ELSEVIER

Food Chemistry 77 (2002) 337–341

Food Chemistry

www.elsevier.com/locate/foodchem

Tocopherol, fatty acids and sterol distributions in wild Ecuadorian *Theobroma subincanum* (Sterculiaceae) seeds

R. Bruni[a], A. Medici[a], A. Guerrini[a], S. Scalia[b], F. Poli[c], C. Romagnoli[d], M. Muzzoli[e], G. Sacchetti[e,*]

[a]*Department of Chemistry, University of Ferrara, Via Luigi Borsari 46, I-44100 Ferrara, Italy*
[b]*Department of Pharmaceutical Sciences, University of Ferrara, Via Fossato di Mortara 17-19, I-44100 Ferrara, Italy*
[c]*Department of Evolutionary and Experimental Biology, University of Bologna, Via Irnerio 42, I-40126 Bologna, Italy*
[d]*Department of Animal Biology, Section of Botanical Garden, University of Modena and Reggio Emilia, v.le Caduti in Guerra 127, I-41100 Modena, Italy*
[e]*Department of Biology, Section of Botany, Pharmaceutical Biology Lab., University of Ferrara, C.so Porta Mare 2, I-44100 Ferrara, Italy*

Received 19 June 2001; received in revised form 15 October 2001; accepted 15 October 2001

Abstract

Ecuadorian *Theobroma subincanum* (Sterculiaceae) seed parts were analyzed to determine quali-quantitative tocopherol distribution. Fatty acids and sterols in the embryos, teguments and endosperm were also evaluated with an aim to better-characterize the plant matrix for its potential use as a source of phytochemicals, for the cocoa butter processing industry and/or as a new source of low cost natural products in the cosmetic, drug and alimentary/nutraceutical industries. HPLC for tocopherols and gas-chromatography (GC), GC–mass spectrometry (MS) analyses for fatty acids profile (FAP) and phytosterols were performed. Tocopherols were particularly abundant in the embryo, with quali-quantitative data similar to wheat germ oil whereas, in the teguments and endosperm, the concentrations of tocopherols were lower. The fatty acid profile and phytosterol characterization of the seed parts showed qualitative homogeneous data. In the endosperm, 80% of the entire FAP consisted of oleic and stearic acid while, among sterols, cycloartenol was more abundant in endosperm than in embryos and teguments. Accordingly, *T. subincanum* seeds can be proposed as possible substitutes in the cocoa processing industry and as a potential source of vitamin E isomers.

Keywords: *Theobroma subincanum*; Sterculiaceae; Tocopherols; Fatty acids profile; Phytosterols

1. Introduction

With the main cultivars of *Theobroma cacao* threatened by the aging of plantations, diseases and parasites (Hunter, 1990; Willson, 1999), there is a growing demand for research on wild species or varieties belonging to the *Theobroma* genus. Those species could have naturally developed resistance against etiological agents and, at the same time, could provide cocoa butter substitutes (Bruni, Bianchini, Bettarello, & Sacchetti, 2000). Moreover, they might provide low-cost renewable resources of high value-added compounds, such as tocopherols and phytosterols.

Theobroma subincanum (Sterculiaceae) is a small subtropical tree growing in the forests of central and south America and its seeds are used by natives for food and fodder (Martinez, 1996; Villacres, 1995). A general analytical comparison between *T. cacao* and *T. subincanum* seeds has already been reported, with emphasis on fatty acid profile, triacylglycerol and phytosterol composition and purinic alkaloid content (Bruni et al., 2000; Carpenter, Hammerstone, Romanczyk, & Aikten, 1994; Hammerstone, Romanczyk, & Aitken, 1994; Marx & Maia, 1991).

Morphologically, *T. subincanum* seeds are characterized by a bigger size, with woody and resistant teguments thicker than those of *T. cacao*. This characteristic could represent a hindrance during the industrial squeezing process, interfering with their effective industrial exploitation. The whole seed or its parts (embryo, endosperm, teguments), like many other lipid rich plant matrices, can be evaluated as low-priced renewable resources of high value-added compounds, such as

* Corresponding author. Fax: +39-0532-208561.
E-mail address: scg@dns.unife.it (G. Sacchetti).

0308-8146/02/$ - see front matter
PII: S0308-8146(01)00357-0

phytosterols and tocopherols. In this paper we report a complete description of the distribution of tocopherols, fatty acids and sterols in the *T. subincanum* seed parts (embryos, teguments, endosperm). The goal is to evaluate and characterize the real potential of the whole seed or its parts as a complementary source of phytochemicals in the cocoa processing industry or as a cocoa substitute in the cosmetic, drug and alimentary/nutraceutical industries.

