Original article

Effect of polyphenol and pH on cocoa Maillard-related flavour precursors in a lipidic model system

S. S. Noor-Soffalina, S. Jinap,* S. Nazamid & S. A. H. Nazimah

Department of Food Science, Faculty of Food Science and Technology, Universiti Putra Malaysia, 43400 UPM, Serdang, Selangor, Malaysia

(*Received 6 May 2007; Accepted in revised form 29 November 2007*)

Summary Polyphenol and pH influence the flavour quality of cocoa beans during roasting. Amino acids and reducing sugars are flavour precursors in cocoa beans, which develop into cocoa-specific aroma through Maillard reactions during roasting. A central composite design was applied to determine the combined effect of polyphenol and pH on the flavour precursors during cocoa roasting at 120 °C for 45 min using a lipidic model system. Polyphenol was added at 40, 80 and 120 g kg^{-1} and pH was adjusted to 4.5, 6 and 7.5. The response surface methodology revealed that a lower concentration of amino acids and reducing sugars was obtained at higher polyphenol concentration (120 g kg^{-1}) and lower pH value (4.5). Based on the constraints set, the best polyphenol concentration of 43–58 g kg^{-1} and pH of 7.0–7.5 was found to be optimum for the formation of flavour precursors in this lipidic model study.

Keywords Cocoa bean, flavour, flavour precursors, pH, polyphenol, roasting.

Introduction

The cocoa beans (*Theobroma cacao*), when roasted, give a great impact on flavour. The quality of the flavour produced is dependent on numerous aspects, such as origin of the beans, the variety of the beans and roasting conditions (Jinap *et al*., 1995; Misnawi *et al*., 2004). Other than that, there are also internal factors in the beans that can affect the flavour produced, such as polyphenol concentration and pH.

During roasting, Maillard reactions play a major role in the formation of cocoa flavour (Ziegleder & Biehl, 1988). Free amino acids are amine flavour precursors in cocoa beans that are produced during fermentation through proteolysis by protease activities (Ziegleder & Biehl, 1988). Reducing sugars are carbonyl flavour precursors mostly formed through hydrolysis of sucrose by the action of invertase (Rohan & Stewart, 1967) and enzymatic hydrolysis of anthocyanins (Hoskin & Dimick, 1994).

Hofmann *et al*. (2000) have demonstrated the formation of flavour compounds involving amino acids and reducing sugars in the Maillard reaction using a model system. Through the Maillard reactions, all of the cocoa aroma precursors interact to produce cocoa flavour components (Hoskin & Dimick, 1994; Jinap *et al*., 1998; Puziah *et al*., 1998b).

Maillard reaction, also known as non-enzymatic browning and involved in the formation of brown pigments, comprises the condensation reaction between reducing carbonyl compounds, including reducing sugars, aldehydes or ketones and compounds with free amino groups, such as amines, amino acids and protein or any nitrogenous compound (Carabasa-Giribet & Ibarz-Ribas, 2000; Macrane *et al*., 1993; Yoo *et al*., 2004). In the early stage of the Maillard reaction, the reducing sugar condenses with free amino group of amino acids or proteins to give a condensation product, *N*-substituted glycosylamine, in the case of an aldose sugar that rearranges into so-called Amadori product or Heyns product if the reducing sugar is a ketose (Martins *et al*., 2000; Brands *et al*., 2002). Time, temperature, pH, reactant concentration and water activity are important variables in determining the nature and quantity of the products (Shibamoto & Bernhard, 1977). Under roasting conditions, the amino acids can be partially racemise and the occurrence of enantiomeric forms is enhanced, especially when the process involves high temperature (Friedman & Liardon, 1985). D-amino acid formation in foodstuff and beverages can be carried out by technological enrichment during processing, such as heat and pH (Friedman, 1999). The D-isomers of amino acids are far less abundant in

*Correspondent: E-mail: jinap@putra.upm.edu.my

doi:10.1111/j.1365-2621.2008.01711.x

nature compared with the L-isomers, which are predominantly found in biological molecules (Takano *et al.*, 2006). Study by Casal *et al.* (2005) found that at roasting temperature of 140–240 °C, the racemisation of D-amino acids still occurred at lower temperature but below 15% racemisation values.

Polyphenols are compounds naturally occurring in cocoa, which are responsible for the astringency and contribute to bitter and green flavours (Bonvehi & Coll, 1997, 2000; Luna *et al.*, 2002). The total amount of soluble polyphenols in the dried fat-free mass of fresh cocoa beans is 15–20% (Wollgast & Anklam, 2000). Cocoa is rich in polyphenol substances, such as (-)-epicatechin, (+)-catechin, quercetin (including its glucoside) and procyanidin (Sanbogi *et al.*, 1998; Hammerstone *et al.*, 1999). Polyphenols are characterised as astringent, and their most important attribute is their propensity to form complexes with protein, polysaccharide and alkaloid (Hagerman & Butler, 1981; Mcmanus *et al.*, 1985; Spencer *et al.*, 1988). Proteins and polyphenolic compounds can combine to form insoluble complexes; these can grow to colloidal size at which they scatter light, and larger still, which can lead to sediment formation. Hydrogen and hydrophobic bonding is involved in the protein–polyphenol interaction (Siebert *et al.*, 1996). It is this capacity to precipitate proteins, in particular, the salivary proteins in the oral cavity, which give the polyphenols their astringent character (Santos-Buelga & Scalbert, 2000).

According to de Brito & Narain (2003), the two most important parameters which influence aroma quality control in cocoa products are pH adjustment before distillation and collection of a definite volume of extract after distillation; higher sensorial aroma values were obtained for each of the responses at a lower pH range (1.5–3). The acid-extracted samples possessed a more intense and chocolate-like aroma than the alkaline ones. Lee *et al.* (2000), reported that polyphenols, i.e. catechins and theaflavins, are unstable under neutral and alkaline conditions.

Response surface methodology (RSM) is currently the most popular optimisation technique in food science owing to its comprehensive theory, reasonably high efficiency and simplicity. It can be used in problems invovling ingredients and/or processing conditions as variables (Arteaga *et al.*, 1994) and has been successfully applied to optimise food-processing operations (King & Zall, 1992; Madamba, 2002; Oomah & Mazza, 2001; Unal *et al.*, 2003).

The aim of the present study was to determine the combined effect of polyphenol concentration and pH on cocoa flavour precursors, namely amino acids and sugars during roasting in a lipidic model system, using RSM.

Materials and methods

Chemicals and standards

The authentic standard of L-amino acids (asparagine, glutamine, serine, glycine, histidine, arginine, threonine, alanine, proline, tyrosine, valine, methionine, cysteine, iso-leucine, leucine, phenyl-alanine and lysine) and D-sugars (sucrose, fructose and glucose) were purchased from Sigma Aldrich Chemical Co. Inorganic reagents and solvents were all commercial products of analytical grade from Fisher Scientific.

Source of cocoa beans for extraction of cocoa butter and crude polyphenol

Dried bulk fermented cocoa beans of mix Malaysian hybrid were obtained from Hilir Perak Cocoa Research and Development Centre, Malaysian Cocoa Board, Hilir Perak, Perak.

Cocoa butter extraction

Fermented cocoa bean was ground in a cocoa breaker (Limprita 19582; John Gordon & Co Ltd, Epping, Essex, England) and separated from its shell using a winnower (Catador CC-1; John Gordon & Co. Ltd, Epping, Essex, England). The ground sample was then refined to reduce its size to < 15 μm in a cocoa triple roll refiner (Pascal Eng., England). The obtained cocoa powder was defatted using Soxhlet apparatus with petroleum ether (bp. 40–60 °C) for 16 h. The collected petroleum ether was then dried in a 250 ml round bottom flask using a rotary evaporater (VV2000; Heidolph, Germany). The collected cocoa butter was then kept for further use in the lipidic model system.

