53	2.07
roasted (135 °C for 3 min)	8	7	8	9	8	10	9	9	8	10	8	12	11	11	13	9.40	1.72
glutamine																	
unroasted	47	44	42	29	29	37	42	33	46	32	44	26	37	31	38	37.13	6.84
roasted (125 °C for 3 min)	13	12	12	15	12	11	7	12	6	14	14	12	11	14	13	11.87	2.48
roasted (135 °C for 3 min)	7	8	7	11	7	8	5	7	4	9	10	9	9	10	9	8	1.89
serine																	
unroasted	24	19	22	14	26	18	18	23	15	17	20	26	18	9	19	19.20	4.60
roasted (125 °C for 3 min)	9	9	5	10	11	9	10	10	10	7	6	11	9	13	8	9.13	2.03
roasted (135 °C for 3 min)	6	4	3	8	9	6	7	6	7	4	4	8	7	8	5	6.13	1.81
histidine																	
unroasted	8	9	8	5	9	7	8	8	6	8	7	13	6	8	8	7.87	1.81
roasted (125 °C for 3 min)	4	3	3	2	4	3	2	4	4	3	2	3	3	3	3	3.07	0.70
roasted (135 °C for 3 min)	2				1			1	1							0.33	0.66
glycine																	
unroasted	12	12	14	10	14	12	15	15	12	12	16	14	13	11	17	13.27	1.94
roasted (125 °C for 3 min)	7	6	4	4	4	5	6	5	7	4	6	7	4	8	5	5.47	1.36
roasted (135 °C for 3 min)	3	2	1	1	1	2	4	2	3	1	3	4	1	5	1	2.27	1.34
threonine																	
unroasted	15	15	21	15	22	18	23	21	15	19	18	22	24	16	18	18.80	3.19
roasted (125 °C for 3 min)	6	5	4	4	5	4	7	7	9	6	4	12	10	12	11	7.07	2.99
roasted (135 °C for 3 min)	2	5	1	2	2	1	4	3	6	3	1	9	9	8	7	4.2	2.93
alanine																	
unroasted	67	68	74	65	70	67	59	81	63	68	65	71	67	73	66	68.27	5.16
roasted (125 °C for 3 min)	23	22	24	19	22	24	25	23	27	23	15	35	31	37	34	25.60	6.14
roasted (135 °C for 3 min)	12	15	16	12	14	16	17	16	15	16	10	27	26	29	28	17.93	6.29
arginine																	
unroasted	60	61	59	42	62	52	51	69	58	57	51	57	55	46	52	55.47	6.74
roasted (125 °C for 3 min)	18	19	23	23	18	23	19	17	21	19	11	31	29	32	31	22.27	6.09
roasted (135 °C for 3 min)	8	12	16	15	16	17	14	12	15	12	7	25	22	23	26	16	5.77
tyrosine																	
unroasted	27	27	22	28	31	26	29	34	25	22	26	6	28	30	32	26.20	6.51
roasted (125 °C for 3 min)	7	9	9	8	8	9	9	8	11	8	7	14	14	16	18	10.33	3.48
roasted (135 °C for 3 min)	2	7	6	5	6	4	6	5	8	5	4	9	10	12	12	6.73	2.94
cystine																	
unroasted	6	6	6	6	6	5	6	6	5	5	6	3	5	8	6	5.67	1.05
roasted (125 °C for 3 min)																	
roasted (135 °C for 3 min)																	
valine																	
unroasted	33	33	26	28	30	28	31	38	28	27	28	30	30	36	32	30.53	3.38
roasted (125 °C for 3 min)	13	11	10	10	8	11	11	14	15	13	8	20	16	18	17	13	3.63
roasted (135 °C for 3 min)	8	6	7	8	6	8	7	9	9	8	5	14	11	13	11	8.67	2.58
methionine																	
unroasted	12	8	8	8	7	9	10	7	8	7	6	6	9	9	10	8.27	1.62
roasted (125 °C for 3 min)	4	3	2	2	3	2	3	2	3	4	3	4	3	3	2	2.87	0.74
roasted (135 °C for 3 min)																	
isoleucine																	
unroasted	14	13	11	11	12	12	14	14	9	12	13	11	12	12	9	11.93	1.58
roasted (125 °C for 3 min)	5	4	5	5	5	4	4	4	3	6	3	6	5	6	5	4.67	0.98
roasted (135 °C for 3 min)	1	2	1	2	2	1	1	1		1		1	2	1	1	1.13	0.64
phenylalanine																	
unroasted	36	29	30	26	33	39	30	33	28	31	29	32	27	32	33	31.40	3.78
roasted (125 °C for 3 min)	12	11	10	10	12	11	11	12	14	11	12	18	17	19	17	13.13	3.07
roasted (135 °C for 3 min)	7	8	7	7	9	8	7	7	9	7	8	12	12	13	12	8.87	2.23
leucine																	
unroasted	25	25	24	24	40	32	22	31	25	28	34	39	28	30	21	28.53	5.79
roasted (125 °C for 3 min)	8	7	8	8	6	8	8	7	10	10	15	15	19	18	16	10.87	4.42
roasted (135 °C for 3 min)	4	3	4	5	4	5	5	4	7	7	11	10	13	11	10	6.87	3.27
lysine																	
unroasted	39	39	38	34	46	29	34	47	32	36	37	44	32	42	40	37.93	5.30
roasted (125 °C for 3 min)	14	14	12	12	14	11	12	13	18	13	17	22	21	23	24	16	4.49
roasted (135 °C for 3 min)	9	9	7	8	9	8	8	8	11	8	12	17	16	16	18	10.93	3.86
proline																	
unroasted	411	410	429	380	392	386	414	421	417	376	479	392	443	403	389	409.5	26.94
roasted (125 °C for 3 min)	37	39	29	32	26	34	24	29	22	35	47	33	43	36	37	33.53	6.85
roasted (135 °C for 3 min)	21	29	21	25	18	25	16	19	17	26	33	26	28	28	28	24	5.04
total																	
unroasted	865	847	866	758	851	802	833	924	828	761	907	813	867	842	818	838.8	45.94
roasted (125 °C for 3 min)	193	188	172	181	171	184	170	180	193	192	182	261	251	273	259	203.3	36.97
roasted (135 °C for 3 min)	100	117	105	118	112	119	110	109	120	117	118	183	177	188	180	131.5	32.05

