

Analytical Methods

Phenolic content and antioxidant capacity of hybrid variety cocoa beans

W.A. Jonfia-Essien, G. West, P.G. Alderson, G. Tucker *

School of Biosciences, University of Nottingham, Sutton Bonington Campus, Loughborough, Leicestershire LE12 5RD, UK

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Abstract

Cocoa (*Theobroma cacao* L.) is a major, economically important, international crop and has been associated with several nutritional benefits including high antioxidant capacity. New cocoa hybrids have been developed in Ghana that exhibit resistance to pest damage during storage. The aim of this work was to assess the phenolic content and antioxidant capacity of these new hybrids in comparison to more traditional cocoa varieties. Total extractable phenolics were similar in all the four hybrids tested ranging from 69.9 to 81.6 FAE g⁻¹. These levels were very similar to that extracted from traditional beans (73.8 ± 2.5 FAE g⁻¹). The “phenolic profile” was determined by HPLC. A total of 25 peaks was observed but there were only minor differences in this profile between traditional and hybrid bean extracts. Antioxidant capacity was determined using the FRAP assay and traditional beans were found to possess 12.4 μmol TE g⁻¹. In comparison the hybrid beans had antioxidant capacities ranging from 21.6 to 45.5 μmol TE g⁻¹, and these were significantly higher than in the traditional beans for three out of the four hybrids. Since the phenolic and antioxidant levels and in these hybrid varieties were either similar to, or higher than, that obtained from traditional beans, the introduction of these new varieties would be unlikely to impact detrimentally on these nutritional components of the beans.

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1. Introduction

Polyphenols have become an intense focus of research interest because of their perceived beneficial effects for health (Wollgast & Anklam, 2000), including anti-carcinogenic, anti-atherogenic, anti-ulcer, anti-thrombotic, anti-inflammatory, immune modulating, anti-microbial, vasodilatory and analgesic effects.

Studies suggest cardiovascular diseases may be preventable by lifestyle modifications, such as exercise and nutrition (Hu & Willett, 2002; Stampfer, Hu, Manson, Rimm, & Willett, 2000; Tanasescu et al., 2002; Weisburger, 2000). A diet containing major sources of antioxidants and polyphenols is recommended for prevention purposes.

Many studies have considered fruits, vegetables and teas as a major sources of dietary antioxidative phenolics, but

Lee, Kim, Lee, and Lee (2003) demonstrated the importance of cocoa. Wines and beverages, such as cocoa, red wine, black tea and green tea, are consumed widely and are known to be rich in phenolic phytochemicals. In particular, theaflavin, epigallocatechin gallate, resveratrol and procyanidin in black tea, green tea, red wine and cocoa respectively, have been considered as major chemo-preventive agents mainly due to their strong antioxidant activities (Lee et al., 2003). Cocoa (*Theobroma cacao* L.) is particularly rich in polyphenols (Wollgast & Anklam, 2000). It is also one of the richest naturally occurring sources of antioxidants. Indeed cocoa products contain greater antioxidant capacity and greater amounts of flavonoids per serving than either tea or red wine (Lee et al., 2003; Steinberg, Bearden, & Keen, 2003). Chocolate, a cocoa product, can also be a major source of dietary antioxidants, and these may have protective effects against cardiovascular disease (Keen, Holt, Oteiza, Fraga, & Schmitz, 2005; Kris-Etherton & Keen, 2002; Steinberg et al., 2003). Chocolate has also been reported to be a good source of dietary

* Corresponding author. Tel.: +44 1559 516126; fax: +44 1559 516122.
E-mail address: gregory.tucker@nottingham.ac.uk (G. Tucker).

catechins, second only to black tea (Arts, Hollman, & Kromhout, 1999).

The polyphenolic composition of cocoa has been characterised and quantified (Sanchez-Rabaneda et al., 2003; Wollgast & Anklam, 2000; Zumbe, 1998). The compounds identified include the catechins – catechin; epicatechin; gallocatechin and epigallocatechin; procyanins; anthocyanins; and flavone and flavonol glycosides such as luteolin-7-*O*-glucoside and quercetin-3-*O*-arabinoside.

