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Characterization of anthocyanins in Kenyan teas: Extraction and identification

L.C. Kerio [a], F.N. Wachira [a,b,*], J.K. Wanyoko [a], M.K. Rotich [c]

[a] Tea Research Foundation of Kenya, P.O. Box 820, 20200 Kericho, Kenya
[b] Egerton University, Biochemistry Department, P.O. Box 536, Egerton, Kenya
[c] Egerton University, Chemistry Department, P.O. Box 536, Egerton, Kenya

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ABSTRACT

Characterization and quantification of anthocyanins in selected tea cultivars processed into black (aerated) and green (unaerated) tea products was carried out in this study. The anthocyanins were extracted from tea products processed from a number of newly bred purple leaf coloured Kenyan tea cultivars (*Camellia sinensis*) using acidified methanol/HCl (99:1 v/v). Extracted anthocyanins were purified by C_{18} solid phase extraction (SPE) catridges and characterised by HPLC-UV–Visible. They were identified according to their HPLC retention times, elution order and comparison with authentic standards that were available. Total monomeric anthocyanins were determined by the pH-differential method. Although the tea cultivars gave different yields of anthocyanins, the unaerated (green) teas had significantly ($p \leqslant 0.05$) higher anthocyanin content than the aerated (black) teas. This was attributed to the degradation of anthocyanins by polyphenol oxidase products (catechin *O*-quinones) formed during the auto-oxidation (fermentation) process of black tea manufacture. Of the six most common natural anthocyanidins, five were identified in the purified extracts from purple leaf coloured tea, in both aerated (black) and unaerated (green) teas namely; delphinidin, cyanidin, pelargonidin, peonidin and malvidin. The most predominant anthocyanidin was malvidin in both tea products. In addition, two anthocyanins namely, cyanidin-3-*O*-galactoside and cyanidin-3-*O*-glucoside were also identified. Tea catechins were also identified in the tea products derived from the purple coloured tea cultivars namely, epigallocatechin (EGC), catechin (+C), epicatechin (EC), epigallocatechin gallate (EGCG), and epicatechin gallate (ECG). Correlation between the total catechins versus the total anthocyanins and anthocyanin concentration in unaerated teas revealed significant negative correlations ($r = -0.723^{*}$ and $r = -0.743^{**}$, $p \leqslant 0.05$ and $p \leqslant 0.01$, respectively). However, in aerated (black) tea the correlations were insignificant ($r = -0.182$ and $r = -0.241$, $p > 0.05$).

1. Introduction

Tea (*Camellia sinensis* (L.) O. Kuntze) is the most important crop species of the genus *Camellia*, that is used to produce the most widely consumed beverage. Tea contains bioactive polyphenols, mainly the catechins, which play an important role in green and black tea quality (Owuor & Obanda, 2006). It is approximated that of all the tea consumed worldwide, about 76–78% is black (aerated) tea (Cabrera, Gimenez, & Lopez, 2003). Black tea is manufactured by a post harvest process which involves withering the leaf to reduce its moisture content, leaf maceration and auto-oxidation and its subsequent drying. The auto-oxidation process is catalysed by the enzyme polyphenol oxidase (PPO) which converts the green leaf catechins to complex coloured compounds, which include the theaflavins (TFs) and thearubigins (TRs), which give the black tea its quality characteristics (Obanda, Owuor, & Mang'oka, 2004). The processing of green tea on the other hand does not involve the oxidation step and the leaf is initially steamed to inactivate the PPO and hence minimize any chemical and biochemical reactions involving the enzyme (Wilson & Clifford, 1992). Tea catechins have been found to be pharmacologically active (Higdon & Frei, 2003; Karori, Wachira, Wanyoko, & Ngure, 2007; Rizvi, Zaid, Anis, & Mishra, 2005; Wachira & Kamunya, 2005). The major catechins in the tea leaf include: (+)-catechin (C), gallocatechin (GC), (−)-epigallocatechin (EGC), (−)-epicatechin (EC), (−)-epicatechin gallate (ECG) and (−)-epigallocatechin gallate (EGCG) with the most abundant in green leaf being EGCG.

Anthocyanins are plant pigments found in many plant species including red grapes (Perez-Rivero, Muniz, & Gonzalez-Sanjos, 2008), berries (blueberry, strawberry, raspberry, blackcurrant, bilberry, cranberry, elderberry) (Nicoue, Savard, & Belkacemi, 2007), eggplant (Azuma et al., 2008), purple fleshed sweet potatoes (Oki et al., 2002) and flowers like Hibiscus (Lo, Huang, Lin, Chien, & Wang, 2007). These pigments have been found to be the largest and most important group of water soluble pigments found in nature and they contribute to the attractive colours of fruits,

* Corresponding author at: Tea Research Foundation of Kenya, P.O. Box 820, 20200 Kericho, Kenya. Tel.: +254 0722 644279; fax: +254 052 20575.
E-mail address: fwachira@yahoo.com (F.N. Wachira).

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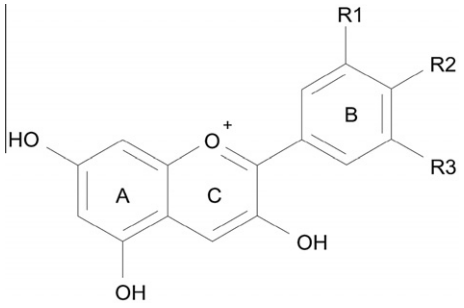

Fig. 1. The basic structure of the anthocyanidin pigment, the flavylium cation.

