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Optimization and Validation of a Simple Spectrophotometric Based DPPH Method for Analysis of Antioxidant Activity in Aerated, Semi-Aerated and Non-Aerated Tea Products

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Abstract

In this study, the simple DPPH UV-spectrophotometric analytical procedures were optimized and validated for the estimation of antioxidant properties in aerated, semi-aerated and non-aerated tea products as per the AOAC guidelines. The method was found to be simple, rapid, accurate and economical in estimating antioxidant properties in the different tea products. A (0.0100 ± 0.0001) g well ground tea sample was extracted directly in 80% v/v methanol in distilled water. The precision of the method had an RSD% of 1.695 with a linear relation equation of $Y = 200.7X + 5.25$ ($r^2 = 0.997$). The method proved to be a reliable and effective tool in analysis of anti-oxidant properties in varied tea cultivars and their processed products.

Keywords

UV-Spectrophotometric, Optimized, Validated, Antioxidant

1. Introduction

Tea (*Camellia sinensis* L.), a widely consumed beverage in the world, comes in a variety of forms, scents and flavors [1]. Green (un-aerated), oolong (semi-aerated) and black (aerated) teas are made from the shoots of tea plants at different degrees of aeration [2] [3]. These beverages have been receiving much attention in the last decade because of their inherent beneficial components, mainly poly-

phenols [4]. Many biological functions of tea polyphenols have been associated with antioxidant activity [5], anti-mutagenic effects [6], cardiovascular diseases [7] and anti-obesity prevention [8]. Furthermore, epi-gallocatechin gallate (EGCG) a polyphenol found in large amounts inun-aerated tea is reported to have anti-HIV activity when bound to cluster of differentiations (CD4) receptor [9]. 2,2-diphenyl-1-picrylhydrazyl (DPPH; $C_{18}H_{12}N_5O_6$, M = 394.33) free radical scavenging method present popular, quick, easy, and affordable approaches for determining antioxidant capacity in material of biological origin [10]. The method was first suggested by Blois (1958) using cysteine as model antioxidant [11]. Brand-Williams *et al.*, 1995 and Bond *et al.*, 1997 introduced modifications to the earlier method of Blois where they applied the term "EC50" (efficient concentration) for the interpretation of results from DPPH method. "EC50" referred to substrate concentration that caused 50% depletion in the DPPH colour [12] [13]. The disadvantage of this factor was that it showed an inverse relation to the substrate antioxidant activity [14]. Furthermore, to overcome the disadvantage, Brand-Williams applied the term "antiradical power" (ARP) the inverse of EC50 and hence, larger the ARP, the more efficient is the antioxidant [15]. In an effort to improve reporting on findings of the DPPH assy, another term that was used to describe the DPPH scavenging capacity was "antiradical efficacy" (AE), with higher AE value indicating higher antioxidant activity [16]. Researchers over the years have worked to improve on determination of the antioxidant potential of a substrate through the DPPH assay since different literatures have presented discrepancies. Recently, Bryan Brummelhaus de Menezes *et al.* through intensive literature search, highlighted two main problems. First, was the absolute determination of the fifty percent inhibitory concentration (IC50) despite the initial concentration of DPPH used and secondly, the determination of the percentage of scavenged DPPH radical (DPPH_), due to the missing measure of the absorbance of the molecular DPPH (DPPH-H) formed in the reaction. This team of researchers observed that the model which is currently being used to calculate the DPPH concentration can lead to an overestimation of up to 7%, as it does not take into account the small contribution of the reaction products. Their work demonstrated that to address the first problem IC50 value needs to be expressed as a function of the DPPH mol number used in the reaction while the reported mathematical model would correct the overestimation of the DPPH concentration [17].

Generally, the principle of DPPH method, is reduction by an antioxidant through the transfer of a proton "plus" electron (a hydride equivalent) resulting in color loss of the DPPH [18]. The DPPH free radical gives a strong absorption maximum at 517 nm and is purple in color [19]. The color turns from purple to yellow as the molar absorptive of the DPPH radical at 517 nm decreases from 9660 to 1640 μM^{-1} cm^{-1} [20]. During this process the DPPH radical odd electron becomes paired with an electron donated from the antioxidant and hydrogen atom to form the reduced DPPH-H [21] (**Figure 1**).

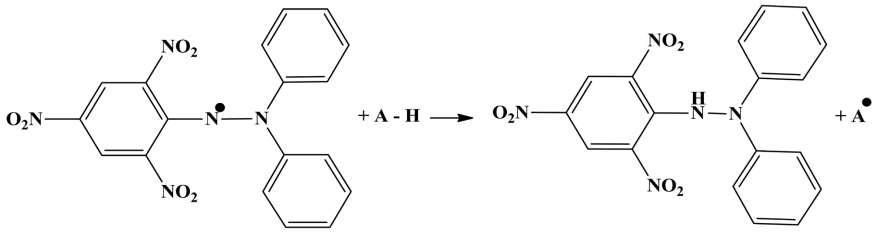

Figure 1. DPPH radical reaction scheme against a hydrogen donor antioxidant molecule.

