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ORIGINAL PAPER

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Effect of roasting on the radical scavenging activity of cocoa beans

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Abstract The free-radical scavenging activity of cocoa samples subjected to different roasting treatments has been determined. The samples (raw, pre-roasted and roasted) were separated into four molecular weight fractions per sample (>30, 30–10, 10–5, and <5 kDa). The free-radical scavenging activity was determined with the $DPPH^{\bullet}$ (1,1-dipheny-2-picrylhydrazyl), and $ABTS^{\bullet+}$ [2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid)] free-radical scavenging assays for all samples. Both tests were compared in terms of sensitivity and measurement precision, at different reaction times. Comparing the results from each test, the free-radical scavenging activity trends were similar for each fraction but with notable differences in the sensitivity of the assays. Analysis of the concentration of reducing substances, such as water soluble phenolics, melanoidins, carbohydrates, etc, in these fractions by the photometric Folin–Ciocalteu assay, showed a similar pattern to the free-radical scavenging activity trend. Moreover, this comparison showed that there were significantly ($P < 0.05$) more reducing substances and free-radical scavenging activity in the 10–5 kDa roasted cocoa bean fraction.

Keywords Cocoa · $ABTS^{\bullet+}$ · $DPPH^{\bullet}$ · Roasting effect · Free-radical scavenging activity

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Introduction

Natural antioxidants in foods may be from (a) endogenous compounds in one or more components of the food; (b) substances formed from reactions during processing; and (c) food additives isolated from natural sources. Most natural antioxidants are from plants (e.g. fruits, vegetables, herbs, beans, tea and coffee) and are primarily polyphenols, such as simple phenolic acids, cinnamic acid derivatives and flavonoids. These polyphenolic substances have gained much attention, due to their antioxidant capacity (as reducing agents, as free-radical scavengers, as metal chelators etc.) and their beneficial implications in human health. One foodstuff rich in antioxidants is cocoa, derived from the beans of *Theobroma cacao*. This tree is native to South America and was considered by the Olmec, Maya and Aztec peoples to have strong medicinal properties [1]. More recently cocoa has been shown to be rich in polyphenols [2], particularly in catechins (flavan-3-ols) and procyanidins [3]. The available methodology for analysis, isolation, purification and identification of flavonoids was covered in a review of polyphenols in cocoa by Wollgast [4]. In addition, antioxidant compounds in processed food and beverages have been described in the literature [5, 6]. During heat treatment, a reaction between reducing sugars and amino acids, known as the Maillard reaction can take place, forming a variety of by-products, intermediates and brown pigments (melanoidins), which may contribute to the flavour, antioxidative activity and colour of food. It has been shown that this non-enzymatic browning reaction produces strongly reducing substances, whose reducing power is responsible for their free-radical scavenging activity [7]. A number of attempts have been undertaken to isolate and purify reducing substances, such as melanoidins, from food by using separation techniques based on differences in either the molecular weight, e.g. dialysis, size-exclusion chromatography and polyacrylamide gel electrophoresis, or the charge of these macromolecules, e.g. ion-exchange chromatography, capillary electrophoresis, or isoelectric focussing [8]. However, difficulties associated with

collecting large amounts of cocoa bean fractions by, for, e.g., gel filtration were noted and so the technique of ultrafiltration was optimised for this work.

Since very little is known about the structure of melanoidins, as it has not been possible to isolate or completely characterise any of them, their investigation became the focus of a European Co-operation in the field of Scientific and Technical Research (COST)—Action 919 [http://cost.cordis.lu]. Most of the available information, to date, about the role of melanoidins in food and health is based on the COST action studies which deal with the reactions between sugars and amino acids at different roasting temperatures and durations. The most widely studied model system is that of glucose/glycine [9–15] while complementary studies were also carried out on genuine food samples such as coffee [16–20] and bread crusts [21]. One important outcome of these series of investigations was that melanoidins were found in the molecular weight range of <30 kDa. However, the measurement of free-radical scavenging activity in model solutions is not as intricate as trying to do the same for genuine food samples.

The usual determination of total polyphenolic content is achieved using the Folin–Ciocalteu procedure of Singleton [22], which is also adopted by the European Communities for wine analysis [23]. This method is based on the chemical oxidation of the reduced molecules by a mixture of the two strong inorganic oxidants phosphotungstic and phosphomolybdic acids.

