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ORIGINAL PAPER

Effect of Fermentation and Drying on Procyanidins, Antiradical Activity and Reducing Properties of Cocoa Beans

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Abstract This work was aimed to determine the effect of fermentation and drying on the content and profile of procyanidins (from monomers P1 to polymers P10) as well as on the antiradical and scavenging properties of cocoa beans. To this purpose, three experiments were carried out: a traditional fermentation process followed by air drying and two pilot-scale fermentation processes by either natural microbiota or starter followed by sun drying. Procyanidins were evaluated by HPLC analysis, while the total polyphenol index (TPI), the antiradical activity as well as the reducing power were determined by means of the reaction with the Folin–Ciocalteu reagent, the decolorization assays of the ABTS radical (TEAC) and the Ferric Reducing Antioxidant Power (FRAP) methods, respectively. Both the traditional and pilot-scale processes resulted to affect the profile and content of the procyanidins fractions as well as the antiradical and reducing power functionality. Drying caused a severe reduction of compounds and thus resulted to be the critical step for the loss of procyanidins and monomers in particular. The indices of functionality generally showed a decreasing trend as a consequence of processing, and their evolution was similar to that observed in procyanidins content. To study the relationship between the individual procyanidins and the antioxidant activity expressed as TEAC, FRAP and TPI, the data set were processed by modified partial least squares regression. The obtained models presented a good predictive ability. Normalised regression coefficients showed that the relative contribution of each single class of compounds to total antioxidant activity resulted as follows: P1>P2>P3>P4>P6>P8>P5>P7>P9 >>P10.

Keywords Cocoa beans · Fermentation · Drying · Procyanidins · FRAP · TPI · TEAC

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Introduction

Chocolate, thanks to its unique and complex structure and flavour, is an extraordinary food usually consumed for pleasure that, in recent times, has been revalued as an important source of healthy compounds.

Cocoa and chocolate products are in fact rich in flavan-3-ol polyphenols, also known as flavanols, which possess antioxidant activity and have been reported to exert a protective effect against cardiovascular diseases, cancer and inflammatory processes in the human body (Engler et al. 2004; Heiss et al. 2005; Serafini et al. 2003; Jourdain et al. 2006; Corti et al. 2009; Cooper et al. 2008).

Procyanidins, flavan-3-ols polymers, also play another important role in cocoa-derived products since they contribute to their taste and acceptability by affecting both bitterness and astringency (Komes et al. 2012; Sun-Waterhouse and Wadhwa 2012). The procyanidins content of cocoa-derived products depends on many factors, some related to the raw material, such as the bean variety and postharvest handlings, and others related to processing, like fermentation, drying and roasting (Wollgast and Anklam 2000; Rusconi and Conti 2010).

Fermentation of the pulp surrounding the beans represents a key step in cocoa processing for the development of the chocolate flavour and taste since it produces aroma precursors. During fermentation, the combination of endogenous and exogenous (microbial) enzymatic activities along with the diffusion of metabolites into and out of the cotyledons allows polyphenols to polymerise and react with other compounds to form complexes. These reactions decrease polyphenols solubility, thus reducing bitterness and astringency and give rise to the typical colour of well-fermented beans (Hansen et al. 1998; Lima et al. 2011).

Fermentation is considered responsible for the decrease of the flavan-3-ol content, (−)-epicatechin in particular, which is often used as an index of the processing extent; the level of reduction resulted proportional to the degree of fermentation and was ascribed to both the oxidation processes and the diffusion of polyphenols into fermentation sweating (Kim and Keeney 1984; de Brito et al. 2000; Camu et al. 2008; Payne et al. 2010).

After fermentation, a drying step is applied to reduce the moisture content and water activity of cocoa beans and to confer them a longer shelf life. Drying can be carried out naturally, by making use of solar energy, or artificially by air. Whatever the process, drying conditions may favour oxidative phenomena that contribute, together with polymerisation reactions, to induce the formation of new compounds implied in the reduction of the negative sensory notes and the development of the peculiar chocolate 'brown' colour. As far as the effect on polyphenolic compounds is concerned, drying is considered to reduce epicatechin and catechin contents with percentages of decrease depending on the processing conditions adopted (de Brito et al. 2000; Payne et al. 2010).

To our knowledge, scarce in literature is the information regarding the effect of fermentation and drying on the procyanidins profile, on their polymerisation from monomers up to oligomers and polymers, and also on some functional properties such as antiradical activity and reducing power (Aikpokpodion and Dongo 2010).

Consumers are becoming more and more aware of health issues, and research is needed to evaluate the effect of processing conditions on food functional properties and to optimise technological procedures that allow the right balance between health, taste and acceptability of the final product.

Based on these premises, this study was thus undertaken to determine the effect of fermentation, as well as drying conditions, on procyanidins profile, on their polymerisation and on some functional parameters of cocoa beans; to this purpose, in parallel to the traditional conditions used in both fermentation and drying, two pilot-scale experiments were carried out and investigated. Moreover, to study the relationship between the individual procyanidins and antioxidant activity, the data set was processed by partial least squares (PLS) regression.

Materials and Methods

Materials

The cocoa beans (Trinitario variety from Costa Rica) were provided by Belcolade, Puratos (Groot-Bijgaarden, Belgium). The analytical determinations were carried out on samples obtained from three cocoa batches of the same batch produced under different process conditions and in particular, one in a large scale (experiment 1—FCC) and two small pilot scale [experiments 2–E2—experiment 2 Blanco (E2B) and Mix (E2M)]. A scheme of the experimental plan of this study is presented in Fig. 1. Process and sampling conditions were carried out as below reported.

FCC

These conditions correspond to the traditional process. Cocoa beans were spontaneously fermented in wooden boxes, having the dimensions to contain about 1,600 kg of fresh cocoa beans and with holes at the bottom to allow drainage of sweating generated during the fermentation. Every 24 h, the beans were mixed by transferring them to another box of the same volume. After 7 days of fermentation, the beans were slowly dried for 4 days on a platform dryer using indirect heated air at 60 °C. The water content of FCC cocoa beans after drying was about 10 %. Samplings of approximately

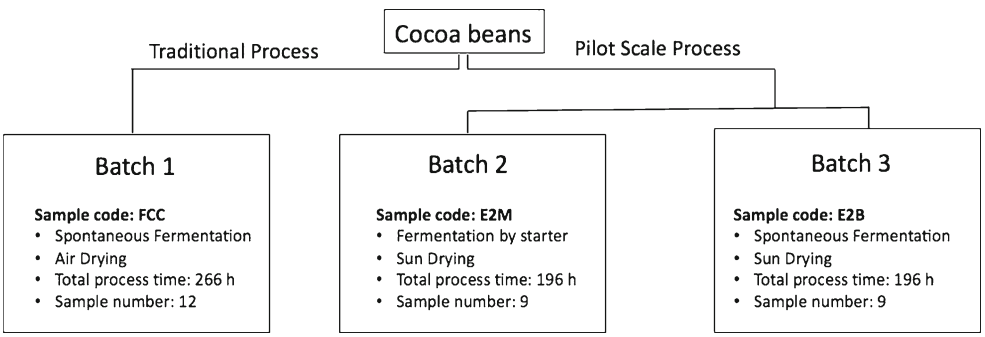

Fig. 1 Experimental design of the samples under investigation

150 g of cocoa beans were taken every 24 h in the centre of the fermenting/drying mass for a total time of 266 h (11 days).

