# Changes in total polyphenols, o-diphenols and anthocyanin concentrations during fermentation of pulp pre-conditioned cocoa (*Theobroma cacao*) beans

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**Abstract:** Investigations were conducted to elucidate changes in total polyphenols, o-diphenols and anthocyanin concentrations during fermentation of pulp preconditioned Ghanaian cocoa beans. A 4x4 factorial experiment was conducted with the principal factors as pod storage and fermentation time, and samples were analyzed using standard analytical methods. Results showed that total polyphenol, o-diphenols and anthocyanin content of beans from the unstored pods were 180.87 mg/g, 24.17 mg/g, and 15.68 mg/kg respectively. Reductions in the concentrations of total polyphenols, o-diphenol and anthocyanin composition of the cocoa beans occurred with increasing pod storage and fermentation time. However, the rates of decreases were more dependent of fermentation time than on pod storage. Both total polyphenol and o-diphenol content reduced only slightly (<10%) in the beans fermented for 6 days after 7 days pod storage. Storage of cocoa pods for up to 7 days after harvesting retained about 88% and 90% of total polyphenols and o-diphenol content respectively after 6 days of fermentation. Further increase in pod storage above 7 days leads to drastic reductions in the polyphenolic content of the fermented beans. The observed changes would result in significant reduction in astringency and bitterness in their derived chocolates products during subsequent industrial manufacture.

**Key words: Cocoa, *Theobroma cacao,* pod storage, pulp pre-conditioning, fermentation, polyphenols, catechin, epicatechin, anthocyanins**

## Introduction

Cocoa beans are the principal raw material for chocolate manufacture. After harvesting, the beans are made to undergo several treatments including pod storage, fermentation, drying and roasting, all of which contribute significantly to the development of the characteristic cocoa flavour which is further transformed into distinct chocolate notes during subsequent conching stage in industrial manufacture (Afoakwa *et al*., 2007; Afoakwa *et al*., 2008; Beckett, 2008; Fowler, 2009; Adeyeye *et al.*, 2010; Owusu *et al*., 2011).

Cocoa beans are a concentrated source of polyphenols and anthocyanin, with flavan-3-ols and their derivatives being present in high concentrations (Schwan and Wheals, 2004; Kyi *et al.*, 2005). During the transformation of fresh cocoa beans to finished products, the concentration of polyphenols and anthocyanin can be affected by a variety of biological and processing conditions. Genetics can cause as much as a 4-fold difference in polyphenolic content of fresh cocoa beans. Fermentation of fresh cocoa beans, although critical for full cocoa flavour, also tends to decrease the flavan-3-ol content (Wollgast and Anklam, 2000; Nazaruddin *et al*., 2001; Rodriguez-Campos *et al.*, 2011). These changes in the total polyphenols and anthocyanin content during post-harvest treatment of the cocoa beans dictates the levels of bitterness, astringency and colour development in finished chocolates.

Polyphenolic cells in cocoa beans are made up of 14–20% dry bean weight, and contain a single large vacuole filled with polyphenols and alkaloids including caffeine (0.1-0.2%), theobromine (2.5-3.2%), and theophylline (Osman *et al.,* 2004). The pigmented polyphenols, when undisturbed, confer a deep purple colour to fresh Forastero cocoa cotyledons. The major polyphenolic compounds contained in cocoa seeds are catechins (3.0-6.0%), leucocyanidins (2.5%) and tannins (2.0-3.5%). The polyphenols have bitter, astringent flavours and their antioxidant properties help protect the seed from damage and disease (Kyi *et al.*, 2005). Nazaruddin et al. (2001) reported that the total polyphenols ranged from 45–52 mg/g in cocoa liquor, 34–60 in beans,

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and 20–62 in powder: (−)-epicatechin contents were 2.53, 4.61, and 3.81 mg/g, respectively. Polyphenol reactions with sugar and amino acids contribute flavour and colour to cocoa bean whereas the alkaloids contribute to the bitterness (Lehrian and Patterson, 1983; Afoakwa and Paterson, 2010).

