

Roasting impact on the contents of clovamide (*N*-caffeoyl-*L*-DOPA) and the antioxidant activity of cocoa beans (*Theobroma cacao* L.)

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Received 7 December 2006; received in revised form 28 June 2007; accepted 4 July 2007

Abstract

The caffeoylated amino acid clovamide [(–)-*N*-[3′-4′-dihydroxy-(*E*)-cinnamoyl]-dihydroxyphenylalanine] was identified in the antioxidant polyphenolic fraction of cocoa (*Theobroma cacao* L.). As a naturally occurring caffeoyl conjugate, clovamide represents an interesting antiradical/antioxidant compound. We have streamlined the synthesis of clovamide, investigating the effect of roasting on its content in different samples of cocoa beans from different geographic origin. Within the samples analyzed, those from Ghana showed the highest clovamide content (2.637 mg/kg, powder from fermented beans, dry weight; 1.264 mg/kg, powder from roasted nibs, dry weight), while the Arriba samples showed the lowest values. A poor correlation existed between the contents of clovamide and the antioxidant properties of cocoa, while roasting caused a dramatic reduction in the clovamide contents (up to 59.13% in Arriba cocoa), paralleled by an overall decrease of the antioxidant properties, as measured by the DPPH[•] method. Taken together, results show that while roasting is detrimental for the clovamide contents of cocoa, no correlation exists between the concentration of this compound and the overall antioxidant properties of cocoa samples, and suggest that clovamide is important but not critical for the antioxidant activity of cocoa.

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Keywords: *Theobroma cacao*; (–)-*N*-(3′-4′-dihydroxy-(*E*)-cinnamoyl)-dihydroxyphenylalanine; Clovamide; Antioxidant properties; Cocoa astringency

1. Introduction

Over the past few years, the antioxidant and health-promoting properties of cocoa (*Theobroma cacao* L.) and cocoa-related products have been thoroughly investigated (Heiss et al., 2005; Lee, Hwang, Kang, Kim, & Lee, 2005; Mao, Van de Water, Keen, Schmitz, & Gershwin, 2002; Ramiro et al., 2005; Sakano et al., 2005; Schroeter et al., 2006; Yamagishi et al., 2001). Polyphenols, widely distributed in plant foods, are the main antioxidant-active fraction of cocoa, and, within polyphenols,

flavanols and procyanidins have been identified as the active antioxidant agents of cocoa and dark plain chocolate. While various monomeric [(–)-epicatechin, (+)-catechin, (+)-gallocatechin, (–)-epigallocatechin and epicatechin 3-*O*-gallate] and oligomeric (3–11 units) flavan-3-ols from cocoa show powerful antioxidant activity (Counet & Collin, 2003), the contribution of other polyphenolics like flavonoids (quercetin, quercetin glycosides, luteolin, naringenin, apigenin) and anthocyanidins has not yet been fully assessed. The antioxidant properties of simple polyphenols have been largely studied by means of *in vitro* DPPH[•] scavenging tests (Lee, Kim, Lee, & Lee, 2003; Othman, Ismail, Abdul Ghani, & Adenan, 2007) and some recent studies have confirmed the protective action of cocoa flavanols and procyanidins using *in vitro* cellular

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models (Kenny et al., 2004; Zhu et al., 2005). Moreover, other benign properties related to the bioactivity of phenolics from cocoa were largely studied. It has recently been shown the protective activity of total methanolic phenolic extract from cocoa on liver cells, by preventing apoptosis induced by celecoxib (probably by inducing the autophagic mechanism) (Arlorio et al., 2006). It has also described the positive antioxidant action of cocoa extracts in a cellular model, showing their protective action toward O₂ deprivation (modulated hypoxemia) (Arlorio et al., 2005).

Sanbogi et al. (1998) reported the occurrence of (–)N-[3'-4'-dihydroxy-(*E*)-cinnamoyl]-L-dihydroxyphenylalanine (clovamide) and (–)N-[4'-hydroxy-(*E*)-cinnamoyl]-L-tyrosine (deoxyclovamide) in cocoa liquor. Clovamide was described for the first time by Yoshihara, Yoshikawa, Sakamura, and Sakuma (1974) as a constituent of red clover; its structure is very similar to that of rosmarinic acid, an ester analogue occurring in various plants from the *Lamiaceae* family, and especially in rosemary, *Rosmarinus officinalis* L. Whereas several studies were conducted about rosmarinic acid, scientific literature is still lacking about clovamide.