Crude polyphenol extraction

Defatted fermented cocoa powder was mixed with chilled 80% aqueous acetone twice followed by 100% chilled acetone. The extract was then centrifuged at 4000 r.p.m. at 4 °C for 15 min. The supernatant was evaporated at a temperature of 45 °C using a Heidolph WB/VV 2000 Rotary Evaporator (Germany) and freeze dried (Labconco Freeze Dry System, Freezone 6, Kansas City, MO, USA) at pressure of $< 133 \times 10^{-3}$ mbar. The polyphenol extract obtained is in the form of dried purplish powder.

Quantification of total polyphenol

Spectrophotometric method was used to determine the total polyphenol according to the method by Singleton and Rossi (1965). Five hundred milligrams of defatted cocoa powder was mixed with 80 mL of 80% aqueous acetone in a 125 mL conical flask. The mixture was then

sonicated for 30 min using a Sonicor C-125 (Sonicor Inst., New York, USA). The extraction mixture was constantly kept cold by adding ice water into the sonicator vessel. The mixture was then passed though a vacuum filter with a Whatman No. 1 filter paper to obtained a clear extract. The residues and all glassware were then washed with the 80% aqueous acetone and the total volume of the extract was made up to 100 mL in a volumetric flask. One millilitre of the extract was pipetted into a 100 mL volumetric flask and diluted with 70 mL of distilled water. The extracted polyphenol was further reacted with 5 mL of 2 N Folin-Ciocalteau's reagent for 2 min to form a blue-coloured solution. Then, 15 mL of saturated sodium carbonate (Na_2CO_3) solution was added to stabilise the colour formed. The blue colour was allowed to develop for at least 2 h and its absorbance was then measured at 765 nm.

Free amino acid analysis

Determination of free amino acids was carried out using the extraction method of Kirchhoff *et al.* (1989) with slight modification followed by an HPLC measurement method of Cohen *et al.* (1990) that was modified by Puziah *et al.* (1998a). Only L-amino acids were quantified as the cocoa flavour precursors. Seven hundred milligrams of defatted powder and 1.4 g of polyvinylpyrolidone (PVP) was homogenised for 5 min at 0 °C in 15 mL of distilled water and adjusted to pH 2.5 using glacial acetic acid. The mixture was then centrifuged at 13 000 ***g*** for 15 min and filtered through Whatman no. 4 filter paper. The filtrate was then made up to 50 mL using distilled water. DL-Alpha-amino-*n*-butyric acid (AABA, internal standard) and 12 mL of acetone were added to 3 mL of the filtrate; the mixture was then mixed thoroughly using Polytron Homogenizer (PT 1200; Kinematica AG, Switzerland), kept at room temperature for 30 min and centrifuged at 13 000 ***g*** for 15 min. Acetone was then removed by streaming with nitrogen gas.

The amino acids were converted into phenylthiocarbanyl (PTC) amino acids using phenylisothiocyanate (PITC) as described by Cohen *et al.* (1990). Twenty microlitres of sample extract were used. The free amino acids were separated using reversed-phase HPLC with gradient elution at a flow rate of 0.8 mL min^{-1}. Free amino acids were detected at 254 nm. Solvent A of the gradient elution was acetate buffer at pH 5.7 and solvent B was acetonitrile:deionized water (60:40); the gradient elutions were as follows: 0 min: 100% A, 0% B; 5 min: 75% A, 25% B; 13 min: 52% A, 48% B; 13.5 min: 0% A, 100% B; 16.5 min: 0% A, 100% B; 17 min: 100% A, 0% B; 22 min: 100% A, 0% B. Waters Pico-Tag free amino acids column (3.9 × 300 mm i.d., Waters, Millipore Corporation, Milford, MA, USA) was used for the analysis which was employed at a temperature of 37 °C. The free amino acids were detected using a Waters Associates Model 486 Tunable Absorbance Detector at 254 nm.

Sugars determination

Sugars were determined using modified method of Hunt *et al.* (1977). One gram of cocoa powder was mixed with 20 mL of 85% methanol in a 50 mL bottle and homogenized for 5 min using Polytron Homogenizer (PT 1200; Kinematica AG) before internal standard of rhamnose was added. The mixture was then heated in a water bath at 90 °C for 30 min, cooled at room temperature and filtered through Whatman no. 1 filter paper. The filtrate was vacuum evaporated at 50 °C to about 10 ml and the evaporated sample was then filtered through a SEP PAK C_{18} cartridge (Waters) and a 0.45 μm Whatman membrane filter. Sugars were separated in a μBondapak-NH_2 (300 × 3.9 mm i.d.) stainless steel tube and detected using HPLC (Waters 600 Controller) refractive index detector (Waters, Model 410). The eluent was acetonitrile: deionised water at 80:20 (v/v) at the flow rate of 2 mL min^{-1}. Ten microlitres of sample extract was used. Sugars in the samples were quantified by comparing peak area of the sample to those obtained from authentic standard of sucrose, glucose and fructose.

Lipidic model system

The experiment was conducted using a lipidic model system of cocoa butter. Reducing sugars or amino acids were mixed with 55% cocoa butter (w/w) with 4% water (w/w). Polyphenols were added and pH was adjusted according to the central composite design (CCD). A magnetic bar was put in the reaction vial containing the mixture with the cocoa butter. Powdered polyphenols were added at 40, 80 and 120 g kg^{-1}. The vial was preheated to 40 °C, to enable the cocoa butter to melt in order to ease the stirring and homogenising. The pH was adjusted to 4.5, 6 and 7.5 using 1 M hydrochloric acid or 1 M sodium hydroxide (Chen *et al.*, 2005).

The mixture was then subjected to roasting in an oven at 120 °C for 45 min before it was rapidly cooled on ice. The roasted samples were then analysed for their reducing sugars and amino acid concentration. The initial amount of the compounds used were; for amino acids: asparagines, 68; glutamine, 113; serine, 73; glycine, 26; histidine, 48; arginine, 160; threonine, 50; alanine, 112; proline, 64; tyrosine, 126; valine, 111; methionine, 52; cycteine, 72; iso-leucine, 87; leucine, 183; phenyl-alanine, 185; and lysine 125 mg per 100 g, whereas for sugars: glucose, 84; fructose, 177; and sucrose, 91 mg per 100 g.

Experimental design

The study used a CCD (face-centered) with two factors and three levels; each factor had three levels of −1, 0 and +1, which corresponds to the low, mid and high level. The factors and respective coded and uncoded levels are shown in Table 1. A total of 13 combinations (including five replicates at the centre point with each value coded as 0) were chosen in random order according to CCD configuration for two factors (Rastogi & Rashimi, 1999). The effect of the two independent variables on the responses (Y) was modelled using a polynomial response surface. The second-order response function for the experiments was predicted by the following equation:

$$Y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_{11} x_1^2 + \beta_{22} x_2^2 + \beta_{12} x_1 x_2$$

where β_0 is the value of the fixed response at the central point of the experiment which is the point (0,0); β_1 and β_2 are linear; β_{11} and β_{22} are quadratic and β_{12} is interaction coefficient. The validity of the models was evaluated as a function of their respective coefficients of determination and by an analysis of the lack of fitness. Calculations were performed using the software package, Minitab Statistical Software 14 (Minitab Inc., PA, USA).

Results and discussion

Model fitting

Response surface methodology is an empirical modelling technique, and was used to estimate the relationship between a set of controllable experimental factors and observed results (Gao *et al*., 2006). The effect of polyphenol concentration and pH on the amount of free amino acids and sugars left after roasting process are shown in Table 2. The independent and dependent variables were fitted to the second-order model equation and examined for the goodness of fit.