During fermentation, oxidative enzyme activity also occurs, being most prevalent in the aerobic phase but continuing well into the drying of cocoa (Thompson *et al*., 2007). The polyphenol oxidase (PPO) is a major oxidative enzyme in cocoa which oxidizes cocoa polyphenols during the fermentation. The process activates PPO as oxygen diffuses into the beans following disruption of the polyphenol storage cells. The polyphenols are first oxidized into o-quinones which then complex with amino acids, proteins and flavonoids resulting in brown, water insoluble tannins (Lopez and Dimick, 1995; Afoakwa *et al*., 2008). Polyphenol oxidase achieves its optimal activity at pH 6 and 35.5°C (Lopez and Dimick, 1995). The activity of the enzyme quickly diminishes during fermentation, probably due to the increase in temperature and inhibitory effect of tannins. Residual polyphenol oxidase is completely inactivated when the beans are dried (Lopez and Dimick, 1995; Kyi *et al*., 2005). A certain reduction in the level of polyphenols is required to achieve cocoa beans with a good flavour this is because the polyphenols are both astringent and bitter (Quesnel and Jugmohunsingh, 1970; Kratzer *et al*., 2009; Afoakwa, 2010; Rodriguez-Campos *et al*., 2011).

The development of the final cocoa bean flavour and quality may also be contributed by non enzymatic changes that occur in the cocoa seeds. A proportion of polyphenols are consumed in tanning reactions with albumin proteins. This renders them insoluble and reduces the astringency of the cocoa beans to a palatable level (Zak and Keeney, 1976; Misnawi *et al*., 2003). The diffusion of (-) epicatechin from the seed into the shell accounts for a significant proportion of polyphenol losses also contributing to the reduction in astringency (Kyi *et al*., 2005). Amino acids and peptides may also diffuse out of the seed accounting for the losses observed in some studies (Schwan and Wheals, 2004).

Fresh cocoa beans contain purple anthocyanidin pigments, 3-β-D-galactosyl- and 3-α-L-arabinosyl-cyanidins. During fermentation, these pigments are hydrolysed by glycosidases, resulting in a paler purple colour and this is referred to as bleaching of the cotyledons. These native enzymes hydrolytically cleave sugar groups from the anthocyanins contained in the seeds. The glycosidases have not been characterised, but maximum destruction of pigments has been described to occur at 45°C in the pH range 3.8-4.5 (Biehl *et al*., 1989). Later in the fermentation process, the free anthocyanidins are oxidised by polyphenol oxidase to quinones. The quinones can complex with amino acids and proteins and polymerize with other flavonoids to form tannins. High-molecular-weight tannins complex with proteins through hydrogen bonding, and the result of these reactions is a brown, water insoluble pigment that gives cocoa and chocolate its characteristic brown colour (Schwan and Wheals, 2004; Aroyeun *et al*., 2006; Kratzer *et al*., 2009; Rodriguez-Campos *et al*., 2011).

The impact of post-harvest treatment on fresh cocoa beans and the effects of these treatments on fermentation and final bean quality have been investigated. Three basic processes have been evaluated for the treatment of fresh cocoa beans prior to fermentation: pod storage, mechanical depulping and enzymatic depulping (Rohan, 1963; Wood and Lass, 1985; Schwan and Wheals, 2004). All of these treatments were developed or investigated in attempts to reduce the problem of acidity in dried fermented cocoa beans. Over acidity in processed cocoa beans has been linked to the production of high levels of lactic and acetic acid during fermentation. By removing a portion of the pulp, or reducing the fermentable sugar content of the beans, it has been shown that less acid is produced during fermentation, leading to less acid beans (Duncan *et al.*, 1989; Sanagi *et al*., 1997). Removal of up to 20% of the cocoa pulp from fresh Brazilian cocoa beans significantly improved the flavour quality of the beans produced (Schwan and Wheals, 2004). As well, pod storage was found to reduce the acidity and polyphenol content of Malaysian cocoa beans (Nazaruddin *et al.,* 2006). Previous studies on the chemical and physical quality characteristics as well as changes in acidification, proteolysis, sugars and free fatty acids concentrations of Ghanaian cocoa as influenced by pulp pre-conditioning and fermentation have been published (Afoakwa *et al*., 2011a,b). However, the extent to which the technique of pulp pre-conditioning influences the total polyphenolic content and anthocyanin concentrations during fermentation of Ghanaian cocoa beans still remains unknown. Thus, the objective of this study was to investigate changes in total polyphenols, o-diphenols and anthocyanin concentrations during fermentation of pulp pre-conditioned Ghanaian cocoa (T*heobroma cacao*) beans.