Various health claims for cocoa polyphenolics have been reported (Ding, Hutfless, Ding, & Girotra, 2006; Sies, Schewe, Heiss, & Kelm, 2005; Keen, 2001; Weisburger, 2001; Wollgast & Anklam, 2000a), but nothing is known on the effective contribution of clovamide to this activity. On the other hand, antioxidants cinnamoyl conjugates of amino acids were identified, along with procyanidins, as the astringent principles of non-fermented cocoa beans and roasted nibs (Ley & Bertram, 2003; Spasova et al., 2005; Stark, Bareuther, & Hofmann, 2005; Stark & Hofmann, 2005). Moreover, it is important to note that clovamide is structurally similar to some beta 2-adrenoceptors (dobutamine, denopamine) capable of inhibiting platelet aggregation via a cAMP mediated process (Park, 2005a; Park, 2005b; Park, 2005c).

Taken together, these observations qualify clovamide as an interesting bioactive compound for nutritional research, but, surprisingly, the effect of industrial practices – namely roasting process – on its concentration in cocoa is still lacking. The cocoa-related products (chocolate, cocoa powders) were industrially transformed using different thermal process; within these processes, roasting could be considered the crucial step on the persistence of clovamide in its active form. In fact, the antiradical and antioxidant properties of roasted cocoa beans/nibs samples are decreased in a significant way by roasting process (Arlorio et al., 2006).

We have streamlined the synthesis of clovamide, next focusing on (i) the study of its occurrence in samples of three batches of fermented beans and roasted nibs (from three different geographical areas: Ecuador, Ivory Coast and Ghana) and (ii) the correlation between the clovamide contents and the DPPH[•] antiradical scavenging test of these samples.

2. Materials and methods

2.1. Cocoa samples

Samples of cocoa (var. Forastero) obtained from different geographical areas (Ghana, Ivory Coast and Ecuador) and their roasted nibs (roasted cocoa beans separated from hull and broken in small bits) were analyzed. Cocoa beans were fermented and dried in country of origin according to local practices, than were conventionally roasted (pre-roasting temperature: 100 °C; roasting temperature: 130 °C) by an Italian company, that kindly provided all cocoa samples. No other details about post-harvest treatment on cocoa beans were known, except they were all from the same harvesting year. The moisture content was determined to express the results on a dry weight (dw) basis. A mixture of hulls obtained from pre-roasted cocoa beans from Ghana, Ivory Coast and Ecuador were also analyzed.

2.2. Reagents and standards

All reagents and solvents used for synthesis of clovamide were obtained from Sigma–Aldrich (Milano, Italy); petrol ether and ethyl acetate were purchased from Carlo Erba (Rodano, Milano, Italy); silica 60 for gravimetric c.c. 70–230 mesh was from Delchimica Scientific glassware (Milano, Italy). Methanol, acetonitrile (all HPLC grade) and formic acid (50%, LC–MS grade), were purchased from Sigma–Aldrich (Milano, Italy). Water was obtained by Milli-*Q* instrument (Millipore Corp., Bedford, MA, USA). All reagents and standard chemicals used for the determination of total phenol content and antiradical activity were purchased from Sigma–Aldrich (Milano, Italy).

2.3. Instrumentation

The moisture content of all cocoa samples was determined using a thermo-balance Sartorius MA30 (Sartorius AG, Goettingen, Germany). Sample extractions were performed using an automatic Soxhlet Büchi Extraction System B-811 (Büchi Labortechnik AG, Flawil, Switzerland). ¹H NMR spectra were recorded using Jeol 300 (¹H: 300 MHz) spectrometer (Jeol, Peabody, MA, USA) in CD₃OD solution. The separations were obtained by an LC–ESI–MS “on-line” using a Surveyor HPLC system (Thermo Finnigan, San Josè, CA, USA) equipped with a quaternary pump, a Surveyor AS autosampler (racks maintained at 25 °C), a vacuum degasser, a Surveyor PDA and an ion-trap LCQ Deca XP Plus MS; this instrumentation was equipped with an Xcalibur[®] system manager data acquisition software. All the UV–VIS analyses were performed using a Kontron UVIKON 930 Spectrophotometer (Kontron Instruments, Milano, Italy).

2.4. Extraction of phenolic compounds

First, cocoa beans, roasted nibs and hulls were finely ground in a mixer and then extracted in automatic Soxhlet (12 h) using dichloromethane to remove the lipid fraction. The phenolic fraction was then extracted from the defatted cocoa powders in automatic Soxhlet apparatus, using methanol as the solvent for 4 h (up to complete decolouration). The solvent was then evaporated to dryness (vacuum, 40 °C) and dry extract was stored at –20 °C until use.