To appreciate the antioxidant property of any sample, the color loss cannot always be taken to completion; therefore, the weight of sample is adjusted so that approximately 50% of DPPH color is lost [22]. This requires a pre-knowledge of the sample or, alternatively, preliminary tests to decide the most appropriate sample weight. Other than DPPH, several techniques have been used for antioxidant measurements [23]. In total peroxyl radical trapping (TRAP) method, thermal decomposition of AAPH produces luminal derived radicals and from it CL signal [24]. TRAP measurement is dependent on period at which sample quench the signal due to the presence of antioxidants [25]. In ABTS technique, interaction is between an antioxidant and pre-generated $ABTS^{\bullet+}$ radical cation that is easily quantified due to bleaching of absorption spectrum characteristic maxima at 414, 417, 645, 734, and 815 nM. [26]

The instrumentation commonly used for anti-oxidant capacity profiling involves spectroscopy, electrochemical and chromatography [27]. In the spectrometric techniques, radical, radical cation or complex reacts with an antioxidant molecule which donates a hydrogen atom [28]. The techniques involves well defined steps including, initiation (LH + R· → L· + RH); propagation (L. + O_2→ LOO.LOO. + LH → L. + LOOH); branching (LOOH→LO. + HO., 2LOOH → LOO. + LO. + H_2O) and termination (LO. + LO., LOO. + LOO., LO. + LOO.) [29]. The electrochemical methods for determination of antioxidant activity are based on cyclic, differential pulse, square wave voltammetry and coulometry [30]. These methods are sensitive, rapid and simple [31]. Evaluation involves direct measurement of electrons transferred by an antioxidant [32]. Chromatographic assays for antioxidant activity determination easily eliminate interferences from photosynthetic pigments occurring naturally in plant extracts [33]. The DPPH-HPLC enables the separation of the DPPH free radical peak and its reduced form (DPPH-H) from other components in the analyte [34]. However, simultaneous analysis of antioxidants and evaluation of their activity is more complicated [35]. Therefore, post-column reaction between the antioxidant and DPPH has been used to enhance efficiency of these methods [36].

In this study attempt has been made to optimize and validate a simple, quick, easy, reliable and affordable DPPH method for use in routine analysis of antioxidant activity in tea and related products. For this purpose, the method has been used to generate anti-oxidant activity levels in cultivars of high, medium and low total catechin contents. Additionally, processed tea products were profiled using

the steps of the method. The finding of the study suggests that, the steps of the method are adequate enough to determine antioxidant activity in tea and related products.

2. Materials and Methods

2.1. Sample Collection

The samples required for analysis using the optimized and validated method were collected at the Timbilil estate fields (0˚22'S, 35˚21'E) with an altitude of 2180 meters (mean above sea level) of Kericho County in Kenya. Two leaves and a bud were hand plucked fresh for the cultivars of TRFK895/22, Ejulu, TRFK895/7, AHP S15/10, TRFK914/39, TN14-3, D99/10, TRFK6/8, ST832, ST831, ST830, ST543, ST536, TRFK306/4, TRFK306/3, TRFK306/2, TRFK306/1, TRFK91/1, TRFK660/1, TRFK597/4, TRI306/FLD/GB/2, TRI306/FLD/GB/3, TRI306/FLD/GB/4, TRI306/FLD/GB/5, TRI306/FLD/GB/6 and TRI306/FLD/GB/3. The different orthodox tea grades of BO-OP1, GO-FP, BO-FOF, BO-FP, GO-OP1, GO-FOP, GO-OPA, GO-FOF, GOFBOE, BO-FBOB and BO-FOP were sourced from selected tea processing factories. The cut, tear and curl (CTC) tea grades of PD, D1, F1, PF1 and BP1 were collected from Kipkebe and Matara tea factories. The samples were kept in well labeled aluminium lined khaki bags for preservation before determination.

2.2. Chemicals and Reagents

The DPPH (2,2-Diphenyl-1-picrylhydrazyl) and HPLC grade methanol were purchased from Sigma-Aldrich Chemie GmbH, Germany through Kobian local suppliers. Distilled water was generated in the laboratory. Spectrophotometric measurements were done using a Shimadzu 1800 series spectrophotometer with 1 cm quertz cuvettes.

2.3. Sample Preparation

The hand plucked fresh two leaves and a bud were sorted for high quality material in each of the cultivars above. The leaf was then steamed for 1 min before being microwaved. The microwaved leaf and the different tea grade samples were finely ground using an electric coffee miller (AR40 Molineux) and packed in aluminium lined brown paper sachets ready for use in antioxidant activity analytical determinations.

2.4. Sample Moisture Content Determination

2.0 ± 0.01 g of the sample was weighed into aluminium dishes and heated in an oven at 103˚C ± 2.0˚C for 4 hours to obtain constant weight. The samples were weighed again and moisture content was then determined by subtracting the final weight from the initial weight, and expressed as a percent. The moisture content obtained was used to determine the sample dry matter content necessary for standardizing the sample weights during analysis.

2.5. Preparation of 0.06 mM DPPH Working Solution

0.0024 g of DPPH was accurately weighed and transferred into a 100 mL volumetric flask. 80% methanol (v/v) in distilled water was added to above half full mark. The mixture was stirred on a magnetic stirrer until all the DPPH dissolved and then toped to the mark with 80% methanol. The DPPH is sensitive to light and hence must be prepared in light controlled environment. Prepare fresh every day.

2.6. Optimization of the Method

Optimization in analytical chemistry involves designating a set of experiments in order to establish the proper conditions for carrying out a method, to achieve the best possible responses and ensure ideal analytical characteristics. For each parameters considered, the focus is to identify the factors for optimization, measure performance under the set conditions, establish trends and visualize improvement. The optimized conditions in this study were, sample weight, extraction solvent concentration, extraction temperature, extraction time, ideal sample extract concentration for DPPH reaction and sample extract-DPPH mixture incubation time.

