Tetrahydro-β-carbolines, Potential Neuroactive Alkaloids, in Chocolate and Cocoa

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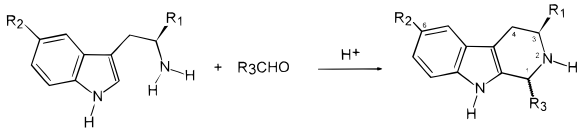
Tetrahydro-β-carbolines (THβCs), potential neuroactive alkaloids, were found in chocolate and cocoa. 6-Hydroxy-1-methyl-1,2,3,4-tetrahydro-β-carboline (6OHMTHβC), 1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (THCA), 1-methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (MTCA) in both diastereoisomers (1*S*,3*S* and 1*R*,3*S*), and 1-methyl-1,2,3,4-tetrahydro-β-carboline (MTHβC), besides serotonin and tryptamine biogenic amines, were identified and quantified in dark chocolate, milk chocolate, cocoa, and chocolate-containing cereals by RP−HPLC−fluorescence and HPLC−MS. For each THβC, the concentration ranges were determined: 6OHMTHβC (0.16−3.92 μg/g), THCA (0.01−0.85 μg/g), 1*S*,3*S*-MTCA (0.35−2 μg/g), 1*R*,3*S*-MTCA (0.14−0.88 μg/g), and MTHβC (nd-0.21 μg/g). The highest content was generally found in chocolates and cocoas, but cereals containing chocolate also showed an appreciable amount of THβCs. The possible biological implications of this novel group of alkaloids in chocolate are discussed.

Keywords: *Chocolate; cocoa; alkaloid; tetrahydro-β-carboline; β-carboline; biogenic amine*

INTRODUCTION

Tetrahydro-β-carbolines (THβCs) (1,2,3,4-tetrahydro-9H-pyrido[3,4-b]indole) are naturally occurring indole alkaloids produced from indoleamines and aldehydes and/or alpha-ketoacids through Pictet−Spengler condensation (Figure 1). Tetrahydro-β-carbolines and β-carbolines have attracted the attention of neurochemists who have pointed out their occurrence under physiological conditions in biological tissues and fluids (Buckholz, 1980; Airaksinen and Kari, 1981; Melchior and Collins, 1982; Rommelspacher et al., 1991; Adachi et al., 1991; Brossi, 1993; Callaway et al., 1994). This has encouraged speculation on their putative role in the central nervous system where they could function as neuromodulators. THβCs inhibit the monoamine oxidase and the monoamine uptake, and bind to the benzodiazepine receptor (Buckholtz, 1980; Airaksinen and Kari, 1981; Braestrup et al., 1980; Melchior and Collins, 1982; Myers, 1989; Rommelspacher et al., 1991; Cox and Cook, 1995). Simultaneously, tetrahydro-β-carbolines have been increasingly studied in relation with alcoholism where they might play a role in the etiology or addiction of alcoholism, or in pathological states (Myers, 1989; Rommelspacher et al., 1991; Adachi et al., 1993; Adell and Myers, 1994). On the other hand, some β-carbolines are comutagens or precursors of mutagens (Wakabayashi et al., 1983; De Meester, 1995, Higashimoto et al., 1996), can cause neuronal cell death in vitro (Brenneman et al., 1993), and can be bioactivated, giving rise to endogenous neurotoxins (Albores et al., 1990; Matsubara et al., 1998).

Because tetrahydro-β-carboline alkaloids exhibit strong biological actions, their availability during food consumption is an interesting matter. Several tetrahydro-β-carbolines are present in food and alcoholic drinks, and this means that at least part of the tetrahydro-β-carbolines in biological tissues surely arise from dietary sources (Herraiz et al., 1993; Herraiz, 1996−2000, Herraiz and Sanchez, 1997; Sen et al., 1995; Tsuchiya et al., 1996b; Gustche and Herderich, 1997).



R_1	R_2	R_3	Name
COOH	H	H	THCA
COOH	H	CH_3	MTCA (1*S*,3*S*; 1*R*,3*S*)
H	OH	CH_3	6OHMTHBC
H	H	CH_3	MTHBC

Figure 1. Pictet-Spengler condensation between indoleamines and aldehydes to give tetrahydro-β-carbolines in chocolate and cocoa.