There are some drawbacks, however, with this method as it is unspecific, with all reducing substances, such as water-soluble phenolics, soluble melanoidins, carbohydrates, etc, being determined. The free-radical scavenging activity assays are carried out using different radicals i.e. $ABTS^{\bullet+}$ (2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid)) and $DPPH^{\bullet}$ (1,1-dipheny-2-picrylhydrazyl). These methods are among the most popular spectrophotometric methods [24–26] for determining the free-radical scavenging capacity of foods and chemical compounds. These two stable radical chromogens (the violet $DPPH^{\bullet}$ radicals and the blue/green $ABTS^{\bullet+}$ radical cations) are easy to use, have a higher level of sensitivity [27, 28] and allow for analysis of a large number of samples within a reasonable time period. Both are relatively stable in certain conditions but show several important differences in their response to antioxidants [29].

This work was carried out to show the effect of roasting on the total quantity of reducing substances in the bulk and cocoa bean fractions as well as the free-radical scavenging activity of the bulk and cocoa bean fractions. A further objective was to compare the relative sensitivity of the $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ radical assays, which are mostly used to described free-radical scavenging activity, when conducted at different reaction times. It is, in fact the effect of roasting of the cocoa beans, in terms of the formation of these Maillard reaction by-products that should be explored.

Materials and methods

Chemicals and Reagents

DPPH (1,1-dipheny-2-picrylhydrazyl), ABTS [2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid)], and Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), were obtained from Sigma-Aldrich (St. Louis, USA). Sodium dihydrogen phosphate monohydrate and methanol 99.93%, were obtained from Fluka (St. Gallen, Germany), ethanol spectrophotometric grade and petroleum ether 30–60° were obtained from Merck (Darmstadt, Germany). Folin-Ciocalteu reagent, gallic acid, catechin and sodium carbonate were obtained from Sigma Aldrich. The cocoa beans (Ghana) were kindly supplied by Lindt & Sprüngli SpA in Varese (Italy). The supplied beans came from three different batches and they were mixed in the laboratory to ensure a representative sample. They had been subjected to two different heat treatments by the supplier, i.e. pre-roasting and roasting. Ultra pure water of >18 MΩ/cm resistivity and total organic carbon of <5ppb was obtained from a MilliQ 185 system (Millipore, France), fed by pure water from a Millipore Elix 5 system, the MQ grade being used to make up all solutions.

Sample preparation

The raw cocoa beans (2 kg) were hand peeled and crushed with a pestle and mortar to obtain small pieces known as nibs. The nibs were cryogenically ground at −198 °C to a fine powder in a Sterilmixer II for 1 min at 17000 rpm. The pre-roasted (1 kg) and roasted (1 kg) cocoa beans were transferred to the grinder directly as they had already been peeled and crushed during processing. The powder was defatted by extraction with petroleum ether and centrifuged using a Beckman Avanti J-20-Rotor JLA 8.1000 at 3000 rpm for 15 min at 4 °C. The supernatant was then discarded. Fresh petroleum ether was added and successively centrifuged (three times). The resulting defatted material was air dried at room temperature (in pre-weighed Petri dishes) and then re-weighed. The fat content was calculated by subtracting final net weights from the original (found to be ∼33%). The defatted bean powder was extracted in hot water using an automatic shaker bath for 20 min at 70 °C. The ratio between cocoa powder and water was 1:8. Subsequently, the three aqueous solutions were filtered through paper filters (Schleicher & Schuell ø 240 mm, grade 0858 1/2and 7–12 μm). The average extraction yields were 17% for roasted, pre-roasted and raw cocoa beans. The filtrate was split into two amounts, one for bulk analysis (∼350 mL) and another for ultrafiltration (∼2100 mL). The bulk was frozen and then freeze dried using a Lyovac GT2 lyophilizer.

The ultrafiltration was carried out using an Amicon 8400 Ultrafiltration device (Millipore) and a regulated N_2 supply

to create a slight positive pressure in the filtration device. Ice packs were used to cool the filtrate reception beaker.

