

## Antioxidant and biological activity of phenolic pigments from *Theobroma cacao* hulls extracted with supercritical CO<sub>2</sub>

M. Arlorio <sup>a,\*</sup>, J.D. Coisson <sup>a</sup>, F. Travaglia <sup>a</sup>, F. Varsaldi <sup>b</sup>,  
G. Miglio <sup>b</sup>, G. Lombardi <sup>b</sup>, A. Martelli <sup>a</sup>

<sup>a</sup> LCBA (Laboratorio di Chimica e Biotecnologie degli Alimenti), Università degli Studi del Piemonte Orientale "A. Avogadro", DiSCAFF, Via Bovio 6, 28100 Novara, Italy

<sup>b</sup> Pharmacology Section, Università degli Studi del Piemonte Orientale "A. Avogadro", DiSCAFF, Via Bovio 6, 28100 Novara, Italy

Received 9 June 2004; accepted 28 March 2005

### Abstract

*Theobroma cacao* L. (Sterculiaceae) and cocoa-derived products are phenolics-rich food; these products are largely studied because of the antioxidant and antiradical in vitro properties of phenolic constituents. Cocoa hulls are the principal by-product of cocoa, separated from the cotyledons during the pre-roasting process or after the roasting process of *T. cacao* beans (de-hulling/de-husking step). This by-product is a matrix rich in fiber (namely insoluble, but also represented by pectins) and phenolics. Supercritical CO<sub>2</sub> is a powerful mild technology able to extract and fractionate from plant or animal foods without the use of organic solvent. This approach was used to extract some phenolics fractions from cocoa hulls. Only two recovered fractions, (150 bar, 50 °C, re-dissolved in acetone; 200 bar, 50 °C, re-dissolved in acetone), apparently free from (-)-epicatechin, catechin and phenolic acids, showed protective action in an in vitro test (SH-SY5Y cells, differentiated to a neuronal phenotype using retinoic acid and then exposed to ischemic damage), similar to the action of cabergoline and vitamin E. We suggest the use of supercritical CO<sub>2</sub> for the isolation of bioactive fractions from cocoa hulls and an in vitro model as a useful model to study the antioxidant/antiradical properties of isolated phenolic pigments.

© 2005 Elsevier Ltd. All rights reserved.

**Keywords:** *Theobroma cacao* L.; Antioxidant; Antiradical; Phenolics; Supercritical CO<sub>2</sub> extraction

### 1. Introduction

Cocoa-derived foods (cacao powders, chocolate, cocoa-related products) are phenolics-rich foods derived from the fermented, roasted and milled seeds of *Theobroma cacao* L. (Sterculiaceae). These products, consumed all over the world, are largely studied because of the antioxidant and antiradical in vitro properties of some phenolic constituents (phenolic acids, procyanidins, flavonoids) (Wollgast & Anklam, 2000a). Pheno-

lics of cocoa (as well as those of other plant species) have been reported in many studies as bio-active compounds (antioxidant, antiradical, anticarcinogenic properties) (Ren, Qiao, Wang, Zhu, & Zhang, 2003; Sanbongi et al., 1998; Wollgast & Anklam, 2000b). Also the anti-microbial properties of cocoa phenolics against some food bacterial pathogens as well as against some cariogenic bacteria (Osawa et al., 1990) were previously shown. The anti-microbial activity is directly correlated to the property to penetrate the bacterial cell wall (Arlorio, Coisson, Turri, & Martelli, 2000; Osawa et al., 1990). The in vivo bio-activity of cocoa phenolics (as well as phenolics from other foods like coffee and vegetables) was well studied. This activity is strictly

\* Corresponding author. Tel.: +39 321689848; fax: +39 321689821.  
E-mail address: [arlorio@pharm.unipmn.it](mailto:arlorio@pharm.unipmn.it) (M. Arlorio).