Table 2. Flavor Index (FI), pH Value, Formol Number (FN), and Ratio (FN/Free Amino Acid Content)[a]

sample	$FI_{(1/2)}$	pH	FN_0	FN_1	FN_2	R_0	R_1	R_2
1	47/69	5.80	0.17	0.10	0.07	0.37	0.64	0.87
2	49/73	5.68	0.15	0.09	0.08	0.34	0.60	0.91
3	52/74	5.67	0.17	0.11	0.08	0.39	0.77	0.95
4	50/74	5.67	0.13	0.08	0.07	0.34	0.54	0.86
5	51/73	5.73	0.16	0.10	0.07	0.35	0.69	0.75
6	52/69	5.79	0.13	0.08	0.07	0.31	0.53	0.75
7	54/71	5.68	0.14	0.09	0.06	0.33	0.62	0.75
8	51/68	5.74	0.19	0.13	0.06	0.38	0.72	0.89
9	52/71	5.60	0.14	0.11	0.09	0.34	0.64	0.87
10	53/68	5.90	0.12	0.08	0.06	0.36	0.51	0.66
11	54/72	5.78	0.18	0.09	0.07	0.42	0.67	0.82
12	28/45	7.92	0.14	0.11	0.08	0.33	0.48	0.51
13	33/45	7.20	0.17	0.12	0.09	0.40	0.58	0.60
14	33/48	7.75	0.15	0.12	0.09	0.34	0.51	0.56
15	32/46	7.40	0.14	0.12	0.09	0.33	0.54	0.59
x	46/64		0.15	0.10	0.075	0.36	0.60	0.76
SD	8.82/11.66		0.020	0.017	0.011	0.031	0.086	0.14

[a] Subscripts: 0, unroasted; 1, roasted (125 °C for 3 min); 2, roasted (135 °C for 3 min).