2.5. Total phenol content

Total phenols determination was obtained using the classic Folin-Ciocalteu assay (Singleton & Rossi, 1965). Folin-Ciocalteu reagent (5 mL) and 10 mL of aqueous Na₂CO₃ (10% w/v) were added to an appropriate volume of sample solution (5 mg/mL methanol) in a 25 mL volumetric flask. After incubation at 65 °C for 20 min the solutions were diluted with water to 25 mL and absorbance was read at 760 nm. Results were expressed as catechin equivalents, through the calibration curve of (±)-catechin monohydrate. The calibration curve linearity range was 24–242 µg ($R^2 = 0.9943$).

2.6. Antiradical activity

The radical scavenging activity was measured using the DPPH[•] (2,2-diphenyl-1-picrylhydrazyl) assay, where the decoloration of solutions is an effect of sample antiradical activity. The sample or MeOH (blank) (700 µL) were added to the same volume of methanolic solution of a 100 µM DPPH[•]. Absorbance was read at 515 nm after 20 min at room temperature and in the dark. Some reference compounds with established antiradical properties (rosmarinic acid, trolox, (–)-epicatechin, quercetin dihydrate, butylated hydroxyanisole (BHA), gallic acid monohydrate, octyl gallate and kaempferol), clovamide and natural cocoa extracts were dissolved in MeOH and diluted to obtain a calibration curve. Results were expressed as EC₅₀ (antioxidant dose required to obtain a 50% inhibition). EC₅₀ was calculated by plotting % inhibition vs sample concentration. R^2 obtained was in the range from 0.9534 to 0.9993, depending on the standard compounds and cocoa samples.

2.7. Synthesis and characterization of clovamide

Under nitrogen atmosphere, thionyl chloride (943 µL, 12.7 mmol) was added to ice bath cooled MeOH (18 mL). After 30 min, L-3,4-dihydroxyphenylalanine (1 g, 5.1 mmol) was added, and the mixture was stirred at room temperature overnight. After removal of the solvent, the residue was taken up in dry pyridine (15 mL) and treated sequentially with caffeic acid (1.01 g, 6.1 mmol, 1.2 equiv.) and DCC (1.25 g, 6.1 mmol, 1.2 equiv.). After stirring at room temperature for 72 h, the reaction was worked up by dilu-

tion with 2N H₂SO₄ and extraction with EtOAc. Removal of the solvent gave a semi-solid residue that was purified by gravity column chromatography on silica gel using a petroleum ether/EtOAc gradient. Fractions eluted with petroleum ether/EtOAc 2:8 (v/v) gave crude clovamide methyl ester as a yellowish powder (1.32 g; yield: 70%). Under a nitrogen atmosphere, a sample of this crude ester (300 mg, 0.8 mmol) was dissolved in tetrahydrofuran/H₂O 2:1 (v/v), and then treated with LiOH · H₂O (84 mg, 2.0 mmol, 2.5 equiv.). The mixture was stirred at 45 °C for 6 h, and then acidified with Dowex[®] 50W × 8 resin, filtered, and evaporated, using toluene to increase water removal. The residue was purified on silica gel column using a petroleum ether/EtOAc gradient to remove some non-polar impurities, then using EtOAc/MeOH 7:3 (v/v) to obtain clovamide (yield: 38%).

The purified clovamide was then characterized by means of NMR, IR and mass analysis.

¹H NMR (300 MHz, CD₃OD): δ 7.36 (1H, d, $J = 15.6$ Hz, H-8'), 6.99 (1H, d, $J = 1.9$ Hz, H-2), 6.89 (1H, dd, $J = 8.2, 1.9$ Hz H-6), 6.75 (1H, d, $J = 8.2$ Hz, H-5), 6.68 (1H, d, $J = 2.5$ Hz, H-2'), 6.66 (1H, d, $J = 8.0$ Hz, H-5'), 6.55 (1H, dd, $J = 8.2$ Hz $J = 1.9$ Hz, H-6'), 6.41 (1H, d, $J = 15.7$ Hz, H-7'), 4.68 (1H, m, H-8), 3.05 (1H, dd, $J = 5.5$ Hz, 8.5 H-7a), 2.89 (1H, dd, $J = 8.5, 5.8$ Hz, H-7b). IR (KBr) cm⁻¹: ν_{\max} 3282 (s), 1718 (s), 1652 (s), 1598 (s), 1522 (s), 1363 (m), 1282 (s), 1198 (s), 1115 (m), 975 (m). MS: m/z 358 [M – H]⁻.

2.8. FIA–ESI–MS

Sample solutions of clovamide (5 µg/mL), dissolved in water/acetonitrile 80/20 (v/v) with 0.1% formic acid, were infused in the ESI source using a syringe pump at a flow-rate of 5 µL/min and the mass scan range was m/z 100–800. Operating conditions on the ion-trap mass spectrometer in negative polarity were as follows: spray voltage, 4.70 kV; source current, 80 µA; capillary temperature, 400 °C; capillary voltage, –6 V; tube lens offset, 0 V; sheath gas flow (N₂), 40 A.U. Data were acquired in negative MS and MS/MS scanning mode; the precursor isolation window was set at 1 atomic mass unit (amu) and the collision energy was optimized 30%.