2. Materials and methods

2.1. Plant material

Theobroma subincanum fruits were harvested on August 2000 on the outskirts of Wasak'entsa reserve in eastern Ecuador (77°.15″ W/2°.35″ S), from trees that the natives call "w*akam*" and identified by applying the wild species identification indications of Villacres (1995) and Martinez (1996). A sample of *T. subincanum* was deposited in the *Herbarium Universitatis Ferrariensis* of the Department of Biology, Section of Botany, University of Ferrara (code 002018 3G1).

The seeds were separated from the rest of the fruit and then manually divided into three parts, splitting the teguments from the rest of the seed and then splitting the embryo from endosperm. All the teguments, embryos and endosperm obtained from the seeds were ground in a blade grinder (Fritsch, Idar-Oberstein, Germany) to pass a 0.2 mm mesh. The powder obtained was stored in the dark, at −20 °C in an inert atmosphere.

2.2. Samples preparation

Ground dry samples (5 g of teguments and endosperm, 1 g of embryos) were suspended in 50 ml of organic solvent (methanol for tocopherols, *n*-hexane for fatty acids and sterols) and extracted for 20 min in an ultrasonic bath (Branson 5200, Danbury, CT, USA). The mixture was then filtered, rinsed with 5 ml of solvent, evaporated under vacuum and collected in a freezer. For tocopherols extraction, the methanolic fractions were processed by a second liquid–liquid extraction with 10 ml of *n*-hexane before being dried under vacuum and stored in the dark at −20 °C.

2.3. Tocopherol analysis: HPLC apparatus and conditions

HPLC analyses were performed using a modular Jasco HPLC unit (Tokyo, Japan) which consisted of a PU-980 pump, a LG-1580-02 ternary gradient unit, a DG-980-503-line degasser, and a UV/vis 975 detector set at an excitation wavelength of 295 nm, linked to an injection valve with a 20 μl sampler loop. A Lichrosorb silica gel Si 60 (5 μm and 25×0.4 cm; Teknokroma, Barcelona, Spain) column was used and the mobile phase was 0.05% isopropanol/hexane at a flow rate of 1 ml/min. The injection volume was 80 μl. All solvents used were of chromatographic grade. Chromatograms were recorded and α-, β-, γ-, δ-tocopherol peaks from samples were identified by comparing their spectra with those of pure standards (Matreya Inc., Pleasant Gap, PA 16823, USA). The peak areas were determined by integration using dedicated Borwin software (Borwin ver. 1.22, JMBS Developments, Grenoble, France). For each extract, quali-quantitative analysis was performed in triplicate.

2.4. Fatty acid profile (FAP): GC apparatus and conditions

The FAP was obtained via gas-chromatographic analyses of the fatty acid methyl esters (FAME). The FAME were prepared by transmethylation using sodium methoxide in the presence of methyl acetate, following the method of Christie (1982). Analyses were performed by employing a Fisons 9160 9000 Series GC equipped with a Fisons EL980 amplifier, a FID detector and a Mega SE52 column (Mega, Legnano, Italy—i.d. = 0.32 mm, length 25 m, film thickness = 0.15 μm). Operating conditions were as follows: injection temperature, 350 °C; detector temperature, 350 °C; split ratio 1:40; carrier gas, helium, at a flow rate of 2 ml/min. Oven temperature was initially 150 °C and then raised to 250 °C at a rate of 5 °C/min, followed by 5 min at 250 °C. One microlitre of each sample was injected. The fatty acid standards were obtained from Alltech (Deerfield, IL, USA).

2.5. Unsaponifiable analysis

Samples of teguments, embryos and endosperm of *T. subincanum* seeds were saponified. Forty milligrammes of each sample were well mixed in 5 ml 1 M methanolic KOH, with the aid of sonication, and then horizontally shaken at 30 °C for 24 h. The unsaponifiable content was then extracted three times by adding 1 ml H_2O, 2 ml *n*-hexane and 0.1 ml ethanol. The aqueous fractions were then collected and extracted with the same mixture as earlier. All unsaponifiable fractions were finally collected and taken to dryness under vacuum.