Table 1
The six most common anthocyanins in nature.

Anthocyanidin	R1	R2	R3
Cyanidin	OH	OH	H
Delphinidin	OH	OH	OH
Malvidin	OCH_3	OH	OCH_3
Pelargonidin	H	OH	H
Peonidin	OCH_3	OH	H
Petunidin	OCH_3	OH	OH

vegetables and flowers imparting red, orange, purple, violet and blue colours (Field, Lee, & Holbrook, 2001). Anthocyanins in plants normally accumulate in the vacuoles of the epidermal and subepidermal cells (Steyn, Wand, Holcroft, & Jacobs, 2002). The colours of these pigments are pH-dependent (Mazza & Miniati, 1993). Interest in anthocyanins has recently increased owing to their potential health benefits (Kong, Chia, Goh, Chia, & Brouillard, 2003) and their use as an alternative source of synthetic colourants/dyes (Jackman, Yada, Tung, & Speers, 1987).

Because of the sedentary nature of plants, they are prone to UV-B irradiation which can cause oxidative stress. Anthocyanins protect plants against such irradiation. Their biosynthesis has been demonstrated to be upregulated when the plant is exposed to UV-B irradiation (Merzlyak, Chivkunova, Solovchenko, & Naqvi, 2008). Anthocyanins also aid to elevate the plants leaf temperature during winter or chilling (Hughes, Neufield, & Burkey, 2005), apart from providing protection against invasion from pests and herbivores (Chalker-Scott, 1999). Industrially, anthocyanins may be used as food colourants (in jams, juices and confectioneries) and preservatives, in the manufacture of cosmetics (soaps, shampoos, lotions) and in the pharmaceutical industry for tablet/capsule coatings, syrups and as health concentrates. Because of their colour, they are also important in the making of red wine (Rivero-Perez et al., 2008; Rigo et al., 2000).

Chemically, anthocyanins are glycoside moieties of anthocyanidins derived from the flavylium (2-phenylbenzopyrilium) cation (Fig. 1). There are several anthocyanidins described in nature but among these, six are widespread in fruits and vegetables namely; pelargonidin, cyanidin, peonidin, delphinidin, petunidin and malvidin (Table 1). Because of their polar nature, anthocyanins are soluble in polar solvents, such as methanol (MEOH), ethanol and water. The initial step in their isolation therefore involves solvent extraction, which includes the use of acidified methanol or ethanol. The use of acid stabilizes anthocyanins in the flavylium cation (Fig. 1) form, which is red at low pH (McGhie & Walton, 2007).

Recent research has shown that anthocyanins have numerous health beneficial properties, which include antioxidant (Bae & Suh, 2007), anticarcinogenic (Lee et al., 2009), anti-angiogenic (Bagchi, Sen, Bagchi, & Atalay, 2004), antimicrobial (Viskelis et al., 2009), antiapoptotic (Elisia & Kitts, 2008) and pro-apoptotic (Lo et al., 2007) properties. Despite their health enhancing properties, no work had been carried out to determine the presence of these coloured polyphenols in newly bred purple leaf coloured tea clones (Fig. 2b).

The biochemical composition of the newly developed coloured tea clones had not been studied though it was thought that they may be rich in anthocyanins. Being products of the same pathways i.e. the shikimate and phenylpropanoid pathways, the impact of the presence of the anthocyanins in these novel clones on the levels of catechin was also determined. It was also expected that the presence of anthocyanins in addition to the catechins would contribute to new and unique tea products. This study aimed at characterizing for the first time the anthocyanins in the tea products processed from the newly developed Kenyan purple coloured tea cultivars.

2. Materials and methods

2.1. Tea samples

An experiment was established at the Tea Research Foundation of Kenya's Kangaita substation in Kirinyaga (0° 26′S and 37° 15′E, elevation 2020 m a.m.s.l) in 2002. It comprised of 30 selected cultivars of newly bred teas. Of these, 18 were purple coloured tea cultivars (Fig. 2b) and 12 were popular ordinary green coloured tea clones used for processing of black tea (Fig. 2a). The green coloured tea clones served as controls in this study. Young tender shoots comprising of the youngest two leaves plus a bud were harvested and processed into black (aerated) and green (unaerated) tea. Black tea was manufactured by physically withering the leaf for 18 h to reduce moisture content to between 50% and 65% and maceration using a Crush, Tear and Curl (CTC) machine. The

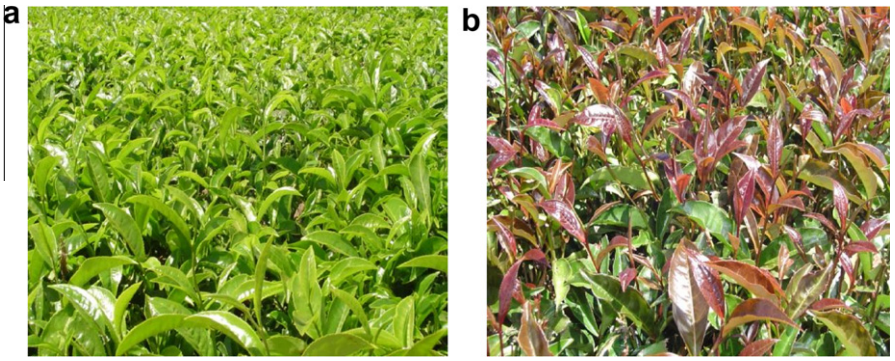

Fig. 2. (a) Ordinary green coloured tea plants. (b) Anthocyanin rich (purple coloured) tea plants. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

crushed leaf was then aerated for 90 min and then dried at 120–150 °C in a fluidised bed drier (Tea Craft, UK) for 20–25 min. Green (unaerated) tea was manufactured by steaming the leaf at 100 °C for 1 min, macerated and dried at the same temperature as for black tea. Both tea products were then milled and stored at 25 °C in sealed aluminium packets for further analysis. The fresh eggplant was purchased from a vegetable market.