2.9. HPLC–DAD–ESI–MS/MS

HPLC analyses were conducted using the method described in Sanchez-Rabaneda et al. (2003) with some modifications. The chromatographic separation was performed on a Phenomenex Luna C18 (2) (150 × 2 mm i.d., with particle size of 5 µm, Waters corporation, Milford, MA, USA) maintained at 25 °C. The mobile phase consisted of water-formic acid 0.1% (eluent A) and acetonitrile-formic acid 0.1% (eluent B) using the following program gradient: from 6% to 16.5% B (14 min), from 16.5% to 17% B (2 min), from 17% to 17.5% B (2 min), isocratic 17.5% B (2 min), from 17.5% to 18.5% B

(2 min), isocratic 18.5% B (2 min), from 18.5% to 20% B (3 min), from 20% to 100% B (19 min), from 100% to 6% B (2 min), isocratic 6% B (5 min). Total run time was 53 min, at a constant flow-rate of 400 $\mu\text{L}/\text{min}$. Chromatograms were recorded at three different wavelength (280, 320 and 365 nm). The injection volume was 10 μL . The eluate was injected into the electrospray ion source with a splitting of 40% and the MS and MS/MS spectra were acquired and interpreted using the software Xcalibur[®]. For the quantification, a methanolic stock solution of clovamide (1 mg/mL) was prepared; this solution was tested to be stable at $-20\text{ }^{\circ}\text{C}$ for at least 3 months. The stock solution was then opportunely diluted in the same solvent to the five different curve concentrations (0.1, 0.5, 1, 2.5, 5 $\mu\text{g}/\text{mL}$). Quantification of clovamide in samples was performed dissolving from 30 mg to 60 mg of dry extracts in 1 mL of MeOH.

2.10. Statistical analysis

The results are expressed as mean \pm standard deviation (SD) of three independent experiments. Statistical significance was determined by analysis of variance (ANOVA) using Microsoft[®] Excel 2002 statistical software for Windows XP. Differences were considered significant at $p < 0.05$.

3. Results and discussion

3.1. Synthesis and characterization of clovamide

Clovamide is not commercially available, but several syntheses have been reported (Ley & Bertram, 2003; Tebayashi, Ishihara, Tsuda, & Iwamura, 2000; Rajan et al., 2001). We prepared clovamide from commercial *L*-phenylalanine and caffeic acid, via DCC (dicyclohexylcarbodiimide)-mediated amidation of the methyl ester of the amino acid and deprotection (Fig. 1). This synthetic scheme does not involve phenolic protection, and the purification of the final product could be streamlined by using

gravity column chromatography on silica gel. The final product was fully characterized by NMR and direct mass analyses, and its purity was assessed by HPLC (see Section 2).

The flow injection analysis (FIA) of clovamide was necessary to determine the best conditions of analysis and to understand the fragmentation patterns in MS/MS experiments. The analysis was made in negative ion mode for the presence of the carboxylic acid. The negative full scan in fact clearly showed the presence of the parent ion with a m/z of 358 corresponding to the $[\text{M} - \text{H}]^{-}$. The parent ion was then fragmented for 90% with a collision energy of 30% in four prevalent daughter ions: m/z 222, 178, 161 and 133, with a relative abundance of 100, 25, 10 and 2, respectively. The major daughter ion produced (m/z 222) suggests that fragmentation occurred on the α - β unsature linkage with loss of the vinyl-catecholic fragment $[\text{M} - \text{H}-136]^{-}$. Fig. 2 shows the MS/MS spectrum obtained by FIA on m/z 358 and the hypotheses for the fragmentation pattern.

From the LC-DAD-ESI-MS/MS it was determined the retention time of the clovamide (14.46 min) with the chromatographic method described above. The MS/MS profile demonstrated the presence of a peak at the same retention time with the same spectrum obtained in infusion.