Table 1
Extraction yelds (%) of *Theobroma subincanum* seed parts

Samples	*n*-Hexane[a]	Methanol/*n*-hexane[b]
Teguments	3.62± 0.47	0.57±0.07
Embryos	8.67±1.13	3.71±0.48
Endosperm	7.24±0.94	4.29±0.56

The results are the average of three determinations ± standard deviation.
[a] Solvent employed for fatty acid and sterol extractions.
[b] Solvents employed for tocopherol extractions.

Table 2
Tocopherols content (mg/100 g) of *Theobroma subincanum* seed parts

Samples	α-Tocopherol	β-Tocopherol	γ-Tocopherol	δ-Tocopherol	Total tocopherols
Teguments	54.7±7.15	8.2±1.07	43.82±5.71	10.0±1.31	117±15.2
Embryos	32.2±4.20	4.84±0.63	175±22.8	9.46±1.23	222±28.9
Endosperm	2.21±0.28	1.21±0.16	38.54±5.02	5.34±0.69	47.30±6.18

The results are the average of three determinations±standard deviation.

Table 3
Fatty acid profile (%) of *Theobroma subincanum* seeds parts

Samples	Palmitic	Linoleic	Oleic	Stearic	Arachidic	Behenic	Lignoceric	Squalene
Teguments	8.89±1.16	26.3±3.43	34.6±4.5	27.2±3.55	1.5±0.19	0.78±0.12	Traces	0.71±0.11
Embryos	15.7±2.04	22.9±2.99	32.2±4.19	24.3±3.16	2.74±0.36	1.13±0.18	1.07±0.17	Traces
Endosperm	7.49±0.97	3.19±0.42	46.4±6.04	40.7±5.30	1.31±0.21	–	–	–

The results are the average of three determinations±standard deviation.

Table 4
Unsaponifiable fraction (%) of *Theobroma subincanum* seed parts

Samples	Squalene	Campesterol	Stigmasterol	Sitosterol	Δ5-Avenasterol	Cycloartenol	24-Methylene-cycloartanol
Teguments	14.0±2.19	10.7±1.67	15.9±2.38	56.7±8.87	–	2.75±0.43	–
Embryos	4.4±0.68	10.0±1.57	6.53±1.02	71.7±11.2	–	7.36±1.15	–
Endosperm	2.58±0.40	4.03±0.63	12.2±1.90	46.1±7.21	2.87±0.45	29.7±4.65	2.59±0.41

The results are the average of three determinations±standard deviation.

The residue was then silanized for gas chromatography (GC)-analyses with 2 ml of silanizing mixture (pyridine:hexamethyldisilazane:trimethylclorosilane 5:2:1). After 1 h, the samples were dried under a nitrogen stream at 70 °C and dissolved with 0.3 ml of *n*-hexane. Before the recovery of the supernatant, the samples were sonicated for 5 min and centrifuged for 10 min at 1800 ***g***. The GC apparatus conditions were the same as reported earlier except for oven temperature which was initially 230 °C and then raised at 320 °C at a rate of 5 °C/min.

2.6. GC–mass spectroscopy analysis

GC–MS analysis was performed on a Fisons 8060 8000 series GC equipped with a MEGA SE54 column (Mega, Legnano, Italy—i.d. = 0.32 mm, length 25 m, film thickness = 0.15 μm) and coupled to a Fisons HD800 mass spectrometer with a MassLab data system. Helium was used as a carrier gas at an inlet pressure of 30 kPa. The injector temperature was 300 °C and the samples were injected under the same conditions as reported earlier for GC analyses. The mass spectra were recorded at an electron energy of 70 eV and the ion source temperature was 300 °C. Qualitative analysis was based on a comparison of the retention times and of the mass spectra with the corresponding data in the literature (Carpenter et al., 1994; Dutta & Normen, 1998). Mass spectra were also compared with those of mass spectra libraries.

3. Results and discussion

Recently, the cosmetic, drug and alimentary/nutraceutical industries have focussed attention on low-cost renewable resources, rich in lipid-related compounds such as tocols and phytosterols. One of the main goals of industry is to identify plant matrices rich in antioxidant compounds, for the preparation of new phyto-derived products. Strict legislation about the use of synthetic food additives, such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), (mainly because of their known carcinogenic activity, and consumer attitudes) has shifted the attention of manufacturers from synthetic to natural antioxidants (Amarowicz, Naczk, & Shahidi, 2000; Dapkevicius, Venskutonis, van Beek, & Linssen, 1998). Tocopherols (vitamin E isomers) are well known natural antioxidants and their presence in oilseeds is often correlated with a relative abundance of unsaturated fatty acids.