## Materials and Methods

### Materials

Ripe cocoa pods from mixed hybrids (Amelonado and Amazonica cultivars) of Forastero variety were harvested from the experimental plots of Cocoa Research Institute of Ghana (CRIG), Tafo in the Eastern Region of Ghana. The cocoa pods were selected according to their ripeness and maturity levels. The beans were pulp preconditioned by storing the harvested pods for a period of time before splitting. About 1200 pods were stored (on the cocoa plantation) at ambient temperature (25-28°C) and relative humidity of 85-100% for periods of 0, 7, 14 and 21 days respectively. The respective pods were then split after these predetermined storage times and fermented using the traditional heap method.

The fermentation was done by heaping about 50 kg of the extracted cocoa beans on the fermenting platform covered with banana leaves. The heaped beans were again covered with banana leaves and fermented for six days with consecutive openings and turnings after every 48 h. Samples of the unfermented beans were picked into a sterile polythene bag and after every 2 days of fermentation till the end of the sixth days, for drying and subsequent analysis. After each sampling time , the samples were immediately transported to the laboratory for drying by spreading the cocoa beans approximately 5 cm deep on metal trays (40 cm × 60 cm), and placed in a temperature controlled, forced air oven for about 24 h at a temperature of 45-50°C until dried (to moisture content below 8%). The dried beans were bagged in airtight black plastic bags and stored at ambient temperature (25-28°C) in a dark room free from strong odours until used. Prior to the polyphenolic compounds and anthocyanin analyses, the dried samples were milled using a hammer mill (Model 2A, Christy and Norris Ltd., Chelmsford, England) and the resulting liquor packed in a black polyethylene bags and used.

### Experimental design

The studies were conducted using a 4 x 4 full factorial design with experimental factors as pod storage (0, 7, 14 and 21 days) and fermentation time (0, 2, 4 and 6 days). The polyphenolic (total polyphenols and 0-diphenols) concentrations and anthocyanins content of the fermented dried beans were studied. All treatments and analyses were conducted in triplicates.

### *Methods*
### Determination of total polyphenols

Cocoa nibs were defatted by extracting the fat with petroleum ether (40-60°C) in a Soxhlet apparatus. Total contents of polyphenolic compounds in the cocoa bean extracts were determined according to the Folin–Ciocalteau procedure (EEC, 1990). About 0.2 g of dry defatted cocoa nibs was extracted with Methanolic HCl (80% MeOH containing 1% HCl) with shaking for 2 h at room temperature. This was centrifuged at 1000 rpm for 15 mins, about 1.0 ml of supernatant was taken to develop colour reaction with 5.0 ml folin-ciocalteau reagent. To the supernatant 4.0 ml of $Na_2CO_3$ was added and allowed to stand for 1 h at 30°C, then 1 h at 0°C. The absorbance was read at 760 nm. The analysis was conducted in triplicates and the mean values reported. A working standard cathechin solution of blank (0), 0.2, 0.4, 0.6, 0.8 and 1 mL was prepared and made up to 1.0 mL volume with distilled water. The colour was developed, the absorbances read at 760 nm and a standard curve drawn. From the standard graph, the amount of polyphenol present in the sample preparation was calculated.

### Determination of o-diphenols

O-diphenols content was determined with Arnow's reagent (10 g $NaNO_2$, 10 g $Na_2MoO_4$ in 100 ml $H_2O$). To 1 ml of extract (ethanol or methanol extract), 1 ml 0.5N HCl, 1 ml Arnow's reagent, 10 ml $H_2O$ and 2 ml 1N NaOH was added. The solution was mixed and the absorbance was read at 515 nm (520) after 30 sec. The analysis was conducted in triplicates and the mean values reported. A working standard cathechol solution of blank (0), 0.2, 0.4, 0.6, 0.8 and 1 mL was prepared and made up to 1.0 mL volume with distilled water. The colour was developed, the absorbances read at 520nm and a standard curve drawn. From the standard graph, the amount of o-diphenol present in the sample preparation was calculated.