To obtain four samples with different molecular weight (MW) ranges, three ultrafiltration membranes (Millipore): 30 kDa (PLCC ø 76 mm); 10 kDa (PLCC ø 76 mm); 5 kDa (PLCC ø 76 mm) were used. The filtrate from the first 30 kDa filter was filtered successively through the 10 and 5 kDa filters. In this way four samples with different MW ranges, >30, 30–10, 10–5 and <5 kDa were obtained for each type of cocoa: raw, pre-roasted and roasted. Aliquots of the filtrates were then transferred to aluminium dishes, frozen at –30°C and then freeze-dried. The lyophilised samples were placed in plastic containers and stored in a desiccator over silica gel at room temperature. The lyophilised samples were completely dissolved in hot water and then cooled down before free-radical scavenging activity measurements. The percent presence of single fractions with respect to bulk was then calculated. The data from reducing substances and free-radical scavenging activity of the fractions were than reported with respect to content of the original material.

Total reducing substance assay

The total reducing substance content of the cocoa and MW fractions was determined using the Folin Ciocalteu method [20]. Twenty milligrams of lyophilised cocoa sample was dissolved in warm (∼40–50°C) water (4 mL). 0.1 mL cool sample solution was added to 10 mL of deionised water, 1.25 mL of Folin-Ciocalteu reagent and to 2.5 mL of saturated sodium carbonate solution. The mixture was left to stand for 2 h at room temperature in the dark and the absorbance was measured at 730 nm (Unicam UV–vis 500 spectrophotometer). A blank sample (water plus reagents) was also prepared.

Quantification (milligram per gram cocoa extract) was obtained by reporting the absorbance in the calibration curve of gallic acid used as standard. The determination was repeated three times for each sample solution.

Free-radical scavenging activity determination

Two chromogen radicals, $DPPH^{\bullet}$ and $ABTS^{\bullet+}$, were used to determine the free-radical scavenging activity of the cocoa samples. $DPPH^{\bullet}$ is a commercially available free radical which is soluble and stable in ethanol and the free-radical scavenging activity may be determined by measuring the decrease in absorbance at 517 nm [24]. On the other hand, the $ABTS^{\bullet+}$ radical cation is generated in-situ by the addition of potassium persulphate as described by Pellegrini [26]. This radical is soluble in water and the free-radical scavenging activity is determined by measuring the decrease in absorbance at 734 nm. Both $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ measurements were carried out using a Unicam UV–Vis 500 spectrophotometer.

$DPPH^{\bullet}$ assay: Twenty-five milligrams of freeze dried sample was dissolved in a volumetric flask (5 mL) of warm (∼40–50 °C) type 1 water and mixed in an ultrasonic bath for 1 min. Each sample was diluted twofold before analysis. The sample solution (0.1 mL) was diluted twofold with ethanol directly in the cuvette (1 cm). Ethanol (1.9 mL) and ethanol solution (0.4 mL) containing $DPPH^{\bullet}$ of 0.5 mM, and 0.1 mL of water were added. The control sample was prepared using 1.9 mL ethanol, 0.4 mL of the same ethanolic solution containing $DPPH^{\bullet}$, 0.1 mL of water and 0.1 mL of a solution ethanol–water 1:2. The absorbance was read after 2.5, 5, 10, 15 and 20 min. A standard solution of 0.7 mM Trolox in ethanol was prepared and standard calibration curves were constructed by plotting percent free-radical scavenging activity as a function of the concentration of Trolox considering the control value as 0%. A standard solution of 0.7 mM catechin was treated in the same way as the samples to check the effectiveness and the precision of free-radical scavenging. The free-radical scavenging activity of the samples was expressed in Trolox milli-equivalent/milligram cocoa extract. All measurements were carried out at room temperature (25 °C). The standard solutions, the DPPH/ethanol solutions and the samples were used on the day of preparation. Each determination was done in triplicate.

$ABTS^{\bullet+}$ assay: Twenty-five milligrams of freeze dried sample were dissolved in a volumetric flask (5 mL) of warm (∼40–50 °C) type 1 water and mixed in an ultrasonic bath for 1 min. Each sample was diluted 1:5 before analysis. The activated $ABTS^{\bullet+}$ was diluted 1:88 with ethanol (working solution) to have an absorbance of 0.7–0.8 units. Standard solutions of 0.5 mM Trolox in ethanol were prepared. One-hundred microlitres of diluted standard (or sample) was added to 1 mL of the $ABTS^{\bullet+}$ radical working solution in a reaction tube and vortexed for 30 s. The absorbance was read at different reaction times as mentioned above. Appropriate blanks were run in each assay (1 mL of the $ABTS^{\bullet+}$ radical working solution and 100 μL of ethanol). The $ABTS^{\bullet+}$ working solution, ethanol, samples and standard solutions of Trolox were kept in a beaker of crushed ice during all measurements. A standard solution of Trolox (0.5 mM in ethanol) was prepared and a standard calibration curve was constructed by plotting percent inhibition values as a function of the concentration of Trolox, as previously described for $DPPH^{\bullet}$. A standard solution of 0.7 mM catechin was treated in the same way as the samples to check the effectiveness and the precision of free-radical scavenging. Triplicate determinations (at 734 nm) were made at each dilution of the standard.