3.2. Total polyphenolic content and antioxidant activity

Polyphenols are compounds produced in secondary metabolism of many plants and play an important role for instance in the defence against micro-organism, as signalling compounds, etc. In past few years, they have gained much more attention and have become an important focus of research interest, owing to their antioxidant activities, and their various beneficial effects on human health, such as in the treatment and prevention of cancer, cardiovascular diseases, antimicrobial and other pathologies. Cocoa beans are rich in polyphenols, that are stored in the pigment cells of the cotyledons. The presence of polyphenols in plants is dependent of several factors including degree

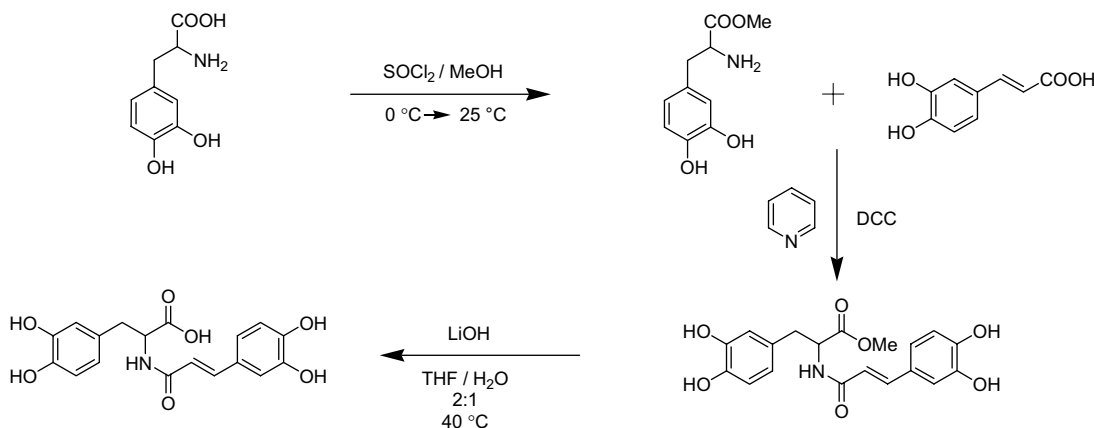


Fig. 1. Scheme of clovamide synthesis.

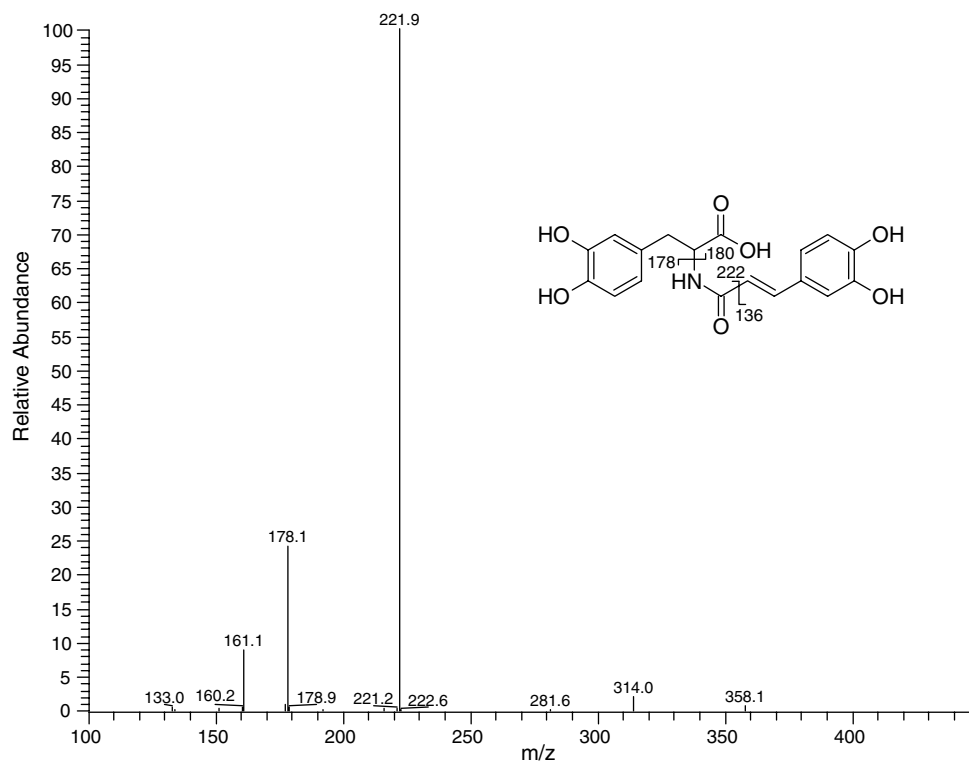


Fig. 2. Fragmentation pattern and relative abundance of fragments of clovamide.