In RSM, it is always necessary to examine the fitted model to ensure that it provides an adequate approximation to the true system and verifies that none of the least squares regression assumptions are violated. Usually, the regression models are fitted to data from a designed experiment. Replication of the centre point is useful in conducting a formal test for lack of fit on the regression model (Myers & Montgomery, 2002).

Table 1 Independent variables and their levels in central composite design

		Coded values		
Independent variables	**Symbol**	**−1**	**0**	**1**
Polyphenol concentration (g kg^{-1})	X_1	40	80	120
pH	X_2	4.5	6	7.5

The analysis of variance for the effect of polyphenol concentration and pH on the degradation of cocoa flavour precursors; amino acids and sugars as linear, quadratic and interaction terms on the response variables are shown in Table 3. The results indicated that the models for all the responses are highly adequate because their R^2 values were higher than 0.70, indicating that the regression model explained the reaction well except for cysteine. The closer the value of R^2 to unity, the better the empirical model fits the actual data. The smaller the R^2, the less influence the dependent variables in the model have in explaining the behaviour variation (Puziah *et al*., 1998b). The models and their respective surfaces were adjusted by excluding the statistically non-significant parameters at $P \leq 0.05$. The validity of the models was evaluated as a function of their respective coefficients of determination and by an analysis of the lack of fitness (de Brito & Narain, 2003).

Results of the study showed that there was no significant lack of fit ($P \leq 0.05$) in responses for sucrose, asparagine, arginine, histidine, alanine, iso-leucine, tyrosine, valine, glycine, methionine, serine, threonine and cysteine (Table 3). A model with significant lack of fit can be attributed to the fact that all appropriate function of independent variables was not included. However, when large amount of data were included in the analysis, a model with a significant lack of fit could still be used. Although the responses for glucose, fructose, glutamine, lysine, leucine, phenyl-alanine and proline were not significant, they showed a high coefficient of determination R^2 and the models were significant ($P \leq 0.05$) at different form of transformation (Table 3).

Effect of polyphenol concentration and pH on sugars

Table 3 shows independent variables as linear, quadratic and interaction terms for the cocoa flavour precursors. The polyphenol concentration and pH were found to be significant factors ($P \leq 0.001$) for sucrose and showed significant contribution to the glucose and fructose although their models were significantly lack of fit. The polyphenol concentration had a linear negative effect on glucose, fructose and sucrose, which indicated that higher polyphenol concentration will decrease the sugar amount (Table 4). pH was found to have a linear effect on the amount of sucrose (Fig. 1a); plane surface was observed as only the linear terms were significant ($P \leq 0.001$). However, Bonvehi & Coll (2002) found that degradation of sucrose during roasting was negligible; thus, they concluded that sucrose concentration in unroasted cocoa beans is not an important variable in

Table 2 Central composite design and experimental result for the response function

	Run no			1	2	3	4	5	6	7	8	9	10	11	12	13
Independent variables	**Polyphenol concentration (g kg^{-1})**			**0**	**1**	**0**	**−1**	**0**	**−1**	**1**	**0**	**0**	**0**	**0**	**1**	**−1**
	pH			**0**	**1**	**0**	**1**	**0**	**0**	**−1**	**0**	**−1**	**0**	**1**	**0**	**−1**
Responses	Sugars (mg per 100 g)	Glucose		42.35	38.17	41.87	51.42	42.88	49.25	27.26	41.73	33.21	43.12	41.22	37.81	36.18
		Fructose		100.80	101.14	100.83	111.21	98.72	108.97	74.79	101.36	92.25	100.87	104.17	96.56	101.14
		Sucrose		40.46	30.12	41.06	51.56	40.82	49.15	28.30	38.72	34.15	40.04	44.53	31.48	44.14
	Amino acids (mg per 100 g)	Acidic	Asparagine	37.26	35.13	38.03	43.24	36.56	40.17	39.25	36.82	44.18	36.16	42.19	33.04	47.12
			Glutamine	72.27	68.79	72.02	83.14	73.26	75.22	79.65	73.11	81.68	71.91	68.34	68.37	84.17
		Basic	Lysine	68.23	70.44	67.20	83.92	68.55	73.27	69.52	68.02	73.25	67.82	80.72	62.23	75.48
			Arginine	98.48	102.56	99.11	110.47	98.06	103.73	91.89	97.72	96.14	99.43	108.98	93.51	104.48
			Histidine	26.21	25.61	26.08	34.88	25.72	30.22	23.86	24.30	25.88	27.81	30.05	24.08	27.64
		Hydrophobic	Alanine	52.72	49.23	54.92	62.41	51.08	61.32	46.23	51.76	48.18	52.82	51.35	48.05	58.28
			Iso-leucine	54.47	49.12	53.22	55.18	53.96	56.03	46.17	56.11	49.17	54.16	51.92	54.62	54.42
			Leucine	96.43	82.23	95.19	99.13	95.02	104.28	69.50	95.72	84.25	95.14	91.77	75.47	95.91
			Phenyl-alanine	86.81	86.91	85.44	104.33	86.32	97.70	69.75	87.10	81.05	85.92	97.45	80.26	93.51
			Tyrosine	51.17	48.95	52.88	61.17	49.16	58.93	38.01	50.15	44.26	50.91	53.27	45.22	57.67
			Valine	52.19	48.14	50.74	61.90	53.42	58.38	39.51	52.19	49.86	52.88	55.92	44.28	52.06
			Proline	37.26	32.45	36.88	40.83	36.59	41.62	36.28	37.09	40.86	37.44	35.20	35.15	43.03
		Others	Glycine	20.83	18.20	20.12	23.19	19.78	21.52	16.85	21.01	18.23	20.51	19.43	19.43	21.07
			Methionine	26.54	25.18	25.11	32.41	27.19	30.91	23.41	26.06	24.14	26.33	30.19	21.92	29.11
			Serine	38.91	37.42	38.18	40.13	37.49	43.22	34.04	38.67	35.23	37.34	40.09	36.61	37.27
			Threonine	37.62	37.21	37.10	44.19	35.44	43.20	33.21	37.81	34.41	37.52	38.66	35.83	35.11
			Cysteine	42.86	38.24	40.54	43.39	40.18	41.61	38.84	40.85	40.27	40.88	37.86	37.12	42.39

Table 3 Analysis of variance on the independent variables as linear, quadratic and interaction terms on the response variables

		Source		Regression	Linear	Square	Interaction	Residual error	Lack of fit	Pure error	Total	R^2 (%)
		df		5	2	2	1	7	3	4	12	
Sequential sum of squares	Sugars	Glucose		465.199*	382.608*	77.895*	4.696	10.079	8.606‡	1.473	475.277	97.9
		Fructose		898.701*	786.871*	45.482	66.349†	36.613	32.383‡	4.230	935.315	96.1
		Sucrose		582.486*	567.540*	7.098	7.848	15.343	11.950	3.393	597.829	97.4
	Amino acids	Acidic	Asparagine	189.743*	105.616*	84.113*	0.013	5.514	3.441	2.073	195.256	97.2
			Glutamine	338.436†	216.337*	97.888†	24.211	36.865	35.302†	1.563	375.301	90.2
		Basic	Lysine	409.223*	202.047*	193.016*	14.160†	6.782	5.759‡	1.023	416.004	98.4
			Arginine	355.368*	302.195*	47.680†	5.492	8.864	6.841	2.023	364.232	97.6
			Histidine	107.371*	90.204*	9.671	7.497‡	7.193	0.902	6.291	114.564	93.7
		Hydrophobic	Alanine	292.823*	264.519*	27.987‡	0.317	12.132	3.674	8.458	304.955	96.0
			Iso-leucine	94.431†	48.168†	45.075†	1.188	15.896	11.325	4.571	110.327	85.6
			Leucine	1163.65*	958.55*	182.48*	22.63	31.35	29.98†	1.37	1195.00	97.4
			Phenyl-alanine	924.253*	901.055*	13.156	10.043	14.469	12.681‡	1.787	938.722	98.5
			Tyrosine	463.577*	438.112*	11.620	13.846‡	11.784	4.208	7.576	475.362	97.5
			Valine	382.658*	372.388*	9.900‡	0.370	9.355	5.285	4.071	392.014	97.6
			Proline	104.132*	100.479*	2.990	0.663	4.174	3.734‡	0.439	108.306	96.1
		Others	Glycine	28.7427†	24.9104†	3.6848	0.1475	3.8546	2.8422	1.0124	32.5973	88.2
			Methionine	105.446*	100.664*	4.203	0.579	8.582	6.280	2.302	114.029	92.5
			Serine	53.3839‡	46.7057†	6.6102	0.0681	10.8164	8.8791	1.9373	64.2004	83.2
			Threonine	109.652*	93.966*	9.237	6.449	9.884	6.192	3.692	119.536	91.7
			Cysteine	30.8137	29.6109‡	0.5644	0.6384	14.3406	9.9843	4.3562	45.1542	68.2