2.2. Anthocyanin extraction and purification

Five grams (5 g) of ground tea samples were weighed into 250 ml conical flasks covered with foil and mixed with 50 ml MeOH/HCl (99:1v/v) and magnetically stirred at 900 rpm for 4 h at room temperature. The resultant solution was filtered and evaporated to dryness using a Rotavapour (Buchi Rotavapour R-300, Switzerland) under reduced pressure at 35 °C. The extract was dissolved in 10 ml distilled water and passed through a membrane filter 0.45 μM and kept in an icebath for analysis.

The extracts were passed through reverse phase (RP) C_{18} solid phase extraction (SUPELCO, SPE) (Sigma–Aldrich, USA) catridges previously activated with 10% MeOH/HCl. Anthocyanins were adsorbed into the column while sugars, acids and other water-soluble compounds were washed out using 0.01% HCl in distilled water. Anthocyanins were then recovered using acidified methanol (10% Formic acid v/v). The catridges were washed with ethyl acetate (Fischer Scientific, UK) to remove phenolic compounds other than anthocyanins. The purified extracts were stored at −10 °C until further analysis.

The eggplant was peeled to a weight of 5 g and its peels ground with mortar and pestle in 10 ml acidified methanol, filtered and stored in a 250 ml conical flask. The extraction was repeated five times and the combined filtrates were evaporated and purified like the tea samples.

2.3. Determination of total monomeric anthocyanin content

The total monomeric anthocyanin content in the processed black (aerated) and green (unaerated) tea samples was determined on 13 purple coloured cultivars and three high quality green coloured clones (randomly selected to serve as controls) and the eggplant peels. The analysis was carried out in triplicate using the pH differential method (Giusti & Wrolstad, 2001) using two buffer systems; 0.025 M potassium chloride (KCl) buffer at pH 1.0 and 0.4 M sodium acetate ($NaC_2H_3O_2$) buffer at pH 4.5. Two hundred microlitres (200 μl) of the anthocyanin sample was mixed separately with 1.8 ml of potassium chloride and sodium acetate buffer and the absorbance at 520 nm and 700 nm determined. The difference in absorbance of the sample was determined as follows.

$$A = (A\ 520\ \text{nm} - A\ 700\ \text{nm})\ \text{at pH}\ 1.0 - (A\ 520\ \text{nm} - A\ 700\ \text{nm})\ \text{at pH}\ 4.5$$

The monomeric anthocyanin pigment concentration (mg/l) in the original sample was determined using the formula:

$$\text{Monomeric anthocyanins pigment(mg/l)} = (A \times \text{MW} \times \text{DF} \times 1000)/(\varepsilon \times 1),$$

where A – absorbance (absorbance difference in the two pH ranges); ε – cyanidin-3-glucoside molar absorbance (26,900); MW – molecular weight of anthocyanin (449.2); DF – dilution factor.

2.4. Fractionation of anthocyanins by HPLC

The tea samples were further selected for anthocyanin profiling and only 10 purple coloured and one green coloured (control) were used. The tea products were characterised by HPLC using a Shimadzu LC 20 AT HPLC system fitted with a SIL 20A autosampler and a SPD-20 UV–Visible detector with a class LC10 chromatography workstation. UV detection was set at 520 nm using a Gemini C_{18} ODS (4.0 mm × 4.6 mm i.d.) (Phenomenex Inc. Torrance CA, USA) fitted with a Gemini C_6 ODS column (4.0 mm × 3.0 mm i.d.) (Phenomenex Inc. Torrance CA, USA) guard column. The column temperature was at 35°C. The eluents were mobile phase A (water/acetonitrile/formic acid-87/3/10 v/v/v) and mobile phase B (100% HPLC grade Acetonitrile). The chromatographic conditions were: 3% B in A at the time of injection, at 45 min; 25% B in A, at 46 min; 30% B in A and at 47 min; 3% B in A (initial conditions).The flow rate of the mobile phase was 1 ml/min and injection volume of 20 μl. The anthocyanin standards Cyanidin-3-*O*-glucoside, Cyanidin-3-*O*-galactoside, Cyanidin chloride, Peonidin chloride, Pelargonidin chloride, Delphinidin chloride and Malvidin chloride (Sigma Aldrich, UK) were used as standards for the identification and quantification of anthocyanin fractions in the processed tea samples.

2.5. Determination of dry matter content

Five grams (5 g) each of the aerated and unaerated tea products were weighed to the nearest 0.001 g, placed in pre-weighed aluminium dishes and dried in an oven (Oven Memmert, UND300, Germany) at 103 ± 2 °C for 16 h to constant weight. The percentage dry matter (DM) content for each sample was calculated from the weight differences.