Ghana is one of the largest producers of high quality cocoa. However, whilst the consumption of cocoa has increased over the last decade, the yield of the crop in the country has been on the decline. To improve yield, hybrids have been developed from crosses between Amazon, Trinitario and Amelonado genotypes to augment the Series II hybrids (Adu-Ampomah & Sersah, 1987/1988) already grown by farmers (Adu-Ampomah, 1996). Some of these hybrids have been selected on the basis of their yields, which are comparable to the traditional planting materials of Amelonado and local Trinitario genotypes of cocoa, or on their disease and pest resistance. Whilst these are not currently in commercial use they are likely to be introduced in the near future. However, very little is known about the level of key nutrients such as antioxidants and phenolics in these hybrids. The aim of this study was to determine the phenolic content and antioxidant capacity of these hybrids with the aim of ascertaining any potential impact on the content of these important nutrients.

2. Materials and methods

2.1. Materials

A range of new hybrid varieties of cocoa have been developed by the Cocoa Research Institute of Ghana. Four of these hybrids (HV1–HV4) (Table 1) have been selected for further study based on their high yields and were grown under tropical conditions at the experimental farm of the Cocoa Research Institute of Ghana at Tafo, a town in the Eastern Region of Ghana. Beans were harvested in November 2002. Traditional beans (TV) were grown under the same conditions but at different farms, and were harvested at exactly the same time as the hybrids. The harvested pods were broken to extract the beans, which were then fermented for a period of six days. After fermentation the beans were sun-dried on mats raised off the ground. Flat or broken beans were removed after drying and the beans were bagged in jute sacks. The beans were then sent

to the Research Department of the Quality Control Division for quality checking, packaging and short-term transit storage before being air-lifted to the UK in March 2003 to commence the experiment.

The dry cocoa beans were sieved to remove dirt. Beans were bulked together and split into four equal parts diagonally. One part was randomly selected for the first sample and a 500 g batch weighed out. The remaining beans were bulked together and the process repeated twice more so as to give triplicate samples from each variety. Each sample was stored in a proto-type jute sacks. Jute sacks were used so as to conform to the approved standard of storing cocoa beans. The cocoa beans were then stored in a controlled environment cabinet at 30 ± 2 °C and relative humidity of $70 \pm 2\%$, based on the prevailing conditions at the cocoa warehouses in Ghana. Samples for analysis were taken from store after 31 days.

2.2. Methanolic extraction

A 10 g sample of cocoa bean nibs was milled with a coffee blender and 2 g of the resultant cocoa powder was homogenized in 50 mL of 80% methanol for 1 min in a flat bottom flask using a polytron homogenizer at 25,000 rpm. The cocoa suspension was refluxed for 30 min and then filtered.

This extract was used for determination of both antioxidant capacity and total phenolic content.

2.3. Antioxidant assay

Antioxidant capacity was measured using the ferric reduction antioxidant potential (FRAP) assay as described by Benzie and Strain (1996), using trolox (0–20 nmol) as a standard. Micro-assay plates were prepared by putting 10 μ L of ethanol (blank), trolox standard or sample solution into wells of a microtitre plate followed by the addition of 100 μ L of FRAP assay reagent to all the wells. Absorbance at 630 nm was recorded using a plate reader (Bio-Rad 550) and the antioxidant capacity of the sample calculated as trolox equivalents.

2.4. Phenolic assay

Total phenolic content was measured using the Folin & Ciocalteu's assay (Forrest & Bendall, 1969). One mL of the filtered refluxed sample was diluted with 49.0 mL of distilled water. Folin–Ciocalteu's phenol reagent was diluted to 50%, then 0.25 mL of the 50% reagent was added to 0.25 mL of the sample and incubated in a water bath for 3 min at 25 °C. This was followed by the addition of 0.5 mL saturated aqueous Na_2CO_3 solution and further incubation in the water bath for 60 min. The absorbance at 750 nm was then recorded. Results are expressed as ferulic acid equivalents (FAE) using a standard curve of ferulic acid (0–20 μ g).

Table 1
Genetic lineage of the cocoa used in this study

Cocoa type	Variety
Amazon/Trinitario hybrids	HV1
Inter Amazon hybrids (Amazon/Amazon)	HV2 and HV3
Amazon/Amelonado Hybrids	HV4
Traditional cocoa type	TV

2.5. Phenolic profiling

Phenolic profiles were determined using High Performance Liquid Chromatography (Perkin–Elmer LC 200). The HPLC was fitted with a Supelco C18 Discovery column, 4.6 mm × 15 cm, and a diode array detector with absorbance set at 280 nm.