### Determination of anthocyanins content

Anthocyanin content was determined using a method described by Misnawi *et al*. (2002). The extract obtained for total polyphenol analysis was filtered using Whatman 4 filter paper, and the supernatants were read spectrophotometrically for total absorbance (TOD) at 535nm. The content of total anthocyanins was calculated as follows;

*Total anthocyanins (mg/kg) = TOD\*1000/ (AvE530)1cm/10*

Where TOD is the total optical density (absorbance) and (AvE530)1cm is the average extinction coefficient for total anthocyanin when a 1 cm cuvette and 1% (10 mg/ml) standards are used; the value is 982. The analysis was conducted in triplicates and the mean values reported.
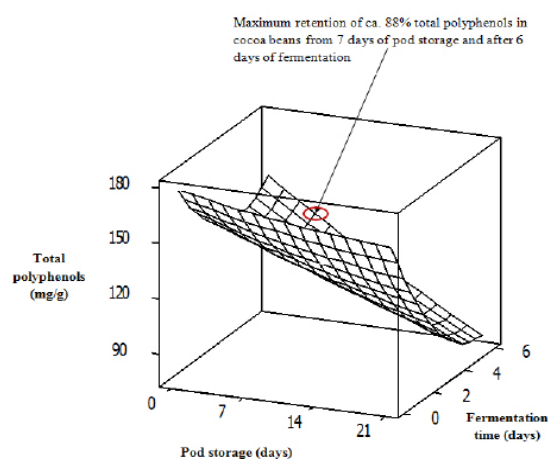
**Figure 1.** Response surface plot showing changes in total polyphenol content of cocoa beans as affected by pod storage and fermentation time

*Statistical analyses*

The data were analysed using Statgraphics software version 4.0 (STSC Inc, Rockville, MD, USA) for analysis of variance (ANOVA). Least significant difference (LSD) was used to separate and compare the means and significance was accepted at 5% level (p<0.05). Further analyses were conducted to evaluate the combined effect of pulp preconditioning and fermentation time on the polyphenol and anthocyanin concentrations using response surface methodology. The data obtained were studied using stepwise multiple regression procedures. Models were developed to relate pulp preconditioning and fermentation time on the polyphenolic and anthocyanin concentrations in cocoa beans. Table 1 shows the coefficients of the variables in the models and their contribution to the model's variation. $R^2$ values were used to judge the adequacy of the models. The $R^2$ of a model refers to the proportion of variation in the response attributed to the model rather than random error. For a good fit of a model, an $R^2$ of at least 80% was used. Malcolmson *et al.* (1993), commented that R of 80% is perfect for a good model study and but recommended that an $R^2$ of 60% can be used for a preliminary study.

**Results and Discussion**

*Changes in total polyphenols content*

Cocoa bean polyphenols, comprising 12-18% of the whole bean weight, have long been associated with the flavour and colour of chocolate (Kim and Keeney, 1984). Polyphenols impart an astringent taste to cocoa beans, which is perceived as a dry feeling in the mouth along with a coarse puckering of the oral tissue. This taste has been associated with the effect of polyphenol-protein interaction in the saliva to form precipitates or aggregates. In this study, the total polyphenolic content of the unstored cocoa samples ranged from 190.87 mg/g to 140.34 mg/g.

