



## The content of protein and non-protein (free and protein-bound) tryptophan in *Theobroma cacao* beans

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### ABSTRACT

The contents of protein and non-protein (free and protein-bound) tryptophan and of proteins in cocoa beans of various origin were determined. Protein concentrations varied from 11.8 g/100 g in beans from the Dominican Republic to 15.7 g/100 g in roasted beans from the Ivory Coast. The highest protein tryptophan content was found in cocoa beans from Ecuador. Madagascar beans had the highest value of free tryptophan and Echeandia the lowest (17.26 and 6.39 mg/100 g, respectively). Tryptophan was bound to water-soluble proteins as well as to proteins soluble in buffer solution (pH 8.9) and in 70% ethanol. In particular, Dominican Republic cocoa contained the highest amount of tryptophan bound to water-soluble proteins. Very little tryptophan was linked to proteins soluble in alkaline or ethanol solutions, and values ranged from 0.96 to 3.04 and from 0.24 to 1.21 mg/100 g of dry defatted cocoa sample, respectively.

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### 1. Introduction

Cocoa beans derive from the tropical tree *Theobroma cacao* L. (*Sterculiaceae* family). Various subspecies, e.g., *Criollo*, *Forastero* and *Trinitario*, are cultivated in Central West Africa, South America and Asia.

Cocoa and cocoa-based products like chocolate are widely consumed in many countries, consumptions ranging from 2.8 g/person/day (Great Britain) to 27.8 g/person/day (Switzerland) (Rusconi & Conti, 2010).

Cocoa beans contain 10–15% protein, and the four predominant fractions, representing 95% (w/w) of total seed proteins, are albumins (water-soluble), globulins (salt-soluble), prolamins (alcohol-soluble) and glutelins (soluble in dilute acids and alkali) (Zak & Keeney, 1976). Voigt, Biehl, and Wazir (1993) found that the protein content in cocoa beans was composed of 52% and 43% of albumin and globulin fractions, respectively.

Cocoa albumin, amino acid content and therefore biological protein value are all highly affected by the extent of roasting (Abecia-Soria, Pezosa-Garcia, & Amaya-Farfan, 2005). During drying and roasting, peptides and free amino acids, together with reducing sugars also present in fermented cocoa beans, undergo a Maillard reaction, responsible for the typical cocoa aroma. Cocoa globulin, mainly composed of vicilin-class globulin (Voigt et al., 1993), also undergoes extensive degradation during fermentation (Amin, Jinap, & Jamilah, 1997), leading to the production of hydrophobic

amino acids and peptides, considered cocoa-specific flavour precursors (Voigt et al., 1994). The increase in the concentrations of hydrophobic amino acids, such as leucine, alanine, phenylalanine and tyrosine, is explained by the activity of two cocoa proteases: carboxypeptidase, which releases single hydrophobic amino acids, and aspartic endoprotease, which attacks proteins preferentially at the sites of hydrophobic amino acids (Hashim, Selamat, Muhamad, & Ali, 1998; Voigt et al., 1994). Thus, fermentation and roasting, together with type of soil, climate, harvest conditions and drying, greatly affect cocoa characteristics. In particular, fermentation involves microbiological and enzymatic reactions, which lead to extensive breakdown of cocoa proteins.

In assessing the nutritional value of food for human nutrition, it is important to analyse its protein quality, which depends mainly on the composition of essential amino acids. In particular, tryptophan (Trp) is the second most deficient amino acid, after lysine, in cereals, which represent 60–70% of human diet. Trp is necessary not only for protein synthesis but also for most biogenetic and biosynthetic pathways, being the precursor of alkaloids, phytohormones (indoleacetic acid), NAD coenzymes, and other important biological substances, such as serotonin and melatonin (Musajo & Benassi, 1964; Reiter et al., 2003). It is also present in food as non-protein Trp, both protein-bound and free, which is more easily absorbable and available to the brain, being the only form able to cross the blood brain barrier (Allegrì, Biasiolo, Costa, Bettero, & Bertazzo, 1993; Comai et al., 2007a,b).