A sample (5 g) of the milled cocoa bean nibs was placed into a beaker and 200 mL of 70% methanol was added. Hesperitin (1 mg) was added as an internal standard to monitor recovery of phenolics. The mixture was stirred with a magnetic stirrer for 2 h then filtered under vacuum through Whatman GF/A paper. The filtrate was transferred quantitatively to a round bottom flask and the methanol evaporated using a rotary evaporator, R-3000, at ~170 rpm and 30 °C to leave approximately 60 mL aqueous solution. Sodium hydroxide (50 mL × 2 N) was added and left to hydrolyse overnight at room temperature. The mixture was then transferred quantitatively to 3 × 50 mL centrifuge tubes, and centrifuged at 2000g for 15 min. The supernatant was decanted from the pellet and filtered through Whatman No. 4 paper into a separating funnel. 80 mL of ether was added, shaken and the solution left to partition. The aqueous phase was retained. This partitioning step was repeated two more times. The final aqueous extract was then acidified to pH 1.5 with hydrochloric acid and filtered through Whatman No. 1 paper into a separating funnel. 80 mL of diethyl ether was added, shaken and left to partition. The ether phase was collected. This partitioning was repeated twice more and the resultant three ether extracts were pooled. The pooled ether extract was dried with MgSO₄ (anhydrous), filtered through Whatman No. 1 paper into a round bottom flask and rotary evaporated until only a few mL of ether remained. This was transferred quantitatively to a small sample tube and the ether evaporated to dryness in a stream of nitrogen.

The sample was taken up in 2 mL of a mixture of HPLC grade methanol (25%) and 0.02 M phosphate buffer pH 2.4 (75%), filtered through a 0.2 µm syringe filter and 10 µL injected into the HPLC. It was then eluted with a linear gradient of HPLC grade methanol and 0.02 M phosphate buffer pH 2.4 over 25 min starting with 20% methanol and 80% buffer and finishing with 80% methanol and 20% buffer.

2.6. Statistics

All measurements were made in triplicate and the results analysed by ANOVA using the Genstat 3.1 statistical programme.

3. Results and discussion

Traditional and hybrid cocoa beans were sampled and phenolics extracted into 70% methanol. The total extracted phenolics were then quantified (Fig. 1). All of the varieties of beans tested contained relatively high – around 70 to

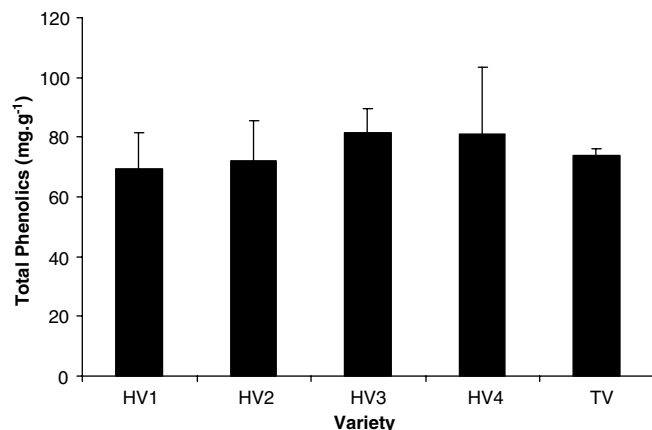


Fig. 1. Total phenolics extractable from traditional or hybrid cocoa beans. Milled nibs from either traditional (TV) or hybrid (HV1–HV4) cocoa beans were extracted into 70% methanol and total phenolics measured using the Folin–Ciocalteu's assay. Results are expressed as ferulic acid equivalents and presented as the mean and SE ($n = 3$). There were no statistically significant differences between any of the beans analysed.

80 mg g⁻¹ – levels of phenolics. The total phenolic content of cocoa beans has been reported to range between 67 and 149 and from 101 to 144 mg g⁻¹ for freshly harvested and 2 day fermented cocoa beans, respectively (Niemenak, Rohsius, Elwers, Ndoumoua, & Lieberei, 2006). The slightly lower values in this instance could be attributed either to the fact that beans had been stored prior to analysis or to varietal differences. There were, however, no significant differences observed between the levels in the four new hybrid varieties and that of the traditional mixture. Thus it is probably the case that these four hybrids are no different to the traditional beans in terms of total phenolics.