*Significant at $P \leq 0.001$.
†Significant at $P \leq 0.01$.
‡Significant at $P \leq 0.05$.

Table 4 Regression coefficients of the second-order polynomial for the response variables

	Coefficients		β_0	β_1	β_2	β_{11}	β_{22}	β_{12}
Sugars	Glucose		42.404	−5.602*	5.691*	1.093	−5.222*	−1.084
	Fructose		100.984*	−8.139*	8.056	0.615	−3.943‡	4.073†
	Sucrose		40.345	−9.160*	3.269*	−0.341	−1.318	−1.401
Amino acids	Acidic	Asparagine	37.191	−3.851*	−1.665*	−1.153	5.429*	−0.058
		Glutamine	71.872	−4.287†	−4.205†	1.526	4.739‡	−2.460
	Basic	Lysine	68.229	−5.081*	2.804*	−1.148	8.094*	−1.881‡
		Arginine	98.600	−5.118*	4.916*	−0.071	3.868*	1.172
		Histidine	26.176	−3.198*	2.193*	0.595	1.414	−1.369‡
	Hydrophobic	Alanine	52.348	−6.414*	1.717‡	3.111†	−1.810	−0.282
		Iso-leucine	54.420	−2.621†	1.076	0.818	−3.969†	0.545
		Leucine	94.91	−12.02*	3.91†	−3.55‡	−5.41†	2.38
		Phenyl-alanine	86.771	−9.770*	7.397*	1.075	1.347	1.584
		Tyrosine	50.649	−7.599*	3.909*	1.929‡	−1.379	1.860‡
		Valine	52.494	−6.736*	4.086*	−1.695‡	−0.134	−0.304
		Proline	37.222	−3.599*	−1.949*	0.742	0.392	−0.407
	Others	Glycine	20.316	−1.882*	0.781‡	0.496	−1.153‡	−0.192
		Methionine	26.219	−3.653*	1.853†	0.262	1.015	−0.380
		Serine	38.426	−2.090†	1.849†	0.717	−1.541	0.131
		Threonine	37.307	−2.706*	−2.196†	1.685	−1.299	−1.270
		Cysteine	40.598	2.887*	−0.335	−0.079	−0.381	−0.399

*Significant at $P \leq 0.001$.
†Significant at $P \leq 0.01$.
‡Significant at $P \leq 0.05$.

the generation of alkylpyrazines. Hoskin & Dimick (1994) reported that the yield of roasting process is dependent on heat and pH; if the pH is neutral the the components of the reaction form reductones. Carbohydrate adsorption by polyphenols could be explained by cooperative hydrogen bonding between the oxygen atom of the carbohydrate and the phenolic hydroxyl group and by hydrophobic interactions. Electrostatic and inonic forces, which in general involve charged molecules, do not appear to be determinant as polyphenols have negligible negative charges (Vernhet *et al.*, 1996).

Effect of polyphenol concentration and pH on amino acids

In accordance with the fitted models, a response surface was constructed for the amino acids (Fig. 1b–l). Amino acids were grouped into acidic, basic, hydrophobic and others. Acidic amino acids, such as asparagine, showed negative linear and positive non-linear effect with pH as both linear and quadratic terms (β_2 and β_{22}) were significant at $P \leq 0.001$ (Fig. 1b). Under acidic conditions, the rate of racemisation of asparagine correlates with the negative inductive strength of its undissociated β-carboxylic group (Liardon & Jost, 1981). The acidic amino acid, asparagine and glutamine are amino acids most prone to racemisation (Stenberg *et al.*, 2002). It is suggested that racemisation can be a factor in lowering the amount of these amino acids available for the flavour formation. However, the temperature used in this study is not in the high range that can cause aggressive loss of these acidic amino acids by racemisation. Caligiani *et al.* (2007), reported that the level of racemisation is highly dependent on the roasting temperature of the cocoa, whereby it is only significant if temperature applied are higher than 180 °C.

The variation of basic amino acids, i.e. arginine and histidine, with respect to polyphenol concentration and pH is presented in Fig. 1c and d. As shown in Table 4, arginine and histidine were significantly ($P \leq 0.001$) affected by the first-order (linear) term of both variables. The surface plots (Fig. 1c and d) indicate that increasing the polyphenol concentration would decrease the concentration of arginine and histidine, while increasing the pH increase would increase their concentration. Lysine and arginine have significant linear ($P \leq 0.001$) and quadratic ($P \leq 0.001$) effects giving an overall curvilinear effect. However, only arginine does not show significant interaction term in the basic amino acid group.

Hydrophobic amino acids, i.e. alanine, iso-leucine, tyrosine and valine, were found to have no significant lack of fit (Table 3). In the first-order polynomial, it was found that polyphenol had a negative effect whereas pH had a positive effect on the hydrophobic amino acids (Fig 1e–h). Alanine and tyrosine had positive effect in quadratic terms of pH, while valine had negative effect. Only tyrosine showed significant effect ($P \leq 0.05$) on the interaction terms. From the curvature, it can be depicted that the increase of polyphenol resulted in intense