The free-radical scavenging activity of the samples was expressed in Trolox milli-equivalents/milligram cocoa extract. The standard solutions, the $ABTS^{\bullet+}$ /ethanol solution and the samples were used on the day of preparation.

Statistical analysis of the data was carried out using analysis of variance (ANOVA option of Excel, MS Office XP) so as to separate and estimate the different causes of variation. It has been utilised to specifically show whether or not the differences in the free-radical scavenging activity between using different reaction times and within their replicates, for the bulk and various fractions, are significant.

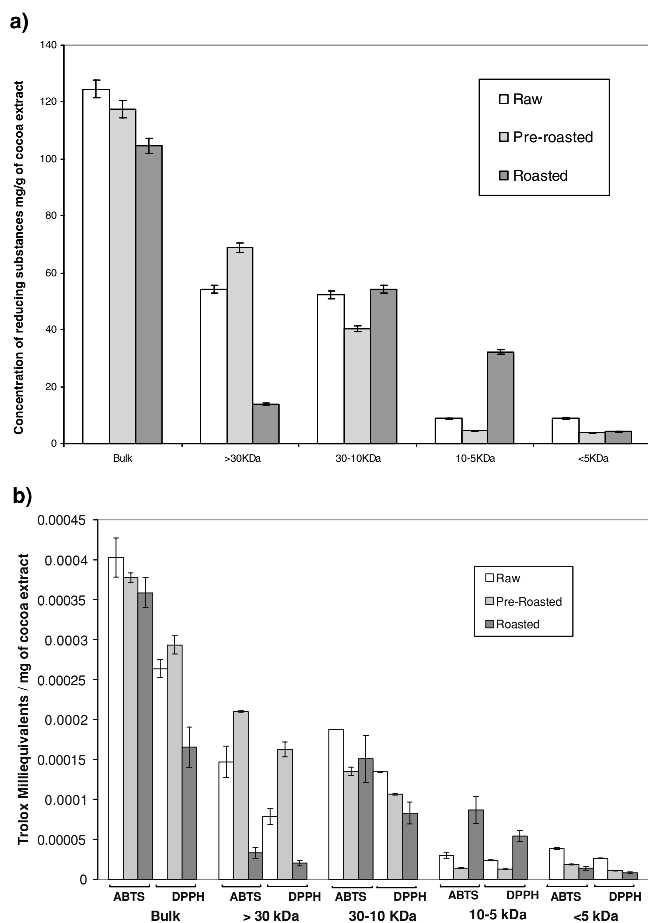

Fig. 1a Reducing substances concentrations determined by Folin–Ciocalteu assay in bulk cocoa and individual cocoa fractions. **b** Free-radical scavenging activity determined by $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ of bulk cocoa and individual cocoa fractions after 10 min

Results and discussion

Effect of roasting

The profile of reducing substances (water soluble phenolics, soluble melanoidins, carbohydrates, etc.) concentration in the bulk cocoa and in the individual fractions is shown in Fig. 1a.

Generally, it can be seen that the concentration of reducing substances in the bulk decreases progressively with pre-roasting and roasting. The most noticeable changes in reducing substances concentration are seen in the individual fractions. The concentration in the >30 kDa fraction increases slightly on pre-roasting but then significantly ($P<0.05$) decreases on roasting. In contrast the reducing substance concentration in the 30–10 kDa fraction decreases slightly on pre-roasting and returns to a similar level as the original raw cocoa with roasting. This could be due to the generation of reducing substances in the 30–10 kDa fraction as a result of thermal degradation, possibly occurring during the high temperatures of the roasting process. However, these lower molecular weight polymers appear to be thermally unstable and decompose with the more drastic conditions used for roasting, thus forming lower molecular weight compounds. This fact is confirmed by the profile found in 10–5 kDa fractions: the roasted samples showed the highest presence of Folin–Ciocalteu reactive substances in comparison to raw and pre-roasted ones ($P<0.05$). Fractions <5 kDa showed a very low index of reducing substances. One could expect that the low molecular weight reducing substances (such as polyphenols) would be found in <5 kDa fractions but the data don't seem to confirm this trend. This could be explained by the fact that the concentration of reducing substances is larger in the 10–5 kDa due to the presence of reducing substances other than polyphenols, such as melanoidins. Since melanoidins are greater than 5 kDa, they are not found in that fraction, thus accounting for the lower concentration of total reducing substances in this fraction in comparison to its neighbouring fraction.