2.7. Validation of the Method

2.7.1. Precision of the Method

The precision of the method was investigated by preparing one sample solution containing the target level of analyte. Ten replicates of this sample solution were analyzed with the mean, standard deviation and relative standard deviation (RSD %) determined. From the measured standard deviation (SD) and mean value, precision as relative standard deviation (% RSD) was calculated.

$$\%\mathrm{RSD}\left(\mathrm{CV}\right)=\frac{\mathrm{SD}}{\mathrm{Mean}}*100 \tag{1}$$

2.7.2. Linearity of the Method

Six different sample concentrations were prepared in triplicate and determined for antioxidant activity using the conditions of the method. The mean anti-oxidant activity in each of the five concentrates was used to generate equations for the regression line and correlation coefficient (R^2) in order to establish linear relationship of the method.

2.7.3. Accuracy of the Method

Accuracy of the analytical method was determined by preparing quality control (QC) materials of gallic acid and a non-aerated tea sample of TRI 6/8 cultivar in 10 mL of 80% (v/v) methanol in distilled water. The anti-oxidant activity (AA) of the QC materials was accurately determined against the DPPH solution. The QC material was used as the standard to spike the tea sample in order to determine the recovery percent of the method.

$$\%\mathrm{Recovery}=\frac{\mathrm{AA\ sample\ spiked}-\mathrm{AA\ sample}}{\mathrm{AA\ gallic\ acid}}\times 100 \tag{2}$$

2.7.4. Limit of Detection (LOD) and Quantitation (LOQ) of the Method

LOD and LOQ were obtained from the standard deviation (σ) of the blank response (n = 6) and slope (S) of calibration curve using the formula 3.3 σ/S and 10 σ/S, respectively.

2.8. Application of the Method

All the samples were evaluated for anti-oxidant activity using the optimized and validated conditions of the method.

2.9. Statistical Determinations

Statistical analysis was carried out using SAS® V 9.1 for windows statistical software. ANOVA was used to determine the means, coefficient of variation (CV) and Least Significance Difference (LSD). The probability limit was set at $p \leq 0.05$ significant levels. Results of the parameter determined were expressed as a mean of the triplicate determination.

3. Results and Discussion

3.1. Optimization of the Method

The influences of sample weight and solvent concentration for the method were tested. The sample weight was optimized on the basis of a 0.06 mM DPPH concentration. From literature search, DPPH concentration of 0.06 mM is extensively used in analytical methods where DPPH is the scavenged free radical [37]. The tea sample weight was evaluated in the range of 0.001 - 1.000 g. It was found out that a 0.0100 ± 0.0001 g sample weight contained in a 10mL extracting solvent and diluted fivefold was optimal for the method. Plank *et al.*, 2012 while working on a collaborative study on food and beverage reaction with DPPH, suggested that for antioxidant property of any sample, the DPPH color loss cannot always be taken to completion; therefore, the weight of sample is adjusted so that approximately 50% of DPPH color is lost [22]. A medium total catechins cultivar of ST 543 was selected as reference material for moderating the DPPH depletion by sample antioxidants to obtain a consistent end point of approximately 50 ± 2AA%. Methanol was used as the extraction solvent due to its high extraction efficiency in extracting both lipophilic and hydrophilic molecules [38]. The solvent concentration range was optimized at 50% - 100% methanol: water (v/v) and 80% methanol was found ideal since it gave a good yield of the antioxidants. A study by Chigayo *et al.*, 2016 concluded that 80% methanol was the best solvent to use for extraction of phytochemicals and antioxidant properties of different solvent extracts of *Kirkia wilmsii* tubers [39]. Furthermore, elevated temperature of 70˚C for the reaction mixture gave improved antioxidant activity in a short extraction time of 10 min. Finally, a spectrophotometric scan of DPPH colour change in a DPPH-sample matrix, was done over a period of 90 min (**Figure 2**) in order to establish the reaction time end point. It was observed that after 30 min, recorded change in absorbance levels had little or no change.

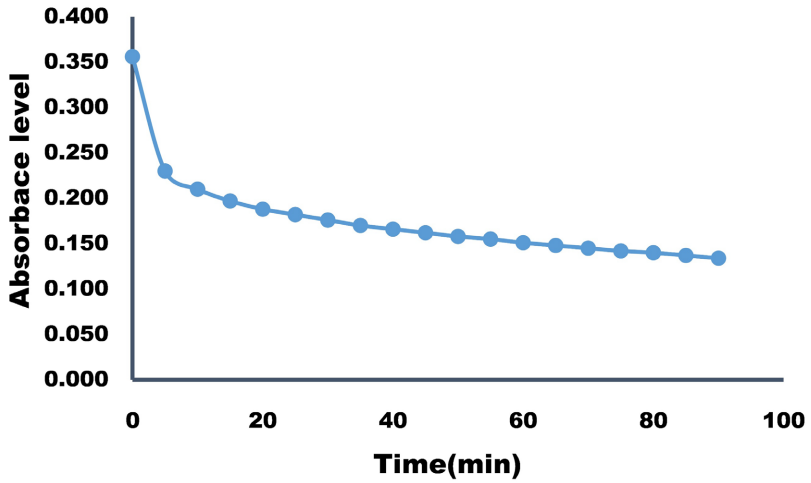

Figure 2. 0.06 mM DPPH depletion curve against 0.2 mg/mL tea sample in 80% methanol.

3.2. Validation of the Method

The proposed method was validated as per the Association of Official Analytical Chemists (AOAC) guidelines [40]. Tests for precision, linearity, accuracy, LOD and LOQ for the method were carried out according to earlier adopted procedures in Section 2.7 above.