Whilst total phenolics may be a useful indicator of potential nutritional benefit, the actual profile of phenolics within the bean is likely to be more important. Thus the profile of the extractable phenolics, following alkaline hydrolysis, from either the hybrid or traditional beans was determined by HPLC. A typical HPLC profile is shown in Fig. 2A. A total of 25 peaks were observed within the varieties tested. It was not possible to assign identities to these peaks. A similar extraction procedure, but without the alkaline hydrolysis (Niemenak et al., 2006), managed to resolve 8 peaks, three of which were identified as theobromide, epicatechin and caffeine. A comparative analysis was carried out by comparing the relative peak areas between the five different types of beans. Thus each of the 25 peak areas, after correction for the internal standard, was expressed as a percentage of the whole. There were only minor differences evident between the traditional type and the hybrid varieties (Fig. 2B). The values for peaks 1 and 3 were higher in all of the hybrid varieties than in the traditional type. Peak 11 was absent in beans of the hybrid varieties but was also very low in the traditional type. Peaks 17, 18, 19 and 20, whilst low in all the hybrid

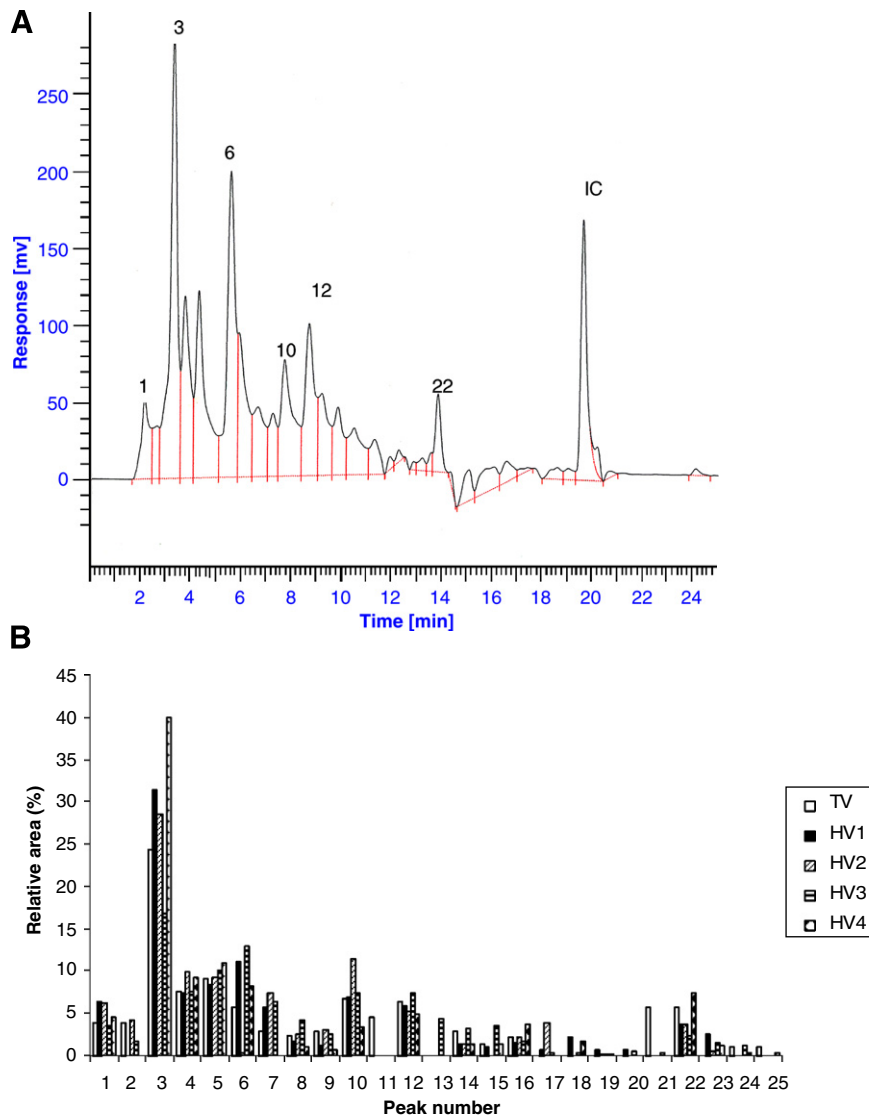


Fig. 2. Phenolic profile of methanolic extracts from dry cocoa beans. Milled cocoa bean nibs were extracted into 70% methanol and the resultant extractant dried and subjected to alkaline hydrolysis prior to separation by HPLC and detection at 280 nm. (A) Typical HPLC trace for a hybrid variety showing the numbering of the major observed peaks. IC = Internal control (hesperitin). (B) Relative contribution of each peak to total phenolics content. Values are presented as % of total of the integrated area of peaks after correlation for the internal control.

varieties, were undetectable in the traditional type. There were no consistent changes in the hybrids compared to the traditional type beans and as such it is unlikely that levels of any individual phenolic compounds are significantly altered between the hybrid and traditional beans.

The antioxidant capacity of the beans, was determined using the FRAP assay (Fig. 3). This was found to be $12.4 \pm 7.3 \mu\text{mol TE g}^{-1}$ for the traditional variety, whilst the values for the hybrids ranged from 21.6 ± 2.7 to $45.5 \pm 2.86 \mu\text{mol TE g}^{-1}$. The values for HV1 ($p = 0.001$), HV2 ($p = 0.004$) and HV3 ($p = 0.002$) were all significantly higher than that of the traditional beans. The value for HV4, whilst numerically higher than for the traditional beans, was not statistically significant ($p = 0.055$). It is difficult to compare these antioxidant capacity values with those quoted by previous workers due to differences in both extraction and assay methodologies. However, Gu, House,