correlated with their absorption and metabolism (Shahidi & Naczki, 2003). In 1994, the estimated per head consumption of cocoa/chocolate and chocolate confectionery in European Union ranged from 1.3 (Portugal) to 8.8 kg/year (Germany). Chocolate (particularly dark chocolate) can be seen as a relevant source for phenolic antioxidants. About bioavailability of polyphenols, above all flavonols, reliable data on the real content of polyphenols in food are still scarce. Recently, the flavonoids intake in Dutch diet (based on five flavonols and flavonons) was calculated as 23 mg/day. This estimation didn't include catechins and proanthocyanidins. Anyway, the bioavailability of flavonols (as well as all phenolics) is still largely discussed. For example, independently of the doses of chocolate and cacao ingested, only 0.5% of catechin was recovered in the free unbound form in the plasma and in the urine (Wollgast & Anklam, 2000b).

Phenolic compounds of cacao (*T. cacao* L.) belong to many classes of molecules: catechins, epicatechins, anthocyanins, pro-anthocyanidins, phenolic acids, condensed tannins, other flavonoids and some minor compounds (Sánchez-Rabeneda et al., 2003; Wollgast & Anklam, 2000a).

(-)-Epicatechin is quantitatively the main compound of cocoa phenolics (approximately 35% of polyphenol content of unfermented Forastero cocoa beans). Total soluble phenolics of good fermented dried cocoa beans ranges from 2 to 6%, strictly depending on the variety as well as the geographic origin. Forastero typical content is about 6%; soluble phenolics content in Criollo cacao is about 2/3 of Forastero. Major content are often an index of bad fermentation. The fermentation step involves some changes in phenolic content of cocoa nibs: a strong decrease of total soluble phenolic content and the polymerisation of some constituent (above all (-)-epicatechin with one other (-)-epicatechin or with anthocyanidins, to form high molecular weight tannins) occurs (Shahidi & Naczki, 2003). Free low molecular weight polyphenols still present in chocolate are responsible of astringent and bitter taste. Some polyphenolic compounds are clearly involved in colour development of *T. cacao* beans, as well as other molecular classes (mainly proteins and reducing sugars involved in Maillard reactions) that act during the fermentation step and during the roasting process. "Cocoa red" is the common name of the typical pigment colour of "good fermented cocoa beans", obtained by mean of different kind of natural microbial fermentation. So, roasting process involves some changes in cacao nibs colour; also in this case some inter-variety difference exist (different phenolics and different natural colorant chemotypes).

The antioxidant properties of cocoa were largely studied during last years by mean of different approaches: chemical characterization of involved anti-

oxidant species (HPLC, HPLC-MS) (Adamson et al., 1999), in vitro chemical studies (in order to study the ability to scavenging some stable radicals like DPPH<sup>•</sup>, ABTS<sup>•+</sup>) (Hatano et al., 2002), in vitro biological studies and nutrigenomic-based studies (bioavailability, in vivo interaction with cellular/molecular species) (Motohashi et al., 1999). Some bio-active properties of cocoa are strictly related to phenolic content as well as to some compounds from the Maillard reactions (non-enzymatic brown pigments).

Cocoa hulls are the principal by-product for cocoa industries, commonly used as secondary source of theobromine, caffeine and cocoa lipids (often not considered as "cocoa butter", because of their high acidity and high unsaponifiable content). Cocoa hulls are part of the cocoa bean, separated from the cotyledons together with the germ during pre-roasting process or after the roasting process of *T. cacao* (de-hulling/de-husking step).

Only few studies on *T. cacao* husks and hulls are developed (Martin-Cabrejas, Valiente, Esteban, Molla, & Waldron, 1994). Recently, because of their high content in pectin soluble fiber, a novel use of this by-product has been suggested (Arlorio, Coisson, Restani, & Martelli, 2001). Supercritical CO<sub>2</sub> is a powerful mild technology useful to extract and fractionate without the use of organic solvent. CO<sub>2</sub> in supercritical conditions was previously used to extract from cocoa nibs theobromine, lipids as well as some aromatic compounds. The studies about the extraction of phenolics from hulls by mean of supercritical CO<sub>2</sub> are still lacking.