E2

Two cocoa fermentation processes were carried out at pilot scale both by using plastic barrels able to contain about 50 kg of beans. The vessels were able to turn around a central axis and were holed at the bottom for liquid drainage. Both fermentors and basins were cleaned with chlorine water and rinsed with tap water before being filled with fresh cocoa beans. Mixing and aeration of the cocoa mass were achieved by turning the barrels every 24 h. In parallel, two different procedures of fermentation in different basins were applied. In the E2 Mix experiment (E2M), ripe cocoa fruits were washed in chlorine water and opened manually using sterile machetes. The seeds and pulp were removed from the pods using sterile rubber gloves and transferred to a sterile reservoir. The cocoa bean mass was inoculated with a mix of starter cultures using a sprayer when cocoa beans and pulp were transferred to the fermentor. The Mix inoculum (750 mL) consisted of strains *Saccharomyces cerevisiae* MEA 50 (2.8E+7 CFU/mL) and *Lactobacillus vaginalis* MRS 94 (2.8E+8 CFU/mL). Both strains were isolated from spontaneous Costa Rican cocoa fermentation and grown in 250-mL sterile MEA and MRS both for 24 h, respectively. A reference fermentation experiment (E2 Blanco) was set up in the second vessel to allow growth of the natural microbiota; pods and utensils were therefore not washed with chlorine water and no starter culture was added. Beans from both E2 experiments at the end of the fermentation step were allowed to dry under the sun for 4 days to a final moisture content of 2 %. Samplings were carried out approximately every 24 h for a total time of 196 h (8 days).

The degree of fermentation was followed on the basis of anthocyanins content as indicated by Gourieva and Tserrevitinov (1979); samples fermentation index at end of the fermentation of all the processes was 0.8±0.1.

Commercial cocoa samples (n=24) were purchased on the local market and used for validation of PLS model.

Sample Preparation

Cocoa beans, after pulp and skin removal, were freeze-dried and stored under vacuum packaging until analysis. The final water content was determined by a moisture analyser (Sartorius MA150, Goettingen, Germany). The average water content of the freeze-dried samples was less than 2 %.

Prior to analysis, beans were grounded and defatted with three cycles of hexane washing (8 g=50 mL). Each time, the mixture was centrifuged and the supernatant was discharged. The lipid-free solids were air-dried at room temperature until complete removal of hexane.

Sample Extraction

Sample extraction was carried out according to Gu et al. (2006) with some modifications. One gram of freeze-dried and defatted sample was extracted with 5 mL of 70:29.5:0.5 acetone/water/acetic acid; after 1 min of vortexing, the mixture was sonicated in an ultrasonic bath at 20 °C for 10 min. Sample extracts were clarified by centrifugation (4,000 rpm for 10 min) and filtrated through cellulose filters.

This extract was used for the evaluation of total polyphenol index, radical scavenging activities and ferric reducing properties. For procyanidins analysis, samples were extracted, kept at −32 °C and analysed on the same day of extraction; the extract was diluted (1:5) and submitted to a further filtration step by means of nylon filters (0.45 μm).

pH

The pH of the nibs was determined according to Jinap and Thien (1994). Ground nibs (10 g) were homogenised in 200 mL distilled water; the homogenate was filtered (Whatman number 4) and pH of the supernatant was measured by a Jenway (Stone, UK) pH meter.

Procyanidins Separation and Quantification

Chromatographic analysis was performed on a 1200 Agilent Series HPLC (Agilent Technologies, Milan, Italy), equipped with a G1322A degasser, a G1311A quaternary pump, a G1316A Column Thermostat, a G1328B manual injection system and a G13115D diode array detector. The system was controlled with Agilent ChemStation for Windows (Agilent Technologies).

The sample (20 μL) was injected onto a Phenomenex (Bologna, Italy) 5 μm normal-phase Luna Silica column, 100 Å, 250 mm×4.6 mm, at 25 °C; the column was equipped with a 4×3.0 mm silica security guard cartridge.

According to Counet and Collin (2003), the separation of procyanidins were carried out at a flow rate of 1 mL min^{-1} by using a three-component mobile phase with a gradient elution from solvent A (dichloromethane) to B (methanol) and a constant 4 % level of C (acetic acid and water, 1:1, v/v). Gradient elution was as follows: from 14 % to 28 % B from 0 to 30 min, from 28 % to 50 % B from 30 to 60 min, from 50 % to 86 % B from 60 to 65 min and isocratic from 65 to 70 min.

Identification of the compounds was previously made according to retention times and confirmed by HPLC–MS/MS analyses by using a Perkin Elmer (Perkin Elmer, Norwalk, CT, USA) Series 200 equipment. Since procyanidins show a similar absorption coefficient (Counet and Collin 2003), a calibration curve made with (−)-epicatechin was used for their quantification and the values of each procyanidins fraction

were expressed as milligrams of epicatechin equivalents per gram of dry weight (d.w.). The total procyanidins content was calculated as the sum of P1–P10 fractions.

The accuracy of the method was evaluated by means of a spiking and recovery procedure on the cocoa beans performing five replicates. The initial endogenous procyanidin content of the cocoa beans samples has been determined five times. After spiking with (−)-epicatechin, the samples were subject to the whole analytical procedure.

The accuracy of the extraction method was evaluated by means and coefficient of variation (CV, %) on the five replicates of cocoa beans sample. The uncertainties of the extraction method were as follows: P1±4 %, P2±3 %, P3±5 %, P4±5 %, P5±8 %, P6±10 %, P7±6 % and P8±4 %.

The precision/accuracy of the method was also performed: the limit of detection (LOD) and quantification (LOQ), set on the samples at the signal-to-noise ratio (S/N) of 3 and 10, respectively, were as follows: LOD, 0.14 mg epicatechin equivalents per gram of dry weight (d.w.); LOQ, 0.18 mg epicatechin equivalents per grams of dry weight (d.w.).

Radical Scavenging Activity

The radical scavenging activity was measured according to the method described by Re et al. (1999) with some modifications. ABTS (Fluka, Buchs, Switzerland) was dissolved in water to a 7 mM concentration; the ABTS radical was formed by reacting ABTS stock solution with 2.45 mM potassium persulphate and allowing the mixture to stand in the dark at room temperature for 12–16 h before use. The ABTS radical solution was diluted with deionised water to reach an absorbance of 0.70±0.02 at 734 nm and at 30 °C. Thirty microliters of differently diluted extracts (from 1:25 to 1:100 in the extraction media) was added to 2.97 mL of diluted ABTS radical solution. The absorbance at 734 nm was evaluated by a Perkin Elmer (Boston, MA, USA) Lambda Bio 20 spectrophotometer. The time of analysis, 5 min, was chosen after preliminary tests and represented the time necessary to reach 80 % of the overall inhibition of the ABTS radical at a given concentration. For each dilution, the percentage of inhibition (I%) was plotted as a function of concentration and the TEAC (Trolox Equivalent Antioxidant Capacity) and calculated by the ratio of the linear regression coefficient of the sample and that of the Trolox standard (Fluka). Results were expressed as micromoles of Trolox equivalents per gram of dry weight.

The coefficient of variation of the method was below 5 %.