3.2.1. Precision of the Method

The precision of the validated method was expressed as percent relative standard deviation %RSD (CV). The results showed an RSD of less than 2% (**Table 1**). This demonstrates that the present method is sufficiently precise for samples tested and can be used to get good predictive antioxidant activity (AA) results in tea.

Table 1. Precision studies.

n	Maximum AA%	Minimum AA%	Mean AA%	SD	%RSD (CV)
10	49.43	47.22	48.30	0.819	1.695

3.2.2. Linearity of the Method

Well ground microwaved samples of cultivar ST 543 was accurately weighed to generate concentrations of 0.025, 0.038, 0.050, 0.083, 0.125 and 0.250 mg/g sample on dry weight basis in 10 mL of 80% methanol in distilled water. Antioxidant activity against DPPH was determined using the conditions of the optimized and validated method before a calibration curve of percent antioxidant activity against concentration was generated (**Table 2**). The linear regression data for the calibration curve showed good linear relationship over the concentration range of 0.025 - 0.250 mg/g sample dry.wt. Additionally, the linear equation was found to be $Y = 200.7X + 5.25$ ($r^2 = 0.997$). This showed that the method met the requirements of validation based on this parameter and hence fit for application during analysis.

Table 2. Linearity studies.

Concentration (mg/g sample dry.wt)	% AA (mean ± SD) $n = 6$	%RSD
0.025	9.62 ± 0.14	1.46
0.038	12.40 ± 0.16	1.30
0.050	14.88 ± 0.28	1.88
0.083	22.91 ± 0.70	3.06
0.125	31.65 ± 0.42	1.33
0.250	54.67 ± 1.27	2.32
Regression equation	$Y = 200.7X + 5.25$ ($r^2 = 0.997$)	

3.2.3. Accuracy of the Method

Among known polyphenols, gallic acid (GA), a naturally occurring low molecular weight tri-phenolic compound has emerged as a strong antioxidant. Therefore, it was used as the QC material for spiking known concentrations of the tea sample to evaluate recovery of the method. The mean antioxidant activity (n = 3) for pre-analyzed sample, GA standard and GA spiked sample were 48.40, 18.25 and 65.58%respectively (Table 3). The recovery for the method was found to be 94%a demonstration that the method is satisfactory for the intended purpose and adequate for routine analysis.

Table 3. Accuracy studies.

n	Sample type	% AA (mean ± SD)	%RSD
3	Pre-analyzed sample	48.40 ± 0.83	1.72
3	GA standard	18.25 ± 0.30	1.64
3	GA spiked sample	65.58 ± 0.83	1.30
	Recovery %		94

3.2.4. Limit of Detection (LOD) and Quantitation (LOQ) of the Method

Limit of detection (LOD) is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value whereas the quantitation limit (LOQ) of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. The LOD found was 0.0017 mg/mL whereas LOQ was 0.0053 mg/mL. The low levels indicate that the method is capable of detecting significantly low antioxidant activity in tea matrices and tea based products. Moreover, the conditions of the validated method can be used in making decisions in tea related product formulations which would otherwise require very low levels of tea lacing to enhance flavor and strength.

3.3. Procedures of the Optimized and Validated Method

3.3.1. Sample Extraction

Finely ground tea test samples were weighed (0.0100 ± 0.0001) g into extraction

tubes using analytical balance (BL-3200 HL, Shimadzu, Japan). 5.0 mL of hot methanol/water (8:2 v/v) extraction mixture was dispensed into the extraction tubes containing the sample before being stoppered. Mixing was done with the help of a vortex mixer (MV-1000, Taiwan Region) for 1 min and the mixture was placed in a water bath set at 70˚C. The heating continued for 10 min, mixing on the vortex mixer at the intervals of 5 min and 10 min. The extraction tubes from the water bath were removed, allowed to cool to room temperature and the stoppers removed. The tubes were placed in a centrifuge at 3500 rpm for 10 min after which the supernatant was carefully decanted into graduated tubes. The extraction steps were repeated resulting in two extracts. The two extracts were combined and made to 10 mL with cold methanol/water (8:2 v/v). The extracted sample was subjected to DPPH assay.

3.3.2. DPPH Assaying

The sample extract at room temperature (20˚C - 25˚C) was diluted fivefold withmethanol/water (8:2 v/v) and vortexed. A 0.1 mL of the diluted sample aliquot was well mixed with 3.9 mL of 0.06 mM DPPH. The working solution (Ws) was a mixture of 0.1 mL methanol/water (8:2 v/v) and 3.9 mL of 0.06 mM DPPH. Both working solution and sample/DPPH mixture were vortexed for 0.5 min before being incubated at 25˚C for 30 min in the dark. Absorbance (ABS) was then recorded at 517 nm using a Shimadzu 1800 series spectrophotometer. Methanol/water (8:2 v/v) mixturewas used as the blank.

The calculation of the sample antioxidant activity was done using the equation:

$$\%AA = \frac{ABS(Ws) - ABS(sample)}{ABS(Ws)} \times 100 \quad (2)$$

3.4. Applications of the Optimized and Validated Method

With the development of functional foods having beneficial effects on human health, the interest of scientists, consumers and industrialists in raw materials rich in antioxidants has increased considerably over the last few years. Moreover, consumers require more accurate information on the composition of the food which they eat. Natural antioxidants such as phenolic compounds have been reported to possess beneficial bioactivities due to their capacity to act as antioxidants. In tea varietal type, processing and storage are factors that have considerably affected the antioxidant activity in the products.

