Effect of cacao bean quality grade on cacao quality evaluation by cut test and correlations with free amino acids and polyphenols profiles

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Abstract Cacao fermentation induces biochemical changes in the beans that lead to different cacao grades such as slaty (unfermented), violet (under fermented) and brown (fully fermented) beans. This grade heterogeneity in a sample set can impact the quality of the end-products. In the present study, amino acids and polyphenol contents of slaty, violet and brown beans were evaluated. Free amino acids were derivatized from *o*-phthalaldehyde and detected with a fluorescence spectrophotometer coupled with a reverse phase HPLC system. Polyphenols were analysed at 280 nm by HPLC using a Photodiode Array Detector. Amino acids content in the violet beans were significantly higher ($11,165 \pm 4281 \text{ mg kg}^{-1}$ fat free dry material, ffdm) than that of the slaty beans ($4304.5 \pm 1927.6 \text{ mg kg}^{-1}$ ffdm), meanwhile there was no significant difference between violet and brown beans. Epicatechin, catechin, cyanidin-3-arabinoside and cyanidin-3-galactoside contents were significantly lower in violet and brown beans when compared to slaty beans. Our results have shown that a mixture of violet and brown

beans is suitable to obtain polyphenols and amino acids in the development of cacao products.

Keywords Cacao quality · Cut-test · Fermentation processes · Free amino acids · Polyphenols

Introduction

The quality of raw cacao beans depends essentially on the degree of fermentation due to the generation of aroma precursors during the process. Besides fermentation, other factors such as climatic conditions, soil type and maturity at harvest, as well as genotype, drying and roasting conditions are important. The quality assessment of dried seeds is closely related to their cotyledon appearance. Cotyledon color is modified in the course of acidification of seeds during fermentation which provides the conditions for biochemical changes, inducing activation of a set of specific enzymes. At the early fermentation stage (anaerobic stage), yeast activities on the cacao pulp produce ethanol which is subsequently oxidised by bacteria to lactic acid and acetic acid in the aerobic stage (De Roos and De Vuyst 2018; Romanens et al. 2018). The oxidation process generates heat that causes seeds death and concomitantly the acetic acid penetrates slowly into the cotyledons. Low pH (< 5) jeopardizes cotyledon cell integrity and allows several enzymes to interact with their substrates (da Veiga Moreira et al. 2013). Thus, storage proteins are hydrolysed by aspartic proteinase and carboxypeptidase (Voigt et al. 1994; Biehl et al. 1993) and generate free amino acids and peptides while invertase cleaves sucrose into fructose and glucose (Lopez et al. 1978). Under aerobic conditions and high pH values, polyphenoloxidase catalyses the oxidation of various phenolic metabolites to quinones. The quinones

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react with each other as well as with other electron donors in the altered tissue, to form brown insoluble polymers. At the same time, anthocyanins are hydrolysed by β -glycosidases to produce anthocyanidins, galactose and arabinose. Furthermore, dimerisation of leucocyanidins and exudation of flavonoids from the seeds occurs. Subsequently, during drying, the amount of polyphenols is substantially reduced by enzymatic browning (Wollgast and Anklam 2000). While enzymatic oxidation removes the astringent and bitter taste of fresh seeds, brown color is one of the indicators of well fermented dried cacao.

Depending on the duration of fermentation, the homogeneity of seeds size and the position in the heap/box, the frequency of mixing and the temperature, the biochemical state of the cacao seeds vary. This variability can be characterized visually by using the cut test, which enables the distinction between well fermented seeds displaying brown color; under-fermented seeds appear violet to violet–brown and non-fermented seeds are slaty. These three groups are often found in traded cacao (Lieberei and Rohsius 2003).

Cacao manufacturers blend beans from various origins that have been processed differently with the aim of having uniform and constant raw materials to produce chocolate. The processing parameters to produce chocolate are then adjusted on the expected characteristic of the blend of beans, quite often only based on simple indicators such as the origins of the cacao (Saltini et al. 2013). Several studies dealing with the biochemical changes occurring during fermentation, drying and roasting of cacao from different origins and genotypes have been conducted in cacao bulk as a whole not accounting for the composition of the lot (Lieberei et al. 2013; Niemenak et al. 2006; Rohsius et al. 2006; Kirchhoff et al. 1989). Moreover, many studies focus on how some specific cacao farming practices or different conditions influence specific parameters of the cacao beans. However, such information rarely reaches the chocolate manufacturers. One of the challenges arising from the existence of different amounts of fermented beans within a lot is how this can affect the chocolate end-product. It is therefore important to obtain information on the composition of slaty, violet and brown beans found in traded cacao for the purpose of adjusting the flavour and colour. In this study, we collected raw cacao samples from different farmers, and evaluated their physical quality via cut test score. Phenolic compounds and amino acids contents of each slaty, violet and brown bean were characterized in order to measure the impact, in terms of biochemical determinants and distribution in a cacao sample set. Correlation between the physical and biochemical attributes of cacao quality was established. Such biochemical variables could help chocolate manufacturers optimize their processes based on the characteristics of the raw materials.