The research on wild species or varieties belonging to the *Theobroma* genus is mainly directed toward those plants which have naturally developed an effective resistance to etiological agents and which could offer—at the same time—substitutes for the cocoa processing industry. In fact, the aging of the plantations, and the continuous strong selections aimed at satisfying the increasing demand, have determined a progressively lower capacity of the main cultivars of *T. cacao* to resist deseases and parasites (Hunter, 1990; Willson, 1999).

Table 5
IE mass spectra for *Theobroma subincanum* sterol–TMS ether derivatives

Sterol–TMS ether derivative	*m/z* (relative intensity)
Campesterol–TMS ether	M+ 472 (5.2), 457 (5.3), 382 (12.2), 367 (12.4), 343 (35.2), 261 (8.8), 255 (11.9), 213 (12.6), 159 (15.3), 147 (25.4), 145 (28.3), 129 (100), 107 (31.1)
Stigmasterol–TMS ether	M+ 484 (5.7), 469 (3.2), 394 (12.5), 379 (8.8), 355 (13.1), 255 (32.4), 213 (18.6), 173 (13.9), 159 (28.1), 147 (34.3), 145 (33.9), 129 (100), 107 (32.2), 105 (30.5)
β-Sitosterol–TMS ether	M+ 486 (5.4), 471 (5.3), 396 (15.8), 381 (13.9), 357 (30.1), 275 (8.8), 255 (11.3), 213 (11.5), 159 (18.2), 145 (28.6), 129 (100), 107 (26.7), 105 (24.3)
Cycloartenol–TMS ether	M+ 498 (0.0) 483 (4.9), 408 (13.3), 393 (49.8), 365 (32.6), 339 (32.1), 175 (42.1), 173 (29.7), 159 (29.6), 149 (37.5), 135 (100), 121 (85.2), 107 (82.4), 105 (81.9)
24-Methylene-cycloartanol–TMS ether	M+ 512 (0.0) 422 (8.8), 407 (24.3), 379 (16.7), 353 (11.0), 203 (21.2), 175 (35.9), 159 (30.2), 147 (56.5), 135 (100), 121 (62.3), 107 (73.4)

EI, electron ionization; TMS, trimethylsilyl.

T. subincanum endosperm, teguments and embryos were therefore processed to tocopherols extraction (Table 1) and then the extracts subjected to HPLC-normal phase analyses. Overall results were interesting. In fact, the total tocopherol content was higher in embryos (Table 2) and the values were similar to those reported for wheat germ oil, known to be the main industrial source of vitamin E isomers (Beliz & Grosch, 1999). Gamma-tocopherol was the predominant isomer (79%) with values quantitatively triple those generally reported for corn and soya oils, which are known—among commercial oils—to contain higher amounts of this compound. The α-tocopherol content, although being minor, was comparable with that of cottonseed oil. In the oil extracted from the endosperm, an analogous distribution emerged, although with lower amounts. Overall, the oil obtained from the endosperm showed a content of tocopherols, similar to edible oils known as industrial sources of these compounds, such as palm and rapeseed oil (Beliz & Grosch, 1999).

In the teguments, where the total tocopherol content was approximately half of that detected in the embryos (and only lower than wheat germ oil), the distribution of single tocopherols showed a different pattern. In fact, α-tocopherol represented 46% of the total and was, together with γ-tocopherol, the prevalent vitamin E isomer.

The different distribution found for tocopherols in the analyzed seed parts can be correlated with different fatty acid profiles (Table 3). In fact, whereas teguments and embryos had considerable amounts of linoleic acid, the presence of this essential fatty acid was actually lower than in the endosperm. In the oil obtained from the teguments and embryos, the fatty acid profile was uniform, with oleic, stearic, linoleic and palmitic acids being prevalent. In the endosperm, however, oleic and stearic acids constituted approximately 80% of the entire FAP, showing the same pattern reported for other edible oils (Beliz & Grosch, 1999). In the teguments and embryos, very low amounts of behenic and lignoceric acids—which instead were not detected in the endosperm—were found.

Within the total transmethylated lipid fraction, through a single GC analysis, it was possible to detect small amounts of squalene in the teguments as the unsaponifiable fraction of the oil (Table 4). The characterization of the unsaponifiable fraction did not show any remarkable differences among the different seed parts analyzed. In fact, sitosterol was always the main phytosterol and a lower percentage of other sterols—generally reported to be present in *Theobroma* genus—such as stigmasterol and campesterol, was present (Carpenter et al., 1994).

