Fermentation of cocoa beans: influence of microbial activities and polyphenol concentrations on the flavour of chocolate

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Abstract

BACKGROUND: Spontaneous cocoa bean fermentation is characterised by a succession of microbial activities. Cocoa flavour precursors are developed during fermentation and drying of cocoa beans. Polyphenols and alkaloids contribute to astringency and bitterness of cocoa and chocolate.

RESULTS: Population dynamics, metabolite target analyses, and chocolate production were performed for seven independent spontaneous cocoa bean heap fermentations in Ghana. Although the same micro-organisms were involved in these heaps, carried out at different farms or in different seasons, heap temperatures and microbial metabolite concentrations were different. This could be due to heterogeneity and size of the heaps, but was mainly ascribed to microbial variability. Indeed, differences in microbial activity could be linked with the flavour of chocolates made from the corresponding dried, fermented cocoa beans. Whereas the polyphenol and alkaloid contents of cocoa beans were crop- and heap-dependent, epicatechin and theobromine levels decreased during fermentation due to diffusion out of the bean cotyledons and polyphenol oxidation and condensation. Residual levels were responsible for the degree of bitterness of the final chocolates.

CONCLUSION: Differences in microbial activities between different heap fermentations can result in dried fermented cocoa beans and chocolates with different flavour characteristics. Hence, fermentation control may direct the flavour of chocolate.

Keywords: cocoa fermentation; polyphenols; alkaloids; chocolate production; epicatechin; catechin

INTRODUCTION

The popularity of cocoa and cocoa-derived products, in particular chocolate, can be ascribed to the unique and complex flavours of these delicious foods.[1–3] The flavours and, in particular, the flavour precursors of cocoa are developed during primary processing of the cocoa beans, i.e., fermentation and drying. This development of flavour precursors involves the action of various micro-organisms in the cocoa pulp and the action of enzymes on carbohydrates, proteins and polyphenols in the cocoa beans. Unlike many other fermented raw materials, endogenous enzymes play a crucial role in cocoa flavour development. However, there is no flavour in cocoa beans without fermentation.[4] During cocoa bean fermentation, the role of micro-organisms is limited to removal of the pulp that surrounds the fresh beans and the production of indispensable metabolites.[5] The former includes pectin depolymerisation by yeasts. The latter encompasses anaerobic yeast fermentation of sugars to ethanol, microaerophilic fermentation of sugars and citric acid to lactic acid, acetic acid and mannitol by lactic acid bacteria (LAB), and aerobic exothermic bioconversion of ethanol into acetic acid by acetic acid bacteria (AAB). These microbial activities result in the death of the bean due to penetration of mainly ethanol and acetic acid through the husk into the cotyledons, and the creation of an environment, i.e., a decrease of internal pH from 6.5 to 4.8, an increased bean temperature up to 50 °C and a damaged internal cocoa bean structure, for development of flavour precursors and pigment degradation by endogenous enzymes, such as invertase, glycosidases, proteases and polyphenol oxidase.[6–9] Other microbial metabolites,

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such as esters and pyrazines, may enter the bean cotyledons and act as flavour precursors or directly as flavour compounds. As a result of these biochemical reactions in the cocoa beans an additional number of flavour precursors are formed, in particular reducing sugars, peptides, and amino acids, that are further modified through Maillard reactions during roasting of well-fermented, dried cocoa beans.[8,10] For instance, storage proteins are hydrolysed by an endogenous aspartic endoprotease and carboxypeptidase with different pH and temperature optima, releasing hydrophilic oligopeptides and free hydrophobic amino acids. The sugars come from sucrose and its hydrolysis products, glucose and fructose, by both cotyledon and pulp invertase activity, in addition to being released from glycosides.

Alkaloids, in particular the methylxanthines caffeine and theobromine, and polyphenolic compounds, in particular proanthocyanidins and flavan-3-ols (epicatechin and catechin), impart bitterness and astringency in cocoa.[1,3,9,11] However, there is a significant reduction in bitterness and astringency as a result of diffusion of alkaloids (30% fall) and polyphenols (20% fall) out of the beans during fermentation. Cocoa bean polyphenols are stored in so-called polyphenol storage cells or pigment cells of the cotyledons. These pigment cells are white to deep purple, depending on the amount of anthocyanins, a minor class of cocoa bean polyphenols. During fermentation of the cocoa beans, polyphenols diffuse with cell liquids from their storage cells and undergo oxidation and complexation into high molecular mass, mostly insoluble tannins. Anthocyanins are rapidly hydrolysed to anthocyanidins and sugars (galactose and arabinose) by glycosidases. This accounts for bleaching of the purple colour of the cotyledons. Polyphenol oxidases convert the polyphenols (mainly epicatechin and free anthocyanidins) into quinones. Polyphenols and quinones form complexes with other polyphenols, proteins, and peptides. This decreases their solubility and astringency and gives rise to the brown coloration of the beans that is typical of well-fermented cocoa beans. In quality control applications, anthocyanin content has been considered as a good marker for fermentation of cocoa beans, along with the formation of a brown colour. Also, changes of cocoa bean colour are widely used to predict flavour potential of cocoa beans and hence their suitability for chocolate manufacture. Therefore, cut test and fermentation index measurements are based on colour changes in cotyledons during fermentation.[12,13]

The cocoa flavour precursors are developed into chocolate flavour by roasting the dried, deshelled beans or nibs, in particular through Maillard reactions.[3,14] Roasted beans are then further processed into cocoa liquor, cocoa butter, and cocoa powder, whereby conching (to knead the mixture of cocoa mass and sugar and remove acidic volatiles) is an important step.[15] Cocoa processing in user countries and, in particular, the manufacture of chocolate in relation to changes in composition and content of flavours has so far not been much object of investigation. However, it is well known that the cocoa bean type influences cocoa flavour and that, for instance, higher processing temperatures and/or longer processing times reduce the amount of polyphenols available in cocoa components.[7,11,16] Besides the cocoa bean type, cocoa flavour is influenced by fermentation (post-harvest pod storage, bean packing or spreading, fermentation method, and duration).[6,16–20] For instance, pod storage and duration of fermentation will affect pH and temperature during fermentation, thus influencing enzyme activities and flavour development and hence acidity, bitterness and astringency of the processed cocoa beans.[8,17] It is indeed well known that the time period of enzyme action is short during fermentation, as enzymes are strongly inactivated (aminopeptidase, invertase and polypenol oxidase) or partly inactivated (carboxypeptidase) upon fermentation, except for endoprotease and glycosidases that remain active during the whole fermentation process.[8] For instance, if the pH becomes too acid too soon ($pH < 4.5$) there will be both a final reduction in flavour precursors and an over-acid final product.