Materials and methods

The materials used consisted of fermented and dried cacao beans. Samples were collected during the 2009–2010 cacao campaign in the South (latitude 2°55'; longitude 11°07'), Center (latitude 4°34'; longitude 11°28') and Littoral (latitude 9°33'; longitude 4°29') regions of Cameroon. From each region, 30 samples were collected from 30 different farmers. Samples of about 1–2 kg per farmer were collected from each site, sealed in plastic bags and transported to Germany. One hundred seeds per sample were cut lengthwise with a cacao Guillotine (Type MAGRA, Swiss company TESERBA). The appearance of the beans was evaluated according to the category i.e. slaty, violet, brown and white beans (data not shown). Beans of different colors from each region were combined and the shells and radicles were removed. Fifty grams were coarsely crushed with a blender and the particles were stored at -20°C . Sampling was done in triplicates for each region.

Free amino acids and phenolic compounds analysis

Free amino acid contents were analyzed according to Rohsius et al. (2006). Two grams of crushed cotyledon from each class of beans (slaty, violet and brown) were milled with 10 ml hexane. Fat was removed by spraying the powder with 100 ml petrol ether of 40–60° using a funnel vacuum. The powder was dried under vacuum at room temperature for 30 min. and stored at -20°C for further analysis (for more details, see Supplementary 1). The determination of catechin, epicatechin, cyanidin-3-galactoside and cyanidin-3-arabinoside were performed according to Stoll et al. (2017) (for more details, see Supplementary 2).

Statistical analysis

All data were analyzed using R-studio version 0.96.122. Statistical significance was assessed by analysis of variance (ANOVA) and results presented as Box-plot. The least significant difference (LSD) was used to separate and compare the means and significance was accepted at 5% level ($P < 0.05$). R-studio was also used for principal component analysis (PCA).

Results and discussion

Cacao quality assessment is generally quickly determined via cut test score. From the results, the samples were classified into three principally distinguishable grades; slaty, violet and brown, and their transitional stages

(Fig. S3). The slaty corresponds to unfermented, the violet to under-fermented and the brown to well fermented beans. This classification excluded premium cacao from the Criollo group which lack anthocyanin and their seeds are white (Rohsius 2007; Lieberei and Rohsius 2003). Brown beans are generally recognized as high quality because of the reduction in bitterness, astringency and acidity and the formation of aroma precursors during fermentation and drying. However, depending on the use, slaty or violet beans may be desired according to their biochemical features.

The protein amino acids were presented according to the classification of Kirchhoff (1993), which encompassed hydrophobic amino acid (Ala, Leu, Phe, Tyr, Val, Ile), acidic amino acid (Asn, Asp, Glu, Gln, His) and others (Tryp, Lys, Ser, Gly, Arg, Threo). In addition to the protein amino acids, the levels of the non-protein amino acid; γ -aminobutyric acid (GABA), were recorded. The individual and total free amino acid (Table 1) levels increased from slaty to brown and white beans ($P < 0.05$). There was no significant difference in the amount of individual free amino acids between slaty and slaty-violet beans, and

between violet and brown beans. However, the content of free amino acid was higher in violet, brown and white beans than in their slaty counterparts. Total hydrophobic amino acids content ranged from 2242 mg kg^{-1} to $7812.17 \text{ mg kg}^{-1}$ and was significantly lower in slaty beans than in the other classes of beans. In the violet, brown and white beans, Leu and Phe were the dominant amino acids of the hydrophobic group accounting for 50%. Total acidic amino acid content was in the range of 1616.94 to $4259.69 \text{ mg kg}^{-1}$. Glu and Asn were the dominant amino acids in this group, representing more than 60%. There was a significant difference on the acidic amino acid concentration values of slaty, brown and violet beans when compared, which increased by 50%. When the violet, brown and white beans were compared, there was no significant difference in the acidic amino acid concentration. The other amino acids were observed to be 2- to 2.5-fold higher in violet and brown beans than in the slaty beans. Chocolate flavour precursors, for instance, have been shown to be composed of small peptides, free amino acids and reducing sugars (Kadow et al. 2015). This examination showed that free amino acids content varies