Particularly relevant was the high percentage of cycloartenol (about 30%) detected in the endosperm. This type of evidence underlines the differentiation between *T. subincanum* and other species belonging to *Theobroma* genus. In fact, a similar abundance of this compound might be considered as characteristic of the chemotype analyzed or might be linked to its role as a precursor in biosynthetic sterol pathways. 24-Methylene-cycloartanol has been also detected in the endosperm while it was absent in the embryos and in the teguments. A GC–MS analysis was also performed on the entire unsaponifiable fraction (Table 5).

According to the described results and to those already reported for this plant matrix (Bruni et al., 2000), *T. subincanum* seeds can therefore be proposed for several uses. The high tocopherol content, in particular in the embryos and similar to that of commercial wheat germ oil, suggests the exploitation of *T. subincanum* seeds or their parts as a low cost renewable source of vitamin E isomers for industrial processing in the fields of cosmetics, drugs and nutraceuticals. Moreover, even if the teguments of *T. subincanum* are woody and more resistant than those of *T. cacao*, this would not compromise the quality of the possible *T. subincanum* products derived from industrial processing of the whole seed, the reason being the qualitative homogeneous fatty acid and phytosterol compositions of *T. subincanum* teguments, embryos and endosperm. In addition, the recently opened commercialization of cocoa substitutes for the cocoa-processing industry, by

European legislation, suggests a good exploitation potentiality of this Ecuadorian plant matrix.

Acknowledgements

The authors wish to thank the Ministero dell'Istruzione, dell'Università e della Ricerca Scientifica e Tecnologica (MIUR-URST) of Italy. Especial thanks go to Orna Crinion for the English suggestions on writing the manuscript.

References

Amarowicz, R., Naczk, M., & Shahidi, F. (2000). Antioxidant activity of crude tannins of canola and rapeseed hulls. *Journal of the American Oil Chemists' Society*, *77*, 957–961.

Beliz, H., & Grosch, W. (1999). *Food Chemistry* (2nd ed.). Berlin: Springer-Verlag.

Bruni, R., Bianchini, E., Bettarello, L., & Sacchetti, G. (2000). Lipid composition of wild Ecuadorian *Theobroma subincanum* Mart. seeds and comparison with two varieties of *Theobroma cacao* L. *Journal of Agricultural and Food Chemistry*, *48*, 691–694.

Carpenter, D. R., Hammerstone, J. F. Jr., Romanczyk, L. J. Jr., & Aikten, W. M. (1994). Lipid composition of *Herrania* and *Theobroma* seeds. *Journal of the American Oil Chemists' Society*, *71*, 845–851.

Christie, W. W. (1982). A simple procedure for rapid transmethylation of glycerolipids and cholesteryl esters. *Journal of Lipid Research*, *23*, 1072–1075.

Dapkevicius, A., Venskutonis, R., van Beek, T. A., & Linssen, J. P. H. (1998). Antioxidant activity of extracts obtained by different isolation procedure from some aromatic herbs grown in Lithuania. *Journal of the Science of Food and Agriculture*, *77*, 140–146.

Dutta, P. C., & Normen, L. (1998). Capillary column gas-liquid chromatographic separation of Δ5-unsaturated and saturated phytosterols. *Journal of Chromatography*, *A816*, 177–184.

Hammerstone, J. F. Jr., Romanczyk, L. J. Jr., & Aitken, W. M. (1994). Purine alkaloid distribution within *Herrania* and *Theobroma*. *Phytochemistry*, *35*, 1237–1240.

Hunter, J. R. (1990). The status of Cacao (*Theobroma cacao*, Sterculiaceae) in the Western Hemisphere. *Economic Botany*, *44*, 425–439.

Martinez, C. C. (1996). *Etno Botanica del Ecuador*. Quito, Ecuador: Abya Yala.

Marx, F., & Maia, J. G. S. (1991). Purine alkaloids in seeds of *Theobroma* species from the Amazon. *Zeitschrift für Lebensmittel-Untersuchung und -Forschung*, *193*, 460–461.

Villacres, O. V. (1995). *Bioactividad de plantas amazonicas*. Quito, Ecuador: Abya Yala.

Willson, K. C. (1999). *Coffee, cocoa and tea*. New York: CABI Publishing.