The aim of the present study was to assess the influence of fermented, dried cocoa beans from heap fermentations performed by two different farmers in two different seasons on final chocolate production to establish a potential relationship between cocoa and chocolate flavour generated, fermentation metabolites produced, and microbial species present.

EXPERIMENTAL METHODS

Spontaneous cocoa bean fermentation and sampling

Seven spontaneous cocoa bean fermentations of 250–500 kg of wet beans (heap method) were carried out at two small farms (A and B), located about 15 km from each other near New Tafo and Old Tafo, respectively, in Ghana, in 2004. During the mid-crop, fermentations at farm A were followed twice (heaps 1 and 3) and those at farm B once (heap 2), while during the main-crop fermentations at both farm A (heaps 4 and 6) and B (heaps 5 and 7) were followed twice. An overview of important heap parameters, including fermentation and drying, is given in Table 1. For a detailed description of the fermentation set-up, sampling and analysis (microbiological analysis, including cell count enumeration, isolate identification, and culture-independent 16S rRNA PCR-DGGE, and physico-chemical analysis, including temperature, pH, rain fall, residual sugars, and amounts of citric acid, ethanol, lactic acid, acetic acid, succinic acid, and mannitol produced), see Camu *et al*.[21] During the present study, polyphenol, theobromine and caffeine determinations on samples taken at different time points during fermentation were analysed (see below). Also, 30 kg of fermented, dried beans were shipped to

Table 1. Characteristics of the seven spontaneous cocoa bean heap fermentations carried out. A questionnaire was used to obtain the necessary information from the farmers regarding the harvest of their cocoa pods, fermentation heaps and drying time. Heap size (initial and final weight), heap dimensions (diameter and height), rainfall, and fermentation temperatures were measured

	Heap dimensions							
Heap number	Diameter (cm)	Height (cm)	Post-harvest pod age (days)	Rainfall (L m^{-2} in 6 days)	Drying time (days)	Final weight (kg)	Initial temp (°C)	Max fermentation temp (°C)
1	170	55	2–3	16	14	60	30.0	42.2
2	135	46	2–3	18	14	42	25.7	42.6
3	105	45	2	56	10	40	28.7	42.8
4	120	52	2–3	10	10	52	24.0	44.7
5	180	64	2–3	28.5	10	200	23.0	44.3
6	95	40	3	29	10	33	26.0	44.1
7	135	40	3	12	10	63	26.4	44.3

Barry Callebaut France for chocolate production (see below).

Quality assessment of fermented, dried cocoa beans

Fermented, dried cocoa beans were checked for appearance (bean count per 300 g of beans, bean size, physical damage, insect penetration) and quality in Ghana (Quality Division of the cocoa board; COCOBOD, Accra, Ghana) and by Barry Callebaut Belgium, making use of the cut test.[12] Therefore, a total of 300 beans were cut lengthwise through the middle to expose the maximum cut surface of the cotyledons. Both halves were examined in full daylight and placed in one of the following categories: fully brown (fermented); partly brown, partly purple (partly fermented); purple (under-fermented); slaty (not fermented); insect damaged; mouldy; or germinated.

Metabolite target analysis of fermented, dried cocoa beans

Fermentation samples were analysed for polyphenols, theobromine and caffeine. Both well-fermented cocoa beans and fermented, dried beans were analysed for sugars, organic acids (citric acid, lactic acid, and acetic acid), polyphenols, caffeine and theobromine.

Sugars and organic acid concentrations were determined by high-performance anion exchange chromatography (HPAEC) with pulsed amperometric detection (PAD) and conductivity measurement under ion suppression (CIS), respectively, according to the methods described previously.[21]

Polyphenols and alkaloids were determined on extracts of dried, defatted nibs by high pressure liquid chromatography (HPLC) using a Nucleosil 100-5 C18 column (250 × 4 mm, particle size of 5 μm; AIT, Nucleosil, France). Defatting was performed as follows. Nib samples were milled in a coffee mill to a powder of particles of 2 mm size; powder (5 g) was suspended in heated (60–80 °C) petroleum ether (20 mL) for 3 h and centrifuged (1500 × *g*, 15 min). After removal of the petroleum ether layer, the previous step was repeated twice. The defatted residue (cocoa powder) was air-dried and stored at −20 °C before being extracted in boiling water for 1 h at a concentration of 20 mg mL^{-1}. After cooling to room temperature, the samples were centrifuged, yielding the final extract for analysis.

The total polyphenol content was quantified by oxidation of polyphenols (0.1 mL of extract) with Folin–Ciocalteu reagent.[22] The absorbance of the solution was measured at 760 nm against a reference sample. Results are expressed in milligrams of epicatechin equivalents per gram of dry mass of defatted material, as linear standard curves were obtained for a solution of epicatechin in the concentration range of 5–20 mg L^{-1}.

Concentrations of epicatechin and catechin were determined by HPLC and fluorescence detection (excitation at 274 nm and emission at 322 nm). Before analysis, defatted cocoa (4 g L^{-1}) was dissolved in 90% (vol/vol) water plus 2% (vol/vol) acetic acid (pH 2.5) and 10% (vol/vol) acetonitrile, placed in an ultrasonic bath for 10 min, and filtered with a 0.45 μm cellulose filter. The standards were treated under the same conditions at 200 and 400 mg L^{-1}. The mobile phase, at a flow rate of 1.0 mL min^{-1}, consisted of water plus acetic acid (pH 2.5, eluent A) and acetonitrile (eluent B) with the following gradient: 0.0 min, 90% A and 10% B; 20.0 min, 85% A and 15% B. Quantification was performed by external calibration with standard solutions of epicatechin (52 mg L^{-1}) and catechin (22 mg L^{-1}). Results are expressed in milligram components per gram of cocoa product.

Concentrations of theobromine and caffeine were determined using a modified version of the HPLC method described by Kreiser and Martin.[23] A mobile phase of water/acetonitrile (80:20, vol/vol) at a flow rate of 1 mL min^{-1} and UV detection at 254 nm were applied. Quantification was performed by external calibration with standard solutions of theobromine (0.08 mg mL^{-1}) and caffeine (0.02 mg mL^{-1}).