The free-radical scavenging activity of the bulk cocoa and fractions determined by the $DPPH^{\bullet}$ assay is shown in Fig. 1b.

The trend of the graph is very similar to the reducing substances concentration shown in Fig. 1a, hence the change in reducing substances concentration seems to be closely related to the changes seen in free-radical scavenging activity of the bulk and individual fractions with pre-roasting and roasting. The pattern of the roasted fractions, with low activity in >30 kDa and the higher value found in 10–5 kDa, was considered very interesting. This trend supports the notion that the higher activity in the 10–5 kDa could be due to a combined contribution of both polyphenols, melanoidins and other low MW water soluble reducing substances.

The free-radical scavenging activity of the same samples measured with the $ABTS^{\bullet+}$ assay is displayed also in Fig. 1b. The profile of the graph is very similar to that obtained by the $DPPH^{\bullet}$ assay. The higher activity in the roasted samples is confirmed for 10–5 kDa fractions: this was also noted in a paper dealing with the free-radical scavenging activity of coffee fractions [17]. However, the $ABTS^{\bullet+}$assay appears to be relatively more sensitive, due to the higher Trolox milli-equivalent values found in all bulk samples. This higher sensitivity could be due to the fact that this radical is water soluble, as are the samples, which is not the case of the $DPPH^{\bullet}$, which needs to be dissolved in ethanol [30], explaining how the $ABTS^{\bullet+}$ assay may have more affinity with the genuine samples.

The $ABTS^{\bullet+}$ activity profile in bulk samples very closely matches the amount of reducing substances in the bulk samples while the free-radical scavenging activities of the $DPPH^{\bullet}$ bulk (Fig. 1b) are quite different, with a similar value for raw and pre-roasted, and a lower value in roasted. Looking at the whole range i.e. bulk to <5 kDa, for each of the three assays, one can see that there is a much closer relationship between the concentration of reducing substances (Fig. 1a) with the free-radical scavenging activity via the $ABTS^{\bullet+}$ assay than the $DPPH^{\bullet}$ free-radical scavenging activity assay (Fig. 1b).

Table 1 Comparison of results (± SD, $n = 5$) on the use of the two radicals for a catechin standard solution

	Reaction time (min)		
Radical	2.5	5	10
$ABTS^{\bullet+}$	6.39±0.23 (3.6)	6.53±0.22 (3.4)	6.83±0.26 (3.8)
$DPPH^{\bullet}$	2.29±0.07 (3.1)	2.52±0.07 (2.8)	2.95±0.08 (2.7)

Note: See text for explanation of use of correction factor of 60. Relative standard deviations (%) are given in brackets. Results expressed in milli-equivalent Trolox/mg cathechin (60×10^4)

In an effort to understand the differences noted between the two free-radical scavenging activity assays, apart from the solubility issue mentioned above, the effect of the reaction time for three different matrices was investigated using the synthetic Trolox standard, a polyphenol standard (catechin) and genuine samples.

Reaction time effect for the Trolox standard

The reaction time effect on the free-radical scavenging activity determination, measured by spectrophotometry, was investigated when changing this factor from 2.5 to 20.0 min. A standard solution of 0.7 mM Trolox was used to obtain similar Trolox concentrations (in the cuvette) for both radicals. Successive dilutions were then carried out for creation of the respective response curves.

No statistically significant difference has been observed at the 95% confidence level (P=0.05) on the different response curves (not shown) established using the above mentioned reaction times which lead us to draw the conclusion that time does not have a crucial effect on the Trolox standard and therefore any of these response curves could be used for the conversion of the free-radical scavenging activity on different cocoa bean varieties to Trolox milli-equivalents (response curve for 10 min reaction time was then selected for both radicals).

Reaction time/radical effect for the polyphenol standard

A catechin aqueous standard solution (50 μg g^{-1}) was prepared and measured when using the two radicals at different reaction times, i.e. 2.5, 5.0 and 10.0 min. Results are presented in milli-equivalent Trolox (Table 1).