3.4.1. Variation in Levels of Antioxidant Activity with Varietal Type Using the Validated DPPH Method

The procedures of the optimized and validated DPPH method for determination of antioxidant activity in aerated, semi-aerated and non-aerated tea products were used in twenty six (26) selected cultivars of KALRO-TRI germplasm in Kenya. The antioxidant activity of the cultivars is as shown in **Table 4**.

Table 4. Variation in levels of antioxidant activity with varietal type.

Cultivars	Mean AA%	Cultivars	Mean AA%
TRFK306/1	62.60^{edf}	306/FLD/GB/3	50.19^{f}
TRFK306/2	62.03^{b}	306/FLD/GB/4	50.58^{f}
TRFK306/3	60.79^{f}	306/FLD/GB/5	50.23^{f}
TRFK306/4	58.49^{c}	306/FLD/GB/6	44.91^{g}
TRFK91/1	61.73^{b}	306/FLD/GB/13	59.91^{cb}
TRFK660/1	60.88^{cb}	**Mean**	**56.24**
TRFK597/4	68.61^{a}	**CV%**	**2.99**
TRFK895/7	54.76^{d}	**LSD ($p < 0.005$)**	**2.75**
TRFK895/22	54.42^{d}		
AHP S15/10	58.49^{c}		
TRFK914/39	54.40^{d}		
TRFK6/8	59.65^{cb}		
Ejulu	61.76^{b}		
BBK TN14/3	60.88^{cb}		
D99/10	60.66^{cb}		
ST 536	47.09^{j}		
ST 543	50.93^{f}		
ST 830	53.73^{ed}		
ST 831	61.76^{b}		
ST 832	61.45^{b}		
306/FLD/GB/2	51.14^{ef}		

Means with the same letters are not significantly different at $p < 0.005$.

Cultivars TRI 306/1, 306/2, 306/3, 306/4, 91/1, 660/1 and 597/4 are purple pigmented. Furthermore, in addition to the individual phenolic biochemicals of the green pigmented tea cultivars, they are rich in anthocyanins and GHG a special polyphenol. These attributes are replicated in the cultivars of 306/FLD/GB/3, 4, 5, 6 and 13 second generation cultivars of the TRI 306 series. The remaining cultivars are all green pigmented with the cultivars ST536, 543, 830, 831, and 832 having their origin from China. The antioxidant activity ranged from 44.91% - 68.61%. TRFK597/4 had the highest antioxidant activity while 306/FLD/GB/6 gave the lowest levels. Different Kenyan tea cultivars have different polyphenolic composition which impacts on their unique biochemical qualities [41]. Antioxidant activity of any tea product is a function of its total polyphenol content. Thus, the higher the total polyphenol content in a product, the higher the antioxidant activity [42]. Additionally, a study carried out by Cleverdon *et al.* while comparing total polyphenol content in infusions of black, green, red rooibos, chamomile and peppermint over different steep times gave an observation that the absolute polyphenol content and prediction of

total antioxidant capacity was dependent on the type of tea product used [43]. The optimized and validated method unequivocally distinguishes the different tea cultivars in terms of their antioxidant levels and hence can be used in routine analyses.

3.4.2. Variation in Levels of Antioxidant Activity with Processed Tea Type Using the Validated DPPH Method

Differently processed teas were sourced from selected factories across the tea growing regions in Kenya. The orthodox tea grades of BO-OP1, GO-FP, BO-FOF, BO-FP, GO-OP1, GO-FOP, GO-OPA, GO-FOF, GOFBOE, BO-FBOB and BO-FOP were processed as aerated, semi aerated and unaerated according to the optimized manufacture conditions in each of the different factories. Additionally, the cut, tear and curl (CTC) grades of PD, D1, F1, PF1 and BP1 were fully aerated before drying and grading accordingly. All the teas were subjected to the procedures of the optimized and validated DPPH method in determining their antioxidant capacity. The antioxidant activity of the teas is as shown in Table 5.

Table 5. Variation in levels of antioxidant activity with processed tea type.

Tea grade	Mean AA%	Tea grade	Mean AA%
Kipkebe PD	36.48^{ji}	Matara BP1	38.95^{hg}
Kipkebe D1	37.98^{hgi}	Matara PF1	30.37^{hgi}
Kipkebe F1	31.08^{k}	Matara PD	32.27^{k}
Kipkebe PF1	38.12^{hg}	**Mean**	**44.71**
Kipkebe BP1	42.18^{f}	**CV%**	**2.27**
Kangaita BO-OP1	46.22^{e}	**LSD ($p < 0.005$)**	**2.12**
Kangaita GO-FP	61.22^{c}		
Gitugi BO-FOF	39.47^{g}		
Gitugi BO-FP	38.05^{hgi}		
Kiru GO-OP1	57.77^{c}		
Kiru GO-OPA	55.83^{d}		
Itumbe GO-FOP	65.61^{ba}		
Itumbe BO-OPA	37.00^{h}ji		
Itumbe GO-FOF	64.12^{b}		
Itumbe GO-FBOE	66.71^{a}		
Michimikuru BO-FBOB	39.12^{g}		
Mutunwa BO-FOP	35.49^{j}		
Matara D1	37.23^{hgi}		

Means with the same letters are not significantly different at $p < 0.005$.

Aerated CTC teas in both Kipkebe and Matara tea factories ranged from 30.37% - 42.18%. The lowest antioxidant activity in black orthodox teas was

37.00% in Itumbe BO-OPA while the highest was 46.22% in Kangaita BO-OP1. Green orthodox teas had generally high antioxidant activity (55.83% - 66.71%) with Kangaita GO-FP being the highest.