of ripeness, variety, stress reactions, processing and storage. Fermentation and drying of cocoa beans, post-harvest treatment essential to develop suitable flavours and/or flavour precursors, greatly vary the total polyphenolic content. During fermentation of cocoa beans, polyphenols diffuse with cells liquids from their storage cells and undergo oxidation to condensed high molecular tannins, by reactions both non-enzymatic and catalyzed by the polyphenol oxidase, even this enzyme is quickly inactivated. Furthermore, during drying process, non-enzymatic oxidations could decrease the polyphenols content. Further process that produce alterations in the content and the composition of polyphenolic fraction of cocoa are various heat-based pre-treatment steps (pre-roasting, conventionally performed at just below at 100 °C given by hot air, steam or infra-red heat) which conduce to loosen the hulls that cover cocoa seeds, and the real roasting step. Roasting of whole beans (more suitable to obtain a good flavoured product, even if the technology and the quality of the fermented cocoa are two fundamental parameters able to influence the aroma development) or crushed nibs is essential in the manufacture of chocolate and cocoa solids since contribute to develop further typical chocolate aroma, which should already exist as precursors from the correct fermentation and drying. As well-known, both proteins and polyphenolic fractions must be considered precursor of cocoa aroma. Moreover, phenolics (and caffeaates too) are strictly related to the bitterness and the astringency of the cocoa (Nazaruddin, Seng, Hassan, & Said, 2006; Voigt & Biehl, 1995; Wollgast & Anklam, 2000b).

In this section of work we have investigated the influence of high temperatures produced during roasting on total polyphenols content in cocoa beans and in a sample of pre-roasted hulls.

Table 1 shows the total phenols content, expressed as catechin equivalents (g/100 g on a dry weight basis). The moisture content of fermented unroasted cocoa beans, determined to express the results on a dry weight (dw) basis, is in line with data published in the literature (Ghana: $6.97 \pm 0.14\%$; Arriba: $6.87 \pm 0.14\%$; Ivory Coast: $6.18 \pm 0.18\%$), to demonstrate the correct fermentation and drying of cocoa beans analyzed (Wollgast & Anklam, 2000b). Total phenols ranged from 1.423 g/100 g dw in powder obtained from Ghana to 1.715 g/100 g dw in powder from “Arriba” beans (Ecuador). Unroasted Ghana samples resulted not significantly different from Ivory Coast samples. High temperatures produced during

Table 1

Total phenolic content expressed as g catechin equivalents/100 g powder, dw (mean \pm SD, $n = 3$)

Sample	Unroasted	Roasted
Ghana	1.423 \pm 0.080 ^a	0.644 \pm 0.019 ^c
Arriba	1.716 \pm 0.014 ^b	1.156 \pm 0.179 ^{c,d}
Ivory coast	1.500 \pm 0.001 ^a	0.927 \pm 0.016 ^d
Hulls	0.023 \pm 0.002	

Means \pm SD followed by the same letter are not significantly different ($p > 0.05$). Statistical analysis was performed among samples from different origin and, for each cocoa type, also considering unroasted samples *vs* roasted ones.

roasting practise severely affects not only the water content (Ghana: $3.66 \pm 0.26\%$; Arriba: $3.32 \pm 0.12\%$; Ivory Coast: $2.87 \pm 0.18\%$), but also the polyphenolic content (not-oxidized polyphenols), decreased in all roasted samples (Table 1). In fact, the phenols decrease ranged from 32.63% (cocoa beans Arriba) to 54.74% (cocoa beans Ghana).

The loss of polyphenolic compounds in cocoa is strictly correlated to the oxidation of these compounds to corresponding quinones, allowing the polymerization and the formation of insoluble high-molecular-weight pigment compounds. Also their reaction with proteins contributes to the total polyphenols content decrease. Moreover, under analytical point of view, it is important to note that polyphenols extraction efficiency depends on many factors, as cocoa variety, beans ripening degree, solvent used in extraction and method of extraction (Azizah, Ruslawati Nik, & Swee Tee, 1999). Finally, significant differences existed between seeds and hulls phenols content: results show, like expectable, that hulls mixture (moisture content: $4.09 \pm 0.13\%$) contains lower level than seeds. The analysis performed on cocoa hulls (by-products obtained by pre-roasting treatment), previously characterized by Arlorio, Coisson, Restani, and Martelli (2001) about the proximate composition and pectin content, showed in this case a low content of total polyphenolic substances (0.023 g/100 g dw) and then a lower potential antioxidant capacity than beans.

As previously reported, polyphenols have been the subject of numerous investigations for their several properties and antioxidant capacity (free-radical scavengers, reducing agents, metal chelators, etc.) and cocoa has been shown to be rich in polyphenols, especially catechins (ca. 37%) and proanthocyanidins (ca. 58%) (Wollgast & Anklam, 2000b).

The radical scavenging test based on the decoloration of DPPH \cdot solution elicited a high antiradical/antioxidant activity of methanolic extracts from all cacao samples. Table 2 shows the results obtained measuring the antioxidant properties of three different samples of cocoa before and after roasting practises. Unroasted Arriba and unroasted Ivory Coast samples resulted not significantly different. For roasted samples, Arriba and Ivory Coast results were not significantly different when expressed on the basis of methanolic extracts, but were significantly different when expressed on the basis of powder dw.