Response surface plot generated from the regression model is as shown (Figure 1). Polyphenol content reduced slightly with pod storage at the beginning of fermentation. However as fermentation time increased the total polyphenol content decreased sharply with increasing pod storage. Fermentation of cocoa beans is crucial for the development of precursor for chocolate flavour. Complex interactions among polyphenols to form high molecular weight tannins and their interactions with protein impact on the overall quality of fermented cocoa beans for chocolate production. During fermentation of cocoa beans, polyphenols diffuse with cell liquids from their storage cells and are oxidized enzymatically by the polphenol oxidase to condensed high molecular mostly insoluble tannins. The sharp decreases observed (Figure 1) reflect the onset of these phenomena during fermentation. Thus, astringency and bitterness associated with polyphenols may be reduced drastically by the combined effect of pod storage and fermentation prior to chocolate manufacturing since roasting, conching, as well as tempering and other processes during chocolate manufacturing, cannot remove the excessive amount of astringency in cocoa beans (Fowler, 1995; Afoakwa *et al.*, 2008). However, in modern times, the health benefits of polyphenols in cocoa products have significant influence on their acceptability and marketing. The response curve (Figure 1) showed that only minimal losses in total polyphenols were observed in the beans from pods stored for 7 days and made to undergo 6 days of fermentation. Further increases in pod storage times above 7 days led to drastic reductions in the polyphenolic content of the beans. This suggest that storage of cocoa pods for up to 7 days and fermenting for 6 days would help retain about ~83% of the polyphenolic content of the beans and this would have significant commercial implications as their derived chocolate and cocoa products would provide appreciable health benefits to consumers.

The model developed to predict the polyphenol content had an $R^2$ of 0.85 implying that it could explain 85% of the variations in polyphenol content. The regression coefficients showed that the linear terms of pod storage and fermentation time as well as the quadratic term of fermentation time had a significant influence on polyphenol content (Table 1). There was also a significant (p<0.05) interaction between pod storage and fermentation time (Table 1). The implication of this finding is that total polyphenols content at every fermentation time depends on the

**Table 1.** Regression coefficients and their adjusted $R^2$ values in the models for total polyphenols, *o*-diphenol and anthocyanin in cocoa beans

| Variables | Coefficients | | |
|---|---|---|---|
| | **Total polyphenols** | ***o*-diphenols** | **Anthocyanins** |
| Constant | 177.817* | 24.2500* | 17.0108* |
| $X_1$ | -1.1937* | -0.00958* | -0.17877 |
| $X_2$ | -0.8028* | -0.09981* | -5.14831* |
| $X_1^2$ | 0.02143 | 0.00438 | 0.00497 |
| $X_2^2$ | 0.0042* | 0.00054* | 0.536797* |
| $X_1 * X_2$ | -0.0182* | -0.00380* | -0.00964 |
| $R^2$ | 0.851 | 0.855 | 0.963 |

* Significant at $p < 0.05$; $X_1$ = Pod storage; $X_2$ = Fermentation time
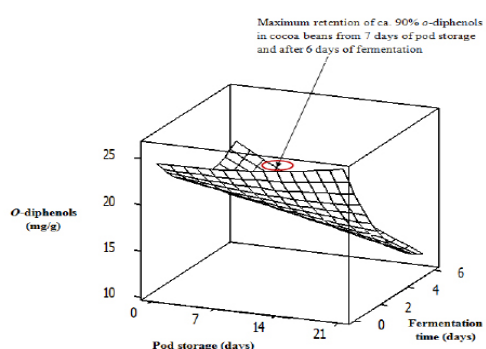


**Figure 2.** Response surface plot showing changes in *o*-diphenol content of cocoa beans as affected by pod storage and fermentation time

duration of pod storage. To optimize maximum retention (~83%) of the total polyphenols, the pods could be stored for up to 7 days after which the beans could be fermented for 6 days.

*Changes in o-diphenols content*

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) (Wollgast and Anklam, 2000). In living tissues, the phenolic substrates and the enzymes 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 (cathechins and epicathechins) and further complexation of o-quinones (Camu *et al*., 2008). There was a significant ($p < 0.05$) influence of the linear factors of pod storage and fermentation time, but only the quadratic factor of fermentation time was significant ($p < 0.05$) on the o-diphenol content of the beans. The model could

explain 85% of the variations in o-diphenol content meaning only 15% of the variations were due to other factors which were not included in the model (Table 1).