Table 1 Profile of individual, hydrophobic, acidic and total free amino acids in different cacao beans fermentation stages: slaty, slaty-violet, violet, brown and white cacao beans

	Cacao beans fermentation stages				
	Slaty	Slaty-Violet	Violet	Brown	White
Val	280.13 ± 152.732^a	433.14 ± 139.796^a	856.39 ± 310.524^b	868.62 ± 317.383^b	1555.26 ± 616.251^c
Leu	341.10 ± 171.334^a	667.67 ± 359.275^a	1716.24 ± 753.896^b	1546.29 ± 390.177^b	2192.63 ± 286.233^b
Ile	297.13 ± 111.123^a	405.29 ± 112.373^a	708.77 ± 294.718^b	674.69 ± 189.113^b	852.10 ± 261.295^b
Ala	502.50 ± 165.136^a	754.10 ± 340.879^{ab}	1176.16 ± 298.435^{cd}	1051.93 ± 230.776^c	1394.19 ± 360.175^d
Tyr	441.00 ± 148.176^a	–	747.70 ± 367.252^b	844.12 ± 132.525^b	–
Phe	380.31 ± 201.157^a	589.51 ± 400.739^a	1686.88 ± 669.102^b	1426.26 ± 419.088^b	1817.99 ± 695.903^b
Total of hydrophobic	2242.17 ± 949.658^a	2849.71 ± 1353.062^a	6892.14 ± 2693.927^b	6411.91 ± 1679.062^b	7812.17 ± 2219.857^b
Asp	118.56 ± 52.854^a	173.15 ± 79.325^{ab}	251.94 ± 85.912^b	259.82 ± 57.519^b	393.33 ± 155.740^c
Glu	595.28 ± 256.579^a	680.49 ± 314.955^a	1149.78 ± 333.126^b	1075.82 ± 184.320^b	1344.13 ± 447.553^b
Asn	527.05 ± 255.145^a	810.41 ± 567.521^a	926.08 ± 370.028^{ab}	905.94 ± 318.249^{ab}	1299.00 ± 578.311^b
Gln	219.43 ± 89.713^a	172.49 ± 68.260^a	247.95 ± 76.751^a	212.41 ± 74.815^a	291.92 ± 190.878^a
His	155.62 ± 128.203^a	282.84 ± 223.993^{ab}	619.48 ± 354.368^{bc}	733.61 ± 214.562^c	931.31 ± 386.053^c
Total of acidic	1615.94 ± 782.494^a	$2119.38 \pm 1254.054^{ab}$	$3195.23 \pm 1220.185^{bc}$	3187.6 ± 849.465^c	4259.69 ± 1758.535^c
Thr	152.70 ± 62.560^a	203.14 ± 82.897^a	359.38 ± 123.703^b	315.20 ± 60.144^b	504.06 ± 118.922^c
Gly	124.75 ± 35.544^a	154.09 ± 35.336^{ab}	260.33 ± 86.364^c	219.48 ± 46.874^{bc}	343.81 ± 81.972^d
Ser	168.91 ± 91.320^a	241.42 ± 107.942^a	457.77 ± 156.893^b	429.08 ± 69.617^b	645.88 ± 223.507^c
Total of others	446.36 ± 189.424^a	598.65 ± 226.175^a	1077.48 ± 366.96^b	963.76 ± 176.635^b	1493.75 ± 424.40^d
Total of free AA	4304.47 ± 1921.576^a	5567.74 ± 2833.291^a	$11,164.85 \pm 4281.072^b$	$10,563.27 \pm 2705.162^b$	$13,565.61 \pm 4402.793^b$

Values with different letters based on the cacao beans stages are significantly different (estimated by analysis of variance (ANOVA) followed by Tukey's 'honest significant difference' test, $P < 0.05$). Values are expressed as mg kg^{-1} fat free dry material (ffdm) and are means of $n = 18$ (nine biological replicates and two technical replicates)

greatly between slaty and the violet and brown beans. It is known that well fermented cacao (brown) contains about 8000 to 14,000 mg kg⁻¹ ffdm and unfermented cacao (slaty) contains as low as 2000 to 4000 mg kg⁻¹ ffdm of free amino acid (Rohsius et al. 2006). Our study displayed that violet beans (under-fermented) have relatively the same amount of free amino acid as brown beans. Caligiani et al. (2014, 2016) found that hydrophobic amino acids were higher in violet beans than in brown beans while similar contents of peptides were measured in violet and brown beans, with a slightly higher amount of peptides in violet beans. In fact, during fermentation, significant amounts of soluble components from the seeds leak into the surrounding pulp. Up to 33% of the total nitrogen of the seeds is lost during fermentation (Kirchhoff et al. 1989). Especially after the death of the seeds due to increase of temperature and the penetration of acetic acid which jeopardizes cells integrity, free amino acids can be released in the surrounding environment (Biehl and Passern 1982).