Ferric Reducing Antioxidant Power (FRAP)

The reducing activity of the samples was determined according to the method described by Benzie and Strain (1996) with some modifications. One hundred microliters of opportunely diluted sample extract was added to 2,900 μL of the FRAP reagent obtained by mixing acetate buffer (300 mM, pH 3.6), TPTZ (2,4,6-tripyridyl-*s*-triazine) 10 mM solubilised in HCl 40 mM and $FeCl_3$ 20 mM in the ratio 10:1:1. The absorbance change was followed at 593 nm for 6 min. A calibration plot based on $FeSO_4 \cdot 7H_2O$ was used and results were expressed as micromoles of Fe^{2+} per gram of dry weight.

The coefficient of variation of the method was below 3 %.

Total Polyphenols Index (TPI)

The TPI was determined according to a procedure modified from Singleton and Rossi (1965). The sample extract (0.1 mL) was diluted with deionised water to a volume of 5 mL and then 500 μL of Folin–Ciocalteu (FC) reagent was added; after 3 min, 1.5 mL of a 25 % Na_2CO_3 solution was added and then deionised water up to 10 mL final volume. Solutions were maintained at room temperature under dark conditions for 60 min and the total polyphenols content was determined at 765 nm using a Perkin Elmer Lambda Bio 20 spectrophotometer. Gallic acid standard (Fluka) solutions were used for calibration purposes. Results were expressed as milligrams of gallic acid equivalents (GAE) per gram of dry weight.

The coefficient of variation of the method was below 3 %.

Statistical Analysis

Data presented are the average of at least six measurements taken from two different repeats of each sample type and reported as mean and standard deviation. One-way ANOVA was applied to experimental data to determine the significance of effects (fermentation and drying time). Differences between means were tested by the least significant difference test.

Relationships between individual procyanidins (P1–P10) and antioxidant activity indices (TPI, TEAC and FRAP) were tested by PLS regression which was applied to study the cause–effect relationship between procyanidins (predictor variables) and antioxidant activity (response variable). PLS regression was carried out using the SIMPLS algorithm (de Jong 1993) and choosing a sigma restricted procedure and a model with no intercept.

The final PLS model was a linear model:

$$Y = a_1X_1 + a_2X_2 + \ldots + a_nX_n + \varepsilon$$

where Y is an *n* cases (30) by *m* (*m*=1, antioxidant activity) variables response matrix, X is an *n* cases by *p* (*p*=10, procyanidin polymers) variables predictor matrix, a is a *p* by *m* regression coefficient matrix, and ε is a noise term for the model which has the same dimensions as Y. The final

PLS model was computed after factors extraction and the recalculation of the regression coefficient.

The model was validated by full cross-validation and the optimal level of extracted factors was calculated by the root mean square error of prediction (RMSEP). All of the independent variables were used to calculate the final model which was eventually validated on an external set of data from 24 commercial cocoa samples. The adequacy of the final model was expressed by the optimum number of extracted factors, the determination coefficient R^2 and the RMSEP.

Data were processed using the STATISTICA for Windows (StatSoft^{TM}, Tulsa, OK, USA) package.

Results and Discussion

Effect of Fermentation and Drying on Procyanidin Content

Freeze-dried and defatted cocoa beans sampled at different stages of the fermentation and drying process underwent the extraction of the procyanidin fractions and the extracts were submitted to HPLC analysis for separation and quantification. In Fig. 2, the evolution of the monomers (P1) upon fermentation and drying is shown; P1, which consists of epicatechin and catechin, has often been used as an index of processing degree. Fermentation caused a reduction of the content of monomers over time ($p<0.01$), even though they did not decrease linearly but showed a fluctuating evolution, in particular in the E2M and E2B samples sets.

It is interesting to note that at the end of fermentation, which was reached after 120 h of processing for E2M and E2B and 140 h for FCC series, the samples showed a comparable monomers content, in spite of the differences in processing conditions, including the size of the batch (50 vs 1,600 kg) and the fermenting microbiota (starter vs natural in the E2 processes).

Drying time significantly affected the P1 content ($p<0.01$). The decreasing trend observed upon processing for the monomeric fraction (Fig. 2) was also observed in other studies for (−)-epicatechin (Kim and Keeney 1984; Kealey et al. 1998; Camu et al. 2008) which is the main component of the monomeric fraction. During the drying step, the content in monomers of E2M and E2B samples showed a sharper decrease if compared to FCC samples, but the final P1 content of the latter was lower than those observed in the E2 batches. This result could be due to the fact that sun drying initially favoured the condensation reactions, but then the faster moisture loss in this step reduced the enzymatic activity and made the diffusion and migration of substrates and other compounds difficult, thus limiting any further procyanidin degradation. Other studies on drying showed that the drying rate is the most relevant factor for preserving the polyphenol content of vegetables (Mrkić et al. 2006).

In Fig. 3a–c, the procyanidin content of the samples under investigation along with the procyanidins profile before processing (Fig. 2a) and as a consequence of fermentation (Fig. 2b) and drying (Fig. 2c) is shown. It can be noted that in the three sets of samples, all the P1–P9 fractions generally showed a decrease upon processing. P1, P3, P4, P5 and P9 fraction values decreased significantly ($p<0.01$) in all samples after the fermentation process whilst the P10 ones significantly increased ($p<0.01$). Drying determined a significant ($p<0.01$) reduction of all the procyanidins.

If the relative contribution of each single P1–P10 class to the total procyanidin content is taken into consideration, interesting observations come out. In the FCC series, the P1 monomers in the initial cocoa beans accounted for 35 % of the total content, and upon processing time their contribution was kept quite constant in the first phases of fermentation, slightly increased at the end of fermentation and then significantly decreased ($p<0.01$) upon drying up to 30 %. A similar behaviour was observed for the pentamers P5 which

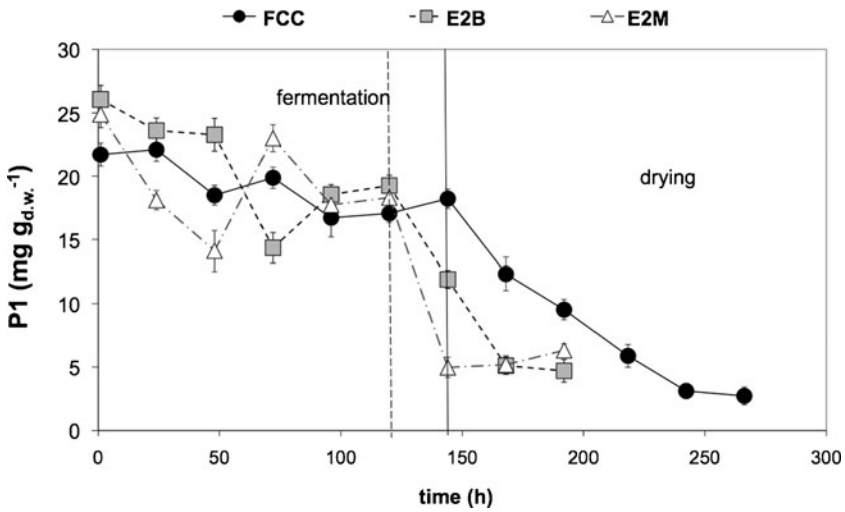

Fig. 2 Changes in monomers content of cocoa beans during fermentation and drying. The *dotted* and *straight lines* indicate end fermentation for FCC and E2 samples, respectively