Ischemia-induced neuronal degeneration is a good model to evaluate the in vitro activity of phenolic bio-active molecules. In fact, dopamine has a role in the ischemic damage; the dopamine released by damaged cells could be oxidized by non-enzymatic or enzymatic systems; both processes generate free radicals. Moreover, the dopamine released induces the glutamate release; also glutamate could be the reason of the secondary production of free radical species. A drug (cabergoline, an ergot-related compounds, a dopamine D2 receptor agonist) as well as an antioxidant bio-active molecule (vitamin E) both showed a protective antioxidant activity in an in vitro model, based on the use of human neuroblastoma cells (SH-SY5Y) (Miglio et al., 2004). This in vitro approach is a useful method to assess the bioactivity of antioxidant molecular species.

Aims of this work were (i) to apply the SFE (Supercritical Fluid Extraction) using CO<sub>2</sub> to extract some pigmented phenolic fractions from cocoa hulls, (ii) to characterize their composition by HPLC and (iii) to evaluate the anti-oxidative/protective effect using an in vitro model able to simulate the cellular ischemia.

## 2. Materials and method

### 2.1. Samples and chemical analyses

*T. cacao* L. (Sterculiaceae) hulls (obtained by pre-roasted beans) were kindly obtained from Ferrero SpA (Alba, Italy). Cocoa hulls were grinded and sieved before the analysis; the powders obtained were directly used for the chemical characterisation as well as for the in vitro studies. Cocoa hulls were chemically characterised (total nitrogen content, % proteins, fats content, total dietary fiber, total phenolic content, ashes and moisture). The methods and protocols used for these analyses are reported in Arlorio et al. (2001).

### 2.2. SFE conditions

A pilot-plant for supercritical CO<sub>2</sub> extraction was employed; the conditions of extraction (from 50 g of cocoa hulls) are reported in Table 2, as well as the relative phenolic fractions recovered in different solvents (C1–C5). The supercritical extraction did not require the use of organic *entrainers*. The same table reports the solvents used to dissolve the extract after the CO<sub>2</sub> fractionation.

### 2.3. Antiradical/antioxidant activity

Antiradical/antioxidant property was evaluated by mean of the scavenging of the DPPH<sub>•</sub> radical, as described by Brand-Williams, Cuvelier, and Berset (1995). The antioxidant properties were expressed as IP (inhibition percentage; %); the IP of DPPH radical was calculated as:  $IP = (Absorbance_{t=0 \text{ min}} - Absorbance_{t=15 \text{ min}}) / Absorbance_{t=0 \text{ min}} * 100$ .

### 2.4. HPLC analysis

The analyses of methanolic extract from cocoa hulls as well as all extracts obtained by supercritical CO<sub>2</sub>, were performed as described in a previous work, using a Shimadzu Class VP 5 HPLC system (Coïsson et al., 2003).

### 2.5. Biological activity test

Human neuroblastoma cells (SH-SY5Y), differentiated into a neuronal phenotype using retinoic acid (10 μM) for 15 days, were exposed to ischemic damage by mean of deprivation of oxygen and glucose for 5 h (oxygen glucose deprivation–OGD-phase) and then re-oxygenated (20 h). All the extracts were evaporated under vacuum (RVC 2-18–Christ, GMBH) and re-dissolved in 500 μl of pure ethanol (1 μl/ml). After filtration (0.45 μm syringe filter) a dilution (1:1000) was prepared using the culture buffer (glucose-free, oxygen-glucose

deprivation buffer: NaCl 154 mM, KCl 5.6 mM, HEPES 5.0 mM, NaHCO<sub>3</sub> 3.6 mM, CaCl<sub>2</sub> 2.3 mM, pH 7.4, bubbled with an anaerobic gas mixture of 95% N<sub>2</sub>, 5% CO<sub>2</sub> for at least 2 h before use) for each sample. Ethanol diluted with buffer, used as positive–negative control, did not show effect in all tests (data not showed).