In fermented foods, tryptophan is transformed into biogenic amines, such as tryptamine and 5-hydroxytryptamine (serotonin) (Kang, Kang, Lee, & Back, 2007; Santos, 1996), psychoactive and

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vasoactive substances, which can affect central nervous system functions (Shalaby, 1996). The presence of these two amines has also been reported in cocoa (Baker, Wong, Coutts, & Pasutto, 1987; Hurst & Toomey, 1981; Pastore et al., 2005).

Although much work has been carried out on amino acid compositions, there is only very limited information regarding the content of protein Trp and above all of free and protein-bound Trp in foods.

Our previous studies demonstrated that Trp, besides being present as a constituent of proteins, is also found in non-protein forms in milk (Allegri et al., 1993), cereals (Comai et al., 2007a) and legumes (Comai et al., 2007b). Green grains of *Coffee arabica* L. and *Coffee canephora* also contain non-protein Trp (Martins & Gloria, 2010). However, detailed information about the content of free Trp in foods is still lacking.

In this work, we examined the presence of protein and non-protein tryptophan (free and protein-bound) in cocoa beans of various origin. Results showed that protein Trp content varies slightly according to origin, whereas the fractions of Trp, free and bound to various soluble proteins, differ considerably.

## 2. Materials and methods

### 2.1. Materials

All chemicals used were of analytical-reagent grade, obtained from Sigma (St. Louis, MO).

### 2.2. Cocoa samples

Samples of fermented, dried and roasted cocoa beans of the main cocoa varieties (*T. cacao* L.) cultivated in Peru, Venezuela, Madagascar, Ecuador, the Dominican Republic and Ivory Coast were studied. All fruits were harvested in 2007 and purchased from Domori (Genova, Italy).

Two cocoa bean samples, originating from the same cultivar of Echeandia were also analysed: one fermented and dried, and the other fermented, dried and roasted.

Dried cocoa seeds were ground to a fine powder in a coffee mill and the flour was passed through a 300- $\mu$ m sieve.

### 2.3. Extraction and analysis of non-protein tryptophan

This procedure was carried out as previously reported by Comai et al. (2007a), with some modifications.

In brief, 2 g of dry, powdered cocoa samples were defatted with ethyl ether at 40 °C for 48 h in a Soxhlet apparatus. 0.1 g of each dried and defatted sample was extracted twice with 8 ml of distilled water for 60 min at 37 °C with shaking, and then centrifuged for 40 min in an IEC-Centra-SR centrifuge at 0 °C and 12,000 rpm. The clear supernatant containing the free Trp and the water-soluble protein fraction was then collected. A 5-ml aliquot of water extract was ultrafiltered through an Amicon model 12 ultrafiltration cell with an XM-50 Diaflo membrane (Amicon, Oosterhout, Holland), and the first 500  $\mu$ l of ultrafiltrate collected were used to determine the free fraction of Trp by a combined HPLC-fluorescence method, according to Comai et al. (2007a). The remaining supernatant part was analysed to determine total water-soluble non-protein Trp. The resulting sediment was re-suspended in 5 ml of 0.1 M potassium phosphate buffer, pH 8.9, shaken for 30 min at 37 °C, and then centrifuged. The supernatant was analysed by HPLC, to determine any non-protein Trp bound to buffer-soluble proteins. The sediment thus obtained was then re-suspended in 5 ml of 70% ethanol, stirred for 30 min at 37 °C and centrifuged, and the supernatant was analysed to verify whether

non-protein Trp was also present, bound to ethanol-soluble proteins.

In order to evaluate the purification level, one HPLC fraction of each sample, i.e., free and protein-bound Trp, eluting at the same retention time as the standard, was analysed by mass spectrometry (MS) on an API-TOF Mariner spectrometer (PerSeptive Biosystems, Stratford, TX).

### 2.4. Protein content

Nitrogen was determined in cocoa seed powder by the micro-Kjeldahl method and the nitrogen percentage was converted to crude protein by multiplying by 6.25 (AOAC International., 2000).