2.6. HPLC analysis of catechins

Analysis of catechins by HPLC was done according to the ISO 14502 procedure (ISO 14502-2-2005E). One ml (1 ml) of the sample was pipetted into separate tubes and diluted to 5 ml with stabilizing solution, filtered and loaded into vials. Reverse phase HPLC analysis was used. A Shimadzu LC 20 AT HPLC fitted with a SIL 20A autosampler and a SPD-20 UV–Visible detector with a class LC10 chromatography workstation with UV detection at 278 nm using a Gemini C_{18} ODS (4.0 mm × 4.6 mm i.d.) column (Phenomenex Inc. Torrance CA, USA) fitted with a Gemini C_6 ODS guard column (4.0 mm × 3.0 mm i.d.) (Phenomenex Inc. Torrance CA, USA) were used. The column temperature was set at 35 °C. The flow rate of the mobile phase was 1 ml/min and the injection volume was 20 μl. Binary gradient elution conditions were as follows: 100% mobile phase A for 10 min, then over 15 min a linear gradient to 68% mobile phase A, 32% mobile phase B, and held at this composition for 10 min. It was then reset to 100% mobile phase A and allowed to equilibrate before the next injection. Individual catechins were identified by comparing the retention times of unknown peaks with those identified from the peaks obtained from the mixed catechin standards. Catechin quantification was done using a caffeine calibration curve together with the consensus relative response factors (RRFs), with respect to caffeine, calculated on a dry matter basis. The total catechin content of the teas as a percentage mass on a sample dry matter basis was determined by the summation of individual catechins as follows:

$$\%\ \text{Total catechins} = (\%\ \text{EGC}) + (\% + \text{C}) + (\%\ \text{EC}) + (\%\ \text{EGCG}) + (\%\ \text{ECG}).$$

2.7. Statistical analysis

All the determinations were carried out in triplicate and the data were subjected to analysis of variance, and the means separated by the least significant difference (LSD) test using MSTAT Version 2.10.

3. Results

3.1. Anthocyanins

3.1.1. Total monomeric anthocyanins-pH differential method

The contents of the total monomeric anthocyanins in the tea products (aerated and anaerated tea) from the assayed tea cultivars are presented in Table 2. The results were expressed as cyanidin-3-glucoside equivalents. Unaerated (green) tea had significantly ($p \leqslant 0.05$) higher total monomeric anthocyanins than the aerated (black) tea. The control clones GW Ejulu-L, TRFK 31/8 and TRFK 6/8, which had green coloured leaves had low levels of total monomeric anthocyanin contents, which ranged between 0.45 mg/l in aerated tea to 4.0 mg/l in unaerated tea of GW Ejulu-L. However, processed unaerated teas from the purple coloured tea clones TRFK 306/1, TRFK 306/2, TRFK 306/3 and TRFK 306/4 had total monomeric anthocyanin concentration of 98.86 mg/l, 91.73 mg/l, 108.26 mg/l and 75.03 mg/l, respectively, compared to their aerated teas, which had 23.99 mg/l, 30.28 mg/l, 45.48 mg/l and 14.14 mg/l, respectively. Processed unaerated teas from clones TRFK 73/1-7 series had much lower contents of total monomeric anthocyanin, ranging from 12.96 mg/l to 23.66 mg/l in anaerated tea, and between 0.67 mg/l and 7.57 mg/l for black tea derivatives. The peels of the eggplant (*Solanum melongena*) were found to be very rich in anthocyanins with 249.40 mg/l monomeric anthocyanins.

3.1.2. Total anthocyanin- HPLC method

The anthocyanin data obtained by HPLC analysis also showed a similar trend with that obtained by the pH differential method though the former gave higher levels of anthocyanins for all the assayed germplasm. By the HPLC method, the anthocyanin concentration ranged from 198 μg/ml in unaerated tea for clone TRFK 6/8 to 1193.4 μg/ml in unaerated tea for clone TRFK 306/3. In the processed black (aerated) teas, anthocyanins ranged from 124.5 μg/ml for clone TRFK 6/8 to 590 μg/ml for clone TRFK 306/3 (Table 3). The green leaf coloured clone TRFK 6/8, a high black tea quality clone used as an internal standard, was found to have a single cyanidin peak in addition to the catechins present in the tea.

3.1.3. Individual anthocyanin profiles

The anthocyanidin fractions in the processed unaerated and aerated tea products derived from the purple coloured tea clones were identified and quantified by HPLC using pure anthocyanin standards. The order of elution of the anthocyanidin standards was as follows; cyanidin-3-*O*-galactoside < cyanidin-3-*O*-glucoside < delphinidin < cyanidin < pelargonidin < peonidin < malvidin. A representative HPLC chromatogram of aerated tea from a purple tea clone is presented in Fig. 3. The major anthocyanidins found in nature were identified in the processed tea with malvidin being the most predominant in both the aerated and unaerated tea products from the purple coloured tea clones (Tables 4a and 4b). However, there were significant ($p \leqslant 0.05$) differences in the anthocyanidin concentrations in the two types of tea products and between the products from the different purple coloured clones. The different anthocyanidins were higher in unaerated than in aerated tea.