Systematically higher results were obtained with the use of $ABTS^{\bullet+}$. The $ABTS^{\bullet+}$ radical is approximately twice as sensitive as the $DPPH^{\bullet}$ radical as also described by Kim et al. [30]. Conversely, the precision was higher when using $DPPH^{\bullet}$ as a radical. Since the initial catechin solution was not in the same range as the samples, a factor of 60 was used in order to make the catechin concentration range comparable to reported values for cocoa beans (2.96–3.27 mgg^{-1}) [30]. Values given in Table 1 were then used as "reference" values against which, measured free-radical

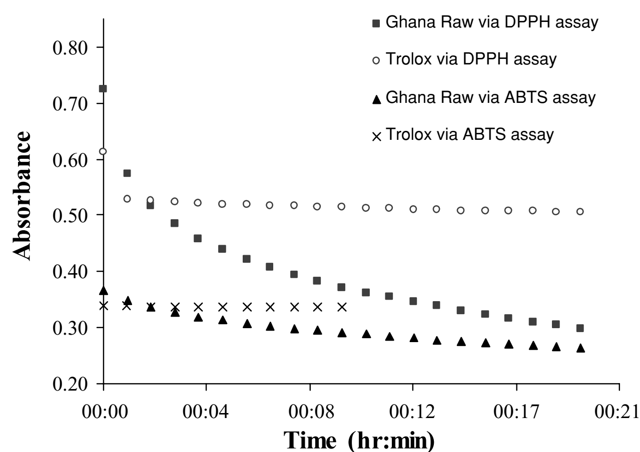

Fig. 2 Consumption of $DPPH^{\bullet}$ and $ABTS^{\bullet+}$-derived radicals after increasing reaction time for Ghana raw (■;▲) and Trolox (○×)

scavenging activity values of the cocoa bean samples were compared to.

Reaction time / radical effect for the cocoa samples

Due to the fact that the kinetic profile of the reaction between phenols and other reducing substances with $ABTS^{\bullet+}$ (and/or $DPPH^{\bullet}$) is very much dependant on the characteristics of the substrate, an illustration of the consumption of $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ derived radicals following the addition of (a) Trolox and (b) Ghana raw cocoa bean (bulk) is shown (Fig. 2) as examples.

As can be seen in Fig. 2, Trolox reacts with the $ABTS^{\bullet+}$ or $DPPH^{\bullet}$ derived radicals almost instantaneously, presenting a smooth decay. The higher complexity of the food matrix (Ghana raw) shows a higher consumption than the Trolox, in each assay, of the corresponding radical, which cannot easily be fitted. This makes interpretation and/or prediction of the free-radical scavenging activity of the various cocoa bean fractions difficult. However, in most cases and depending on the absorbance of the blank (radical without the sample or Trolox having been added), we found that the usual relationship holds true, i.e. Free-radical scavenging activity % = (100–(Abs∗100)/blank), which in actual terms means that lower absorbance equals higher free-radical scavenging activity and vice versa, i.e. higher absorbance equals lower free-radical scavenging activity.

The effect of the reaction time for each assay and for each sample type was then examined. To do this, the free-radical scavenging activity results obtained when using the two different radicals were compared for all three cocoa bean heat treated samples and for the different molecular weight fractions. Given results are accompanied with their respective expanded uncertainties (U) which were computed from combining standard uncertainties (means 1 SD) for all relevant components of the equation and using a coverage factor k of 2 which corresponds approximately to a level of confidence of 95% [31].

Table 2 Comparison of results for the different MW fractions of Ghana raw, pre-roasted and roasted cocoa beans when using the two radicals