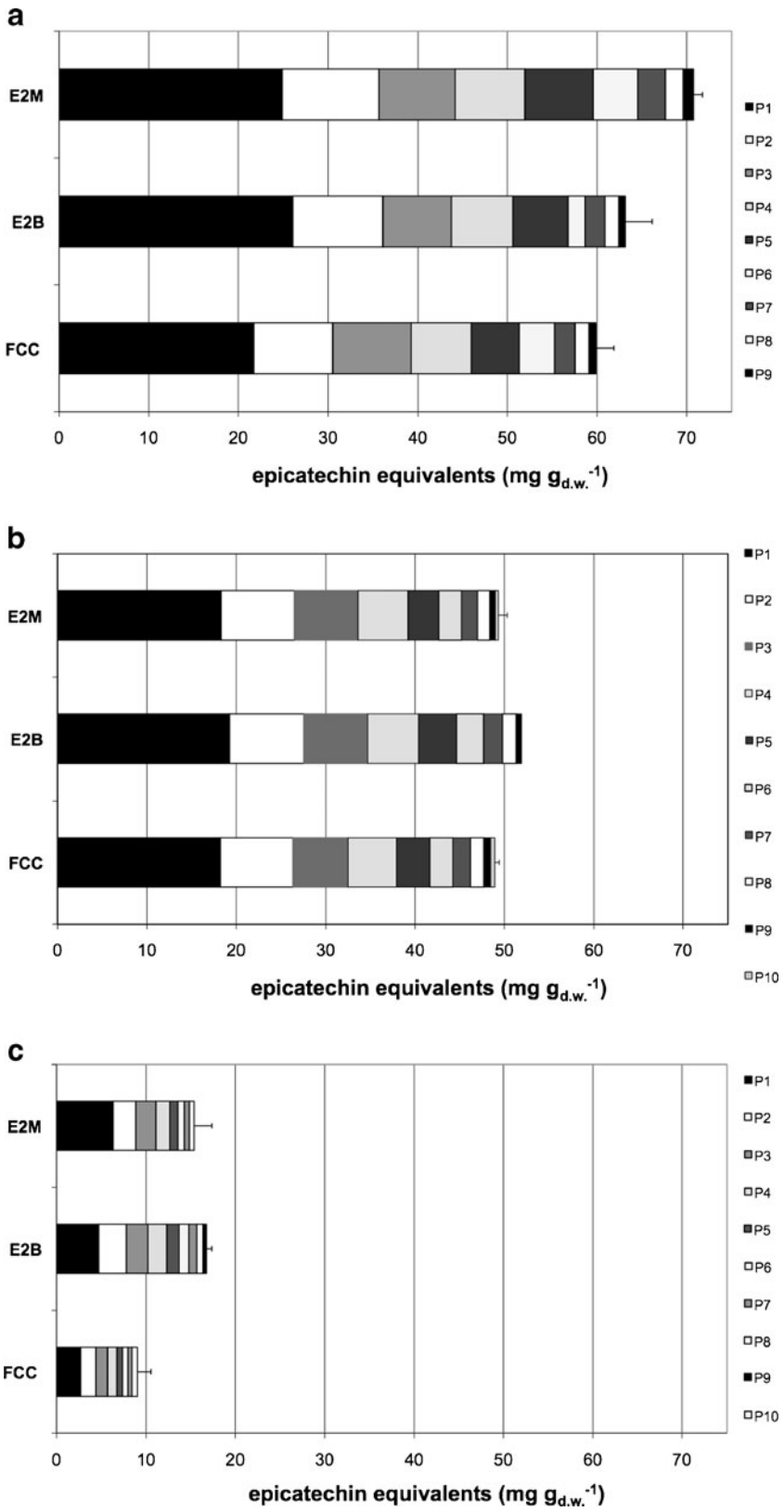

Fig. 3 Procyanidin content and profile of cocoa beans FCC and experiment 2 Blanco and Mix after harvesting (**a**) and upon fermentation (**b**) and drying (**c**)

showed a slight decrease of their relative contribution (from 9 % to 7 %) after drying. Regarding the other fractions, the relative contributions of P2–P4 fractions did not change, while the P7–P10 fractions showed a tendency to an increase especially in the latest steps of the drying process likely due to oxidation and polymerisation phenomena.

Similar results were observed for the E2B samples, where the relative contribution of P1 and P5 fractions decreased upon fermentation and drying ($p<0.01$) whilst a general increase of the other fractions occurred. A different behaviour was observed in E2M samples. In this case, the relative contribution of the P1 fraction increased upon processing (with a relative contribution from ca. 35 % to 41 % at the end of drying), the P5 and P6 fractions showed a decrease and the remaining fractions presented non-significant changes in the overall contribution to the procyanidins content over process time.

It is interesting to observe that at the end of the fermentation step (Fig. 2b), which corresponded to a fermentation index of 0.8±0.1, FCC, E2M and E2B samples resulted characterised by similar monomers content (see Fig. 2) and procyanidin composition.

If the sole P1 fraction (index of degree of processing) is taken into account, it can be observed that upon the entire process (fermentation + drying), the percentage of reduction of the monomers was 87 % for FCC samples while 82 % and 75 % for E2B and E2M samples, respectively; similar percentages of loss were found for total procyanidins. However, if the loss of P1 fraction, due to the sole fermentation step, is considered, the percentage of reduction is 16 % for FCC sample and 26 % for E2B and E2M samples. This data is significantly lower than that found by other authors for (−)-epicatechin (Camu et al. 2008; Nazaruddin et al. 2006; Payne et al. 2010).

On the basis of these results, it can be asserted that P1, which was mainly constituted of (−)-epicatechin and (+)-catechin monomers, represented the fraction that resulted more susceptible to degradation during both the fermentation and drying steps.

According to the results of this study, most of the loss can be ascribed to drying which thus resulted to be the process step which was more critical for the loss of monomers and, more in general, of procyanidins with a reduction of 65–70 %. These results are in agreement with those found by Kyi et al. (2005), despite the differences in processing conditions and analytical methods adopted for phenolic extraction and quantification, whilst they are higher than those obtained by de Brito et al. (2000) and Payne et al. (2010). The latter authors found no significant variation in epicatechin content between freshly harvested freeze-dried beans and sun-dried ones. In this case, however, unfermented beans were submitted to sun drying, and it is likely that cocoa beans presented very few structural modifications with a limited effect on the evolution of chemical and enzymatic reactions.

Effect of Fermentation and Drying on Antioxidant Activity

The antioxidant activity was determined on the same sample extracts by means of the decolorization assays of the ABTS radical (TEAC method), the reaction with the Folin–Ciocalteu reagent (TPI method) and the reduction of Fe^{3+} (FRAP method). These methods differ in several aspects such as the mechanism of action (radical or redox reaction) and the environmental conditions (solvent polarity and pH).

The TPI is based on the capacity of phenolic compounds to react with the Folin–Ciocalteu reagent under basic conditions and thus has been extensively used as a method for the estimation of total phenolics; nonetheless, taking into account that polyphenols show different reactivity with the Folin–Ciocalteu reagent (Naczk and Shahidi 2004) and that the mechanism is based on a oxidation/reduction reaction, TPI can also be considered an antioxidant method (Prior et al. 2005).