Chocolate is a popular food, enjoyed largely for its great sensory properties. One of the most pleasant effects of eating chocolate is the "good feeling" that many people experience. However, chocolate craving is still incompletely understood (Rozin et al., 1991) and active research has attempted to find possible unknown bioactive substances that might be involved in any physiological behavior responsible for cravings (Di Tomaso et al., 1996). Chocolate contains several biologically active constituents (methylxanthines, biogenic amines, and cannabinoid-like substances), all of which potentially cause abnormal behavior and psychological sensation that parallel those of other addictive substances (Bruinsma and Taren, 1999). In this regard, this paper reports the identification and occurrence of novel THβC neuroactive alkaloids in chocolates and cocoas, and briefly discusses their origin and possible biological implications.

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MATERIALS AND METHODS

Reference Compounds and Samples. 1-Methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (MTCA) was purchased from Sigma Chemical Co. (St. Louis, MO) and synthesized from L-tryptophan and acetaldehyde, giving rise to two diastereoisomers (1*S*,3*S*, major compound and 1*R*,3*S*, minor compound) (Brossi et al., 1973; Herraiz and Ough, 1993). 1,2,3,4-Tetrahydro-β-carboline-3-carboxylic acid (THCA) and 1-ethyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (ETCA) were synthesized from L-tryptophan, and formaldehyde and propionaldehyde, respectively (Herraiz, 1997). 6-Hydroxy-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (6OHMTHβC) was synthesized from serotonin oxalate (Sigma) and acetaldehyde, whereas 1-methyl-1,2,3,4-tetrahydro-β-carboline (MTHβC) was synthesized from tryptamine (Sigma) and acetaldehyde by a Pictet−Spengler condensation. Data of NMR, MS, and GC−MS (trifluoroacetyl and methoxycarbonyl methyl ester derivatives) were consistent with the structures of the synthesized compounds (Herraiz and Ough, 1994; Herraiz, 1997, Herraiz and Sanchez, 1997).

Commercial samples of dark chocolate (50−85% cocoa), milk chocolate (up to 30% cocoa), commercial cocoa powders, and chocolate breakfast cereals were purchased in local supermarkets and subsequently analyzed for THβCs as indicated below.

Isolation of Tetrahydro-β-carbolines. THβCs in chocolate products and cocoas were isolated by using a SCX−solid-phase extraction method (Adachi et al., 1991; Herraiz et al., 1993). Aliquots of 2−5 g were homogenized in 0.6 M $HClO_4$ (15−20 mL) containing 1 mg/mL of semicarbazide and centrifuged (5000*g*, 10−15 min, 0−5 °C). An aliquot of supernatant was spiked with 0.5 mL of ETCA solution (5 mg/L) used as internal standard (IS), and loaded onto benzenesulfonic acid-derivatized silica SCX columns (Bond Elut, 3 mL size, Varian, Harbor City, CA). After they were washed with 6 mL of 0.1 N HCl, 2 mL of methanol, and 6 mL of HPLC water, and rinsed with 2 mL of 0.4M phosphate buffer (pH 9.1), the THβCs were eluted with 4 mL of methanol with 0.4 M phosphate buffer pH 9.1 (1:1). The eluates were injected into the HPLC.

Chromatographic and Quantitative Analysis. Chromatographic analysis was performed using a 1050 high-performance liquid chromatograph (HPLC) with a 1046A fluorescence detector and a 3365-Series II HP Chemstation (Hewlett-Packard, Santa Clara, CA). A 150 mm × 3.9 mm, 5μ, Novapak C18 column (Waters) was used for HPLC separation. Fluorescence detection was carried out at 270 nm for excitation and 343 nm for emission. Eluents were 50 mM ammonium phosphate buffer adjusted to pH 3 with phosphoric acid (Eluent A) and 20% of A in acetonitrile (Eluent B). Two gradients were used; first, 0% B to 32% B in 8 min, then 90% B at 18 min, and 100% B at 20 min; and second, 0% B to 75% B in 60 min. The flow rate was 1 mL/min, the oven temperature was 40 °C, and the injection volume was 20 μL.

Quantitative analysis of THβCs in chocolate and cocoas was calculated from calibration curves obtained with standard solutions of known concentration against ETCA used as an internal standard, and carried through the entire isolation procedure. For quantitation of MTCA, the same response factor of MTCA (Sigma) was used for its two diastereoisomers. The SCX method has already shown a good reliability (Herraiz, 1996). Recoveries were 98% (MTCA), 95% (THCA), 92.3% (6OHMTHβC), 68% (MTHβC), 93.4% (serotonin), and 81.6% (tryptamine). Analysis of a sample of cocoa ($n = 4$) gave the following relative standard deviation (RSD), in percent: 0.66 (MTCA), 4.10 (THCA), 0.9 (6OHMTHβC), 4.8 (MTHβC), 2.7 (serotonin), and 7.4 (tryptamine). In addition to using semicarbazide to avoid artifacts, blank and control samples spiked with standards of serotonin, tryptamine and tryptophan (5 and 20 mg/L) did not give any significant artifacts of THβCs after analysis. Also, several samples of cocoa and chocolate that were homogenized, filtered, and injected into the HPLC without any sample preparation appear to give peaks of THβCs.