### 2.5. Analysis of protein tryptophan

Tryptophan was analysed in triplicate by HPLC, according to the method of Slump, Flissebaalje, and Haaksman (1991), based on alkaline hydrolysis of cocoa powder in Ba(OH)<sub>2</sub>.

The liquid chromatographic equipment consisted of an automatic injector (AS 3000), a pump (P 400) and a fluorescence detector (FL 3000) (Spectra System, Thermo Finningan, Waltham, MA). The column was a Zorbax extended C18 (3  $\times$  250 mm) type. The eluting solvent was 0.1 M Na acetate (42.5%)/0.1 M acetic acid (42.5%)/methanol (15%). The purity of protein Trp was assessed by MS, as reported above. Mean values are expressed in mg of Trp per 100 g of flour (dry matter).

## 3. Results and discussion

### 3.1. Protein and lipid content

The values of both protein and lipid contents and protein Trp in cocoa seeds are listed in Table 1. Data are expressed in g/100 g dry weight (DW) of ground seeds for protein and lipid contents, and in mg/100 g dry weight of sample or mg/100 g of protein for protein Trp. Values are means  $\pm$  S.E.M. of three separate analyses. Protein content was estimated by applying a nitrogen/protein conversion factor of 6.25 on dry matter. As shown, protein content greatly differed among the cocoa samples, reaching the highest value for Ivory Coast cocoa (15.7 g/100 g DW) and the lowest was for that from the Dominican Republic (11.8 g/100 g DW). The protein levels

**Table 1**

Protein and lipid (g/100 g dry weight) contents and protein tryptophan (mg/100 g dry weight and mg/100 g protein) in cocoa seeds of various geographical origin (mean values  $\pm$  S.E.M.).

Cocoa beans origin	Protein <sup>a</sup>	Lipid <sup>a</sup>	Protein tryptophan <sup>b</sup>	
	g/100 g dry matter	g/100 g dry matter	mg/100 g dry weight	mg/100 g protein
Peru	14.8 $\pm$ 1.12	53.6 $\pm$ 2.7	226 $\pm$ 4	1527 $\pm$ 18
Venezuela	14.1 $\pm$ 0.43	52.0 $\pm$ 4.8	201 $\pm$ 19	1422 $\pm$ 21
Madagascar	13.1 $\pm$ 0.51	49.2 $\pm$ 3.0	218 $\pm$ 11	1661 $\pm$ 30
Ecuador	13.8 $\pm$ 0.17	50.7 $\pm$ 2.0	235 $\pm$ 14	1702 $\pm$ 25
Dominican Republic	11.8 $\pm$ 0.54	46.2 $\pm$ 2.1	200 $\pm$ 10	1695 $\pm$ 18
Ivory Coast	15.7 $\pm$ 0.50	49.5 $\pm$ 3.0	221 $\pm$ 9	1407 $\pm$ 15
Ecuador Echeandia, roasted	12.3 $\pm$ 0.61	51.4 $\pm$ 1.8	180 $\pm$ 11	1395 $\pm$ 21
Ecuador Echeandia, unroasted	12.0 $\pm$ 0.72	52.3 $\pm$ 2.5	208 $\pm$ 12	1733 $\pm$ 17

<sup>a</sup> Values are averages of three separate determinations. Protein content was calculated applying a nitrogen protein conversion factor of 6.25.

<sup>b</sup> Determined in triplicate by HPLC analysis after alkaline hydrolysis of cocoa powder.

we found were similar to values reported in the literature (Aremu, Agiang, & Ayatse, 1995).

Lipid content also varied, ranging from 46.2 g/100 g DW (Dominican Republic) to 53.6 g/100 g DW (Peru). Chemical composition differed according to cultivar and also geographical conditions (Borchers, Keen, Hannum, & Gershwin, 2000; Lipp & Anklam, 1998).

### 3.2. Quantification of protein tryptophan

Mass spectrometric analyses of HPLC fractions with the same retention time as the Trp standard identified a single species at  $m/z$  205.2, corresponding to the protonated amino acid  $[M + H]^+$ , thus revealing that protein and non-protein Trp is totally pure.