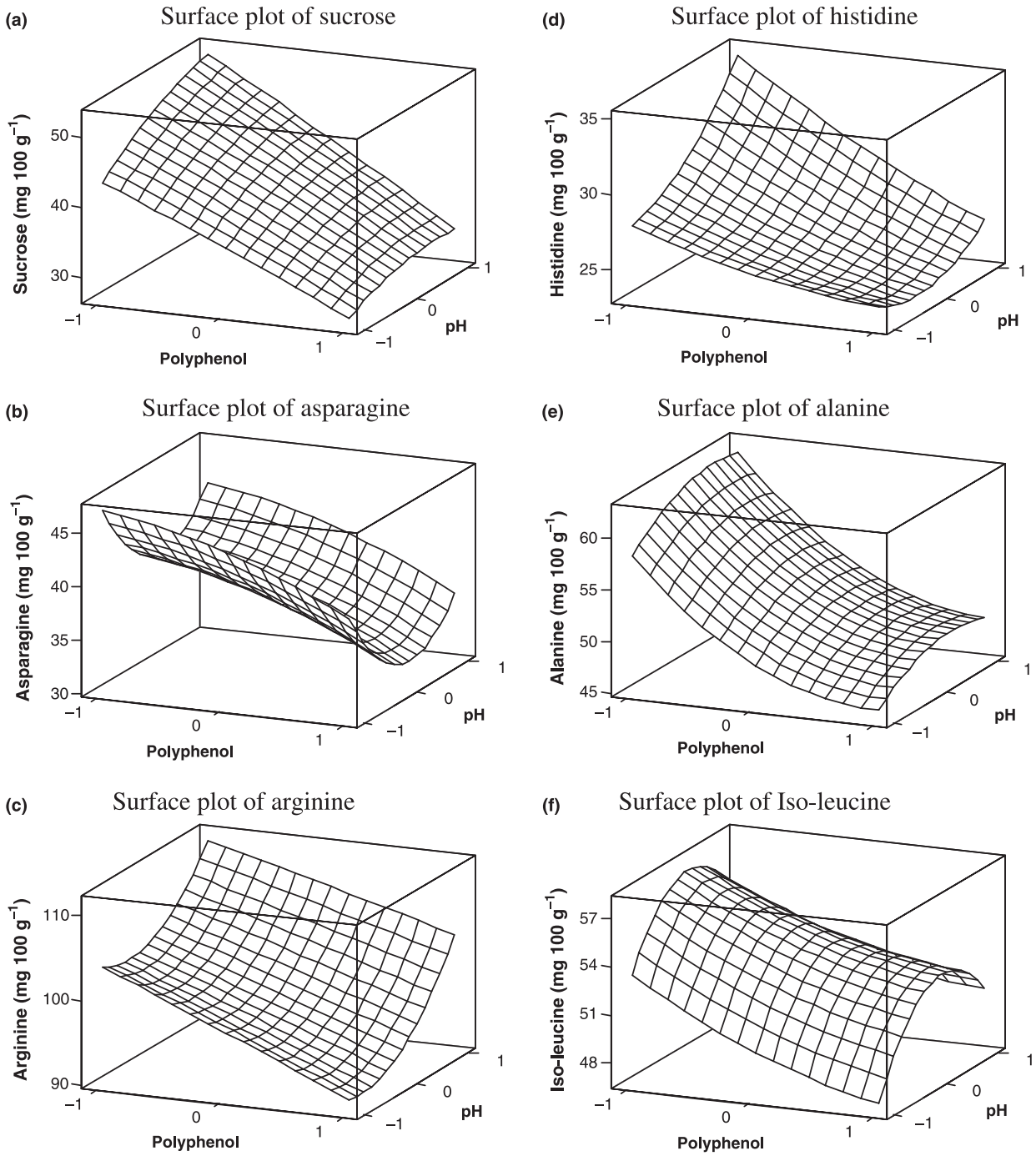

Figure 1 The surface plots of fructose, asparagine, arginine, histidine, alanine, iso-leucine, tyrosine, valine, glycine, methionine, serine and threonine as affected by the polyphenol concentration and pH.

reduction of alanine and tyrosine. Hydrophobic amino acids, *viz*. alanine, tyrosine, valine, iso-leucine, leucine and phenylalanine, are the specific aroma precursors for the formation of cocoa aroma (Voigt *et al*., 1993). Even though proline model was lack of fit, the linear terms of polyphenol and pH was highly significant ($P \leq 0.001$). Polyphenol–proline-rich protein interaction can cause astringency and reduce lubricating action of the saliva,

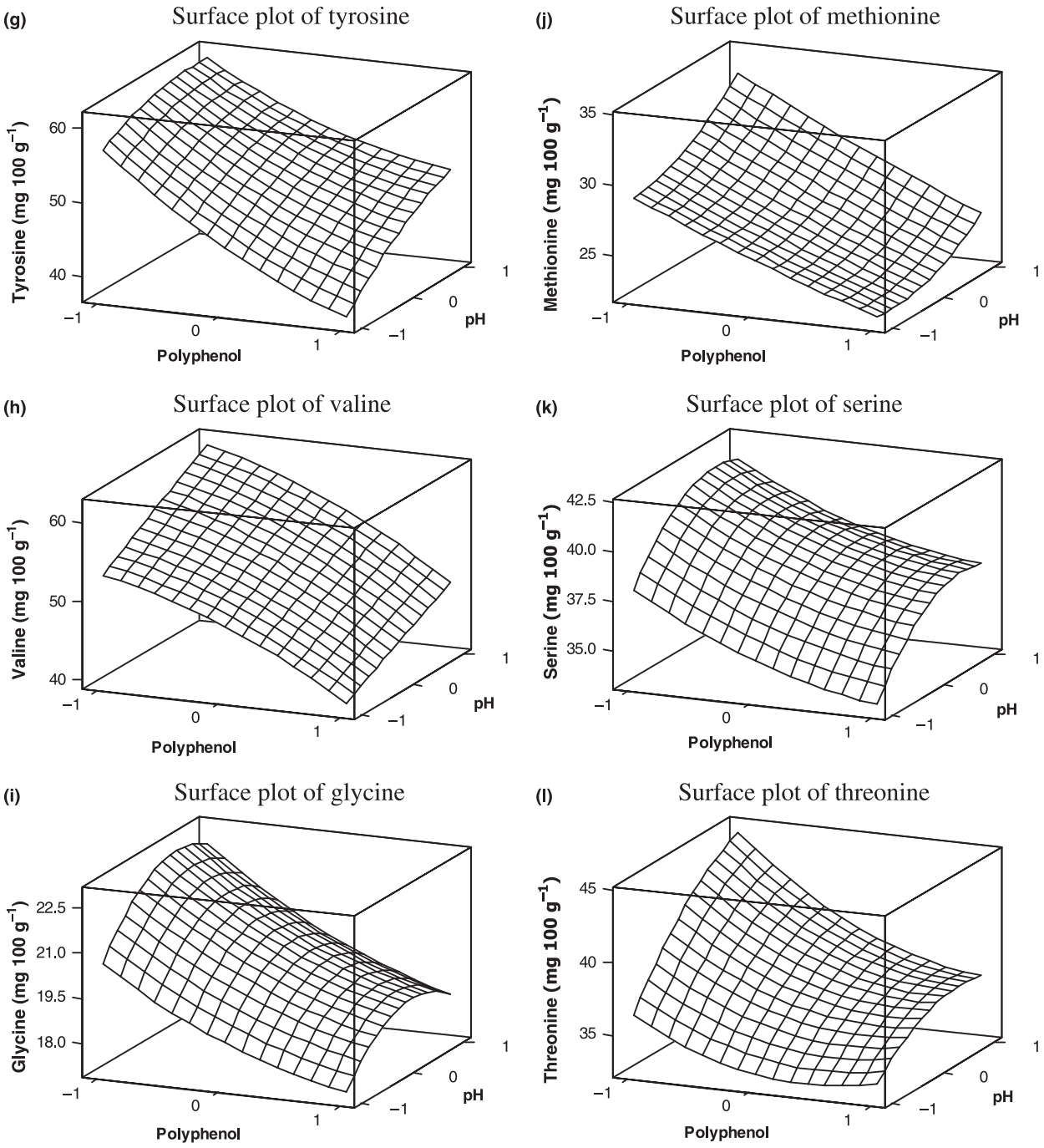

Figure 1 Continued.

owing to the formation of precipitates or aggregates. In addition to astringent taste, it also gives a puckering sensation and causes a less viscous sensation on the tongue (Clifford, 1985; Lindsay, 1996). The effect of polyphenol on these hydrophobic amino acids was explained by Charlton *et al.* (2002) that stated intermolecular binding of polyphenol–amino acid is dominated by staking of polyphenolic onto planar hydrophobic surface and strengthened by multiple cooperative binding of polyphenolic rings. Most of the literature suggests that hydrophobic and hydrogen bonding are responsible for polyphenol–protein interactions (Spencer *et al.*,

1988; Haslam & Lilley, 1988). Beart *et al.* (1985) have also suggested the ability of polyphenols to interact covalently with proteins.