Studies have shown that green tea has higher levels of polyphenols when compared to black tea [44]. However, the type and amount of flavonoids they contain differ [45]. For example, green tea contains a much higher amount of epigallocatechin-3-gallate (EGCG), whereas black tea is a rich source of theaflavins [46]. The findings on variation of antioxidant activity with different tea grades in the present study congruent well with work by Erol and Velioglu, 2010 on antioxidant activity of different grades of Turkish black tea [47]. Furthermore, differences in the total antioxidant potential obtained in green, black and earl grey teas, have demonstrated that not only the production method (fermentation or its absence), but also individual technological processes utilized by different manufacturers, as well as the origin of the tea itself, can impact the antioxidant properties of the final product which reaches the consumer [48].

4. Conclusion

This optimized and validated spectrophotometric DPPH method for analysis of antioxidant activity in aerated, semi-aerated and non-aerated tea products is simple, accurate, precise, reproducible and sensitive. The validation procedure confirms that this is a reliable method for determination of antioxidant activity in tea and related products. Furthermore, the method is adequate for application in routine analysis. Additionally, it is a proven alternative for companies and universities since it is quick, does not have extra costs with solvents or inert gas flows, and does not require intense training for operation.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

References

[1] Kingori, S.M., Ochanda, S.O. and Koech, R.K. (2021) Variation in Levels of Flavonols Myricetin, Quercetin and Kaempferol—In Kenyan Tea (*Camellia sinensis L.*) with Processed Tea Types and Geographic Location. *Open Journal of Applied Sciences*, **11**, 736-749. https://doi.org/10.4236/ojapps.2021.116054

[2] Kamunya, S., Ochanda, S., Cheramgoi, E., Chalo, R., Sitienei, K., Muku, O., Kirui, W. and Bore, J.K. (2019) Tea Growers Guide. The Kenya Agricultural and Livestock Research Organization.

[3] Too, J.C. (2016) Profiling for Antihypertensive Amino Acid (Theanine) in Selected Kenyan Tea Clones. Master's Thesis, Egerton University, Nakuru.

[4] Caleja, C., Ribeiro, A., Barreiro, M.F. and Ferreira, I.C.F.R. (2017) Phenolic Compounds as Nutraceuticals or Functional Food Ingredients. *Current Pharmaceutical Design*, **23**, 2787-2806. https://doi.org/10.2174/1381612822666161227153906

[5] Yan, Z., Zhong, Y., Duan, Y., Chen, Q. and Li, F. (2020) Antioxidant Mechanism of

Tea Polyphenols and Its Impact on Health Benefits. *Animal Nutrition*, **6**, 115-123. https://doi.org/10.1016/j.aninu.2020.01.001

[6] Massounga Bora, A.F., Ma, S., Li, X. and Liu, L. (2018) Application of Microencapsulation for the Safe Delivery of Green Tea Polyphenols in Food Systems: Review and Recent Advances. *Food Research International*, **105**, 241-249. https://doi.org/10.1016/j.foodres.2017.11.047

[7] Quiñones, M., Miguel, M. and Aleixandre, A. (2013) Beneficial Effects of Polyphenols on Cardiovascular Disease. *Pharmacological Research*, **68**, 125-131. https://doi.org/10.1016/j.phrs.2012.10.018

[8] Boccellino, M. and D'Angelo, S. (2020) Anti-Obesity Effects of Polyphenol Intake: Current Status and Future Possibilities. *International Journal of Molecular Sciences*, **21**, Article 5642. https://doi.org/10.3390/ijms21165642

[9] Sobhani, M., Farzaei, M.H., Kiani, S. and Khodarahmi, R. (2020) Immunomodulatory; Anti-Inflammatory/antioxidant Effects of Polyphenols: A Comparative Review on the Parental Compounds and Their Metabolites. *Food Reviews International*, **37**, 759-811. https://doi.org/10.1080/87559129.2020.1717523

[10] Ndongo, D. (2017) Antibacterial, Antioxidant and Phytochemical Investigation of Acacia Arenaria, Aloe Esculenta and Pechuel-Loeschea Leubnitziae. Master's Thesis, The University of Namibia.

[11] Amzad Hossain, M. and Shah, M.D. (2015) A Study on the Total Phenols Content and Antioxidant Activity of Essential Oil and Different Solvent Extracts of Endemic Plant Merremia Borneensis. *Arabian Journal of Chemistry*, **8**, 66-71. https://doi.org/10.1016/j.arabjc.2011.01.007

[12] Brand-Williams, W., Cuvelier, M.E. and Berset, C. (1995) Use of a Free Radical Method to Evaluate Antioxidant Activity. *LWT—Food Science and Technology*, **28**, 25-30. https://doi.org/10.1016/s0023-6438(95)80008-5

[13] Bondet, V., Brand-Williams, W. and Berset, C. (1997) Kinetics and Mechanisms of Antioxidant Activity Using the DPPH·Free Radical Method. *LWT—Food Science and Technology*, **30**, 609-615. https://doi.org/10.1006/fstl.1997.0240

[14] Apak, R., Özyürek, M., Güçlü, K. and Çapanoğlu, E. (2016) Antioxidant Activity/capacity Measurement. 2. Hydrogen Atom Transfer (HAT)-Based, Mixed-Mode (Electron Transfer (ET)/HAT), and Lipid Peroxidation Assays. *Journal of Agricultural and Food Chemistry*, **64**, 1028-1045. https://doi.org/10.1021/acs.jafc.5b04743