3.2. Total catechins

The catechin contents of the processed tea products from the ordinary green coloured and purple coloured tea cultivars were determined by HPLC using pure catechin standards (Table 5). Catechins that are normally present in the green leaf coloured tea plants were also present in the purple coloured tea cultivars namely; epigallocatechin (EGC), catechin (+C), epicatechin (EC), epigallocatechin gallate (EGCG) and epicatechin gallate (EGC) (data not shown). As expected, unaerated tea products had significantly ($p \leqslant 0.05$) higher total catechin content than the aerated tea products. The percent catechin content in the tea products from most of the purple leaf coloured clones was the same as that in the green leaf coloured clones. However, the catechin levels in the unaerated products from the anthocyanin rich clones TRFK 306/1, TRFK, 306/2, TRFK 306/3 and TRFK 306/4 were much lower compared to the TRFK 73/1-5 and 7 series and the widely cultivated green leaf coloured high black tea quality clones GW Ejulu-L, TRFK 31/8 and TRFK 6/8 (Table 5). The highest level of the total catechin content was recorded in the unaerated leaf from the control clone GW Ejulu-L (15.86%). The highest total catechin content in aerated tea was recorded in products derived from clones TRFK 31/8 (8.44%) and TRFK 6/8 (7.39%).

Table 2
Total monomeric anthocyanins (mg/l) in aerated and unaerated teas derived from ordinary green coloured and purple coloured tea germplasm obtained by the pH differential method. The results are expressed as cyanidin-3-glucoside equivalents.

Clone	Anthocyanin conc. (mg/l)	
	Green (unaerated)	Black (aerated)
Green leaf coloured		
GW Ejulu-L	4.01	0.45
TRFK 31/8	1.73	1.67
TRFK 6/8	3.23	1.34
Purple leaf coloured		
TRFK 306/1	98.86	23.99
TRFK 306/2	91.73	30.28
TRFK 306/3	108.26	45.48
TRFK 306/4	75.03	14.13
TRFK 73/1	22.10	2.78
TRFK 73/2	18.10	3.67
TRFK 73/3	16.14	2.89
TRFK 73/4	19.54	7.57
TRFK 73/5	12.97	0.82
TRFK 73/7	23.66	7.35
TRFK K-purple	46.98	7.35
TRFK KS1	12.49	13.47
TRFK KS3	2.25	1.89
Eggplant[a]	249.40	
Mean	35.03	10.32
LSD ($P \leqslant 0.05$)	12.01	5.48

[a] The eggplant was used as a positive control for anthocyanins.

Table 3
Concentration of anthocyanins (μg/ml) in processed black and green tea from selected green and purple coloured tea clones by HPLC.

Clone	Anthocyanin conc. (μg/ml)	
	Green (unaerated)	Black (aerated)
Green leaf coloured		
TRFK 6/8	198.0	124.5
Purple leaf coloured		
TRFK 306/1	928.4	450.2
TRFK 306/2	1040.7	458.7
TRFK 306/3	1193.4	590.6
TRFK 306/4	814.4	240.8
TRFK 73/1	472.6	208
TRFK 73/2	389.7	193.9
TRFK 73/3	269.2	187.7
TRFK 73/4	428.4	183.1
TRFK 73/5	339.3	220.4
TRFK 73/7	321.4	200.5
Mean	581.403	278.03
CV%	1.52	3.11
LSD ($P \leqslant 0.05$)	15.0	14.8

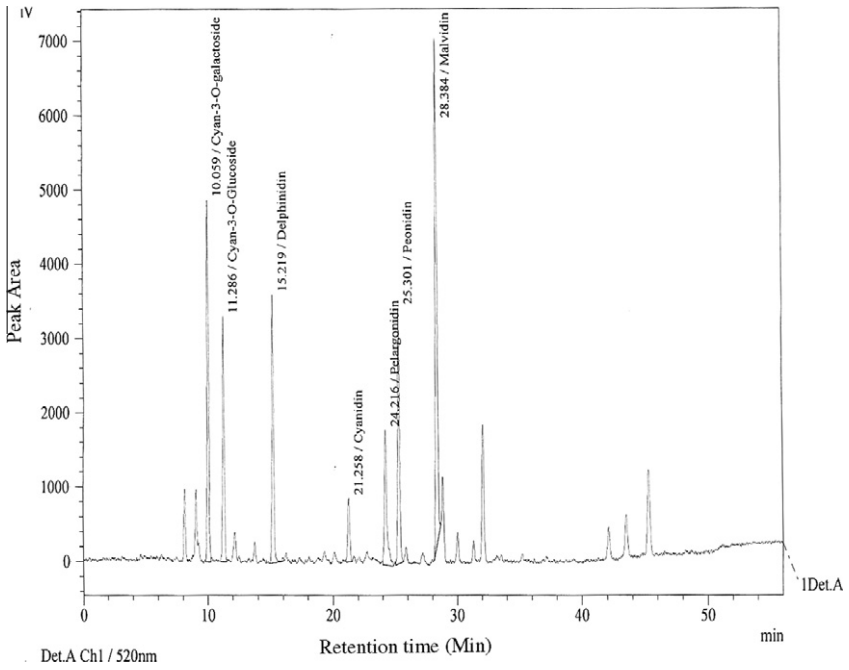

Fig. 3. A representative HPLC Chromatogram of aerated (black) tea from the purple coloured clone TRFK 306/1.

Table 4a
Anthocyanidin concentrations (μg/ml) in processed aerated (black) tea derived from purple and green leaf coloured clones.