The response surface plot generated (Figure 2) showed that pod storage and fermentation time all had significant effect on o-diphenol content of the cocoa beans with significant interaction between these factors. It showed a curvilinear plot with fermentation time. This implies that the o-diphenol content reduced considerably during the fermentation and increased slightly by the end of the fermentation. Generally, increasing pod storage and fermentation time caused drastic reduction in the content of o-diphenol compounds (cathechin and epicathechin) in the beans. Nazarrudin *et al*. (2006) reported that the reduction in pulp volume during pod storage may have facilitated the oxidation and polymerization of o-diphenols and its oxidation products as clearly demonstrated by the beans from 14 and 21 day pod storage. The occurrence of condensation reactions is confirmed by the sharp decrease of o-diphenols content between the second and sixth day of fermentation for beans stored for 14 and 21 days (Figure 2). Epicatechin and catechin content, is reduced to approximately 10–70% during fermentation. This is not only due to the oxidation process but also caused by diffusing of polyphenols into fermentation sweatings (Kim and Keeney, 1984). Earlier study on the effect of pod storage on the content of (−)-epicatechin, from different clones also demonstrated almost the same pattern (Clapperton *et al*., 1992). The reduction in the o-diphenols might help to lower the level of astringency in the cocoa beans. Previous work by other researchers also show similar trend (Biehl *et al*., 1989; Meyer *et al*., 1989; Nazaruddin *et al.,* 2006).

Even though the reduction in o-diphenols during fermentation of cocoa beans might impact on levels of astringency and bitterness in the fermented beans, retention of the o-diphenol concentrations in the beans would enhance the potential health benefits of its chocolate and cocoa products. The combined effects of pod storage and fermentation times could be manipulated to maximize retention of the o-diphenols in the fermented beans. The response curve (Figure 2) showed that losses in o-diphenols noted in the beans from pods stored for 7 days and made to undergo 6 days of fermentation were very minimal and the process can retain ~90% of the o-diphenol content of the fermented beans. Further increases pod storage times above 7 days led to drastic reductions in the o-diphenol content of the beans, and these would have significant commercial and health implications for both the chocolate manufacturing industry and

consumers.

*Changes in anthocyanin concentration*

Anthocyanins give the purple colour to unfermented Forastero beans, but are absent from Criollo beans (Rohan and Stewart, 1967; Biehl *et al.,* 1989). The model developed for predicting anthocyanin content, could explain 96.3% of the variation observed in this index. The variables which significantly affected the anthocyanin content of the cocoa bean were the linear and quadratic terms of fermentation time (Table 1). Significant interaction between pod storage and fermentation time was not found and these findings give an indication that anthocyanin content of pulp preconditioned fermented beans is dependent only on fermentation time.

The response surface plots generated (Figure 2) showed that the anthocyanin content of the pulp preconditioned cocoa beans decreased drastically with increasing fermentation at all pod storage levels. This drastic reduction in anthocyanin content (~95%) occurred by the end of the fourth day of fermentation, beyond which no considerable changes in anthocyanin content were observed for all the pod storage treatments. Wollgast and Anklam (2000) and Nazaruddin *et al*. (2006) have all reported that anthocyanins usually disappear rapidly during fermentation process (93% loss after 4 days). The observed changes might have been due to hydrolysis of anthocyanins to anthocyanidins with the latter compounds being polymerised along with simple catechins to form complex tannins during fermentation. Pod storage caused only marginal and insignificant ($p > 0.05$) decreases in anthocyanin content at all periods of fermentation.

## Conclusion

The processes of fermentation and pod storage decreases the polyphenolic content of cocoa beans but the rates of decrease are more dependent of fermentation time than on pod storage. Total polyphenol and o-diphenol content reduced marginally (<10%) in the cocoa beans fermented for 6 days after 7 days of pod storage. Increasing pod storage beyond 7 days led to drastic reduction in the polyphenolic content at the end of the 6 days of fermentation. Similarly, anthocyanin content of beans from the 7 days of pod storage decreased by ~90% by the fourth day of fermentation beyond which only slight changes were observed till the end of the fermentation. Pod storage caused only marginal and insignificant decreases in anthocyanin content at all periods of fermentation. Storage of cocoa pods for up to 7 days after harvesting helps to retain about 88% and 90% of total polyphenols and o-diphenol content respectively after 6 days of fermentation.

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