[15] Mendonça, J.D.S., Guimarães, R.D.C.A., Zorgetto-Pinheiro, V.A., Fernandes, C.D.P., Marcelino, G., Bogo, D., *et al.* (2022) Natural Antioxidant Evaluation: A Review of Detection Methods. *Molecules*, **27**, Article 3563. https://doi.org/10.3390/molecules27113563

[16] Sharoyko, V.V., Shemchuk, O.S., Meshcheriakov, A.A., Vasina, L.V., Iamalova, N.R., Luttsev, M.D., *et al.* (2022) Biocompatibility, Antioxidant Activity and Collagen Photoprotection Properties of C60 Fullerene Adduct with L-Methionine. *Nanomedicine*: *Nanotechnology*, *Biology and Medicine*, **40**, Article ID: 102500. https://doi.org/10.1016/j.nano.2021.102500

[17] de Menezes, B.B., Frescura, L.M., Duarte, R., Villetti, M.A. and da Rosa, M.B. (2021) A Critical Examination of the DPPH Method: Mistakes and Inconsistencies in Stoichiometry and IC_{50} Determination by UV-Vis Spectroscopy. *Analytica Chimica Acta*, **1157**, Article ID: 338398. https://doi.org/10.1016/j.aca.2021.338398

[18] Slimani, I., Chakchouk-Mtibaa, A., Mansour, L., Mellouli, L., Özdemir, I., Gürbüzd, N., *et al.* (2020) Synthesis, Characterization, Biological Determination and Catalytic Evaluation of Ruthenium(ii) Complexes Bearing Benzimidazole-Based NHC Lig-

ands in Transfer Hydrogenation Catalysis. *New Journal of Chemistry*, **44**, 5309-5323. https://doi.org/10.1039/d0nj00311e

[19] Baliyan, S., Mukherjee, R., Priyadarshini, A., Vibhuti, A., Gupta, A., Pandey, R.P., *et al.* (2022) Determination of Antioxidants by DPPH Radical Scavenging Activity and Quantitative Phytochemical Analysis of Ficus Religiosa. *Molecules*, **27**, Article 1326. https://doi.org/10.3390/molecules27041326

[20] Duan, Y., Sangani, C.B. and Ameta, R.K. (2019) Thermal, SEM, AFM, BET and Biological Analysis of Newly Synthesized Fe^{2+}/Fe^{3+} Based MOIFs. *Journal of Molecular Liquids*, **295**, Article ID: 111709. https://doi.org/10.1016/j.molliq.2019.111709

[21] Leaves, L. and Leaves, L. (2014) Antioxidant Activity by DPPH Radical Scavenging Method of Ageratum Conyzoides. *American Journal of Ethnomedicine*, **1**, 244-249.

[22] Plank, D.W., *et al.* (2012) Determination of Antioxidant Activity in Foods and Beverages by Reaction with 2, 2'-Diphenyl-1-Picrylhydrazyl (DPPH): Collaborative Study First Action 2012.04. *Journal of AOAC INTERNATIONAL*, **95**, 1562-1569.

[23] Flieger, J. and Flieger, M. (2020) The [DPPH·/DPPH-H]-HPLC-DAD Method on Tracking the Antioxidant Activity of Pure Antioxidants and Goutweed (*Aegopodium podagraria* L.) Hydroalcoholic Extracts. *Molecules*, **25**, Article 6005. https://doi.org/10.3390/molecules25246005

[24] Xiao, F., Xu, T., Lu, B. and Liu, R. (2020) Guidelines for Antioxidant Assays for Food Components. *Food Frontiers*, **1**, 60-69. https://doi.org/10.1002/fft2.10

[25] Ahmad, S., Arshad, M.A., Ijaz, S., Khurshid, U., Rashid, F. and Azam, R. (2014) Review on Methods Used to Determine Antioxidant Activity. *International Journal of Multidisciplinary Research and Development*, **1**, 35-40.

[26] Ilyasov, I.R., Beloborodov, V.L., Selivanova, I.A. and Terekhov, R.P. (2020) ABTS/PP Decolorization Assay of Antioxidant Capacity Reaction Pathways. *International Journal of Molecular Sciences*, **21**, Article 1131. https://doi.org/10.3390/ijms21031131

[27] Kheyar-Kraouche, N., da Silva, A.B., Serra, A.T., Bedjou, F. and Bronze, M.R. (2018) Characterization by Liquid Chromatography-Mass Spectrometry and Antioxidant Activity of an Ethanolic Extract of *Inula viscosa* Leaves. *Journal of Pharmaceutical and Biomedical Analysis*, **156**, 297-306. https://doi.org/10.1016/j.jpba.2018.04.047

[28] Munteanu, I.G. and Apetrei, C. (2021) Analytical Methods Used in Determining Antioxidant Activity: A Review. *International Journal of Molecular Sciences*, **22**, Article 3380. https://doi.org/10.3390/ijms22073380

[29] Aytul, K.K. (2010) Antimicrobial and Antioxidant Activities of Olive Leafextract and Its Food Applications. Master's Thesis, Izmir Institute of Technology.