In Fig. 4, the TPI of the three series of cocoa bean samples as a function of process time is reported. This index showed a significant ($p<0.05$) non-linear decrease during

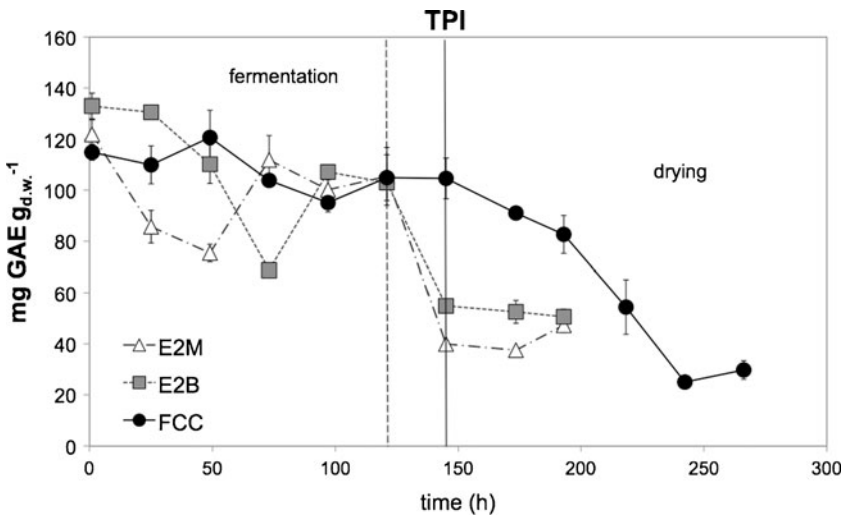

Fig. 4 Total polyphenols index of the samples under investigation as a function of process time. The *dotted* and *straight lines* indicate end fermentation for FCC and E2 samples, respectively

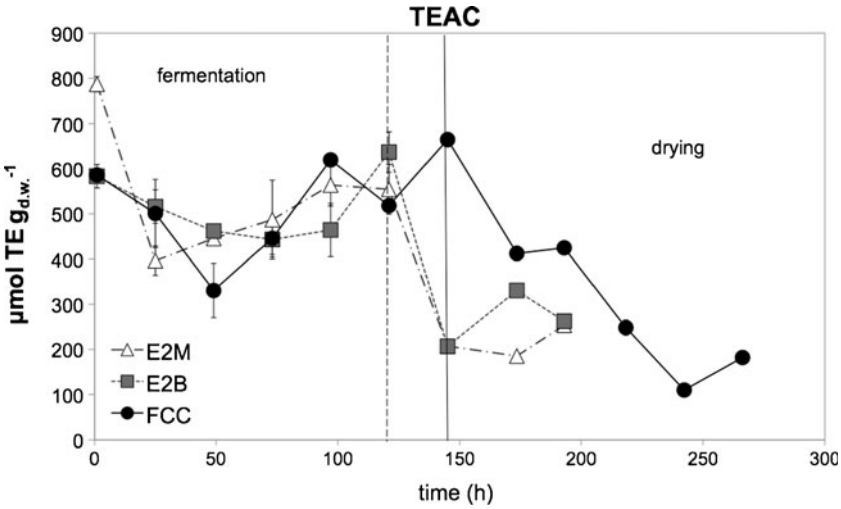

Fig. 5 Evolution of the radical scavenging activity upon fermentation and processing. The *dotted* and *straight lines* indicate end fermentation for FCC and E2 samples, respectively

fermentation, somehow similar to that occurred to the procyanidin fractions. TPI values of E2B and E2M samples showed a decrease upon drying higher than that observed in the FCC series ones, and after drying, similar TPI indexes were found in both E2M and E2B final samples. During the drying step, the TPI index of the FCC sample decreased more slowly than that of E2M and E2B, but the final total polyphenol index was lower in the former sample.

Similar results were achieved for the antiradical properties evaluated by the ABTS radical decolorization assay which works on a mechanism based on both electron and hydrogen transfer (Prior et al. 2005). Results, expressed as TEAC, are illustrated in Fig. 5. E2M showed a significantly higher initial TEAC compared to both FCC and E2B, which in turn were characterised by similar values. The antiradical activity of the samples FCC and E2M described an uneven evolution as a consequence of fermentation, with a slight increase at the end of the process; contrarily, a significant ($p<0.01$) reduction of 30 % of the antiradical activity of E2M samples was observed. No significant differences were evidenced between the final TEAC values of FCC and E2B cocoa beans. Drying affected the antioxidant capacity by causing an average reduction of 57 % of the activity determined at the end of the fermentation step, but, interestingly, no significant differences were found among the percentages of reduction of the samples. After processing, E2M and E2B samples presented very similar TEAC values and showed the highest antiradical activity (254±2 and 263±13 µmol Trolox equivalents $g^{-1}_{d.w.}$).

In Fig. 6, the reducing capacity of the samples, measured by means of the FRAP assay, is shown. The FCC series showed the lowest initial FRAP value while E2B and E2M series were similar. Upon fermentation, a complex trend could be observed in FRAP while during drying a decrease occurred in all the samples similarly to what has already been observed in Figs. 2 and 3.

It must be pointed out that at the end of drying, similar final results were found in the case of FRAP, TEAC and TPI indices: E2B and E2M samples presented final values non-significantly different among them and in both cases significantly higher than those determined in FCC samples.

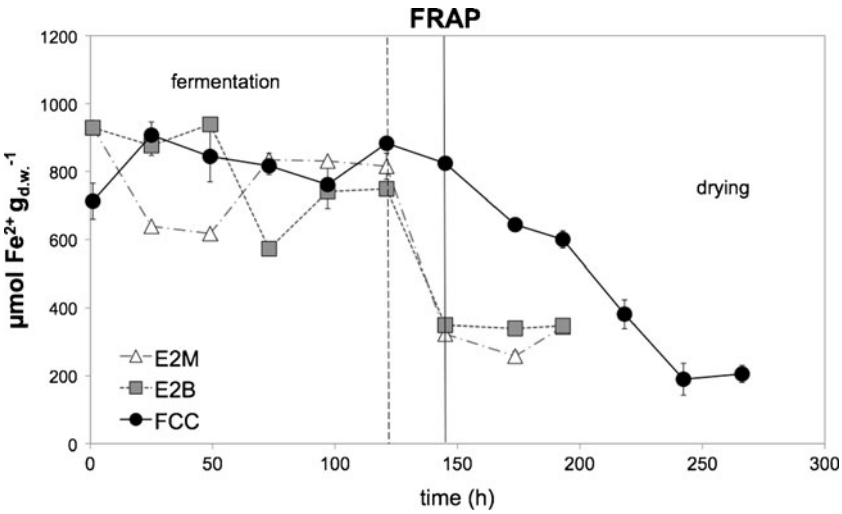

Fig. 6 Ferric reducing antioxidant properties values of the samples over process time. The *dotted* and *straight lines* indicate end fermentation for FCC and E2 samples, respectively

Table 1 pH values of cocoa beans as a consequence of fermentation

Time (h)	FCC	E2M	E2B
0	6.01	6.56	6.60
25	5.93	6.06	6.59
49	5.68	6.03	5.82
73	5.20	5.18	5.67
97	4.89	–	5.24
121	4.89	–	–
145	4.80	–	–

Data are averaged on three replicates (average coefficient of variation <8 %)

By the results of all the evaluations, it could be pointed out that during fermentation both procyanidins and functional parameters presented a general decreasing, even though non-linear, trend. This behaviour might be related to both polymerisation and enzymatic reactions which occur simultaneously as a consequence of the changes in pH (Table 1), temperature and moisture during cocoa fermentation (Thomson et al. 2001; Wollgast and Anklam 2000) along with structural changes which allowed reactants diffusion (de Brito et al. 2000). It must be pointed out again that, despite the huge differences in the batch size, fermenting microbiota and the difficulties in the management of the technological parameters upon fermentation, similar results were achieved since the samples at end fermentation were characterised by comparable procyanidins contents, profile and antioxidant activity.