Sample	Time (min)	DPPH	U, k=2	RU%	ABTS	U, k=2	RU%	Diff
G. Raw	2.5	1.61	0.10	**6.3**	4.53	0.14	**3.2**	2.91
	5.0	1.95	0.13	6.6	4.75	0.17	3.5	**2.81**
	10.0	2.22	0.17	7.5	5.09	0.19	3.7	2.87
	15.0	2.39	0.21	8.6	5.32	0.21	4.0	2.93
	20.0	2.52	0.24	9.5	5.52	0.21	3.7	3.00
G. Raw >30 KDa	2.5	0.95	0.07	7.4	4.96	0.57	**11.4**	4.01
	5.0	1.28	0.09	**7.1**	5.22	0.67	12.9	**3.93**
	10.0	1.55	0.12	7.5	5.55	0.76	13.7	4.00
	15.0	1.71	0.14	8.0	5.77	0.85	14.7	4.06
	20.0	1.83	0.15	8.2	5.95	0.95	16.0	4.12
G. Raw 30-10 KDa	2.5	1.71	0.09	5.4	5.47	0.53	9.6	3.76
	5.0	2.23	0.11	**5.1**	5.70	0.49	**8.6**	3.47
	10.0	2.60	0.15	5.8	6.04	0.54	8.9	**3.45**
	15.0	2.80	0.19	6.7	6.27	0.66	10.5	3.47
	20.0	2.94	0.24	8.1	6.45	0.76	11.9	3.51
G. Raw 10-5 KDa	2.5	1.75	0.10	**5.5**	4.77	0.40	**8.3**	3.01
	5.0	2.25	0.15	6.7	5.00	0.47	9.4	2.74
	10.0	2.61	0.22	8.5	5.33	0.54	10.2	2.72
	15.0	2.80	0.28	10.1	5.55	0.61	11.0	2.74
	20.0	2.94	0.34	11.5	5.72	0.67	11.7	2.77
G. Raw <5 KDa	2.5	1.45	0.08	**5.8**	4.53	0.57	**12.6**	**3.07**
	5.0	1.67	0.10	6.0	4.79	0.74	15.5	3.12
	10.0	1.88	0.12	6.3	5.15	0.91	17.7	3.26
	15.0	2.03	0.14	7.0	5.38	1.02	19.0	3.34
	20.0	2.15	0.16	7.6	5.59	1.17	21.0	3.43
G. P-R	2.5	2.04	0.24	**11.5**	3.39	0.09	2.5	1.35
	5.0	2.41	0.41	17.0	3.57	0.09	**2.4**	1.16
	10.0	2.70	0.63	23.5	3.83	0.11	2.9	**1.14**
G.P-R >30 KDa	2.5	1.72	0.09	**5.5**	4.22	0.34	8.1	2.50
	5.0	2.16	0.18	8.2	4.42	0.33	7.6	2.27
	10.0	2.55	0.33	12.9	4.72	0.36	**7.5**	**2.17**
G. P-R 30-10 KDa	2.5	1.88	0.08	**4.0**	5.11	0.18	**3.6**	3.22
	5.0	2.35	0.16	6.7	5.30	0.26	4.9	2.95
	10.0	2.67	0.28	10.5	5.59	0.39	6.9	**2.92**
G. P-R 10-5 KDa	2.5	1.87	0.17	**9.0**	4.76	0.07	**1.6**	2.89
	5.0	2.30	0.28	12.3	4.96	0.11	2.2	**2.66**
	10.0	2.62	0.43	16.4	5.27	0.18	3.4	2.66
G. P-R <5 KDa	2.5	1.32	0.04	2.7	3.96	0.30	**7.6**	2.63
	5.0	1.53	0.04	**2.5**	4.11	0.33	8.1	**2.58**
	10.0	1.73	0.04	2.5	4.37	0.40	9.1	2.64
G. Roasted	2.5	1.39	0.09	**6.8**	2.92	0.05	1.7	1.53
	5.0	1.67	0.12	7.4	3.07	0.05	1.7	**1.40**
	10.0	1.90	0.16	8.6	3.29	0.03	**0.9**	1.40
G. Roasted >30 KDa	2.5	1.41	0.06	**4.0**	3.17	0.18	**5.6**	1.76
	5.0	1.78	0.09	4.8	3.35	0.23	6.7	1.58
	10.0	2.06	0.13	6.5	3.58	0.27	7.5	**1.52**
G. Roasted 30-10 KDa	2.5	1.81	0.13	**7.4**	4.90	0.19	**3.8**	3.08
	5.0	2.33	0.21	8.9	5.11	0.22	4.3	2.78
	10.0	2.71	0.28	10.2	5.42	0.27	5.0	**2.71**
G. Roasted 10-5 KDa	2.5	1.79	0.10	**5.6**	4.57	0.17	**3.7**	2.78
	5.0	2.27	0.17	7.5	4.77	0.20	4.2	2.50
	10.0	2.61	0.30	11.3	5.08	0.24	4.7	**2.47**
G. Roasted <5 KDa	2.5	1.19	0.04	**3.2**	3.52	0.10	2.8	2.34
	5.0	1.39	0.06	4.3	3.71	0.16	4.3	**2.32**
	10.0	1.54	0.05	3.2	3.90	0.10	**2.6**	2.37

The free-radical scavenging activity is expressed in Trolox milli-equivalent/milligram cocoa extract ($\times 10^4$, n= 3). Diff. meaning the difference of the results obtained with $ABTS^{\bullet +}$-$DPPH^{\bullet}$ radicals

Statistically significant difference has been ascribed in all cases since the higher result minus its associated expanded uncertainty does not reach the lower result (for each reaction time). The free-radical scavenging activity (FRSA) is expressed in Trolox milli-equivalents/milligram cocoa extract. Table 2 for Ghana cocoa beans and its respective MW fractions. The averaged mean of the three replicates was used for both radicals.