Clones	Cy-3-*O*-galac	Cy-3-*O*-glu	Delphinidin	Cyanidin	Pelargonidin	Peonidin	Malvidin
Green leaf coloured							
TRFK 6/8	–	–	–	50.0	–	–	–
Purple leaf coloured							
TRFK 306/1	52.60	23.77	73.13	41.93	60.60	44.93	153.27
TRFK 306/2	59.27	26.63	85.50	40.53	49.87	44.63	152.23
TRFK 306/3	85.23	36.37	113.00	41.47	69.10	51.63	193.80
TRFK 306/4	27.27	13.40	23.30	38.27	29.80	40.43	68.30
TRFK 73/1	8.70	4.50	45.73	39.47	26.10	40.93	42.57
TRFK73/2	8.47	4.30	36.33	39.57	24.37	38.70	42.17
TRFK73/3	4.80	5.97	42.47	37.73	20.50	39.43	36.77
TRFK 73/4	7.77	4.30	27.33	37.73	25.03	38.70	43.27
TRFK 73/5	14.57	6.07	51.97	38.00	26.83	40.57	42.40
TRFK 73/7	12.77	4.80	34.53	39.37	32.83	39.60	36.57
Mean	28.04	13.01	53.33	40.49	36.50	41.96	81.23
CV%	3.75	3.26	16.62	2.42	5.15	4.30	3.29
LSD ($P \leqslant 0.05$)	1.80	0.73	15.21	1.67	3.22	3.10	4.58

Table 4b
Anthocyanidin concentrations (μg/ml) in processed unaerated (green) tea derived from purple leaf coloured clones.

Clones	Cy-3-*O*-galac	Cy-3-*O*-glu	Delphinidin	Cyanidin	Pelargonidin	Peonidin	Malvidin
Green leaf coloured							
TRFK 6/8	–	–	–	51.2	–	–	–
Purple leaf coloured							
TRFK 306/1	138.10	56.03	56.03	54.53	89.37	50.77	483.60
TRFK 306/2	156.10	60.57	92.10	59.27	178.57	51.37	442.70
TRFK 306/3	184.07	73.90	111.73	56.83	220.50	50.87	495.47
TRFK 306/4	143.50	58.57	62.47	52.03	142.57	46.70	308.57
TRFK 73/1	37.87	8.77	76.47	42.27	135.70	44.33	127.23
TRFK73/2	32.70	7.57	61.60	46.23	96.00	43.47	102.07
TRFK73/3	13.43	14.93	49.03	38.87	43.43	39.67	70.13
TRFK 73/4	26.90	8.83	49.63	45.30	125.10	44.13	127.90
TRFK 73/5	28.47	9.57	42.43	39.67	75.77	41.73	101.97
TRFK 73/7	27.13	6.07	26.03	43.10	106.73	40.07	70.50
Mean	78.83	30.48	62.76	48.01	121.37	45.50	233.01
CV%	1.56	1.64	10.26	3.00	3.03	2.32	2.45
LSD ($P \leqslant 0.05$)	2.12	0.86	11.05	2.45	6.31	1.81	9.81

Table 5
Total catechin content (%) in the aerated and unaerated tea products from 30 tea cultivars including green leaf coloured high quality black tea clones, clones for high quality green tea and purple leaf coloured clones.

Clones	Green (unaerated)	Black (aerated)
Green leaf coloured		
GW Ejulu-L	15.86	6.84
AHP S15/10	11.97	4.19
TRFK 31/8	13.58	8.44
TRFK 31/11	11.44	2.81
TRFK 6/8	15.04	7.39
TRFK 301/2	7.65	2.99
EPK 14-3	9.23	4.40
TRFK 303/577	13.70	2.89
TRFK 303/216	9.57	4.84
Yutakamidori+	11.80	2.79
Yabukita+	7.49	2.76
TRFK 301/1	7.82	4.26
Purple leaf coloured		
TRFK 306/1	8.68	5.51
TRFK 306/2	9.98	4.29
TRFK 306/3	8.69	4.15
TRFK 306/4	11.20	3.85
TRFK 73/1	12.55	4.23
TRFK 73/2	12.37	3.14
TRFK 73/3	12.24	4.68
TRFK 73/4	12.07	5.25
TRFK 73/5	9.13	4.16
TRFK 73/7	14.24	5.61
TRFK K-purple	10.87	4.80
TRFK KS1	9.88	3.29
TRFK KS2	14.03	3.60
TRFK KS3	6.10	2.55
TRFK 91/1	9.07	4.79
TRFK 91/2	11.81	4.85
TRFK 83/1	11.18	3.03
TRFK 14/1	12.15	2.92
Mean	11.07	4.31
CV%	17.6	15.6
LSD ($P \leqslant 0.05$)	3.20	1.10

3.3. Relationship between total catechins, total monomeric anthocyanins and anthocyanin concentrations

Total catechins, total monomeric anthocyanins and anthocyanin concentrations by HPLC analysis correlated negatively with both the unaerated (green) teas and the aerated (black) teas (Table 6). The correlation coefficients were however non-significant ($p > 0.05$) for the aerated tea. The negative correlations signify an inverse relationship, where, as the levels of anthocyanins increase, the levels of catechins decrease.