During all the OGD phases, the cells were exposed to cocoa hulls extracts (C1–C5) obtained by mean of supercritical CO<sub>2</sub> extraction as well as to some control molecules with antioxidant/protective properties, in standard conditions fixed in previously works (α-tocopherol: 50 μM; cabergoline: 10 μM). The contact between cocoa hulls extracts and cells was maintained throughout reoxygenation phase.

The cellular vitality (expressed as % of viable cells) was measured after the re-oxygenation step, using a colorimetric method (MTT), described by Mosmann (1983).

## 3. Results and discussion

The proximate composition (average of three different analyses on different batch samples) of the cocoa hulls is reported in Table 1. As previously reported, the main hulls component is fiber, principally composed by insoluble fiber and also containing pectin characterized by with high methylation degree. A mean of 1.8% of total phenolic compounds was detected from the hulls by means of Folin Ciocalteu's method (Arlorio et al., 2001).

The evaluation of total antioxidant activity of the “whole” phenolic extract from cocoa hulls, obtained with methanol using Soxhlet apparatus, was then performed using DPPH<sub>•</sub> method. The scavenging properties of the methanolic extract of phenolic pigments from cocoa hulls are shown in Table 2. A methanolic extract (1:500 diluted in methanol) from cocoa hulls showed an inhibition percent (IP%) similar to those obtained with BHA (butyl-hydroxy-anisole) 10<sup>-5</sup>M (66.06 and 54.08 for hulls and BHA, respectively). The highest radical scavenging activity has been showed by the

Table 1  
Proximate composition of cocoa hulls (g kg<sup>-1</sup> ± SD, dry weight)

	Composition
Fat	68.1 ± 2.5
Nitrogen	29.0 ± 1.3
Proteins (N × 6.25)	181.2 ± 8.1
Ashes	81 ± 3.9
Fiber	606 ± 6.4
Phenolics (Folin-Ciocalteu's method)	18.2 ± 8.4
Phytic acid (spectrophotometric method)	5.9 ± 0.6
Moisture	101.2 ± 6.0
Theobromine (HPLC)	12.9 ± 1.8

Table 2  
Antiradical activity measured with DPPH<sup>•</sup> method (IP%)

Samples and reference antioxidants	IP (%)
Trolox ( $10^{-3}$ M)	97.90
Caffeic acid ( $10^{-3}$ M)	97.70
BHA ( $10^{-5}$ M)	54.08
Cocoa bean (methanolic extract, Arriba) 1:500	85.58
Cocoa bean (methanolic extract, Ivory Coast) 1:500	80.14
Cocoa bean (methanolic extract, Ghana) 1:500	96.55
Cocoa hulls (methanolic extract) 1:500	66.06
C1 (SFE Conditions: CO <sub>2</sub> 100 bar, 50 °C) (dissolved in methanol)	33.33
C2 (SFE Conditions: CO <sub>2</sub> 150 bar, 50 °C) (dissolved in methanol)	71.71
C3 (SFE Conditions: CO <sub>2</sub> 150 bar, 50 °C) (dissolved in acetone)	40.40
C4 (SFE Conditions: CO <sub>2</sub> 200 bar, 50 °C) (dissolved in methanol)	69.60
C5 (SFE Conditions: CO <sub>2</sub> 200 bar, 50 °C) (dissolved in acetone) dil. 1:200	2.50

methanolic extracts obtained from whole fermented cocoa beans diluted 1:500 (96.55 and 85.58 for Ghana and Arriba cacao, respectively; Table 2).