As shown, roasted samples have lower activity than unroasted beans, confirming results recently reported by Summa et al. (2006) for the aqueous phenolic fraction. It is important to note that in the case of Ghana samples a significant difference was observed only considering values related to the powder dry weight. For this reason, we suggest as critical the direct consideration of alcoholic extracts values in antioxidant/antiradical capacity evaluation, as commonly reported in many papers (Summa et al., 2006; Othman et al., 2007). Moreover, the approach based on the analysis of aqueous-soluble phenolics is fundamental to assess the real “bioavailable” content of antioxidants from cocoa, but this extraction is clearly not exhaustive. In this work we have considered the methanol-soluble phenolics; in fact, a complete comparative study about different extraction methods for evaluation of roasting influence on antioxidant activity is still lacking in literature. We have also provided to correlate the total phenolic content *vs* EC₅₀ activity (Fig. 3), confirming a complex interaction between phenolic compounds and antioxidant capacity of extracts. We cannot demonstrate *in vitro* a direct correlation between polyphenolic total content and the antiradical activity, also considering the relative concentration of clovamide, as reported below. Our results indicate that high scavenging ability on DPPH \cdot could not be exclusively due to phenols in cocoa extracts. Our results, in agreement with these reported by Othman et al. (2007), suggest that high scavenging ability on DPPH \cdot could not be exclusively due to phenols in cocoa extracts. In fact, as previously described by Othman et al. (2007), other methanol-soluble compounds like methyl xanthines (theobromine and caffeine), minor flavonoids and pigments might influ-

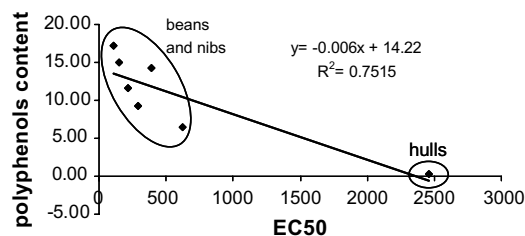


Fig. 3. Relationship between total phenolic content (mg/g) and antioxidant activity (EC₅₀: µg/mL) of cocoa sample (powder, dw).

Table 2
Antioxidant activity of cocoa samples (EC₅₀ expressed as mean \pm SD, $n = 3$)

Sample	Methanolic extracts (µg/mL)		Powder, dw (mg/mL)	
	Unroasted	Roasted	Unroasted	Roasted
Ghana	30.100 \pm 2.417 ^a	34.761 \pm 2.018 ^a	0.392 \pm 0.031 ^A	0.626 \pm 0.036 ^C
Arriba	14.986 \pm 1.348 ^b	26.546 \pm 2.107 ^c	0.111 \pm 0.025 ^B	0.216 \pm 0.020 ^D
Ivory Coast	20.748 \pm 3.566 ^b	27.008 \pm 2.507 ^c	0.152 \pm 0.014 ^B	0.290 \pm 0.023 ^E
Hulls	132.878 \pm 7.494		2.460 \pm 0.139	

Means \pm SD followed by the same letter are not significantly different ($p > 0.05$). Statistical analysis was performed among samples from different origin and, for each cocoa type, also considering unroasted samples *vs* roasted ones; results expressed on the basis of methanolic extracts and those expressed on the basis of powder dw were processed separately.

ence the total antioxidant/antiradical capacity of methanolic cocoa extracts. Moreover, we highlight that the real antioxidant properties of a certain food extracts must be necessarily calculated also considering their bioavailability *in vivo*. For example, the powerful radical scavenging capacity of high-molecular-weight proanthocyanidins (≥ 8 -mers) cannot be considered *in vivo*, because of their negative bioavailability (Rasmussen, Frederiksen, Struntze Krogholm, & Poulsen, 2005; Shoji et al., 2006). This fact is not so clearly focused in many scientific papers that emphasize, in a general way, the high antioxidant properties of all proacyanidins contained in cocoa.

The radical scavenging activity was then measured also for synthesized clovamide, comparing the antiradical activity of some well-known antioxidants (rosmarinic acid, trolox, epicatechin, quercetin, BHA, gallic acid, octyl gallate, kaempferol). Fig. 4 reports EC_{50} data calculated for all substances, showing that clovamide action (EC_{50} : $9.238 \pm 1.054 \mu\text{M}$) was comparable to those of rosmarinic acid (EC_{50} : $8.993 \pm 1.918 \mu\text{M}$), as previously reported by Ley and Bertram (2003). Moreover, our study confirmed that clovamide and rosmarinic acid are very active substances with a high antiradical property.