Gamma-aminobutyric acid (GABA), a non-protein amino acid, content ranging from 336 to 2673 mg kg⁻¹ ffdm with an average of 1667 mg kg⁻¹ (Fig. 1). There was no significant difference between the fermentation stages based on the GABA content. Mean value and median are almost equal in all cases. The amount of 2673 mg kg⁻¹ of GABA found in slaty beans makes raw cacao one of the highest sources of this metabolite in the plant kingdom. Germinated brown rice is known to be a rich source of GABA with its highest content of 445.3 mg kg⁻¹ (Diana et al. 2014; Banchuen et al. 2010). GABA has been widely studied because of its numerous physiological functions and positive effects on many metabolic disorders. One of the most important of these is the hypotensive effect that has been demonstrated in animals and in human intervention trials. Various studies have reported GABA producing ability by lactic acid bacteria (LAB) species/subspecies.

Vast concentrations of GABA production by LAB have also been reported (Diana et al. 2014). Based on this observation, the question on the importance of the microorganisms acting during fermentation on cacao is still debated. Is the GABA accumulation in cacao beans the result of seed acidification, oxygen deficiency and/or the product of LAB? Indeed, a reduction in the pH in the cytosol, as it is found during cacao fermentation, leads to a Ca²⁺ increase. The resulting Ca²⁺/calmodulin complex in the cytosol activates the glutamic acid decarboxylase (GA) to form γ -aminobutyric acid. Obviously, there is a correlation between oxygen depletion and the formation of γ -aminobutyric acid (Bown and Shelp 1997). In the absence of oxygen, the Ca²⁺ level in the cytosol increases, that leads to the activation of glutamate carboxylase. The first phase of the fermentation proceeds under anaerobic conditions, by which the formation of γ -aminobutyric acid can be induced. Attempt to produce GABA enriched food have been proposed base on LAB producing ability. The highest GABA content ever found was in Bathura sourdough bread (2262 mg kg⁻¹) (Bhanwar et al. 2013), followed by GABA-enriched bread (115 ppm) made with exogenous supplementation of recombinant produced GAD from *Yersinia intermedia* as starter culture (Lamberts et al. 2012).

Polyphenol analysis revealed that 1 kg of defatted dried cacao beans provide an average of 30,000 mg, 15,000 mg, 10,000 mg and 8000 mg of epicatechin for slaty, violet, white and brown beans respectively. For 1 kg of defatted dried beans, the average catechin content is as follow: 700 mg (slaty beans), 450 mg (violet beans), 200 mg (white beans) and 300 mg (brown beans). The epicatechin and catechin contents were significantly higher in slaty beans than the other beans classes (Fig. 2). The evaluation of the cyanidin-3-arabinoside and cyanidin-3-galactoside distribution in cacao beans revealed a consistently high preponderance in slaty samples with an amount of

Fig. 1 Variation of γ -aminobutyric acid (GABA) contents in different cacao beans fermentation stages: slaty, slaty-violet (S-V), violet, brown, white and mixed (total) cacao beans. Mixed beans are taken in the lot without sorted according to cotyledon color. Mean values (n = 18) with different letters differed significantly at $P < 0.05$, Post hoc Test: Fisher LSD, $P < 0.05$

Fig. 2 Variation of epicatechin and catechin contents in different cacao beans fermentation stages: slaty, slaty-violet (S-V), violet, brown, white and mixed (total) cacao beans. Milled defatted cacao seeds were extracted in methanol and polyphenol analysis by HPLC. Otherwise as in Fig. 1