Wu, Ou, and Prior (2006) using an ethanolic extraction and an ORAC assay reported values of $826 \pm 103 \mu\text{mol TE g}^{-1}$ for natural cocoa powder. These values are notably higher than those found in this study. Since phenolics represent a major component of the cocoa bean it is postulated that they represent the major source of antioxidant capacity in cocoa beans and their products. Thus Othman, Ismail, Ghani, and Adenan (2007) monitored phenolics and antioxidant capacity in beans from a range of geographical sources, including Ghana, and have demonstrated that there was a significant effect of “region of production” on both these parameters and that there was a good correlation between total phenolics and antioxidant capacity. Similarly, Gu et al. (2006) have demonstrated a good correlation between total phenolics and antioxidant capacity in cocoa beans and their products. It is interesting to note that in this study, whilst total phenolic content was not

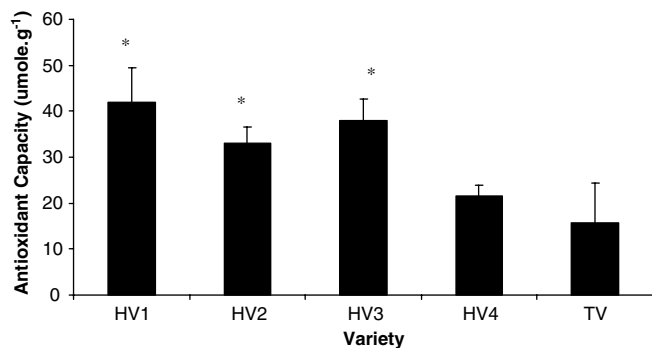


Fig. 3. Total antioxidant capacity extractable from traditional or hybrid cocoa beans. Milled nibs from either traditional (TV) or hybrid (HV1–HV4) cocoa beans were extracted into 70% methanol and total antioxidant capacity measured using the FRAP assay. Results are expressed as trolox equivalents and presented as the mean and SE ($n = 3$). Values marked with (*) represent samples whose antioxidant capacity is significantly ($p < 0.005$) different to TV.

affected in the hybrids, antioxidant capacity has apparently increased. This might suggest the presence of other important antioxidants in the cocoa bean.

In conclusion, there seems to be little or no difference between the hybrid varieties and the traditional type cocoa beans in terms of either their phenolic content or composition. The antioxidant capacity of the hybrids is equivalent to, or may even be higher than, the traditional beans. On the basis of this study, therefore, it is unlikely that the introduction of these hybrid varieties into commercial trade would have any impact on the provision of these key nutrients to the consumer.

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