RESULTS AND DISCUSSION

The data of analyses on α-free amino acid composition in unroasted cocoa beans are presented in **Table 1**. This table also shows the data corcerning changes in individual free amino acids during roasting in processed cocoa powder (125 and 135 °C for 3 min). In all of these samples, the amino acid distributions are quite uniform; 17 amino acids were detected. For each sample, the amino acid content of the solution was determined before and after the roasting process to obtain the initial and final amounts of amino acid compounds. The degradation of amino acids could be the result of Maillard reactions plus oxidative deamination in alkalinized cocoa. The degree of browning of cocoa phenolics is related to the amount of degraded polyphenolic compounds that generate additional products and quinone derivatives. The results showed that proline, alanine, arginine, glutamic acid, lysine, phenylalanine, and valine dominate, followed by aspartic acid. A high percentage of free hydrophobic amino acids (Ala, Phe, Val, and Leu) and also a low percentage of the acidic amino acids (Glu + Gln and Asp + Asn) were detected. The total amount of free amino acids depended on the nib pH during the stage of proteolysis (*27*), but during fermentation considerable amounts of especially soluble components were exuded from the seeds into the pulp. The dominance of hydrophobic amino acids was more pronounced in the exudate produced by the fermentation, and in the total of exudate plus seeds, compared to seeds before fermentation (*1*). The total content of free amino acid, ranges widely (758−924 mg/100 g) in unroasted cocoa, values in agreement with those of Kirchhoff et al. (*1*) and Oberparleiter and Ziegleder (*31*).

A minimum FN of 0.12 was detected in the samples of unroasted cocoa beans according to CEPLAC (*32*). FN is considered to be one of the best parameters indicating the total free amino acid content. It ranged between 0.12 and 0.19, with a standard deviation of 0.020 in unroasted cocoa. Statistical analyses indicated significant variations ($P \leq 0.05$) in FN between the different roasting temperatures of cocoa (**Table 2**). Also, the ratio FN/total free amino acid content seems to be an interesting control parameter between roasted cocoas at different thermal treatments. A considerable variation in FN and FN/total free amino acid content was detected between unroasted and roasted cocoa. Therefore, these parameters may be used as quality indices.

Table 3. Absolute Recoveries of Alkylpyrazines from Spiked Samples and Analytical Relative Standard Deviation (RSD)

component	recovery (%)	RSD (%)
pyrazine	46	4.6
2,5-dimethylpyrazine	87	1.1
2,3,5-trimethylpyrazine	92	1.7
2,3,5,6-tetramethylpyrazine	93	1.8

The results showed that the evolution of total free amino acids content, FN, and FI depended on the temperature of roasting. One can observe in **Table 2** that the more the FI increased, the more the FN decreased, in accordance with Serra Bonvehí and Ventura Coll (*28*). The formation of diketopiperazines (DKPs) is also known to occur in cocoa roasting. Unroasted cocoa has no typical cocoa bitterness and does not contain DKPs (*28*). As expected, both the reducing sugars and the free amino acids, especially the former, decreased in concentration to minimal levels during roasting. Seventy-five percent of the total free amino acid content was consumed by roasting at 125 °C, whereas 84% was consumed by roasting at 135 °C.

Reducing sugars were represented by fructose (0.30 ± 0.08 g/100 g) and glucose (0.19 ± 0.04 g/100 g). Sucrose was present in a large quantity (0.92 ± 0.18 g/100 g). Also, alcohol sugars were detected (e.g., mannitol and inositol) (0.088 g/100 g). Hydrolysis of sucrose during roasting was negligible. This indicated that the sucrose concentration in unroasted cocoa beans is not an important variable in the generation of alkylpyrazines. Ketoses dominated the sugars fraction in the majority of the samples and were responsible for most of the total weight of sugars consumed during roasting. In general, the ketoses were more reactive than aldoses. The greater reactivity of the ketoses might account for the more rapid and greater production of alkylpyrazines. These alkylpyrazines are generally considered to be important components contributing to the flavor of roasted cocoa. The recovery of the procedures employed was determined using solutions that contained variable amounts of selected alkylpyrazine standards. The spiked samples as well as the unspiked controls were analyzed in duplicate. Recoveries were calculated from the differences in the total amount of standard between the spiked and unspiked samples, respectively (**Table 3**). An acceptable recovery range for the determined levels was 46−93%, meeting the requirements of the AOAC Peer-Verified Methods Program (*33*). Analytical relative standard deviations were <4.6% (intraday).