4. Discussion

In this study, an acidified methanolic solution (99:1 v/v) was used to extract anthocyanins from the milled tea products derived from the purple and green leaf coloured tea clones because anthocyanins are more stable in acidic media (Lacobucci & Sweeny, 1983). It is in the acidic medium that the red flavylium cation is most predominant (McGhie & Walton, 2007). Increase in the pH of the medium results in loss of concentration and colour. The loss of colour is ascribed to the loss of the proton caused by the nucleophilic attack of water on the flavylium cation, resulting in the blue quinoidal (carbinol) form and ultimately to the colourless chalcone as the pH continues to rise (Kahnkonen, Heinamaki, Ollialainen, & Heinonen, 2003). The choice of an acidified medium for the extraction of anthocyanins from the test tea varieties in this study therefore ensured maximal isolation. The total monomeric anthocyanin contents in this study were expressed as cyanidin-3-glucoside equivalents because the identities of the pigments were initially unknown and this anthocyanin has been found to be the most abundant in nature (Francis, 1989). The total monomeric anthocyanin content was significantly ($p \leqslant 0.05$) higher in anaerated (green) tea than aerated (black) tea. The black tea processing procedures therefore decreased the levels of total monomeric anthocyanins in the aerated tea. The difference in anthocyanin concentration between the black (aerated) and green (unaerated) teas is hypothesised to be due to anthocyanin degradation during the black tea processing procedure.

Table 6
Correlation coefficients between anthocyanins and total catechins.

	Total catechins (TC)	
	Green (unaerated)	Black (aerated)
Total monomeric anthocyanins (TA)	−0.723*	−0.182, $p > 0.05$
Anthocyanin concentration by HPLC	−0.743**	−0.241, $p > 0.05$

Research on fruits has shown that anthocyanins are rapidly degraded by polyphenol oxidase (PPO) in the presence of other polyphenolic substrates, such as the catechins (Liu, Cao, Xie, Sun, & Wu, 2007). For example it has been reported that the rate of degradation of strawberry anthocyanins by PPO is increased in the presence of catechins (Wesche-Ebeling & Montgomery, 1990). In fruits and vegetables, anthocyanin degradation causes tissue browning, an undesirable factor in food processing (Sadilova, Carle, & Stintzing, 2007). A study on the degradation of the anthocyanin cyanidin-3-*O*-rutinoside by PPO in the presence of (−)-epicatechin has proposed a pathway of oxidation of (−)-epicatechin to epicatechin-*O*-quinone, which then undergoes a nucleophilic attack of (−)-epicatechin to form dehydroepicatechin A (Liu et al., 2007). Though a similar pathway has not as yet been demonstrated in tea, the proposed scheme of degradation can be modified to suit the degradation of anthocyanins in the tea plant during the fermentation (auto-oxidation) process of the catechins to form theaflavins (TFs) and thearubigins (TRs), a process that is catalysed by PPO.The proposed scheme could be as follows:

$$(+)\text{-Catechin (or EC, EGC, ECG, EGCG)} + \text{PPO} \rightarrow \text{Catechin} - O\text{-quinone} \quad \text{(i)}$$

$$\text{Catechin-}O\text{-quinone} + (+)\text{-Catechin} \rightarrow \text{Dehydro-catechin A} \quad \text{(ii)}$$

In the presence of the *O*-quinone, (+)-catechin (or any other catechin) or anthocyanin would be able to combine with the *O*-quinone to form complex compounds. The compounds would compete for *O*-quinone, resulting in the partial regeneration of (+)-catechin from its *O*-quinone by the coupled oxidation of anthocyanin, for example cyanidin-3-glucoside.

$$(+)\text{-Catechin-}O\text{-quinone} + \text{Cyanidin-3-glucoside}$$
$$\downarrow$$
$$(+) - \text{Catechin} + \text{Cyanidin-3-glucoside-}O\text{-quinone}$$

Such coupled oxidation reactions would lead to the gradual degradation of the anthocyanin molecule. During the manufacture of aerated (black) tea, the oxidation process of the catechins can therefore lead to the formation of the *O*-quinones, which would gradually lead to the degradation of the anthocyanins in the black tea. In the processing of unaerated (green) tea, polyphenol oxidase is inactivated by steaming of the leaves and therefore there would be no formation of the reactive *O*-quinones which cause anthocyanin degradation and hence the higher levels of anthocyanins in the unaerated teas. Further, thermal degradation has also been implicated in anthocyanin degradation during processing of anthocyanic foods including strawberry and elderberry (Sadilova, Stintzing, & Carle, 2006; Sadilova et al., 2007). The eggplant peels that

were used as a positive control had much higher anthocyanin content than the tea products since they did not undergo any form of processing.

The HPLC analyses of the tea products derived from the purple coloured tea clones revealed that they contained cyanidin-3-*O*-galactoside, cyanidin-3-*O*-glucoside, delphinidin, cyanidin, peonidin, pelargonidin and malvidin in that order. We hypothesize that the HPLC method is more accurate because it could detect both the anthocyanidins and anthocyanins, whereas the pH-differential method, which is based on the measurement of the flavylium cation mainly measures less stable anthocyanidins. The HPLC assays indeed detected the presence of anthocyanins in the tea samples in addition to the anthocyanins namely, cyanidin-3-*O*-glucoside and cyanidin-3-*O*-galactoside which led to higher levels of total anthocyanins than in the pH-differential method. The elution sequence of the anthocyanins found in this study was in agreement with those of similar studies on plants and biological fluids (Mazza, Cacace, & Kay, 2004). The retention times were found to decrease as a result of increasing polarity, which is directly related to the number of hydroxyl groups in the flavylium nucleus and the sugar substituents. Studies on bilberry anthocyanins confirmed the structural dependence of the HPLC separation pattern of anthocyanins in that the retention of glycosides, in comparison to that of the aglycones, is decreased by the presence of sugars in the order galactoside < glucoside < arabinoside (Ichiyanagi, Hatano, Matsugo, & Konishi, 2004), as also found in this study.