All determinations were performed in triplicate. Standard deviations are indicated below.

Chocolate production and sample analysis

Chocolate was made in the pilot plant of Barry Callebaut France (Louviers, France). Fermented,

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dried beans (30 kg) were roasted for 30 min in a pilot roaster using an air temperature of 130–140 °C. The beans were deshelled by breaking and winnowing the beans, the nibs were ground into cocoa liquor using a ball mill, and passed through a 20-mesh-sized screen. The cocoa liquor was then mixed with cane sugar and passed through a three-roll refiner. Deodorised cocoa butter was melted at 60 °C and partly mixed for 15 min with the sugar–cocoa mixture before passing the refiner. The recipe used for chocolate production consisted of 42% (wt/wt) cocoa liquor, 46% (wt/wt) sugar, and 11.4% (wt/wt) cocoa butter, with the addition of 0.6% (wt/wt) lecithin and 0.03% (wt/wt) vanillin. The chocolate mix was conched at 50 °C for 2 h with addition of cocoa butter, lecithin and vanillin, subsequently tempered, and finally stored at room temperature until further analysis.

Chocolate sensory analysis by a taste panel

Sensory analysis of the chocolates was performed by a trained panel of eight members of Barry Callebaut Belgium. Characteristic flavour notes of the samples were recorded and discussed during the sessions. The flavour descriptors were acid, fruity, cocoa, bitter, flowery, taste intensity, and intensity of after-taste, expressed as numerical values between 0 and 100. As a final parameter, the after-taste was described. Before analysis, chocolate samples were brought to a temperature of 45 °C to allow them to melt before sensory evaluation. A maximum of four chocolate samples were evaluated during each session to reduce perception fatigue. Water was used for rinsing the mouth between different samples. The panel members compared each sample tasted with an internal reference sample. This reference sample has certain scores for the flavour descriptors mentioned above.

RESULTS

Metabolite dynamics during spontaneous cocoa bean fermentation

Fermentation courses of metabolites were heap-dependent, indicating microbial variability at both species and strain level (Table 2).[21] Only slight differences occurred with respect to heap size (except for heap 5, which was twice as big as the other heaps), rainfall (low for heaps 1, 2, 4 and 7; moderate for heaps 5 and 6; and heaviest for heap 3), and fermentation temperature (highest at the end of the

Table 2. Population and metabolite dynamics of spontaneous Ghanaian cocoa bean heap fermentations

	Yeast (log CFU g^{-1})			LAB (log CFU g^{-1})			AAB (log CFU g^{-1})		
Heap number	Begin	Max	End	Begin	Max	End	Begin	Max	End
1	4.98	7.30	0.00	5.66	8.90	5.95	2.48	7.13	5.69
2	6.45	7.86	3.93	6.51	8.89	5.97	2.30	7.53	4.89
3	6.94	7.30	4.76	7.38	8.70	6.38	0.00	6.24	4.95
4	7.05	7.20	4.49	8.05	8.66	6.53	2.48	6.97	5.95
5	6.32	7.81	3.85	6.82	8.68	6.21	2.48	6.89	5.03
6	4.37	7.41	3.08	4.48	8.75	6.03	2.00	6.95	6.22
7	6.74	7.45	3.72	6.98	8.37	6.28	4.58	6.53	6.18

	Citric acid pulp (mg g^{-1})		Ethanol pulp (mg g^{-1})		Lactic acid pulp (mg g^{-1})		Acetic acid pulp (mg g^{-1})	
	Begin	End	Begin	End	Begin	End	Begin	End
1	6.17	0.12	0.25	5.60	0.25	5.60	0.11	3.60
2	6.07	0.31	0.03	5.50	0.03	5.50	0.12	7.25
3	6.33	0.18	0.18	5.50	0.18	5.50	0.18	12.50
4	5.87	0.65	0.18	4.45	0.18	4.45	0.43	9.40
5	7.98	1.26	0.05	8.15	0.05	8.15	0.12	5.95
6	9.00	1.47	0.00	6.40	0.00	6.40	0.07	9.40
7	9.18	1.91	0.00	3.53	0.00	3.53	0.52	4.83

	Citric acid beans (mg g^{-1})		Ethanol beans (mg g^{-1})		Lactic acid beans (mg g^{-1})		Acetic acid beans (mg g^{-1})	
	Begin	End	Begin	End	Begin	End	Begin	End
1	5.32	3.23	0.33	4.02	0.00	0.00	4.75	4.75
2	6.50	3.48	0.07	8.01	0.03	0.03	6.73	6.73
3	3.18	3.27	0.23	1.90	0.00	0.00	11.15	11.15
4	3.98	2.70	0.74	2.65	0.63	0.63	6.65	6.65
5	5.48	3.30	0.50	7.09	0.08	0.08	6.42	6.42
6	4.17	2.78	0.23	5.79	0.30	0.30	9.20	9.20
7	6.00	2.02	0.95	6.57	0.22	0.22	5.20	5.20

heap 4 fermentation and lowest for the fermentations performed during the mid-crop) (Table 1).

At the end of fermentation, the cocoa beans from all heaps contained ethanol, lactic acid, acetic acid and citric acid, their concentrations being dependent on the heap (Table 2). For instance, whereas the cocoa bean concentrations of citric acid and lactic acid (except for heap 7 that showed lower amounts) were comparable for all heaps, cocoa beans from heap 3 showed a high acetic acid content and a concomitant low ethanol content. Whereas the amounts of ethanol and acetic acid were always slightly higher in the pulp than in the beans, the amount of lactic acid was considerably higher in the pulp than in the beans.

During fermentation, the polyphenol content of the beans decreased (Fig. 1). The total content of polyphenols at the start of fermentation ranged from an average of 4.10 to 4.83% (wt/wt) during mid-crop 2004, and from an average of 13.07 to 16.11% (wt/wt) during main-crop 2004. After 144 h of fermentation, the concentration of polyphenols averaged from 2.95 to 4.08% (wt/wt) for the mid-crop fermentations, and from 6.57 to 10.11% (wt/wt) for the main-crop fermentations. However, fermentation had a different influence on the epicatechin and catechin contents of the beans. Concentrations of catechin ($\pm 0.16\,mg\,g^{-1}$) hardly changed during fermentation, whereas epicatechin concentrations started from $\pm 11\,mg\,g^{-1}$ in the beans and showed a linear decrease from the start of the fermentation (Fig. 2). After 144 h of fermentation more than 70% of the initial concentration of epicatechin was lost.