A typical chromatogram of the aroma fraction from natural roasted cocoa (at 135 °C for 3 min) is presented in **Figure 2**. More than 80 peaks have been isolated, and various alkylpyrazines were identified: pyrazine, 2,5-dimethylpyrazine (DMP), 2,6-DMP, 2-ethylpyrazine, 2,3-DMP, 2,3,4-trimethylpyrazine (TrMP), and 2,3,5,6-tetramethylpyrazine (TMP) were evaluated for their sensorial attributes, which contribute to the flavor of cocoa after the roasting process (**Table 4**). The same alkylpyrazines were present in all samples but in different proportions. Major quantitative differences involved primarily the dimethyl-, trimethyl-, and tetramethylpyrazines peaks, whereas other alkylpyrazines appeared only at minor levels. Alkylpyrazine concentration increased rapidly during roasting (125 or 135 °C) to a near maximum value, which remained relatively unchanged for the remainder of the roasting period (**Table 5**). Tetramethylpyrazine was found in unroasted cocoa, but no other pyrazine was identified. The formation of alkylpyrazines and other Maillard products in the reaction between amino acids and fructose has generally been studied in model systems (*34*). Our

Table 4. Identification of Alkylpyrazines: Relative Retention Times (RTT) (Minutes), Content, and Sensorial Attributes[a]

sensorial attributes	compound	RRT	IK	mean concn (mg/kg)
pungent, sweet	pyrazine	0.69 ± 0.02	1129	<0.10
nutty, cocoa, chocolate	2-methylpyrazine	0.72 ± 0.021	1251	0.03–0.50
cocoa, roasted nuts	2,5-DMP	0.84 ± 0.03	1306	0.10–2
nutty, coffee, green	2,6-DMP	0.85 ± 0.02	1312	0.020–0.50
peanut butter, musty, nutty	2-ethylpyrazine	0.86 ± 0.02	1314	<0.10
caramel, cocoa	2,3-DMP	0.91 ± 0.027	1315	0.030–0.50
	4-ethylpyridine	1		
cocoa, roasted nuts, peanut	TrMP	1.04 ± 0.02	1387	0.10–2
chocolate, cocoa, coffee	TMP	1.22 ± 0.04	1438	0.50–2

[a] 4-Ethylpyridine (internal standard); DMP, dimethylpyrazine; TrMP, 2,3,5-trimethylpyrazine; TMP, 2,3,5,6-tetramethylpyrazine; IK, Kovats retention index.

results show that with the presence of hydrophobic free amino acids different alkylpyrazines were generated. On the other hand, only two alkylpyrazines were detected in the case of the glutamic and aspartic acid precursors (*34*, *35*).

The relationship between the values of certain alkylpyrazines generates a high interest in controlling the roasting process of cocoa beans. The sensory evaluation of different roasted cocoa beans showed that a ratio of TMP/TrMP ≃1 would permit us to consider that the degree of roasting was normal (*18*, *22*, *36*). This ratio can be used as a way to evaluate objectively the optimum roasting temperature for cocoa beans. The ratios of roasted cocoa at 125 and 135 °C are presented in **Table 5**. The ratio TMP/TrMP was lower in roasted cocoa at 135 °C than that found at 125 °C, in harmony with normal roasted cocoa. It can be noted that only the samples of natural cocoa beans presented mean values of 1.49 ± 0.16 and 1.08 ± 0.059, respectively. In contrast, in the samples of alkalinized cocoa, the values varied over a wide mean, 1.81 ± 0.051 and 1.65 ± 0.054, respectively.

Table 5. Alkylpyrazines and Brown Compounds Generated in the Roasting Process (125 and 135 °C for 3 min) with Different pH Values[a]