The phenolic composition of the methanolic hulls extract was determined in previous studies (Azizah, Nik Ruslawati, & Swee Tee, 1999; Coisson et al., 2003). All phenolic compounds identified in hulls methanolic extract were described as antioxidant or antiradical molecules; this fact confirms the behaviour of the whole cocoa hulls extract underlined in our in vitro study. The major components in hulls are epicatechin and p-hydroxybenzoic acid (2753 and 2252 ppm, respectively). The HPLC method used in this study showed the presence of some minor “unknown” compounds; we suppose the presence of some anthocyanins and proanthocyanidins. Also these compounds are reported as strong in vitro antioxidant (Adamson et al., 1999). Further studies (HPLC-MS characterization of each phenolic extract) are actually in the course of development.

All fractions obtained by supercritical CO<sub>2</sub> showed a light contamination with lipids; the optimisation of the extraction conditions could be fundamental to limit this co-extraction phenomenon. Nevertheless, the co-extractions of some fats from hulls could be avoided by mean of the previous fat extraction from the matrix. Concerning this point, we have selected the used parameters (50 °C at 100, 150 and 200 bar) considering all works published in this topic, with the aim to limit the co-extraction of lipids (Li & Hartland, 1992; Li & Hartland, 1996; Rossi, Arnoldi, Savioni, & Schiraldi, 1989; Saldaña, Mohamed, & Mazzafera, 2002; Saldaña, Zetzl, Mohamed, & Brunner, 2002). The phenolic extracts fractionated by supercritical CO<sub>2</sub> were analysed by HPLC; contrary to our expectation, all chromatographic profiles did not show the presence of phenolic acids as well as of flavonoid. Only caffeine and some

low-retained high-hydrophilic unidentified peaks were present (data not shown). Caffeine concentration ranged from 0.014 to 0.031 mg/ml (C1: 0.014 mg/ml; C2: 0.031 mg/ml; C4: 0.015 mg/ml). The evaluation of the antioxidant-antiradical activity of the extract fractionated by supercritical CO<sub>2</sub> was then measured by mean of an in vitro model optimised for the evaluation of the protection from ischemic damage, as reported in materials and method section. This test showed that fraction C3 and C5 extracted by CO<sub>2</sub> (150 bar, 200 Bar) and re-dissolved in acetone, exhibited the highest biological activity. The fractions C1, C2 and C4 (rich in caffeine content) did not show positive biological activity, but they have promoted ischemic damage (Fig. 1). In fact, only caffeine-free samples (C3 and C5), showed a protective action from ischemia damage in our in vitro model. The protective effect of C3 and C5 fractions was similar to those obtained by cabergoline (10 µm) and vitamin E (50 µm).

We suppose that some oligomeric polyphenol as procyanidins (as well as some not-polymerized anthocyanins) could be the bio-active molecules involved in damage protection. As recently described (Hatano et al., 2002), pro-anthocyanidin glycosides of cacao show inhibitory effects on nicotinamide adenine dinucleotide phosphate-dependent lipid peroxidation in microsomes and on the auto-oxidation of linoleic acid. These actions were attributed to the high radical scavenging activity in the peroxidation chain reaction. Another hypothesis could be the presence of clovamide, a strong antioxidant compounds recently described in *T. cacao*. Even if the literature recently reported the protective action of caffeine from stroke as well as other ischemic problems (Aronowski, Strong, Shirzadi, & Grotta, 2003), above all if in combination with ethanol (caffeinol), our results

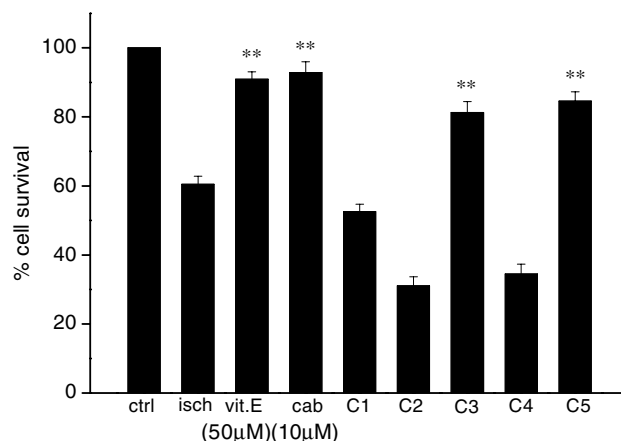


Fig. 1. Effect of protection from ischemic oxidative damage with cocoa hulls extract and some reference antioxidants (\*\*  $P \leq 0.01$ ). Legend: ctrl: control cells; isch: ischemic cells; Vit. E:  $\alpha$ -tocopherol; Cab: cabergoline; C1–C5: fractionated pigment extracts.

confirmed the protective action of the “unknown” substance extracted from hulls.