The contents of theobromine and caffeine at the start of the fermentations ranged from 0.84 to $1.33\,mg\,g^{-1}$ and from 0.06 to $0.10\,mg\,g^{-1}$, respectively, during mid-crop 2004, and from 1.98 to $2.85\,mg\,g^{-1}$ and from 0.18 to $0.29\,mg\,g^{-1}$, respectively, during main-crop 2004 (Fig. 3). Through the entire fermentation process the concentrations of caffeine remained approximately the same ($\pm 0.3\,mg\,g^{-1}$), whereas the amounts of theobromine always decreased upon fermentation. The total loss of theobromine amounted up to 20% after 144 h of fermentation.

Metabolite target analysis of dried fermented cocoa beans

Dried, fermented cocoa beans did not contain sucrose and showed a more or less double amount of fructose than glucose (Table 3). Glucose, fructose and mannitol concentrations ranged from 1.61 to $3.09 \pm 0.08\,mg\,g^{-1}$, from 3.40 to $6.53 \pm 0.14\,mg\,g^{-1}$, and from 0.46 to $2.70 \pm 0.02\,mg\,g^{-1}$, respectively.

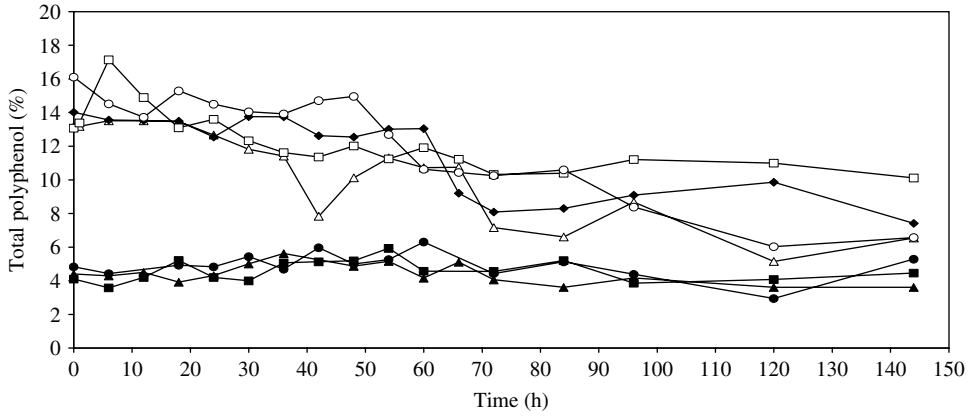

Figure 1. Course of the total polyphenol content (in %, expressed in epicatechin equivalents) during seven spontaneous cocoa bean heap fermentations (Heap 1, ■; Heap 2, ▲; Heap 3, ●; Heap 4, ♦; Heap 5, □; Heap 6, ○; Heap 7,△).

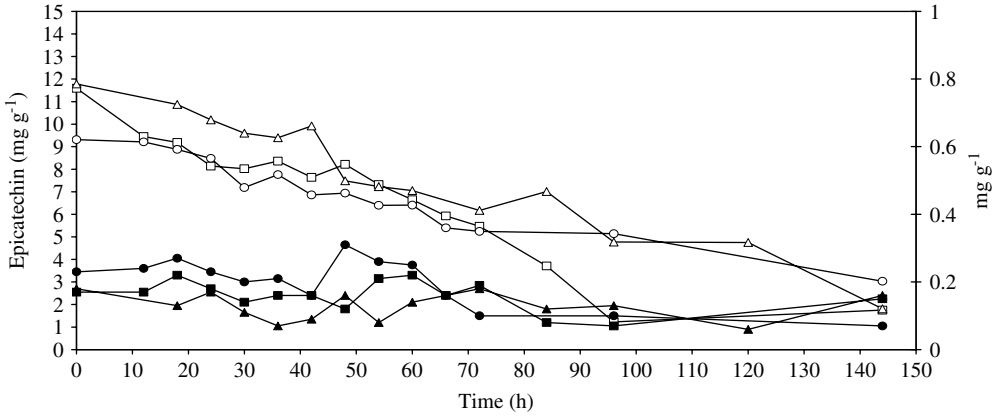

Figure 2. Course of epicatechin (H1 = □, H2 = ○, H3 = △) and catechin (H1 = ■, H2 = ●, H3 = ▲) during the spontaneous cocoa bean heap fermentations 1, 2 and 3.

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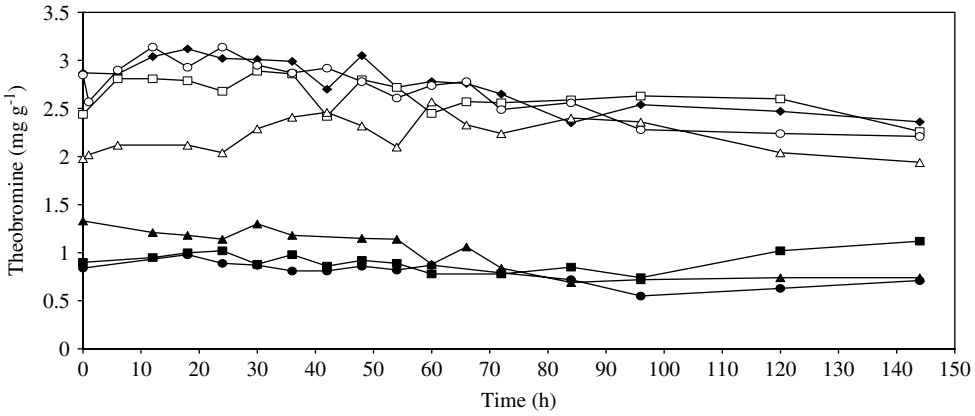

Figure 3. Course of the theobromine content during seven spontaneous cocoa bean heap fermentations (Heap 1, ■; Heap 2, ▲; Heap 3, ●; Heap 4, ♦; Heap 5, □; Heap 6, ○; Heap 7, △).