		alkylpyrazines[a] (mg/kg)						
sample	pH	DMP	TrMP	TMP	DMP/TMP	DMP/TrMP	TMP/TrMP	A_{420nm}
1								
roasted at 125 °C for 3 min	5.80	0.19	0.60	0.77	0.32	0.25	1.28	0.648
roasted at 135 °C for 3 min		0.54	1.05	1.11	0.51	0.49	1.06	0.661
2								
roasted at 125 °C for 3 min	5.68	0.17	0.46	0.69	0.37	0.25	1.50	0.597
roasted at 135 °C for 3 min		0.51	0.88	1.05	0.58	0.49	1.19	0.601
3								
roasted at 125 °C for 3 min	5.67	0.21	0.58	0.75	0.36	0.28	1.29	0.630
roasted at 135 °C for 3 min		0.56	0.93	0.96	0.60	0.58	1.03	0.641
4								
roasted at 125 °C for 3 min	5.67	0.16	0.44	0.68	0.34	0.24	1.55	0.635
roasted at 135 °C for 3 min		0.45	0.85	0.97	0.53	0.46	1.14	0.647
5								
roasted at 125 °C for 3 min	5.73	0.18	0.52	0.73	0.35	0.25	1.40	0.621
roasted at 135 °C for 3 min		0.41	0.84	0.92	0.48	0.45	1.10	0.634
6								
roasted at 125 °C for 3 min	5.79	0.16	0.42	0.68	0.38	0.24	1.62	0.602
roasted at 135 °C for 3 min		0.42	0.79	0.91	0.53	0.46	1.15	0.613
7								
roasted at 125 °C for 3 min	5.68	0.19	0.49	0.77	0.39	0.25	1.57	0.635
roasted at 135 °C for 3 min		0.49	0.89	0.90	0.55	0.55	1.01	0.645
8								
roasted at 125 °C for 3 min	5.74	0.20	0.53	0.81	0.38	0.25	1.53	0.632
roasted at 135 °C for 3 min		0.48	0.87	0.89	0.55	0.54	1.02	0.641
9								
roasted at 125 °C for 3 min	5.60	0.22	0.59	0.84	0.37	0.26	1.42	0.599
roasted at 135 °C for 3 min		0.50	0.89	0.93	0.56	0.54	1.05	0.608
10								
roasted at 125 °C for 3 min	5.90	0.19	0.50	0.74	0.38	0.26	1.48	0.636
roasted at 135 °C for 3 min		0.42	0.81	0.86	0.52	0.49	1.06	0.648
11								
roasted at 125 °C for 3 min	5.78	0.20	0.54	0.76	0.37	0.26	1.41	0.619
roasted at 135 °C for 3 min		0.44	0.85	0.89	0.51	0.49	1.05	0.632
12								
roasted at 125 °C for 3 min	7.92	0.10	0.28	0.52	0.25	0.19	1.86	1.362
roasted at 135 °C for 3 min		0.26	0.43	0.70	0.46	0.34	1.63	1.396
13								
roasted at 125 °C for 3 min	7.20	0.08	0.23	0.41	0.24	0.22	1.78	0.832
roasted at 135 °C for 3 min		0.22	0.38	0.62	0.44	0.31	1.63	0.914
14								
roasted at 125 °C for 3 min	7.75	0.09	0.25	0.46	0.24	0.20	1.84	0.849
roasted at 135 °C for 3 min		0.24	0.40	0.69	0.46	0.31	1.73	0.936
15								
roasted at 125 °C for 3 min	7.40	0.08	0.24	0.42	0.23	0.19	1.75	0.846
roasted at 135 °C for 3 min		0.26	0.41	0.66	0.47	0.35	1.61	0.921
x								
roasted at 125 °C for 3 min		0.16	0.45	0.67	0.33	0.24	1.55	0.716
roasted at 135 °C for 3 min		0.41	0.75	0.87	0.52	0.46	1.23	0.743
SD								
roasted at 125 °C for 3 min		0.049	0.13	0.14	0.060	0.027	0.19	0.201
roasted at 135 °C for 3 min		0.11	0.22	0.14	0.047	0.089	0.27	0.217

[a] DMP, 2,5-dimethylpyrazine; TrMP, 2,3,5-trimethylpyrazine; TMP, 2,3,5,6-tetramethylpyrazine.

Table 6. Effect of Alkali, Temperature, and Air Injected during Roasting of Cocoa Bean

cocoa sample	flavor index
natural (135 °C for 3 min; with no air incorporated)	72
alkali pH 7.40 (135 °C for 3 min; with air incorporated)	46
natural (125 °C for 3 min; with no air incorporated)	51
alkali pH 7.40 (125 °C for 3 min; with air incorporated)	32

Considering the total amount of the principal alkylpyrazines generated (2,6-DMP, TrMP, and TMP) and the ratio TMP/TrMP under each condition revealed quite interesting results. Alkalinized cocoa, as expected, exhibited a very low concentration of alkylpyrazine aroma compounds at 125 and 135 °C roasting treatments (0.79 ± 0.01 and 1.32 ± 0.071 mg/kg, respectively). With regard to natural cocoa, we see that the total concentration increased considerably (1.45 ± 0.12 and 2.30 ± 0.18 mg/kg, respectively). This illustrates that the natural roasting process increased the pyrazine concentration by almost 1.73 times the concentration detected in alkalinized cocoa with the same cocoa bean. The ratio TMP/TrMP in roasted cocoa depends mainly on the initial free α-amino acid content, pH solution, and roasting process (temperature, duration, and amount of alkali and air incorporated in brown cocoa). Also, it is possible that cocoa subjected to an improper roasting procedure generates undesirable flavor compounds and at the same time DKPs. It follows that addition of alkali and air injected may decrease the formation of alkylpyrazines by reducing the activity of the traditional pathways of the Maillard reaction and directly affects color formation (**Table 6**). Thus, the alkalization process during the manufacture of cocoa powder has an additional effect on flavor composition.