In the group of others amino acids, it was found that glycine, methionine, serine and threonine had negative linear effect with polyphenol concentration and positive linear effect with pH (Fig. 1i–l). Negative quadratic term of pH was only found to be significant with glycine. Glycine and serine response surface showed a downward concavity, showing that medium level of pH favours higher concentration of these amino acids. This in agreement with Bates *et al.* (1998) that found low pH is not favourable in Maillard reaction. The pH strongly influences the amount of the amino acid in the unprotonated form, and thus, the initial condensation step of the Maillard reaction is increased by higher pH. Higher pH favours the reductone formation over furfural production from the Amadori products, leading to colour development. Only glycine showed significant function of the linear and quadratic effects of pH. The linear effect ($P \leq 0.05$) was positive, whereas the quadratic effect ($P \leq 0.05$) negative, which resulted in a curvilinear increase of pH.

The reduction of amino acids during roasting can also be attributed to the racemisation of the amino acids. D-Amino acid formation in foodstuff and beverages can be form by technological enrichment during processing, such as heat and pH (Friedman, 1999). Casal *et al.* (2005) found that in coffee roasting, asparagine, glutamine, lysine and alanine were highly racemised. The more stable amino acids, such as tyrosine, valine, leucine, phenylalanine and alanine remained almost constant for all the temperature assayed. Caligiani *et al.* (2007) reported a significant correlation between the level of racemisation and heating temperature of more than 180 °C; thus, the percentage of D-amino acid could be considered as a good chiral marker of the roasting process only if temperatures applied are higher than 180 °C.

The non-enzymatic browning reaction is dependent on temperature and water activity (a_w) of the food in addition to compositional factors, such as concentration and ratio of sugar/amino acids and pH (Özdemir *et al.*, 2001). The effect of polyphenol on the amino acids is attributed to the fact that polyphenol have a high propensity to form complexes with protein, polysaccharide and alkaloid (Hagerman & Butler, 1981). Phenolic hydroxyl group is an excellent hydrogen bond donor and forms bonds with amide carbonyls (Hagerman, 1992). There are five potential types of interaction between polyphenol and protein: hydrogen bonding, π-bonding, hydrophobic, ionic and covalent linkage, in which either one can potentially explain how the polyphenol affected the amino acids. In addition, another suggested means of finding the effect of polyphenol on amino acids is by the oxidation products of polyphenols known as quinones, which can also react with amino acids and proteins, or polymerise with each other to form higher molecular weight complexes, the so-called 'condensed tannin', whereas at molecular weight above 3000, they form complexes with protein through hydrogen bonding (Kattenberg & Kemmink, 1993). Higher polyphenol concentration was found to reduce the cocoa flavour (Misnawi *et al.*, 2004). It is suggested that the reduction of flavour compound was to the result of the binding of polyphenol on aroma precursors and aroma compounds formed during roasting. The flavour precursors binding hypothesis is based on the finding of Misnawi *et al.* (2004), which detected a decrease in free amino acids and reducing sugars in the cocoa bean with the increase in polyphenol concentration. Luna *et al.* (2002) also found that, besides being responsible for astringent and bitter sensations, polyphenol also contributed to green and fruity senses of cocoa liquor.

Maillard reaction is found to be a course of racemisation of amino acids, among which asparagine, glutamine, alanine, serine and phenylalanine were found to racemise fast in the browning reaction tested (Bruckner *et al.*, 2001). D-Alanine however can be found before the roasting process as it can form as characteristic of the bacterial peptidoglycan, indicating that cocoa fermentation can induce D-alanine racemisation (Caligiani *et al.*, 2007; Patzold & Bruckner, 2006).

Reduction of amino acids can also be attributed to the tentative mechanism *via* the formation of a carbanion in the Amadori compound (Patzold & Bruckner, 2004). The release of amino acids from Amadori compounds is reversible until amino acids are finally transferred at advanced stages of the Maillard reaction irreversibly into heterocyclic or polymeric compounds (Ali *et al.*, 2006). Degradation of free amino acids can occur throughout the deamination–decarboxylation steps (Strecker degradation) that will yield branched volatile compounds, including esters, alcohol and ketones (Virgili *et al.*, 2007). During decarboxylation, biogenic amines could also be produced (Bauza *et al.*, 1995; Maynard & Schenker, 1996). In the case of cocoa, the heterocyclic or polymeric compounds are usually flavour volatiles than is mainly pyrazines.

Optimisation of polyphenol concentration and pH for cocoa flavour precursors

The independent variables that have a complex relationship with the responses may have more than one maximum point (Table 4). An optimum condition of polyphenol concentration and pH is needed to have the most amounts of these flavour precursors, amino acids and sugars as they are important constituents in developing chocolate flavour. Figure 1 shows the

contour plots of sucrose, asparagine, arginine, histidine, alanine, iso-leucine, tyrosine, valine, glycine, methionine, serine, and threonine concentration.

Plain chocolate made from low pH (4.75–5.19) and high pH (5.5–5.8) cocoa beans presented low sensory response to chocolate flavour, and more off-flavour descriptors were perceived from samples made from low-pH cocoa beans. On the other hand, chocolate made from medium pH (5.20–5.49) cocoa beans received a high response in strong chocolate flavour (Jinap *et al*., 1995). Sensory response of chocolate flavour was classified as strong, moderate and weak, while other flavour descriptors were classified as acidic, bitter, fruity, burnt, hammy, musty, nutty and sweet. The hammy or smokey flavour could be caused by the adsorption of phenols from smoke during the drying of cocoa or be generated from isovaleric and isobutyric acids. Astringency, bitter and green flavours result from the presence of polyphenol compounds in cocoa (Bonvehi & Coll, 2000; Luna *et al*., 2002).

The approach in the determination of the overall optimum conditions in these multi-response problems is not straightforward. For this purpose, most researchers use the graphical approach of superimposing the different response surfaces and finding the experimental region that will give desired values of the responses (Arteaga *et al*., 1994). The response models obtained from this study can be overlaid with each other to find the polyphenol concentration and pH that will give higher level of the responses. However, owing to the large number of the responses involved, the superimposed contour plot did not yield any region to suggest the best polyphenol concentration and pH. In order to reduce the responses number, the study has only used hydrophobic amino acid responses, as these amino acids are specific to cocoa flavour production. Superimposing the contour maps gave the cross-hatched area shown in Fig. 2. It is suggested that the highest concentration of flavour precursors can be obtained at the optimum polyphenol concentration of 43–58 g kg^{-1} and pH of 7.0–7.5.

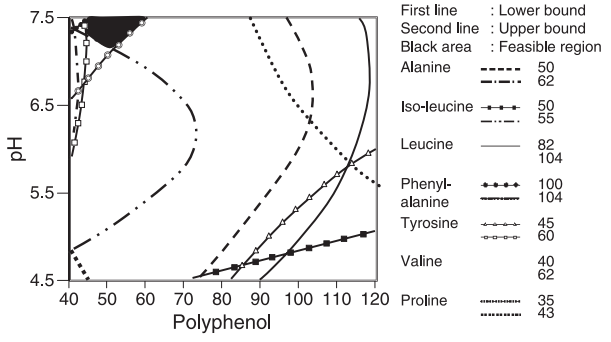

Figure 2 Superimposed contour plot of significant variables.