Our results established these extracts as novel important antioxidant “solvent-free” matrices in food (i.e., dietary, nutraceutical, flavouring) according to other results previously obtained from the use of whole cocoa hulls methanolic extract. The protective bioactivity of these fractions, namely C3 and C5, should be an interesting result about the study of oxidative damage related to the ischemic processes. For this reason, we suggest the performing further analysis (HPLC-MS) to confirm/exclude the presence of other class of phenolic in traces (namely pro-anthocyanidins and free phenolics) with antioxidant properties.

Finally, cocoa hulls phenolic-rich extracts are very interesting bio-active compounds with peculiar colorant and functional properties.

#### 4. Conclusion

The supercritical CO<sub>2</sub> extraction is a powerful technique to obtain some pigmented phenolic fractions from cocoa hulls with bio-active antioxidant and antiradical properties. The use of an in vitro evaluation-test to assess the antioxidant activity of these phenolics from cocoa hulls was optimised. The use of SH-SY5Y cells for the in vitro evaluation of antioxidant properties of cocoa hulls extracts is useful to assess the protection against the OGD damage.

We suggest the better characterization of the bioactivity of phenolic pigments from cocoa hulls for their safe use in food technology as functional colorant ingredients or antioxidant complex extract.

#### Acknowledgements

This work was funded by Università degli Studi del Piemonte Orientale “A. Avogadro”, Italy (FAR funds).

We are grateful to Ferrero SpA (Alba, Italy) to have kindly supplied all cocoa beans and cacao hulls samples.