Table 3. Concentrations of organic acids, sugars, mannitol, ethanol, polyphenols and alkaloids in dried fermented cocoa beans

Heap number	Citric acid (mg g^{-1})	Acetic acid (mg g^{-1})	Lactic acid (mg g^{-1})	Glucose (mg g^{-1})	Fructose (mg g^{-1})	Ethanol (mg g^{-1})	Mannitol (mg g^{-1})
1	5.80	4.12	2.77	1.61	4.72	0.27	1.05
2	3.95	5.07	2.30	1.46	3.74	0.38	1.33
3	4.80	3.32	3.67	1.20	3.92	0.20	0.48
4	4.93	6.95	3.90	1.13	4.68	0.47	2.70
5	5.53	3.55	3.42	1.19	3.40	0.49	0.77
6	5.82	7.87	2.45	2.21	5.65	0.50	0.53
7	7.58	7.73	1.92	3.09	6.53	0.51	0.46

	Fat (%)	Polyphenols (%)	Epicatechin (mg g^{-1})	Catechin (mg g^{-1})	Theobromine (mg g^{-1})	Caffeine (mg g^{-1})	Theobromine/caffeine ratio
1	53.02	3.09	1.02	0.07	0.98	0.11	8.90
2	55.04	2.99	0.75	0.06	0.99	0.13	7.60
3	53.37	2.41	0.51	0.06	0.93	0.10	9.30
4	56.37	2.69	1.18	0.10	1.09	0.09	12.10
5	56.17	2.20	0.50	0.07	1.08	0.08	13.50
6	54.84	2.42	0.70	0.06	1.06	0.09	11.80
7	55.80	1.97	0.46	0.04	1.00	0.08	12.90

After drying, ethanol was barely present in the beans due to its evaporation. The beans from heaps 1 to 3 and 4 to 7 showed similar ethanol levels, $\pm 0.2\,mg\,g^{-1}$ and $\pm 0.48\,mg\,g^{-1}$, respectively. Citric acid concentrations were higher in dried, fermented beans as compared with beans at the end of fermentation, indicating a concentration effect by drying (Table 3, Fig. 4). Acetic acid concentrations after drying were between 3.32 ± 0.09 and $7.87 \pm 0.09\,mg\,g^{-1}$, being highest and lowest for beans from heaps 6 and 2, respectively (Fig. 4). The concentrations of lactic acid were considerably lower than those of acetic acid, except for the dried, fermented beans of heaps 3 and 5, which contained approximately the same amount of lactic acid and acetic acid (Fig. 4). Beans from heaps 4 and 7 showed the highest and lowest concentrations of lactic acid, respectively.

After drying of the beans the polyphenol content of the dried beans further decreased and reached a concentration of an average of 1.97–3.09% (wt/wt), with beans from heap 7 possessing the lowest concentration of polyphenols and those from heap 1 possessing the highest polyphenol concentration (Table 3). During drying another ±50% of epicatechin and ±60% of catechin were lost.

The concentration of theobromine after drying ranged from an average of $0.93–1.09\,mg\,g^{-1}$, cocoa beans from heap 4 possessing the highest concentration and those from heap 1 possessing the lowest concentration (Table 3). The concentration of caffeine after drying ranged from 0.08 to $0.13\,mg\,g^{-1}$, cocoa beans from heap 2 possessing the highest concentration and those from heaps 5 and 7 possessing the lowest concentrations (Table 3). The theobromine/caffeine ratio clearly separated the mid-crop heaps 1–3 from the main-crop heaps 4–7 (Table 3).

Quality assessment of dried, fermented cocoa beans

The fermented, dried cocoa beans were of good quality. All parameters tested were good to excellent. Bean counts varied from 253 to 286 per 300 g of beans,

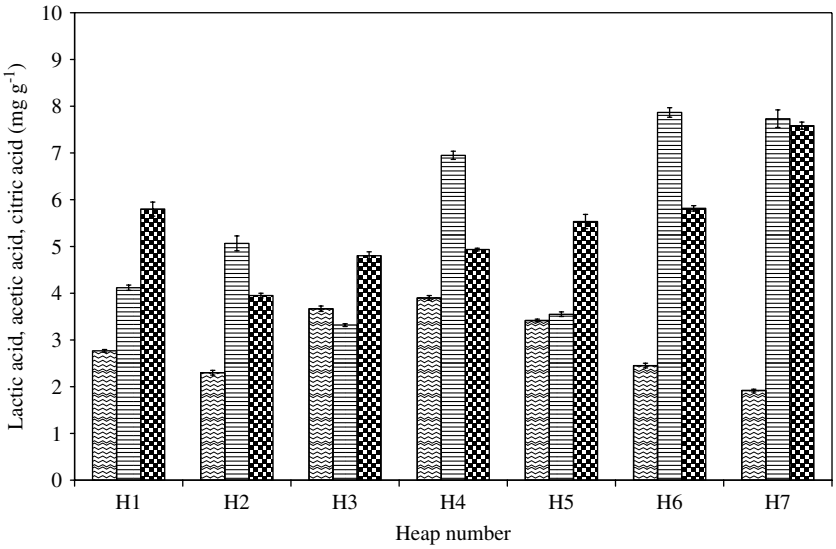

Figure 4. Concentration of lactic acid (▒), acetic acid (▒), and citric acid (▒) in dried fermented cocoa beans.

while broken beans only represented 1.59–3.43 g per 300 g of beans. Slaty beans were not detected. Only very slight differences were observed between the heaps concerning the other cut test parameters. For instance, heap 4 showed a slightly higher percentage of violet beans and heap 6 showed a slightly higher percentage of flat and violet beans (data not shown).

Sensory analysis of chocolate by a taste panel

The results obtained showed that the chocolates prepared from beans of heaps 1–7 differed in flavour (Fig. 5). Chocolate made from beans of heap 6 displayed the highest score for intensity, acidity and after-taste intensity, and the weakest score for fruity, cocoa taste, bitterness and flowery. The after-taste of chocolate from beans of heap 6 was lemon acid. The chocolate flavour of beans from heap 2 displayed the highest score for floweriness and the second highest for fruitiness and bitterness. Chocolate from beans of this heap possessed a flowery after-taste. The chocolate from beans of heap 4 had a slightly flowery after-taste, a high value for taste intensity, but a low value for the after-taste intensity. The chocolate from beans of heap 5 was characterised by a fruity, less acid and intense taste, but had no real cocoa flavour. This was the chocolate with almost no bitter taste and a fruity after-taste. Beans from heap 7 resulted in chocolate with the most cocoa flavour and bitter taste and an impure cocoa after-taste. Chocolate from beans of heaps 1 and 3 showed similar results for all parameters but differed in after-taste, being bitter and common, respectively.