Conclusions

The RSM has been shown to be a useful tool to investigate the optimum concentration of polyphenol and pH on the production of cocoa Maillard-related flavour precursors; these variables are important in the production of high flavour compounds. Cocoa flavours are the result of numerous compounds reacting together and present in the bean. Through the Maillard reactions, varies cocoa aroma precursors interact to produce cocoa flavour components, such as alcohols, ethers, furans, thiazoles, pyrones, acids, esters, aldehydes, imines, amines, oxazoles, pyrazines and pyrroles. However, this study concentrates on two types of precursors involved, namely amino acids and sugars, together with two factors, polyphenol and pH. Polyphenol concentration and pH significantly affect all the responses. The model equation developed can be used for predicting the degradation of amino acids and sugars during roasting. Based on the constraints set, the best polyphenol concentration for the development of the Maillard-related cocoa flavour precursors is between 43 and 58 g kg^{-1} and pH 7.0–7.5.

References

Ali, H., Patzold, R. & Bruckner, H. (2006). Determination of L- and D-amino acids in smokeless tobacco products and tobacco. *Food Chemistry*, **99**, 803–812.

Arteaga, G.E., Li-Chan, E., Vazquez-Arteaga, M.C. & Nakai, S. (1994). Systematic experimental designs for product formula optimization. *Trends in Food Science & Technology*, **5**, 243–254.

Bates, L., Ames, J.M., Mac Dougall, D.B. & Taylor, P.C. (1998). Laboratory reaction cell to model Maillard reaction colour development in a starch-glucose-lysine system. *Journal of Food Science*, **63**, 1991–1996.

Bauza, T., Blaisse, A., Teissedre, P.L., Cabanis, J.C., Kanny, G. & Moneret-Vautrin, D.A. (1995). Les amines biogenes du vin, métabolisme et toxicité. *Bulletin de l'O.I V*., **767–768**, 42–67.

Beart, J.E., Lilley, T.H. & Haslam, E. (1985). Polyphenol interactions. Part 2. Covalent binding of procyanidins to proteins during acid-catalysed decomposition; observations of some polymeric procyanidins. *Journal of the Chemical Society, Perkin Transactions* **2**, 9, 1439–1443.

Bonvehi, J.S. & Coll, F.V. (1997). Evaluation of bitterness and astringency of polyphenolic compounds in cocoa powder. *Food Chemistry*, **60**, 365–370.

Bonvehi, J.S. & Coll, F.V. (2000). Evaluation of purine alkaloids and diketopiperazines contents in processed cocoa powder. *European Food Research and Technology*, **210**, 189–195.

Bonvehi, J.S. & Coll, F.V. (2002). Factor affecting the formation of alkylpyrazines during roasting treatment in natural and alkalized cocoa powder. *Journal of Agricultural and Food Chemistry*, **50**, 3743–3750.

Brands, C.M.J., Wedzicha, B.L. & van Boekel, M.A.J.S. (2002). The use of radiolabelled sugar to estimate the extinction coefficient of melanoidins formed in heated sugar-casein systems. *International Congress Series*, **1245**, 249–253.

de Brito, E. & Narain, N. (2003). Effect of pH and distillate volume on monitoring aroma quality of bittersweet chocolate. *Food Quality and Preference*, **14**, 219–226.

Bruckner, H., Justus, J. & Kirschbaum, J. (2001). accharide induced racemization of amino acids in the course of the Maillard reaction. *Amino Acids*, **21**, 429–433.

Caligiani, A., Cirlini, M., Palla, G., Ravaglia, R. & Arlorio, M. (2007). C-MS detection of chiral markers in cocoa beans of different quality and geographic origin. *Chirality*, **19**, 329–334.

Carabasa-Giribet, M. & Ibarz-Ribas, A. (2000). Kinetics of colour development in aqueous glucose systems at high temperatures. *Journal of Food Engineering*, **44**, 181–189.

Casal, S., Mendes., E., Oliveira, M.B.P.P. & Ferreira, M.A. (2005). oast effects on coffee amino acid enantiomers. *Food Chemisty*, **89**, 333–340.

Charlton, A.J., Baxter, N.J., Khan, M.L. *et al*. (2002). Polyphenol-peptide binding and precipitation. *Journal of Agriculture and Food Chemistry*, **50**, 1953–1601.

Chen, S.-L., Jin, S.-Y. & Chen, C.-S. (2005). Relative reactivities of glucose and galactose in browning and pyruvaldehyde formation in sugar/glycine model systems. *Food Chemistry*, **92**, 597–605.

Clifford, M.N. (1985). Phenol–protein interaction and their possible significance for astringency. In: *Interaction of Food Components* (edited by G.C. Birch & M.G. Lindley). Pp. 143–164. London and New York: Elsevier.

Cohen, S.A., Meys, M. & Tarvin, T.L. (1990). *The Pico-tag method – a manual of advanced techniques for amino acids analysis*. USA: Waters.

Friedman, M. (1999). hemistry, nutrition and microbiology of D-amino acids. *Journal of Agricultural ad Food Chemistry*, **47**, 3457–3479.

Friedman, M. & Liardon, R. (1985). acemization kinetics of amino acid residues in alkali-treated soybean proteins. *Journal of Agricultural and Food Chemistry*, **33**, 666–672.

Gao, Y.-L., Ju, X. -R. & Jiang, H.-H. (2006). Studies on inactivation of *Bacillus subtilis* spores by high hydrostatic pressure and heat using design of experiments. *Journal of Food Engineering*, **77**, 672–679.

Hagerman, A.E. (1992). Tannin-protein interaction. in: *Phenolic Compounds in Food and Their Effect on Health I: Analysis, Occurrence and Chemistry* (edited by C.T. Ho, C.Y. Lee & M.T. Huang). Pp. 237–247. ACS Symposium Series 506.

Hagerman, A.E. & Butler, L.G. (1981). The specificity of the pro-anthocyanins-protein interaction. *Journal of Biological Chemistry*, **256**, 4494–4497.

Hammerstone, J.F., Lazarus, S.A., Mitchell, A.E., Rucker, R. & Schmitz, H.H. (1999). Identification of procyanidins in cocoa (*Theobroma cacao*) and chocolate using high-performance liquid chromatography/mass spectrometry. *Journal of Agricultural and Food Chemistry*, **47**, 490–496.

Haslam, E. & Lilley, T.H. (1988). Natural astringency in foodstuffs – a molecular interpretation. *CRC Critical Reviews in Food Science and Nutrition*, **27**, 1–40.

Hofmann, T., Munch, P. & Schieberle, P. (2000). Quantitative model studies on the formation of aroma-active aldehydes and acids by Strecker-type reactions. *Journal of Agriculture and Food Chemistry*, **48**, 3761–3766.

Hoskin, J.C. & Dimick, P.S. (1994). Chemistry of flavour development in chocolate. In: *Industrial Chocolate Manufacture and Use* (edited by S.T. Beckett), 2nd edn. New York: Van Nostrand Reinhold.

Hunt, R.S., Jackson, P.A., Mortlock, R.E. & Kirtc, R.S. (1977). Quantitative determination of sugars in foodstuff by high performance liquid chromatography. *Analysts*, **102**, 917–920.

Jinap, S., Dimick, P.S. & Hollender, R. (1995). Flavour evaluation of chocolate formulated from cocoa beans from different countries. *Food Control*, **6**, 105–110.

Jinap, S., Wan-Rosli, W.I., Russly, A. R. & Nurdin, L.M. (1998). Effect of roasting time and temperature on volatile components profile during nib roasting of cocoa beans (*Theobroma cacao*). *Journal of the Science and Food Agriculture*, **77**, 441–448.

Kattenberg, H.R. & Kemmink, A. (1993). The flavor of cocoa in relation to the origin and processing of the cocoa beans. In: *Food Flavor, Ingredients and Composition* (edited by G. Charalambous). Pp. 1–22. New York: Elsevier.

King, V.A.-E. & Zall, R.R. (1992). A response surface methodology approach to the optimization of controlled low-temperature vacuum dehydration. *Food Research International*, **25**, 1–8.