As far as drying is concerned, a decrease of all the tested parameters were found in all the samples as a consequence of drying. The procyanidin and antioxidant activity reduction in FCC samples was slower than that of E2 ones likely due to a different heat penetration and water loss in the cocoa bean batches (FCC = 800 kg vs experiment 2 = 50 kg batches). The decrease in the antiradical and reducing power can be attributed to polymerisation and condensation phenomena of the procyanidins due to the relatively high temperatures used in the drying process as well as some residual oxidative enzyme activity.

Contribution of Procyanidins to the Total Antioxidant Activity

In order to study the relationship between individual procyanidins and antioxidant activity, expressed as TEAC, FRAP and TPI, the data set was processed by PLS and the regression models are reported in Table 2. The coefficient of determination of the calibration regressions ranged from 0.88 for TEAC response to 0.92 for FRAP one, and the coefficient of determination of the validation regressions ranged from 0.90 for TEAC to 0.91 for FRAP. RMSEP ranged from 68.4 for TEAC to 86.5 and to 10.7 for FRAP and TPI response, respectively, indicating that all the models have a good predictive ability.

The TEAC model showed a lower predictive ability than the others and this could be explained by the different mechanism of chemical reaction used in the assay. If TPI and FRAP assays exclusively measure the capacity of cocoa extracts to reduce either the Folin–Ciocalteu reagent or iron (Fe^{3+}), in the TEAC assay the ABTS radical could undergo to reduction by both single-electron transfer and hydrogen atom transfer, thus presenting a mixed mechanism of action towards the antioxidants present in cocoa extracts.

All the procyanidins showed a positive coefficient of correlation with the response variables with the same ranking order: P9>P10>P8>P7>P6>P5>P4>P3>P2>P1, and the coefficient of regression of each single compound expressed in GAE per gram of dry weight, TEAC per gram of dry weight and Fe^{2+} per gram of dry weight is reported in Table 3.

In order to compare the three different methods used to test the antioxidant activity (AOA), the coefficients were normalised on their mean value; the normalised regression coefficients generally showed the same ratio among them independently of the method used to test AOA except for

Table 2 Results of partial least square regression analysis between individual procyanidins (X) and antioxidant activity (Y) as determined by TPI, TEAC and FRAP assays: regression coefficient, coefficient of determination of calibration (R^2_{cal}), coefficient of determination of validation (R^2_{val}), root mean square error to predict of the model and optimum number of components (C_{opt}) of each model

Assay	Regression coefficient										R^2_{cal}	R^2_{val}	RMSEP	C_{opt}
	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10				
TPI[a]	0.50	1.14	1.30	1.49	1.85	2.75	3.74	6.01	10.00	6.26	0.914	0.896	10.7	1
TEAC[b]	2.82	6.51	7.25	8.17	10.50	16.09	20.50	33.38	56.74	50.35	0.877	0.904	68.4	1
FRAP[c]	3.97	9.06	10.19	11.57	14.35	21.71	29.01	46.95	81.23	62.08	0.905	0.912	86.5	1

[a] μg GAE/μmol procyanidins

[b] μmol TE/μmol procyanidins

[c] μmol Fe^{2+}/μmol procyanidins

Table 3 Normalised regression coefficient and beta coefficient of each single procyanidin fraction (P1–P10)

Method		P1	P2	P3	P4	P5	P6	P7	P8	P9	P10
TPI[a]	Normalised regression										
	Coefficient	0.143	0.325	0.371	0.425	0.527	0.784	1.069	1.715	2.854	1.787
	Beta coefficient	0.114	0.110	0.111	0.111	0.107	0.108	0.108	0.109	0.100	0.025
TEAC[b]	Normalised regression										
	Coefficient	0.133	0.307	0.341	0.385	0.495	0.758	0.966	1.572	2.673	2.371
	Beta coefficient	0.112	0.110	0.108	0.106	0.105	0.110	0.102	0.105	0.098	0.034
FRAP[c]	Normalised regression										
	Coefficient	0.137	0.312	0.351	0.399	0.495	0.748	1.000	1.618	2.800	2.140
	Beta coefficient	0.114	0.110	0.110	0.108	0.104	0.107	0.105	0.107	0.102	0.031

[a] μg GAE/μmol procyanidins

[b] μmol TE/μmol procyanidins

[c] μmol Fe^{2+}/μmol procyanidins

the P10 coefficient that was much higher in the TEAC model than in the others (Fig. 7).

The higher antioxidant activity of HMW procyanidin was originally ascribed to the chain length or charge delocalisation of the phenoxyl radical formed upon oxidation (Saint-Cricq de Gaulejac et al. 1999; Ursini et al. 2001), but more recent literature ascribed it to the number of catechol groups and the molecular configuration (Steinberg et al. 2002; Muselík et al. 2007).

The TEAC model estimated a TEAC value of 2.82 for P1 which is consistent with that observed on pure compounds; the TEAC value of catechin, as determined by the modified TEAC method used (Re et al. 1999), was of 2.82 in accordance with Di Mattia et al. (2009), and that of epicatechin was of 2.89. Literature TEAC data, 2.40–2.85 for catechin and 2.52–2.93 for epicatechin (Rice-Evans et al. 1996; Muselík et al. 2007), are very similar to those observed in this study; slight differences could be ascribed to the analytical procedure. Muselík et al. (2007) reported TEAC values for procyanidins dimers ranging from 4.37 to 8.36 with an average of 6.36; the TEAC value estimated by the regression coefficient of the model was of 6.50 and also this data confirms the model adequacy. The regression coefficient observed in this study indicated a TEAC value of 7.25 for procyanidins trimers; to the authors' knowledge, the TEAC value of only one procyanidin trimer was determined and it was higher (8.6), but the TEAC of procyanidins trimers could dramatically vary on the basis of different structural configurations as also observed for dimers (Muselík et al. 2007). The antioxidant activity of procyanidins, as evaluated by the TEAC method, has been shown to increase from dimers, trimers to decamers (Huang et al. 2010). Huang and co-workers (2010) found a P10 TEAC value similar to or slightly higher than that of P9; in our study, the P10 regression coefficient in the TEAC method is slightly lower than that of P9 and higher than those estimated by the other methods (Fig. 7). Steinberg et al.