Unlike protein and lipid contents, protein Trp concentrations, calculated as dry matter, did not greatly differ in the proteins of our samples (Table 1). Results for protein Trp are expressed as means  $\pm$  S.E.M. of determinations on three replicate hydrolysates. Cocoa from Ecuador and Peru had higher protein Trp values ( $235 \pm 14$  and  $226 \pm 4$  mg/100 g DW, respectively) and roasted cocoa from Echeandia lower ( $180 \pm 11$  mg/100 g DW) than those of the other samples. Cocoa from Venezuela, Madagascar, Dominican Republic, Ivory Coast and Echeandia (unroasted) had similar values ( $201 \pm 19$ ,  $218 \pm 11$ ,  $200 \pm 10$ ,  $221 \pm 9$  and  $208 \pm 12$  mg/100 g DW, respectively).

Examining the values of protein Trp as mg/100 g of protein (Table 1), large differences were noted: unroasted cocoa from Echeandia had the highest value ( $1733 \pm 17$  mg/100 g protein), not only with respect to the corresponding roasted cocoa beans, which had the lowest value ( $1395 \pm 21$  mg/100 g protein), but also to all the other roasted samples. High values of protein Trp were also found in cocoa from Ecuador, Dominican Republic, Madagascar and Peru ( $1702 \pm 25$ ,  $1695 \pm 18$ ,  $1661 \pm 30$  and  $1527 \pm 18$  mg/100 g protein, respectively), followed by those from Venezuela ( $1422 \pm 21$  mg/100 g) and the Ivory Coast ( $1407 \pm 15$  mg/100 g protein). Similar variations are also reported in the literature (Nielsen & Hurrell, 1985; Offem, 1990).

### 3.3. Determination of free and protein-bound tryptophan

We have recently found that Trp is present as non-protein tryptophan, free and bound to water and basic soluble proteins, in most common vegetable foods such as cereals and legumes (Comai et al., 2007a,b). Trp is also present as free and protein-bound forms in cocoa beans (Table 2). Madagascar cocoa showed the highest content of free Trp ( $17.26 \pm 1.71$  mg/100 g defatted DW powder), followed by that from the Dominican Republic ( $15.65 \pm 2.37$  mg/100 g defat-

ted DW powder). Free Trp concentrations were similar in Venezuela and Ivory Coast cocoa beans ( $13.46 \pm 1.83$  and  $13.66 \pm 1.94$  mg/100 g, defatted DW powder, respectively) followed by Ecuador ( $10.19 \pm 3.10$  mg/100 g). The sample from Peru showed the lowest content ( $8.49 \pm 1.92$  mg/100 g defatted DW powder).

Regarding cocoa beans deriving from the same cultivar in Echeandia, the unroasted sample had a value which was almost double that of the roasted one ( $12.78 \pm 0.63$  and  $6.39 \pm 0.42$  mg/100 g defatted DW powder, respectively), showing the influence of the roasting procedure on amino acid content. It has been shown that roasting time and temperature greatly affect the content of free amino acids, which are involved in Maillard and Strecker reactions, which take place during cocoa bean treatment (de Brito et al., 2001; Granvogl, Bugan, & Schieberle, 2006). In the case of Trp, some authors have recently shown its complete degradation with the intensity of the roasting process of the coffee beans (Martins & Gloria, 2010).

Table 2 also lists the values of total non-protein Trp, i.e., free + protein-bound water-soluble fraction, expressed as mg/100 g powder. This fraction was higher in samples from the Dominican Republic ( $21.62 \pm 1.86$  mg/100 g defatted DW powder) and Madagascar ( $20.84 \pm 3.81$  mg/100 g defatted DW powder), followed by those from Venezuela and the Ivory Coast ( $16.35 \pm 2.42$  and  $14.43 \pm 0.45$  mg/100 g defatted DW powder, respectively). Samples from Peru and Ecuador had lower but similar values ( $10.12 \pm 2.01$  and  $9.71 \pm 0.52$  mg/100 g defatted DW powder, respectively).