The most predominant anthocyanidin in the tea products derived from the purple coloured tea clones was malvidin. Other plants that contain malvidin as the predominant anthocyanidin are Bilberry (*Vaccinium myrtillus*) with malvidin-3-glucoside (Du, Jerz, & Winterhalter, 2004); blueberry with malvidin-3-arabinoside, malvidin-3-glucoside and malvidin-3-galactoside (Lohachoompol, Mulholland, Srzednicki, & Craske, 2008); red grapes with malvidin-3-glucoside (Ghiselli, Nardini, Baldi, & Scaccini, 1998). All edible berries have been found to have malvidin-3-glucoside, malvidin-3-glucoside acetate and malvidin-3-glucoside coumarate (Oh et al., 2008). Products like young red wine (Rivero-Perez et al., 2008), Italian red wine (Ghiselli et al., 1998; Rigo et al., 2000) and Muscal Bailey A grape juice (Oh et al., 2008) also contain malvidin as the predominant anthocyanin. In addition, 3-glucoside anthocyanins of delphinidin, cyanidin, petunidin and malvidin have also been found in red wines (Bakker & Timberlake, 1997). Malvidin is a dimethylated compound and its predominance and existence in the tea plants assayed in this study is of great importance to the food industry because it can be used for making tea based red "wines", just like the grapes that also contain malvidin as the predominant anthocyanin and are used to produce red wines. In addition, methylated anthocyanins have been found to be more stable than their hydroxylated counterparts and hence are suitable for use in the food industry as food colourings in products like jams and juices (Jackman et al., 1987). The presence of catechins in the purple coloured tea plant should be expected to add to the stability of the tea anthocyanins due to the formation of CH–CH_3 bridges with the catechins.

Catechins are the main polyphenolic compounds (flavan-3-ols) in the fresh leaf of the tea plant (*C. sinensis* (L.) O. Kuntze). In this study, the total catechin content in unaerated tea products from the green coloured tea cultivars and the purple coloured clones was significantly higher than the aerated tea products from the same clones. This observation is in agreement with a similar study by Karori et al. (2007) who found that green (unaerated) tea had significantly higher catechin content than black (aerated) tea used in his study. Aerated (black) tea is obtained by a post harvest auto-oxidation reaction (fermentation), which is catalysed by the Polyphenol oxidase (PPO) (EC 1.10.3.1) enzyme while unaerated teas do not undergo the fermentation process since they are initially steamed to inactivate the PPO enzyme. The consequence of enzymatic oxidation on the catechins located in the leaf cell vacuole is the polymerization of the flavan-3-ol monomers to form theaflavins (TFs) and thearubigins (TRs), which are important quality determinants of aerated (black) tea products (Singh et al., 1999). The relationship between total catechins and total anthocyanins revealed negative relationships, as shown by the negative correlation coefficient ($r = -0.723^{*}$, $p \leqslant 0.05$) in unaerated teas and ($r = -0.182$, $p > 0.05$) in aerated teas. The inverse proportionality between total catechins and total anthocyanins in the unaerated tea of the purple coloured cultivars, which most closely represents the situation obtained in the fresh leaf of these cultivars, may be due to the preferential upregulation of the genes responsible for anthocyanin synthase in the pathway for anthocyanins biosynthesis, rather than the one for leucoanthocyanin reductase in the catechin biosynthetic pathway; this was probably due to response to some environmental stimuli (Punyasiri et al., 2004) since both compounds are secondary metabolites derived from the general phenyl propanoid pathway.

Though anthocyanins have for the first time been identified in tea in addition to catechins, much more work needs to be carried out to determine the impact of seasonal variations in weather and geographic variations on the concentrations and profiles of these pigments. Environmental and genetic variations have been known to affect anthocyanins and have been used to obtain anthocyanin fingerprints in other crops including grapes (Ortega-Regules, Romero-Cascales, Lopez-Roca, Ros-Garcia, & Gomez-Plaza, 2006). In grapes, these variations have been used to build a model that could differentiate between the varieties grown in different seasons. Anthocyanins have also been used as chemotaxonomical markers (Ortega-Regules et al., 2006). A similar model can be employed on the newly bred anthocyanin rich tea cultivars as they will be cultivated in different agro-ecological tea growing regions. Further, the impacts of agronomic practises like fertilizers, pruning and plucking standards on the anthocyanin concentration needs to be determined.

The discovery that the major anthocyanins found in nature are also found in the tea plant with the most predominant being malvidin, which also predominates in grapes (Oh et al., 2008), is a positive step towards the diversification of tea products and uses of tea. These purple teas are potential raw material for the production of an anthocyanin rich health drink since anthocyanins are highly soluble in water. In addition they have an added advantage over other polyphenols in that they have been found to be more pharmacologically active due to the positive charge they carry (Bagchi, Roy, Patel, & He, 2006). Since tea grown along the equator produces a lot of dry matter throughout the year, these anthocyanins can be extracted and purified for use in the food industry as an alternative to the existing synthetic colourants. However, because of their instability, there is a need to carry out more studies to establish appropriate methods for stabilization for use in industry.

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