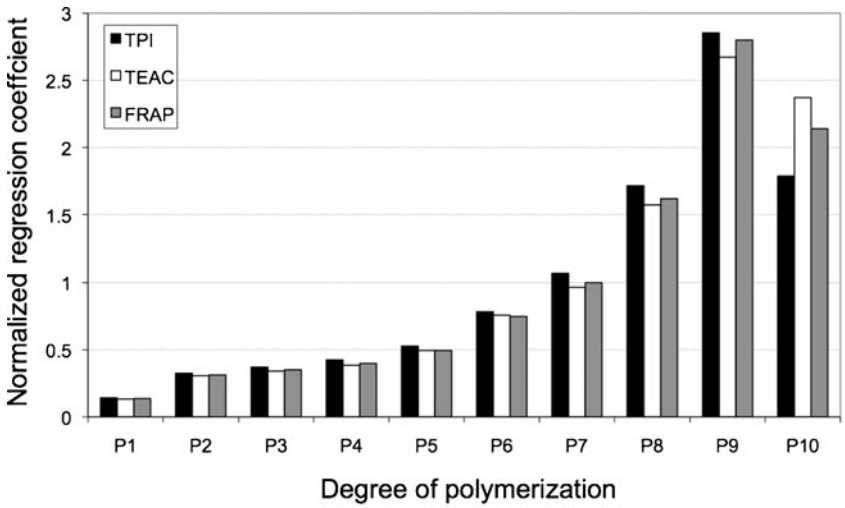

Fig. 7 Normalised coefficients of regression versus degree of polymerisation for the three AOA assays

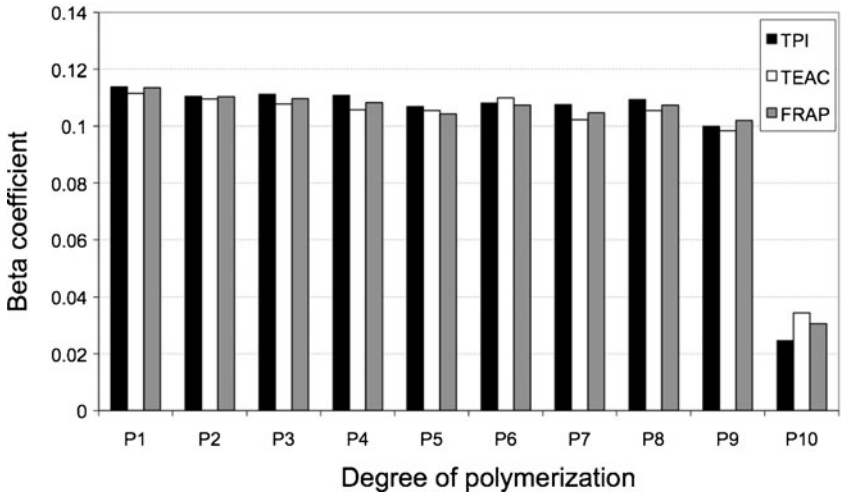

Fig. 8 Relative coefficients of regression as a function of procyanidins degree of polymerisation for the three AOA assays

(2002) reported that dimers and pentamers showed TEAC values comparable to those of monomers contrarily the previously cited studies. Since our model permitted a reliable estimation of the antioxidant activities of pure monomers, dimers and trimers which, in relative terms, show the 33 % of the total antioxidant activity (Fig. 8), HMW procyanidins are likely to act as more efficient antioxidant than LMW; otherwise, the total AOA of cocoa samples would not be explained.

The FRAP model estimated a FRAP value of 3.97 for P1 which is much higher than literature FRAP data; Muselík et al. (2007) reported FRAP values of 2.16 and 2.20 [after data recalculation from micromolar of Trolox to micromolar of Fe(II) eq.] for catechin and epicatechin, respectively. This overestimation of FRAP values is ascribable to the high Fe(III) content of chocolate since the FRAP assay simply consists in a measure of Fe(III) reduction and iron was not selectively removed from the extracts before analysis.

In relative terms, the ratio between the FRAP of dimers and monomers observed in this study (2.28) is consistent with the data reported by Muselík et al. (2007), which was 2.29. The ratio between the FRAP of trimers and monomers observed in this study (2.57) is lower than that reported by Muselík et al. (2007), which was 3.99. However, as previously discussed, the antiradical activity of procyanidins trimers could dramatically vary on the basis of different structural configurations, as also observed for dimers (Muselík et al. 2007).

A part from the possible causes of over or underestimation of analytical data, the results obtained by PLS showed that the relative contribution of different procyanidin fraction to AOA is independent from the method of analysis (except for P10). Moreover, all the PLS models gave an accurate and reliable predictive model for cocoa antioxidant activity.

Even though high molecular weight procyanidins showed higher antioxidant activity values, they are less abundant if compared to monomers and low molecular weight procyanidins. Standardisation of the coefficients was thus performed to understand which of the independent variables (procyanidins) have a greater effect on the antioxidant activity. The standardised regression coefficient (beta coefficient) was thus calculated and resulted to follow the order P1>P2>P3>P4>P6>P8>P5>P7>P9>>P10, as shown in Fig. 8.

Conclusions

The different conditions applied on processing did not cause any significant differences in the procyanidins content and functional properties during the fermentation step whilst differences were evidenced during the drying step.

In particular, drying on smaller batches allowed highest rates of heat penetration and generally preserved the samples from oxidation with the final effect of a higher content of procyanidins, antiradical activity and reducing properties.

Statistical analysis demonstrated that all the P1–P10 procyanidin fractions showed a positive coefficient of regression with all the functional properties as determined by TEAC, TPI and FRAP assays. When the coefficients of regression were standardised, the contribution of the procyanidin classes to the antiradical and reducing properties followed the order P1>P2>P3>P4>P6>P8>P5>P7>P9>>P10.

References

Aikpokpodion, P. E., & Dongo, L. N. (2010). Effect of fermentation intensity on polyphenols and antioxidant capacity of cocoa beans. *International Journal of Sustainable Crop Production, 5*(4), 66–70.

Benzie, I. F. F., & Strain, J. J. (1996). Ferric reducing/antioxidant power assay: direct measure of total antioxidant activity of biological fluids and modified version for simultaneous measurement of total antioxidant power and ascorbic acid concentration. *Methods in Enzymology, 299*, 15–27.

Camu, N., De Winter, T., Addo, S. K., Takrama, J. S., Bernaert, H., & De Vuyst, L. (2008). Fermentation of cocoa beans: influence of microbial activities and polyphenol concentrations on the flavour of chocolate. *Journal of the Science of Food and Agriculture, 88*, 2288–2297.

Cooper, K. A., Donovan, J. L., Waterhouse, A. L., & Williamson, G. (2008). Cocoa and health: a decade of research. *British Journal of Nutrition, 99*, 1–11.

Corti, R., Flammer, A. J., Hollenberg, N. K., & Luscher, T. F. (2009). Cocoa and cardiovascular health. *Circulation, 108*, 1433–1441.

Counet, C., & Collin, S. (2003). Effect of number of flavanol units on the antioxidant activity of procyanidin fractions isolated from chocolate. *Journal of Agricultural and Food Chemistry, 51*, 6816–6822.

de Brito, E. S., Pezoa Garcia, N. H., Gallao, M. I., Corelazzo, A. L., Fevereiro, P. S., & Braga, M. R. (2000). Structural and chemical changes in cocoa (*Theobroma cacao* L.) during fermentation, drying and roasting. *Journal of the Science of Food and Agriculture, 81*, 281–288.

de Jong, S. (1993). SIMPLS: an alternative approach to partial least squares regression. *Chemometrics and Intelligent Laboratory Systems, 18*, 251–263.

Di Mattia, C. D., Sacchetti, G., Mastrocola, D., & Pittia, P. (2009). Effect of phenolic antioxidants on the dispersion state and chemical stability of olive oil O/W emulsions. *Food Research International, 42*, 1163–1170.