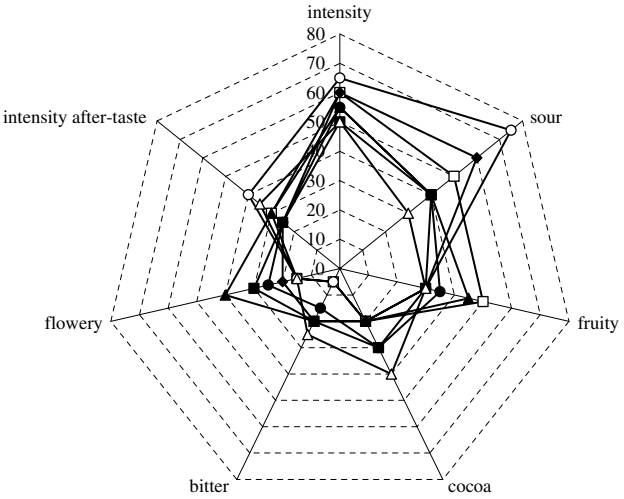

Figure 5. Flavour profiles of seven chocolates produced from cocoa beans of seven independent spontaneous Ghanaian cocoa bean heap fermentations (Heap 1, ■; Heap 2, ▲; Heap 3, ●; Heap 4, ♦; Heap 5, □; Heap 6, ○; Heap 7, △). The centre of the diagram corresponds to the lowest flavour intensity and the perimeter to the highest flavour intensity.

DISCUSSION

Fermentation and roasting are key to developing cocoa and chocolate flavour, respectively. Chocolate flavour is an extremely complex mixture of more than 550 compounds, and as analytical methods improve this number increases.[24] Whereas undesirable bitter and astringent tastes decrease during fermentation, desirable fruity, floral and cocoa flavours develop during fermentation and drying. Essential precursors of the cocoa-specific flavour components are generated during microbial fermentation of the pulp surrounding the beans as well as by enzymatic processes in the beans.[6–8,21,25] The optimal activity of the endogenous enzymes will be influenced by fermentation temperature and pH that increase and decrease in the beans, respectively, over fermentation time, as well as by diffusion of ethanol, acetic acid, and to a lesser extent lactic acid inside the beans and polyphenols and alkaloids outside the beans.[8,11] Thus, with respect to the organic acids that diffuse slowly into the cotyledons, timing of initial entry, duration of the period of optimum pH, and final pH are crucial for optimum flavour development. It may, therefore, be hypothesised that

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these events will be influenced by the micro-organisms involved in the pulp fermentation and their population and metabolite production dynamics. However, as shown before, competitive strains of both LAB (*Lactobacillus plantarum* and *Lactobacillus fermentum*) and AAB (*Acetobacter pasteurianus*) dominate the Ghanaian spontaneous cocoa bean heap fermentation process, and there is no significant influence of heap and farm, season, and size of the heap (for heaps < 500 kg), indicating the importance of the strains involved.[21] Yet, the total amount of ethanol and organic acids present in the beans at the end of fermentation differed from heap to heap, being most pronounced for larger heaps. This implies that heterogeneous heaps and slight, local differences in microbial numbers and sugars available for fermentation in the heaps may play an important role in flavour precursor formation within the beans. This may result in slight flavour differences of the resulting chocolate as observed during this study. Therefore, amount, nature and distribution of the micro-organisms present will determine the speed and intensity of fermentation as well as the quality of the fermented beans and the chocolate made thereof.

Comparison of the total content of polyphenols in freshly harvested beans and fermented beans showed that polyphenol concentrations are heap- and crop-dependent. The total polyphenol content was reduced through fermentation by approximately 10–50% during the main-crop and almost no reduction in total (lower) polyphenol content was found upon fermentation during mid-crop 2004. Although the season will not influence polyphenol contents of freshly harvested cocoa beans, weather conditions (microclimate and sunlight) and light intensity (position of pods on the tree, direct sunlight) seem to be important.[26] It has indeed been shown that the production of polyphenols in plant tissues are stress-related and are produced to protect the plant to, for instance, light intensity.[11] In general, the presence of polyphenols in cocoa beans is dependent on several factors, including degree of pod ripeness, cocoa variety, processing, and storage.[27–29] However, there are no qualitative differences in polyphenol compounds in cocoa beans, in spite of their genetic origin. During fermentation, polyphenol concentrations of the beans decrease due to their diffusion out of the beans (through water release) and further oxidation and condensation of the polyphenol compounds.[9,11,28,30] Oxidase reactions are both non-enzymatic and catalysed by polyphenol oxidase. This polyphenol oxidase is strongly inactivated during the first days of anaerobic (yeast) fermentation at a temperature of 28–35 °C, retaining only 50 and 6% of initial enzyme activity after 1 and 2 days, respectively.[8] Polyphenol oxidase is a copper-dependent enzyme that, in the presence of oxygen, catalyses two different reactions: the hydroxylation of monophenols to *o*-diphenols (monophenolase activity) and the oxidation of *o*-diphenols to *o*-quinones (diphenolase activity).[11] In living tissues, the phenolic substrates and the enzyme are separated within the cell, but upon cell damage (in cocoa beans due to acetic acid penetration) the enzyme and substrate may come into contact, leading to rapid oxidation of *o*-diphenols and further complexation of *o*-quinones. The optimum temperature for polyphenol oxidase activity is around 42–45 °C,[30] which corresponds with cocoa bean fermentation conditions. The occurrence of condensation reactions was confirmed by the sharp decrease of the total polyphenol content between the second and fourth day of the main-crop 2004 cocoa bean heap fermentations, in particular with respect to epicatechin. Losses of polyphenols differed from heap to heap, indicating slight differences in dynamics during fermentation as mentioned above. Although comparable for the cocoa bean fermentations carried out during this study, pod storage may cause a significant reduction in the content of polyphenol compounds.[28] This underlines the importance of pod age for fermentation performance and bean quality.[11,28]

There was a significant difference in the content of theobromine and caffeine of freshly harvested beans according to the harvest season, which may be related to the stress response mentioned above as well. Although no significant effect from pod storage is to be expected,[28] it has been shown that the cocoa genotype determines alkaloid contents of cocoa beans.[31] Reduction of the contents of theobromine and caffeine during fermentation is mainly due to exudation through the bean testa and is genotype-dependent as well.[28,31,32] As for polyphenols, their final levels contribute to the bitterness of roasted beans.