3.3. Quantification of clovamide in cocoa samples

Clovamide, amidic analogue of rosmarinic acid and good natural antioxidant, at present is an interesting compound for nutritional research, but not yet widely investigated. Sanbogi et al. (1998) have firstly identified clovamide in cocoa liquor and they have elicited good properties against lipid peroxidation, subsequently confirmed by Ley and Bertram (2003). Moreover, although changes in phenolic fraction were thoroughly analyzed, no information exists about changes in clovamide content during post-harvest treatments and industrial practises. Starting from hypothesis that high temperatures could affect the content of clovamide, we have quantified it in some extracts obtained from fermented cocoa beans and their industrially-roasted nibs.

Methanolic solutions of cocoa extracts, were injected in HPLC–DAD–ESI–MS/MS system with the same conditions described above in Section 2. The presence of clova-

amide in cocoa samples was confirmed by comparing retention time and considering the MS/MS spectra obtained using synthesized clovamide.

Ghana samples showed the highest clovamide content (2.637 mg/kg, powder from fermented beans, dw; 1.264 mg/kg, powder from roasted nibs, dw), whereas Arriba samples showed the lowest values. The content of clovamide was significantly different among the three cocoa varieties (Table 3). Our results confirmed a strong significant reduction in the content in all roasted samples (up to 59.13% in Arriba cocoa samples) (Table 3), indicating that this compound is easily degraded/oxidized by high temperature processing. Comparing the antioxidant activity these results suggest that, whereas roasting produced a significant decrease of concentration of clovamide, no correlation exists between the concentration of this compound and the overall antioxidant properties of cocoa samples. It is also very important to note that cocoa hulls, that are a by-product of cocoa industry subjected to the pre-roasting, contain levels of clovamide ($1.817 \pm 0.059 \text{ mg/kg}$) higher than those typical of roasted nibs of every variety considered in this work. So, for this reason cocoa hulls can be considered a good low-coast source of antioxidant compounds, namely clovamide and flavonoids.

In conclusion, this work provides the description of a novel high-yield synthesis of clovamide; the antiradical capacity was demonstrated by comparing other antioxidants, showing a good EC_{50} value, comparable to those rendered by rosmarinic acid. The HPLC–DAD–ESI–MS/MS method lead to the easy quantification of clovamide in all samples; hypothesis for the fragmentation pattern of the molecule was also suggested. The quantification of clovamide in cocoa beans and roasted nibs showed that clovamide content could vary depending on geographical origin too, and that roasting process strongly decreases (about 50%) clovamide content in the nibs. These observations suggest that content of clovamide, like total polyphenols content, could be affected by several factors connected to country of origin, as both growing conditions (protective reaction of *T. cacao* plant against stress or molds, as suggested by Alemanno, Ramos, Gargadene, Andary, & Ferriere (2003)) and post-harvest treatments (fermentation and drying steps), and that clovamide content is significantly

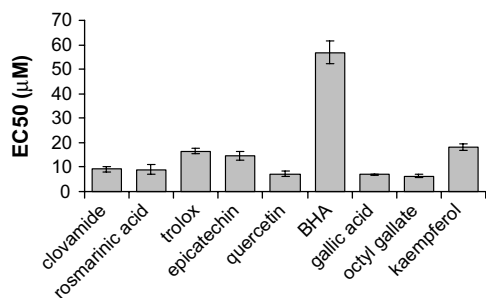


Fig. 4. Antioxidant activity of clovamide and other well-known antioxidant compounds (EC_{50} expressed as μM).

Table 3

Clovamide content expressed as mg/kg of cocoa powder, dw (mean \pm SD, $n = 3$)

Sample	Unroasted	Roasted
Ghana	2.637 ± 0.097^a	1.264 ± 0.037^d
Arriba	1.358 ± 0.054^b	0.555 ± 0.004^e
Ivory Coast	2.157 ± 0.084^c	1.108 ± 0.084^f
Hulls		1.817 ± 0.059

Means \pm SD followed by the same letter are not significantly different ($p > 0.05$). Statistical analysis was performed among samples from different origin and, for each cocoa type, also considering unroasted samples *vs* roasted ones.

reduced in conventionally roasted cocoa by high temperatures.

We could not demonstrate a direct correlation between polyphenols/clovamide content and antioxidant activity, both in fermented beans and roasted nibs. We have demonstrated that cocoa hulls, obtained in an inexpensive method during roasting, after the winnowing process, could be considered a good source of antioxidant phyto-extract containing bioactive clovamide.

Acknowledgement

This work was funded by a grant from the Università degli Studi del Piemonte Orientale (Ricerca Scientifica Locale).

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