Engler, M. B., Engler, M. M., Chen, C. Y., Malloy, M. J., Browne, A., Chiu, E. Y., Kwak, H. K., Milbury, P., Paul, S. M., Blumberg, J., & Mietus-Snyder, M. L. (2004). Flavonoid-rich dark chocolate improves endothelial function and increases plasma epicatechin concentrations in healthy adults. *Journal of the American College of Cardiology, 23*(3), 197–204.

Gourieva, K.B., & Tserrevitinov, O.B. (1979). Method of evaluating the degree of fermentation of cocoa beans. *USSR Patent no.646254*.

Gu, L., House, S. E., Wu, X., Ou, B., & Prior, R. L. (2006). Procyanidin and catechin contents and antioxidant capacity of cocoa and chocolate products. *Journal of Agricultural and Food Chemistry, 54*, 4057–4061.

Hansen, C. E., Del Olmo, M., & Burri, C. (1998). Enzyme activities in cocoa beans during fermentation. *Journal of the Science of Food and Agriculture, 77*, 273–281.

Heiss, C., Kleinbongard, P., Dejam, A., Perrè, S., & Schroeter, H. (2005). Acute consumption of flavonoid-rich cocoa and the reversal of endothelial dysfunction in smokers. *Journal of the American College of Cardiology, 46*, 1276–1283.

Huang, P.-L., Chi, C.-W., & Lin, T.-Y. (2010). Effects of *Areca catechu* L. containing procyanidins on cyclooxygenase-2 expression in vitro and in vivo. *Food and Chemical Toxicology, 48*, 306–313.

Jinap, S., & Thien, J. (1994). Effects of drying on acidity and volatile fatty acids content of cocoa beans. *Journal of the Science of Food and Agriculture, 65*, 67–75.

Jourdain, C., Tenca, G., Deguercy, A., Troplin, P., & Poelman, D. (2006). In vitro effects of polyphenols from cocoa and [beta]-sitosterol on the growth of human prostate cancer and normal cells. *European Journal of Cancer Prevention, 15*(4), 353–361.

Kealey K., Snyder, R., Romanczyk, L. J., Geyer, H. M., Myers, M. E., Whitacre, E. J., Hammerstone, J. F., & Schmitz, H. H. (1998). *Cocoa components, edible products having enhanced polyphenol content, method of making same and medical use*. 1998 Patent Cooperation Treaty (PCT) WO 98/09533, Mars Incorporated, USA.

Kim, H., & Keeney, P. G. (1984). (−)-Epicatechin content in fermented and unfermented cocoa beans. *Journal of Food Science, 49*, 1090–1092.

Komes, D., Belščak-Cvitanović, A., Horžić, D., Drmić, H., Škrabal, S., & Miličević, B. (2012). Bioactive and sensory properties of herbal spirit enriched with cocoa (*Theobroma cacao* L.) polyphenolics. *Food and Bioprocess Technology*. doi:10.1007/s11947-011-0630-7.

Kyi, T. M., Wan Ramli, W. D., Abu Bakar, M., Mohd Wahid, S., Abdul Amir, H. K., & Meor Zainal, M. T. (2005). The kinetics of polyphenol degradation during the drying of Malaysian cocoa beans. *International Journal of Food Science and Technology, 40*, 323–331.

Lima, L. J. R., Almeida, M. H., Nout, R. M. J., & Zwieterig, M. H. (2011). *Theobroma cacao* L., "The Food of the Gods": quality determinants of commercial cocoa beans, with particular reference to the impact of fermentation. *Critical Reviews in Food Science and Nutrition, 51*(8), 731–761.

Mrkić, V., Cocci, E., Dalla Rosa, M., & Sacchetti, G. (2006). Influence of drying conditions on bioactive compounds and antioxidant activity of dried broccoli (*Brassica oleracea* L.). *Journal of the Science of Food and Agriculture, 86*, 1559–1566.

Muselík, J., García-Alonso, M., Martín-López, M. P., Žemlička, M., & Rivas-Gonzalo, J. C. (2007). Measurement of antioxidant activity of wine catechins, procyanidins, anthocyanins and pyranoanthocyanins. *International Journal of Molecular Sciences, 8*, 797–809.

Naczk, M., & Shahidi, F. (2004). Extraction and analysis of phenolics in food. *Journal of Chromatography A, 1054*, 95–111.

Nazaruddin, R., Seng, L. K., Hassan, O., & Said, M. (2006). Effect of pulp preconditioning on the content of polyphenols in cocoa beans (*Theobroma cacao*) during fermentation. *Industrial Crops and Products, 24*, 87–94.

Payne, M. J., Hurst, W. J., Miller, K. B., Rank, C., & Stuart, D. A. (2010). Impact of fermentation, drying, roasting and Dutch processing on epicatechin and catechin content of cocoa beans and cocoa ingredients. *Journal of Agricultural and Food Chemistry, 58*, 10518–10527.

Prior, R. L., Wu, X., & Schaich, K. (2005). Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. *Journal of Agricultural and Food Chemistry, 53*(8), 3101–3113.

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., & Rice-Evans, C. (1999). Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biology & Medicine, 1999*(26), 1231–1237.

Rice-Evans, C. A., Miller, N. J., & Paganga, G. (1996). Structure–antioxidant activity relationships of flavonoids and phenolic acids. *Free Radical Biology & Medicine, 20*, 933–956.

Rusconi, M., & Conti, A. (2010). *Theobroma cacao* L., the food of the Gods: a scientific approach beyond myths and claims. *Pharmacological Research, 61*, 5–13.

Saint-Cricq de Gaulejac, N., Provost, C., & Vivas, N. (1999). Comparative study of polyphenol scavenging activities assessed by different methods. *Journal of Agricultural and Food Chemistry, 47*, 425–431.

Serafini, M., Bugianesi, R., Maiani, M., Valtueña, S., De Santis, S., & Crozier, A. (2003). Plasma antioxidants from chocolate. *Nature, 424*, 1013.

Singleton, V. L., & Rossi, J., Jr. (1965). A colorimetry of total phenolics with phosphomolibdic–phosphotungstic acid reagents. *American Journal of Enology and Viticulture, 16*, 144–158.

Steinberg, F. M., Holt, R. R., Schmitz, H. H., & Keen, C. L. (2002). Cocoa procyanidin chain length does not determine ability to protect LDL from oxidation when monomer units are controlled. *The Journal of Nutritional Biochemistry, 13*, 645–652.

Sun-Waterhouse, D., & Wadhwa, S. S. (2012). Industry-relevant approaches for minimising the bitterness of bioactive compounds in functional foods: a review. *Food and Bioprocess Technology*. doi:10.1007/s11947-012-0829-2.

Thomson, S. S., Miller, K. B., & Lopez, A. S. (2001). Cocoa and coffee. In M. P. Doyle, L. R. Beuchat, & T. J. Montville (Eds.), *Food microbiology: fundamentals and frontiers* (2nd ed.). Washington: ASM.

Ursini, F., Rapuzzi, I., Toniolo, R., Tubaro, F., & Bontempelli, G. (2001). Characterization of antioxidant effect of procyanidins. *Methods in Enzymology, 335*, 338–351.

Wollgast, J., & Anklam, E. (2000). Review on polyphenols in *Theobroma cacao*: changes in composition during the manufacture of chocolate and methodology for identification and quantification. *Food Research International, 33*, 423–447.