Drying of the fermented cocoa beans reduces moisture content from 45% to 7%.[2] Until there is enough moisture, flavour-forming reactions in the beans continue. During drying, strong browning reactions occur that include oxidation of polyphenols with reduction of astringent and bitter taste. In the present paper, metabolite target analysis of dried fermented cocoa beans revealed that all sucrose was hydrolysed by enzymatic (invertase) and non-enzymatic (acid environment) reactions into glucose and fructose. The concentration of citric acid (non-volatile) and acetic acid (volatile) slightly increased and decreased, respectively, because of the drying process, whereas the concentration of lactic acid (non-volatile but diffusable) remained constant. Only when drying is slow, also non-volatile lactic acid may partly be transported by the water from the bean to the husk. The total polyphenol content of the fermented beans decreased further during the drying process. Polyphenols and polyphenol oxidase are sensitive to the drying process.[8] This is not only due to further oxidation, the remaining enzyme activity being only about 2%, but also to migration of polyphenols with vaporising water. The epicatechin concentration further decreased with 50% during drying, which confirms previous assumptions that non-enzymatic

oxidation of polyphenols is also important during the drying process.[7,33] The amount of alkaloids was not influenced by drying of the fermented cocoa beans.

Specific cocoa flavours are produced during roasting, in particular through Maillard reactions, provided that the appropriate precursors generated through fermentation and enzymatic activities are present. It is well known that lactic acid hardly diffuses into the beans, while volatile acetic acid diffused into the beans is eliminated through drying, roasting and conching to a large extent. Pyrazines may be produced during fermentation, due to growth of bacilli, but they are mainly formed during roasting.[18,19,34,35] Although not studied during this work, roasting results in the formation of aldehydes, alcohols, ketones, and esters. Whereas the presence of some of the flavour compounds are influenced by fermentation time, i.e., by flavour precursor development, which depend on microbial activity during fermentation, Maillard compounds are affected by temperature and time of roasting.[2] Hence, the quality of roasted cocoa beans depends on the fermentation and roasting conditions.[10] After roasting, the beans possess the typical chocolate flavour and are less astringent, although remaining unpleasant without further processing and addition of ingredients. Although a higher concentration of polyphenols would decrease the cocoa flavour,[10] efforts are being made to maintain their levels while avoiding taste problems, as there are abundant health claims ascribed to polyphenols.[36–38] A less intense cocoa flavour is possibly due to a lower intensity of flavour compounds in the cocoa liquor and/or masking astringency and bitterness tastes in the mouth, for instance due to their binding to polyphenols. However, it has been shown that polyphenol concentrations have no influence on the acidity, fruity/floral flavour, raw/green, and mouldy/earthy properties of chocolate.[10] The flavour evaluation obtained by the taste panel during this study could be correlated with the above-mentioned data and with the chemical analyses of organic acids, polyphenols and alkaloids obtained previously and during this study.[21] For instance, beans from heap 7 resulted in chocolate with the most cocoa flavour and showed the least lactic acid and the lowest polyphenol content in fermented dried beans. Beans of this heap thus showed the best parameters to produce a good chocolate. Heap 6 was characterised by the highest fermentation temperature and the resulting fermented dried beans indeed reflected an almost complete conversion of ethanol into acetic acid by AAB. A too high acetic acid concentration may have caused a too-rapid decrease of the pH within the beans and hence low endogenous, suboptimal enzyme activities, resulting in a less pronounced cocoa flavour. Conversely, the high ethanol content of beans from heap 2 and concomitant low levels of lactic acid and acetic acid and the lowest fermentation temperature obtained for this heap indicated a pronounced yeast growth during fermentation, resulting in chocolate with a pronounced fruity flavour, probably due to ester formation.

CONCLUSIONS

The main conclusion to be drawn from the results of this research is that even when the fermentations are performed at the same farm, with the same cocoa cultivar, and under the same fermentation conditions, dried fermented beans with different flavour characteristics can be obtained, due to microbial variability, population dynamics, and metabolite kinetics between the heaps. This is reflected in the chocolates made of these beans. These data indicate the importance of mixing of dried fermented beans when produced at small scale, as is carried out in Ghana, or the necessary establishment of reliable controlled fermentation processes, when consistently high quality end products on large scale are to be obtained.

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REFERENCES

1 Jinap S, Dimick PS and Hollender R, Flavour evaluation of chocolate formulated from cocoa beans from different countries. *Food Control* **6**:105–110 (1995).
2 Wood GAR and Lass RA, *Cocoa*, 4th edition. Longman Group, London (1985).
3 Ziegleder G, Composition of flavor extracts of raw and roasted beans. *Z Lebensmitt Unters-Forsch* **192**:521–525 (1991).
4 Lehrian DW and Patterson GR, Cocoa fermentation, in *Biotechnology, a Comprehensive Treatise*, vol. 5, ed. by Reed G. Verlag Chemie, Basel, pp. 529–575 (1983).
5 Schwan RF and Wheals AE, The microbiology of cocoa fermentation and its role in chocolate quality. *Crit Rev Food Sci Nutr* **44**:205–221 (2004).
6 Biehl B, Brunner E, Passern D, Quesnel VC and Adomako D, Acidification, proteolysis and flavour potential in fermenting cocoa beans. *J Sci Food Agric* **36**:583–598 (1985).
7 de Brito ES, Pezoa García NH, Gallão MI, Cortelazzo AL, Fevereiro PS and Braga MR, Structural and chemical changes in cocoa (*Theobroma cacao* L) during fermentation, drying and roasting. *J Sci Food Agric* **81**:281–288 (2000).
8 Hansen CE, del Olmo M and Burri C, Enzyme activities in cocoa beans during fermentation. *J Sci Food Agric* 77:273–281 (1998).
9 Misnawi SJ, Jamilah B and Nazamid S, Effects of incubation and polyphenol oxidase enrichment on colour, fermentation index, procyanidins and astringency of unfermented and partly fermented cocoa beans. *Int J Food Sci Tech* **38**:285–295 (2003).
10 Misnawi SJ, Jamilah B and Nazamid S, Sensory properties of cocoa liquor as affected by polyphenol concentration and duration of roasting. *Food Qual Pref* **15**:403–409 (2004).