The lowest value of water-soluble non-protein Trp was that of roasted cocoa from Echeandia ( $7.30 \pm 0.18$  mg/100 g defatted DW powder). Also in this case, the corresponding unroasted sample had a higher value ( $12.91 \pm 0.11$  mg/100 g defatted DW powder). Trp was also bound to proteins extracted with buffer, pH 8.9, and with 70% ethanol (Table 2). Compared with the protein-bound water-soluble fraction, the amount of Trp bound to basic and ethanol-soluble proteins was lower.

Dominican cocoa again contained more Trp bound to basic proteins ( $3.04 \pm 0.12$  mg/100 g defatted DW powder), followed by those from Madagascar ( $3.01 \pm 0.67$  mg/100 g defatted DW powder). Venezuela, Ivory Coast and Ecuador samples showed similar amounts ( $2.06 \pm 0.45$ ,  $2.02 \pm 0.43$  and  $1.98 \pm 0.61$  mg/100 g defatted DW powder, respectively), and Peru cocoa the lowest ( $0.96 \pm 0.14$  mg/100 g defatted DW powder). Again, for the same cocoa from Echeandia, roasted beans had contents which were half those of the unroasted ones ( $1.16 \pm 0.24$  and  $2.52 \pm 0.15$  mg/100 g defatted DW powder, respectively). Table 2 also lists the levels of Trp bound to the protein fraction soluble in 70% ethanol; the highest value was Madagascar ( $1.21 \pm 0.18$  mg/100 g defatted DW powder) while the lowest was Peru ( $0.24 \pm 0.01$  mg/100 g powder).

**Table 2**

Values (means  $\pm$  S.E.M.) of non-protein tryptophan (Trp) fractions, i.e., free, total (free + bound to H<sub>2</sub>O-soluble protein), bound to proteins soluble in buffer, pH 8.9, and in 70% ethanol in cocoa seeds of various geographical origin.

Origin of cocoa beans	Free Trp (mg/100 g dry defatted sample)	Total protein-bound Trp, H <sub>2</sub> O-soluble fraction (mg/100 g dry defatted sample)	Protein-bound Trp, buffer fraction, pH 8.9 (mg/100 g dry defatted sample)	Protein-bound Trp, 70% ethanol fraction (mg/100 g dry defatted sample)
Peru	$8.49 \pm 1.92$	$10.12 \pm 2.01$	$0.96 \pm 0.14$	$0.24 \pm 0.01$
Venezuela	$13.46 \pm 1.83$	$16.35 \pm 2.42$	$2.06 \pm 0.45$	$0.74 \pm 0.25$
Madagascar	$17.26 \pm 1.71$	$20.84 \pm 3.81$	$3.01 \pm 0.67$	$1.21 \pm 0.18$
Ecuador	$10.19 \pm 3.10$	$9.71 \pm 0.52$	$1.98 \pm 0.61$	$0.48 \pm 0.10$
Dominican Republic	$15.65 \pm 2.37$	$21.62 \pm 1.86$	$3.04 \pm 0.12$	$0.89 \pm 0.23$
Ivory Coast	$13.66 \pm 1.94$	$14.43 \pm 0.45$	$2.02 \pm 0.43$	$1.03 \pm 0.27$
Ecuador	$6.39 \pm 0.42$	$7.30 \pm 0.18$	$1.16 \pm 0.24$	$0.52 \pm 0.09$
Echeandia roasted				
Ecuador	$12.78 \pm 0.63$	$12.91 \pm 0.11$	$2.52 \pm 0.15$	$0.67 \pm 0.21$
Echeandia unroasted				

The fractions analysed by MS showed that non-protein Trp was totally pure.

#### 4. Conclusions

Cocoa is a widely-consumed confectionery food, and is also used as an ingredient in many preparations, such as drinks, cookies, cakes, breads, etc.

The data reported here indicate that cocoa beans contain levels of protein Trp which are higher than those of cereals but similar to those of most legumes (Comai et al., 2007a,b). Cocoa beans also contain non-protein Trp, both free and bound to variously soluble proteins. As tryptophan is one of the amino acids limiting the biological value of vegetable proteins, when determining the nutritional quality of foods, it is necessary to consider non-protein Trp contents as well as amino acid profiles.

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