[30] Haque, M.A., Morozova, K., Ferrentino, G. and Scampicchio, M. (2021) Electrochemical Methods to Evaluate the Antioxidant Activity and Capacity of Foods: A Review. *Electroanalysis*, **33**, 1419-1435. https://doi.org/10.1002/elan.202060600

[31] Gulaboski, R., Mirčeski, V. and Mitrev, S. (2013) Development of a Rapid and Simple Voltammetric Method to Determine Total Antioxidative Capacity of Edible Oils. *Food Chemistry*, **138**, 116-121. https://doi.org/10.1016/j.foodchem.2012.10.050

[32] Hoyos-Arbeláez, J., Vázquez, M. and Contreras-Calderón, J. (2017) Electrochemical Methods as a Tool for Determining the Antioxidant Capacity of Food and Beverages: A Review. *Food Chemistry*, **221**, 1371-1381. https://doi.org/10.1016/j.foodchem.2016.11.017

[33] Butnariu, M. and Coradini, C.Z. (2012) Evaluation of Biologically Active Compounds from Calendula Officinalis Flowers Using Spectrophotometry. *Chemistry Central Journal*, **6**, Article No. 35. https://doi.org/10.1186/1752-153x-6-35

[34] Tatarczak-Michalewska, M. and Flieger, J. (2022) Application of High-Performance Liquid Chromatography with Diode Array Detection to Simultaneous Analysis of Reference Antioxidants and 1, 1-Diphenyl-2-Picrylhydrazyl (DPPH) in Free Radical Scavenging Test. *International Journal of Environmental Research and Public Health*, **19**, Article 8288. https://doi.org/10.3390/ijerph19148288

[35] Pedan, V., Fischer, N. and Rohn, S. (2016) An Online NP-HPLC-DPPH Method for the Determination of the Antioxidant Activity of Condensed Polyphenols in Cocoa. *Food Research International*, **89**, 890-900. https://doi.org/10.1016/j.foodres.2015.10.030

[36] Ou, Z., Schmierer, D.M., Rades, T., Larsen, L. and McDowell, A. (2012) Application of an Online Post-Column Derivatization HPLC-DPPH Assay to Detect Compounds Responsible for Antioxidant Activity in *sonchus Oleraceus* L. Leaf Extracts. *Journal of Pharmacy and Pharmacology*, **65**, 271-279. https://doi.org/10.1111/j.2042-7158.2012.01591.x

[37] Kedare, S.B. and Singh, R.P. (2011) Genesis and Development of DPPH Method of Antioxidant Assay. *Journal of Food Science and Technology*, **48**, 412-422. https://doi.org/10.1007/s13197-011-0251-1

[38] Keddar, M.N., Ballesteros-Gómez, A., Amiali, M., Siles, J.A., Zerrouki, D., Martín, M.A., *et al.* (2020) Efficient Extraction of Hydrophilic and Lipophilic Antioxidants from Microalgae with Supramolecular Solvents. *Separation and Purification Technology*, **251**, Article ID: 117327. https://doi.org/10.1016/j.seppur.2020.117327

[39] Chigayo, K., Mojapelo, P.E.L., Mnyakeni-Moleele, S. and Misihairabgwi, J.M. (2016) Phytochemical and Antioxidant Properties of Different Solvent Extracts of *Kirkia wilmsii* Tubers. *Asian Pacific Journal of Tropical Biomedicine*, **6**, 1037-1043. https://doi.org/10.1016/j.apjtb.2016.10.004

[40] Paez, V., Barrett, W.B., Deng, X., Diaz-Amigo, C., Fiedler, K., Fuerer, C., *et al.* (2016) AOAC SMPR® 2016.002. *Journal of AOAC INTERNATIONAL*, **99**, 1122-1124. https://doi.org/10.5740/jaoacint.smpr2016.002

[41] Karori, S., Wachira, F., Ngure, R. and Mireji, P. (2014) Polyphenolic Composition and Antioxidant Activity of Kenyan Tea Cultivars. *Journal of Pharmacognosy and Phytochemistry*, **3**, 105-116.

[42] Kerio, L.C., Wachira, F.N., Wanyoko, J.K. and Rotich, M.K. (2013) Total Polyphenols, Catechin Profiles and Antioxidant Activity of Tea Products from Purple Leaf Coloured Tea Cultivars. *Food Chemistry*, **136**, 1405-1413. https://doi.org/10.1016/j.foodchem.2012.09.066

[43] Cleverdon, R., Elhalaby, Y., McAlpine, M., Gittings, W. and Ward, W. (2018) Total Polyphenol Content and Antioxidant Capacity of Tea Bags: Comparison of Black, Green, Red Rooibos, Chamomile and Peppermint over Different Steep Times. *Beverages*, **4**, Article 15. https://doi.org/10.3390/beverages4010015

[44] Kaur, A., Kaur, M., Kaur, P., Kaur, H., Kaur, S. and Kaur, K. (2015) Estimation and Comparison of Total Phenolic and Total Antioxidants in Green Tea and Black Tea. *Iobal Journal of Bio-Science and BioTechnology*, **4**, 116-120.

[45] Yang, J. and Liu, R.H. (2012) The Phenolic Profiles and Antioxidant Activity in Different Types of Tea. *International Journal of Food Science & Technology*, **48**, 163-171. https://doi.org/10.1111/j.1365-2621.2012.03173.x

[46] Musial, C., Kuban-Jankowska, A. and Gorska-Ponikowska, M. (2020) Beneficial Properties of Green Tea Catechins. *International Journal of Molecular Sciences*, **21**, Article 1744. https://doi.org/10.3390/ijms21051744

[47] Erol, N.T., Sarı, F. and Velioglu, Y.S. (2010) Polyphenols, Alkaloids and Antioxidant

Activity of Different Grades Turkish Black Tea. *Gida*, **35**, 161-168.

[48] Bartoszek, M., Polak, J. and Chorążewski, M. (2017) Comparison of Antioxidant Capacities of Different Types of Tea Using the Spectroscopy Methods and Semi-Empirical Mathematical Model. *European Food Research and Technology*, **244**, 595-601. https://doi.org/10.1007/s00217-017-2986-z