Kirchhoff, P.M., Biehl, B. & Crone, G. (1989). Peculiarity of the accumulation of free amino acids during cocoa fermentation. *Food Chemistry*, **31**, 295–311.

Lee, M.-J., Prabhu, S., Meng, X., Li, C. & Yang, C.S. (2000). An improved method for the determination of green and black tea polyphenols in biomatrices by high-performance liquid chromatography with coulometric array detection. *Analytical Biochemistry*, **279**, 164–169.

Liardon, R. & Jost, R. (1981). Racemization of free and protein bound amino acids in strong mineral acid. *International Journal of Peptide and Protein Research*, **18**, 500–505.

Lindsay, R.C. (1996). Flavor. In: *Food Chemistry* (edited by O.R. Fennema), 3rd edn. New York: Marcel Dekker.

Luna, F., Crouzillat, D., Cirou, L. & Bucheli, P. (2002). Chemical composition and flavour of Ecuadorian cocoa liquor. *Journal of Agriculture and Food Chemistry*, **50**, 3527–3532.

Macrane, R., Robinson, R.K. & Saadler, M.J. (1993). *Encyclopedia of Food Science, Food Technology and Nutrition (vol. 1)*. Pp. 146–166. London: Academic Press Limited.

Madamba, P.S. (2002). The response surface methodology: an application to optimize dehydration operations of selected agricultural crops. *Lebensmittel-Wissenschaft und Technolgie*, **35**, 584–592.

Martins, S.I.F.S., Jongen, W.M.F. & van Boekel, M.A.J.S. (2000). A review of Maillard reaction in food and implications to kinetic modeling. *Trends in Food Science & Technology*, **11**, 364–373.

Maynard, L.S. & Schenker, V.J. (1996). Monoamine-oxidase inhibition by ethanol in vitro. *Nature*, **196**, 575–576.

Mcmanus, J.P., Davis, K.G., Beart, J.E., Gaffney, S.H., Lilley, T.H. & Haslam, E. (1985). Polyphenol interactions. Part 1. Introduction: Some observations on the reversible complexation of polyphenols with proteins and polysaccharides. *Journal of the Chemical Society, Perkin Transaction* **2**, 9, 1429–1438.

Misnawi, Jinap, S., Jamilah, B. & Nazamid, S. (2004). Sensory properties of cocoa liquor as affected by polyphenol concentration and duration of roasting. *Food Quality and Preference*, **15**, 403–409.

Myers, R.H. & Montgomery, D.C. (2002). *Response surface methodology: process and product optimization using designed experiments*, 2nd edn. Pp. 17–84. New York: John Wiley and Sons Inc.

Oomah, B.D. & Mazza, G. (2001). Optimization of a spray drying process for flaxseed gum. *International Journal of Food Science and Technology*, **36**, 135–143.

Özdemir, M., Seyhan, F.G., Bakan, A.K., İlter, S., Özay, G. & Devres, O. (2001). Analysis of internal browning of roasted hazelnuts. *Food Chemistry*, **73**, 191–196.

Patzold, R. & Bruckner, H. (2004). echanistiche Aspekte und Konsequenzen der Bildung von D-Aminosauren im Verlaufe der Maillardreaktion. *Lebensmittelchemie*, **58**, 100.

Patzold, R. & Bruckner, H. (2006). as chromatographic determination and mechanism of formation of D-amino acids occurring in fermented and roasted cocoa beans, cocoa powder, chocolate and cocoa shells. *Amino Acids*, **31**, 63–72.

Puziah, H., Jinap, S., Sharifah, K.S.M. & Asbi, A. (1998a). Changes in free amino acids, peptide-N, sugar and pyrazine concentration during cocoa fermentation. *Journal of the Science of Food and Agriculture*, **78**, 535–542.

Puziah, H., Jinap, S., Sharifah-Kharidah, S.M. & Asbi, A. (1998b). ffect of mass and turning time on free amino acid, peptide-N, sugar and pyrazine concentration during cocoa fermentation. *Journal of the Science of Food and Agriculture*, **78**, 543–550.

Rastogi, N.K. & Rashimi, K.R. (1999). ptimization of enzymatic liquefaction of mango pulp by response surface methodology. *European Food Research and Technology*, **209**, 57–62.

Rohan, T.A. & Stewart, T. (1967). The precursors of chocolate aroma: production of free amino acids during fermentation of cocoa beans. *Journal of Food Science*, **32**, 395–398.

Sanbogi, C., Osakabe, N., Natsume, M., Takizawa, T., Gomi, S. & Osawa, T. (1998). Antioxidative polyphenols isolated from *Thoebroma cocoa*. *Journal of Agricultural and Food Chemistry*, **46**, 454–457.

Santos-Buelga, C. & Scalbert, A. (2000). Proanthocyanidins and tannin-like compounds: nature, occurrence, dietary intake and effects on nutrition and health. *Journal of the Science of Food and Agriculture*, **80**, 1094–117.

Shibamoto, T. & Bernhard, R.A. (1977). nvestigation of pyrazine formation pathways in glucose-ammonia model systems. *Agricultural Biology and Chemistry*, **41**, 143–153.

Siebert, K.J., Troukhanova, N.V. & Lynn, P.Y. (1996). Nature of polyphenol-protein interactions. *Journal of Agricultural and Food Chemistry*, **44**, 80–85.

Singleton, V.L. & Rossi, J.A. Jr. (1965). Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *American Journal of Enology and Viticulture*, **16**, 144–158.

Spencer, C.M., Cai, Y., Martin, R. *et al*. (1988). Polyphenol complexation: some thoughts and observations. *Phytochemistry*, **27**, 2397–2409.

Stenberg, M., Marko-Varga, G. & Oste, R. (2002). nantioseparation of D- and L-amino acids by a coupled system consisting of an ion-exchange column and a chiral column and determination of D-aspartic acid and glutamic acid in soy product. *Food Chemistry*, **79**, 507–512.

Takano, Y., Koayashi, K., Ishikawa, Y. & Marumo, K. (2006). mergence of the inflection point on racemization rate constant for D- and L-amino acids in the early stages of terrestrial diagenesis. *Organic Geochemistry*, **37**, 334–341.

Unal, B., Metin, S. & Işıklı, N.D. (2003). Use of response surface methodology to describe the combined effect of the storage time, locust bean gum and dry matter of milk on the physical properties of low-fat set yoghurt. *International Dairy Journal*, **13**, 909–916.

Vernhet, A., Pellerin, P., Prieur, C., Osmianski, J. & Moutounet, M. (1996). Change properties of some grape and wine polysaccharide and polyphenolic fractions. *American Journal of Enology and Viticulture*, **47**, 25–30.

Virgili, R., Saccani, G., Gabba, L., Tanzi, E. & Bordini, C.S. (2007). hanges of free amino acids and biogenic amines during extended ageing of Italian dry-cured ham. *LWT*, **40**, 871–878.

Voigt, J., Biehl, B. & Kamaruddin, S. (1993). The major seed protein of *Theobroma cacao* L. *Food Chemistry*, **47**, 145–147.

Wollgast, J. & Anklam, E. (2000). Review on polyphenols in *Theobroma Cacao*: changes in composition during the manufacture of chocolate and methodology for identification and quantification. *Food Research International*, **33**, 423–447.

Yoo, M.A., Kim, H.W., Kim, K.H. & Kang, M.H. (2004). Antioxidant effect of brown substances separated from defatted roasted sesame dregs. *Food Science and Biotechnology*, **13**, 274–278.

Ziegleder, G. & Biehl, B. (1988). Analysis of cocoa flavour components and flavour precursors. In: *Analysis of Non Alcoholic Beverages, Methods of Plant Analysis*, **vol. 8**. (edited by H.F. Lickens & J.F. Jackson). Pp. 321–393. Springer Verlag, Heidelberg, Germany.