2840 mg kg⁻¹ ffdm and 4789 mg kg⁻¹ ffdm respectively (Fig. 3). With respect to the anthocyanins composition, no difference was found between violet, brown and white beans. But the content of anthocyanins was significantly higher in slaty beans when compared to the other beans colors. The amount of cyanidin-3-arabinoside was 2–3 times higher than the amount of cyanidin-3-galactoside. Polyphenols content are important discriminant determinants in many classes of food and food products. Their dynamic is often affected during food processing and depend on food matrix. As an example, hard-to-cook

kidney beans (*Phaseolus vulgaris*) showed a decrease in phenolic leaching during soaking as compared to easy-to-cook grains. Smaller intercellular spaces in the cell wall of hard-to-cook beans explain this behavior. Hard-to-cook beans have also more dark color due to phenolic compounds polymerization (Parmar et al. 2017). Likewise, highest values of phenolic compounds were observed in purple corn grains than their corresponding white grains due to their high amount of anthocyanins. Consequently, dark colored flours derived from purple grains had higher

Fig. 3 Variation of cyanidin-3-galactoside and cyanidin-3-arabinoside contents in different cacao beans fermentation stages: slaty, slaty-violet (S-V), violet, brown, white and mixed (total) cacao beans. Otherwise as in Fig. 1

content of phenolic compounds as compared to the lighter ones (Trehan et al. 2018; Singh et al. 2014).

Principal component (PC) analysis revealed that the cacao beans colors are well separated from each other according to the biochemical constituents. The 2D graphical representation showed a total variance of 87.84% (Fig. S4), the first PC representing 75.92% of the total variability (eigenvalue, 15.94) and the second PC representing 11.93% of the total variability (eigenvalue, 2.5). The variability of the data was generated mostly by the content of amino acids, epicatechin, catechin and cyanidin-3-galactoside. Brown, white and violet beans were strongly correlated with a high level of amino acids and low level of epicatechin and catechin, while slaty samples revealed high level of cyanidin-3-galactoside, epicatechin and catechin. The typical astringency of cacao is mainly due to phenolic compounds. Raw cacao with a low degree of fermentation is characterized by strong unpleasant astringency (Kadow et al. 2015). Moreover, phenolic compounds apparently inhibit the Maillard reaction and thus chocolate flavour formation during roasting by reacting with sugars (Totlani and Peterson 2005). However, due to the antioxidant properties of polyphenols, when it comes to producing health-promoting chocolate, the quality requirement must be oriented towards phenolic ingredients. Therefore, a genuine amount of slaty or slaty-violet beans could be added to the cacao masse derived from well fermented brown beans. As an illustration, a certain amount of purple beans are found and even, wanted in Arriba cacao for their polyphenols richness (Rohsius 2007). In this regard, Rawel et al. (2019) stressed that utilization of partially processed cacao beans (e.g., fermented, conciliatory thermal treatment) may provide a large collection of bioactive potentials that could be included in the designing of functional foods, illuminating an alternative use of cacao especially in the cacao-producing countries to bolster the diets with corresponding positive impact on the health status of the local populations.

The white beans found in our samples are derived certainly from Criollo group, which have been reported in some farm accessions (FA) in Cameroon. Indeed, Efom-bagn et al. (2008) using microsatellite markers, showed the existence of a high level of a mixture in cacao farm accessions with Criollo displaying a proportion of 7%. PC2 separated white beans from violet and brown beans. The discriminating attributes being high levels of amino acid. The Criollo beans which are the source of white beans are well known for their fine or flavour characteristics. Caligiani et al. (2014) showed that Criollo variety is characterized by high levels of amino acids.

Conclusion

This work reveals the amino acid composition and polyphenolic profile of cacao beans from different grades. The cacao bean samples investigated, based on their grade, showed that violet and brown beans have the same biochemical characteristics (free amino acids contents of $11,165 \pm 4281 \text{ mg kg}^{-1} \text{ ffdm}$ and $10,563 \pm 2705 \text{ mg kg}^{-1} \text{ ffdm}$ respectively; epicatechin content as dominant polyphenol of $16,764 \pm 3872 \text{ mg kg}^{-1} \text{ ffdm}$ and $9791 \pm 4776 \text{ mg kg}^{-1} \text{ ffdm}$ respectively). For this reason, industries that use this raw material for the production of cacao foods should consider this fact. The application of biochemical criteria, in addition to traditional assessment methods, which is often based on geographic origin of the beans, will improve the industry's approach to differentiation of raw materials. This will enable new product development on the basis of pre-selected cacao beans in cooperation with producing countries as well as a better influence on the post-harvest processes in the sense of quality certification. When interested in the human's health beneficial attributes related to cacao polyphenols, manufacturers should consider slaty-violet and violet beans while potential flavored products will need well fermented brown beans.

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