Table 2 provides comparative data for Ghana Raw, Ghana pre-roasted and Ghana roasted cocoa beans with respect to their activity at different reaction times.

In this table highlighted values indicate the smaller relative uncertainty (RU, expressed as U×100/result) and the smaller observed difference between the use of the two radicals, respectively. Smaller relative uncertainties were obtained when using DPPH$^{\bullet}$ as radical and for smaller (2.5–5.0 min.) reaction times. This table also illustrates the fact that the results are significantly different when using different reaction times (higher reaction time induces a lower response value). Moreover, a smaller difference on the analytical result when using the two radicals has been observed for smaller reaction times.

Similar relative uncertainties were computed when using both radicals. Furthermore, smaller reaction times (2.5–5.0 min) gives higher precision for both heat-treated cocoa samples, with the only exception when using ABTS$^{\bullet+}$ as radical for Ghana roasted and its <5 kDa MW fraction, whereas 10 min. is necessary to achieve an higher precision. As already mentioned above, smaller differences on the use of the two radicals were obtained when using the smallest to medium (∼10 min.) reaction times.

Estimation of the method precision

Single-factor Analysis of Variance (one-way ANOVA) was used to separate and identify the different sources of variability found for the three different heat treated cocoa bean (Ghana raw, Ghana pre-roasted and roasted) when using three replicates for each reaction time and for each radical. The question is then to see whether there is a statistically significant difference between-time when compared to the within-time (random variation). In all cases when using DPPH$^{\bullet}$ as the radical, the between-time variability was significantly larger than the within-time (random variation) proving the precision of the method when using this radical. Therefore, the difference in results between-time cannot be attributed to (random) variation alone (F-values $\gg$ critical F-value ($P = 0.05$)). The same variability was observed for the different molecular weight fractions i.e., for >30, 10–30, 10-5 and <5 kDa MW fractions.

A different situation was observed when using ABTS$^{\bullet+}$ as the radical and for the MW fractions <5 kDa of Ghana raw, >30 kDa and <5 kDa for Ghana pre-roasted and for the >30 kDa MW fraction of Ghana roasted, where no statistically significant difference was observed between-time and within-time variability (F-values $<$ critical F-values (P=0.05)). Thus, the within-time (variability within a series of replicates) variability is not significantly smaller than the between-time variability.

It appears that these results may have arisen from a higher variability obtained when using ABTS$^{\bullet+}$ as the radical when compared to DPPH$^{\bullet}$.

When assessing the precision obtained on the free-radical scavenging activity results and for the different reaction times, it can be concluded that a smaller reaction time (2–10 min) favours higher precision (smaller SD—see Table 2). These conclusions held true for both heat-treated cocoa beans.

Conclusion

Aqueous cocoa bean extracts showed considerable free-radical scavenging activity, when measured with two well described methods. The free-radical scavenging activity of the various fractions, measured by both assays, correspond very well with the quantification profile obtained for the reducing substances. There is a significant increase in free-radical scavenging activity after roasting in fraction 10–5 kDa, which corresponds to the MW region of melanoidins.

Regarding the sensitivity of the methods, statistical investigation of the data showed that the ABTS$^{\bullet+}$ assay is ∼25% more sensitive than the DPPH$^{\bullet}$ assay.

However the systematic comparison of the use of the two chromogen radicals for cocoa sample determinations indicated that the DPPH$^{\bullet}$ gave consistently higher precision for Ghana raw than for the other two heat-treated cocoa bean samples. This observation is mainly valid at shorter reaction times. Furthermore, higher accuracy has been confirmed for the DPPH$^{\bullet}$ radical when using the catechin standard. Smaller differences in the precision of free-radical scavenging activity results, when using the two chromogen radicals, has been observed in all samples for shorter reaction times.

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