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

12 del Boca C, Cocoa beans: quality requirements and methods of assessment. *Rev Int Chocolaterie* **17**:218–221 (1962).

13 Shamsuddin SB and Dimick PS, Qualitative and quantitative measurements of cacao bean fermentation, in *Proceedings of Cocoa Biotechnology*, ed. by Dimick PS, Department of Food Science, Penn State University, University Park, PA, pp. 55–78 (1986).

14 Ziegleder G and Biehl B, Analysis of cocoa flavour compounds and precursors, in *Modern methods of plant analysis*, vol. 8, ed. by Linkskens HF and Jackson JF. Springer, Berlin, pp. 361–393 (1988).

15 Pontillon J, *Cacao et chocolat. Production, Utilisation, Caractéristiques*. Collection Sciences & Techniques Agroalimentaires, Lavoisier Tec & Doc, Paris, pp. 100–109 (1998).

16 Baker DM, Tomlins KI and Gray C, Survey of Ghanaian cocoa farmer fermentation practices and their influence on cocoa flavour. *Food Chem* **51**:425–431 (1994).

17 Biehl B, Meyer B, Said MB and Samarakoddy RJ, Bean spreading: a method of pulp preconditioning to impair strong nib acidification during cocoa fermentation in Malaysian. *J Food Agric* **51**:35–45 (1990).

18 Hashim P, Selamat J, Muhammad SKS and Ali A, Changes in free amino acid, peptide-N, sugar and pyrazine concentration during cocoa fermentation. *J Sci Food Agric* **78**:535–542 (1998).

19 Hashim P, Selamat J, Muhammad SKS and Ali A, Effect of mass and turning time on free amino acid, peptide-N, sugar and pyrazine concentration during cocoa fermentation. *J Sci Food Agric* **78**:543–550 (1998).

20 Meyer B and Biehl B, Post-harvest pod storage: a method for pulp preconditioning to impair strong nib acidification during cocoa fermentation in Malaysia. *J Sci Food Agric* **48**:285–304 (1989).

21 Camu, N, De Winter T, Verbrugghe K, Cleenwerck I, Vandamme P, Takrama JS, *et al.*, Dynamics and biodiversity of populations of lactic acid bacteria and acetic acid bacteria involved in spontaneous heap fermentation of cocoa beans in Ghana. *Appl Environ Microbiol* **73**:1809–1824 (2007).

22 EEC, Folin-Ciocalteu index. *Off J Eur Communities*, Ch. 41, pp. 178–179 (1990).

23 Kreiser WR and Martin RA, High pressure liquid chromatographic determination of theobromine and caffeine in cocoa and chocolate products. *J Assoc Off Anal Chem* **61**:1424–1427 (1978).

24 Nijssen LM, Visscher CA, Maarse H, Willemsens LCand Boelens MH, *Volatile Compounds in Foods. Qualitative and Quantitative Data*, 7th edition. TNO Nutrition and Food Research Institute, Zeist (1996).

25 Rohan TA, The precursors of chocolate aroma: a comparative study of fermented and unfermented cocoa beans. *J Food Sci* **29**:456–459 (1964).

26 Niemenak N, Rohsius C, Elwers S, Ndoumou DO and Lieberei R, Comparative study of different cocoa (*Theobroma cacao L.*) clones in terms of their phenolics and anthocyanins contents. *J Food Comp Anal* **19**:612–619 (2006).

27 Hammerstone JF, Lazarus S, Mitchell A, Rucker R and Schmitz H, Identification of procyanidins in cocoa (*Theobroma cacao*) and chocolate using high-performance liquid chromatography/mass spectometry. *J Agric Food Chem* **47**:490–496 (1999).

28 Nazaruddin R, Seng LK, Hassan O and Said M, Effect of pulp preconditioning on the content of polyphenols in cocoa beans (*Theobroma cacao*) during fermentation. *Ind Crops Products* **24**:87–94 (2006).

29 Porter LJ, Ma Z and Chan BG, Cacao procyanidins: major flavonoids and identification of some minor metabolites. *Phytochemistry* **30**:1657–1663 (1991).

30 Misnawi SJ, Nazamid S and Jamilah B, Activation of remaining key enzymes in dried under-fermented cocoa beans and its effect on aroma precursors formation. *Food Chem* **78**:407–417 (2002).

31 Brunetto MR, Gutiérrez L, Delgado Y, Gallignani M, Zambrano A, Gómez A, *et al.*, Determination of theobromine, theophylline and caffeine in cocoa samples by a high-performance liquid chromatographic method with on-line sample cleanup in a switching-column system. *Food Chem* **100**:459–467 (2007).

32 Clapperton, J, Hammerstone JF, Romanczyk LJ, Yow S, Lim D and Lockwood R, Polyphenols and cocoa flavour, in *Proceedings, 16th International Conference of Group Polyphenols*, vol. 2, Lisbon, Groupe Polyphenoles, Norbonne, France pp. 112–115 (1992).

33 Kyi TM, Daud WRW, Mohammad AB, Samsudin MW, Kadhum AH and Talib MZM, The kinetics of polyphenol degradation during the drying of Malaysian cocoa beans. *Int J Food Sci Tech* **40**:323–331 (2005).

34 Hashim L and Chaveron H, Extraction and determination of methylpyrazines in cocoa beans using coupled steam distillation-microdistillator. *Food Res Int* **27**:537–544 (1994).

35 Jinap S, Organic acids in cocoa beans, a review. *ASEAN Food J* **9**:3–12 (1994).

36 Schroeter H, Heiss C, Balzer J, Kleinbongard P, Keen CL, Hollenberg NK, *et al.*, (−)-Epicatechin mediates beneficial effects of flavanol-rich cocoa on vascular function in humans. *Proc Natl Acad Sci USA* **103**:1024–1029 (2006).

37 Sies H, Schewe T, Heiss C and Kelm M, Cocoa polyphenols and inflammatory mediators. *Am J Clin Nutr* **81**:304S–312S (2005).

38 Wollgast J and Anklam E, Polyphenols in chocolate: is there a contribution to human health? *Food Res Int* **33**:449–459 (2000).