Major chemical changes to the cocoa beans occur during thermal treatments. Free amino acid concentration in natural cocoas roasted at 135 °C for 3 min decreased by >85%, and reducing sugars were destroyed during roasting. In alkalinized cocoa free amino acid content decreased by >75% and also reducing sugars were destroyed. Model systems indicated that simple alkylpyrazines could be produced in heated cocoa by condensation of sugars and amino acids. The role of the amino acid is primarily one of providing the nitrogen for the ring, and sugars provide the carbon source for pyrazines. Also, the mechanism of alkylpyrazine formation using ammonia is considered in different products (*5*). The roasted cocoa had ~2 times higher concentration of alkylpyrazines than the unroasted cocoa. These results showed that the concentration of alkylpyrazines generated during the roasting of cocoa beans was very important in increasing the flavor index. From a correlation of the results of total alkylpyrazine content with flavor index, coefficients of $r^2 = 0.82$ (125 °C) and $r^2 = 0.88$ (135 °C) were obtained (**Figure 3**). Regression analysis showed that there was a lineal correlation between these measurements, indicating that cocoa flavor quality can be adequately evaluated by these components. The most abundant alkylpyrazine identified was tetramethylpyrazine, which can be present at a possible high concentration in the unroasted cocoa and at only a moderate level in the normal roasted cocoa. If cocoa was moist-thermally treated and dried, the level of Amadori compounds (sugar−amino acid) could be raised and the roasted flavor intensified (*31*). Thus, the ratio TMP/TrMP decreased in a roasting process of between 125 and 135 °C; however, these results were in natural cocoa. In alkalinized cocoa, the ratio TMP/TrMP was not clearly evident. In this product, browning products can cause a partial oxidation (deamination) of free amino acids, increased

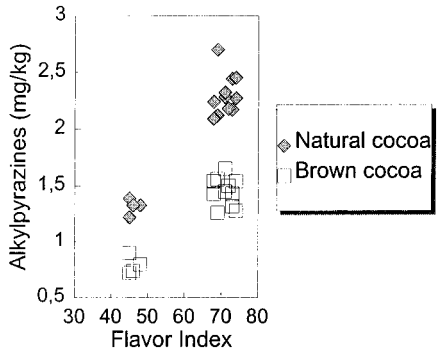

Figure 3. Correlation between total alkylpyrazine content and flavor index.

by the high pH, leading to an underestimation of amino acids actually consumed by the Maillard reaction. Minor changes in ratio value were observed but with considerable degradation in the quantities of amino acid content (>75%). In these cases, it was necessary to separate and quantify simultaneous measurements of alkylpyrazines and brown products generated. The chromatogram of alkalinized cocoa showed minor peaks and also minor alkylpyrazine content. Moreover, the brown compounds did not modify the chromatogram with respect to natural cocoa. The importance of the brown compounds in the roasting process of cocoa was clear. Depending on the phenol (phenolic compounds from the raw cocoa), the generated *o*-quinones showed great differences in stability and darkly colored pigments (brown products). Samples that contained alkali exhibited a greater degree of brown color formation (**Table 5**). The natural cocoa, as expected, gave a lower absorbance reading at 125 or 135 °C (0.623 ± 0.017 and 0.640 ± 0.019, respectively) than brown cocoa (0.972 ± 0.260 and 1.042 ± 0.236, respectively). The results showed that a higher pH value increased the presence of the brown compounds, remaining stable with natural cocoa. An ANOVA test was performed to compare results of the alkylpyrazines and brown compounds obtained in relation to the roasting process; it is shown that significant differences ($P \leq 0.05$) exist between natural and alkali samples.

In conclusion, the separation of alkylpyrazine compounds from different cocoa beans with the same type of roaster demonstrates that the formation of heterocycles by Maillard reactions and destruction of potential anticarcinogenic polyphenolic compounds depend strongly on the technological procedures (temperature and time of roasting) and amounts of alkali and air injected during the roasting process.

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