#### References

- Adamson, G. E., Lazarus, S. A., Mitchell, A. E., Prior, R. L., Cao, G., Jacobs, P. H., et al. (1999). HPLC method for the quantification of procyanidins in cocoa and chocolate samples and correlation to total antioxidant capacity. *Journal of Agricultural and Food Chemistry*, *47*, 4184–4188.
- Arlorio, M., Coisson, J. D., Restani, P., & Martelli, A. (2001). Characterization of pectins and some secondary compounds from *Theobroma cacao* hulls. *Journal of Food Science*, *66*, 653–656.
- Arlorio, M., Coisson, J. D., Turri, A., & Martelli, A. (2000). Effetto anti-batterico di estratti fenolici in *Theobroma cacao*. *La Rivista di Scienza dell’Alimentazione*, *30*(suppl 3), 251–260.
- Aronowski, J., Strong, R., Shirzadi, A., & Grotta, J. C. (2003). Ethanol plus caffeine (caffeinol) for treatment of ischemic stroke: preclinical experience. *Stroke*, *34*(5), 1246–1251.
- Azizah, A. H., Nik Ruslawati, N. M., & Swee Tee, T. (1999). Extraction and characterization of antioxidant from cocoa by-products. *Food Chemistry*, *64*, 199–202.
- Brand-Williams, W., Cuvelier, M. E., & Berset, C. (1995). Use of a radical method to evaluate antioxidant activity. *Lebensm.-Wiss. u.-Technol.*, *28*, 25–30.
- Coisson, J. D., Capasso, M., Travaglia, F., Piana, G., Arlorio, M., & Martelli, A., (2003). Proprietà antiossidanti di estratti fenolici da sottoprodotti di lavorazione di cacao e nocciola. In *Proceedings of 5th National Congress of Food Chemistry* (pp. 154–158). Parma, Morgan Edizioni tecniche, Milano.
- Hatano, T., Miyatake, H., Natsume, M., Osakabe, N., Takizawa, T., Ito, H., et al. (2002). Proanthocyanidin glycosides and related polyphenols from cacao liquor and their antioxidant effects. *Phytochemistry*, *59*, 749–758.
- Li, S., & Hartland, S. (1992). Influence of co-solvents on solubility and selectivity in extraction of xantines and cocoa butter from cocoa beans with supercritical CO<sub>2</sub>. *Journal of Supercritical Fluids*, *5*, 7–12.
- Li, S., & Hartland, S. (1996). A new industrial process for extracting cocoa butter and xantines with supercritical carbon dioxide. *JAOCS*, *73*, 423–429.
- Martin-Cabrejas, M. A., Valiente, C., Esteban, R. M., Molla, E., & Waldron, K. (1994). Cocoa hull: a potential source of dietary fibre. *Journal of the Science of Food and Agriculture*, *66*, 307–311.
- Miglio, G., Varsaldi, F., Francioli, E., Battaglia, A., Canonico, P. L., & Lombardi, G. (2004). Cabergoline protects SH-SY5Y neuronal cells in an in vitro model of ischemia. *European Journal of Pharmacology*, *489*, 157–165.
- Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of Immunological Methods*, *65*, 55–63.
- Motohashi, N., Kawase, M., Kurihara, T., Shirataki, Y., Kamata, K., Nakashima, H., et al. (1999). Relationship between radical intensity and biological activity of cacao husks extracts. *Anticancer Research*, *19*, 1125–1130.
- Osawa, K., Matsumoto, T., Maruyama, T., Naito, Y., Okuda, K., & Takazoe, I. (1990). Inhibitory effects of aqueous extract of cacao bean husk on collagenase of *Bacteroides gingivalis*. *Bulletin of Tokyo dental college*, *31*, 125–128.
- Ren, W., Qiao, Z., Wang, H., Zhu, L., & Zhang, L. (2003). Flavonoids: promising anticancer agents. *Medical Research Review*, *23*, 519–534.
- Rossi, M., Arnoldi, C., Savioni, G., & Schiraldi, A. (1989). Characterization of cocoa extracts obtained with supercritical carbon dioxide. *Italian Journal of Food Science*, *3*, 41–50.
- Saldaña, M. D. A., Mohamed, R. S., & Mazzafera, P. (2002). Extraction of cocoa butter from Brazilian cocoa beans using supercritical CO<sub>2</sub> and ethane. *Fluid Phase Equilibria*, *194–197*, 885–894.
- Saldaña, M. D. A., Zetzl, C., Mohamed, R. S., & Brunner, G. (2002). Extraction of methylxanthines from guaraná seeds, maté leaves, and cocoa beans using supercritical carbon dioxide and ethanol. *Journal of Agricultural and Food Chemistry*, *50*(17), 4820–4826.
- Sanbongi, C., Osakabe, N., Natsume, M., Takizawa, T., Gomi, S., & Osawa, T. (1998). Antioxidative polyphenols isolated from *Theobroma cacao*. *Journal of Agricultural and Food Chemistry*, *46*, 454–457.

- Sánchez-Rababeda, F., Jàuregui, O., Casals, I., Andrès-Lacueva, C., Izquierdo-Pulido, M., & Lamuel Raventòs, R. M. (2003). Liquid chromatographic/electrospray ionization tandem mass spectrometric study of the phenolic composition of cocoa (*Theobroma cacao*). *Journal of Mass Spectrometry*, *38*, 35–42.
- Shahidi, F., & Naczk, M. (2003). *Phenolics in food and nutraceuticals*. Boca Raton, FL, USA: CRC Press.
- Wollgast, J., & Anklam, E. (2000a). 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.
- Wollgast, J., & Anklam, E. (2000b). Polyphenols in chocolate: is there a contribution to human health? *Food Research International*, *33*, 449–459.