The identity of THβCs was confirmed by HPLC retention time and co-injection with authentic standards. Also, the excitation and emission spectra of the HPLC peaks were compared with those of THβCs standards to check peak purity. To achieve that, eluting peaks were trapped into the fluorescence-detector flow cell by stopping the solvent pump, and excitation and emission spectra were recorded.

Chemical Identification by HPLC−MS. Samples of chocolate and cocoa (25 g) were homogenized with 0.6 M $HClO_4$ containing semicarbazide (60 mg/mL), centrifuged (12 000 rpm, 15 min, 0 °C), and SCX-extracted as described above. The eluting fractions corresponding to phosphate buffer and methanol (1:1) were evaporated under vacuum (less than 40 °C) and redissolved in the same buffer prior to HPLC−MS. Chemical identification was accomplished by HPLC−MS on a 3.9 mm × 150 mm Novapak C18 column (Waters), by using an HPLC−MSD series 1100 (Hewlett-Packard) (electrospray, positive-ion mode). Conditions: eluents A, formic acid (0.5%); B, 0.5% formic acid in acetonitrile; 60% B in 60 min; flow, 0.7 mL/min; cone voltage, 50 V; mass range, 50−600 amu.

Oxidation of THβC-3-COOH and Chromatographic Analysis. The samples isolated from SCX were injected into the HPLC and the peaks corresponding to THβC-3-COOH (THCA and MTCA) were collected. They were treated with an $Na_2Cr_2O_7$ solution (80 °C for 1 h), then basified, and extracted with CH_2Cl_2. Under these conditions THβC-3-COOHs are oxidized to their corresponding β-carbolines (Herraiz, 2000). The organic solvent was evaporated under a He stream, redissolved in 0.1M HCl, and injected into RP−HPLC under the same chromatographic conditions as above except for detection (excitation 245 nm, emission 445 nm). β-carbolines were trapped into the detection cell and the spectral characterization accomplished as for THβC-3-COOHs.

RESULTS

THβC alkaloids, along with their respective amino acid and amine precursors (i.e., L-tryptophan, serotonin, and tryptamine), were successfully separated by RP−HPLC (Figure 2a). The possible existence of those alkaloids in chocolate and cocoa was studied in SCX-extracts which provided chromatographic peaks coeluting with 6-hydroxy-1-methyl-1,2,3,4-tetrahydro-β-carboline (6OHMTHβC), 1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (THCA), 1-methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (MTCA) in both diastereoisomers, and 1-methyl-1,2,3,4-tetrahydro-β-carboline (MTHβC) (Figure 2b). THβCs isolated from chocolate and cocoas gave patterns of fluorescence spectra in good agreement with those afforded by standards. This is illustrated in Figure 3 for 6OHMTHβC and 1*S*,3*S*-MTCA. When the corresponding THβCs-3-COOH (i.e., THCA and MTCA) were collected at the end of the fluorescence detector, and subsequently oxidized to check the formation of β-carbolines according to Herraiz (2000), it was recognized that THCA provided norharman, and MTCA (both diastereoisomers, 1*S*,3*S* and 1*R*,3*S*) provided harman as expected (results not shown). All this evidenced the presence of THβCs in chocolate products. A further unequivocal proof was obtained by HPLC−MS. Extracted samples of chocolate and cocoas gave trace ions and mass spectra corresponding to THβCs (Figure 4). The protonated molecular ions were obtained at *m*/*z* 203 for 6OHMTHβC, *m*/*z* 217 for THCA, *m*/*z* 231 for the diastereoisomers 1*S*,3*S*-MTCA and 1*R*,3*S*-MTCA, and *m*/*z* 187 for MTHβC. It also showed the occurrence of the biogenic amines serotonin (*m*/*z* 177 and 160) and tryptamine (*m*/*z* 161 and 144), as well as L-tryptophan (*m*/*z* 205 and 188). Those compounds also coeluted with the corresponding standards under the same conditions.

Table 1 lists the amount of THβC alkaloids in commercial chocolates, cocoas, and cereals. The major

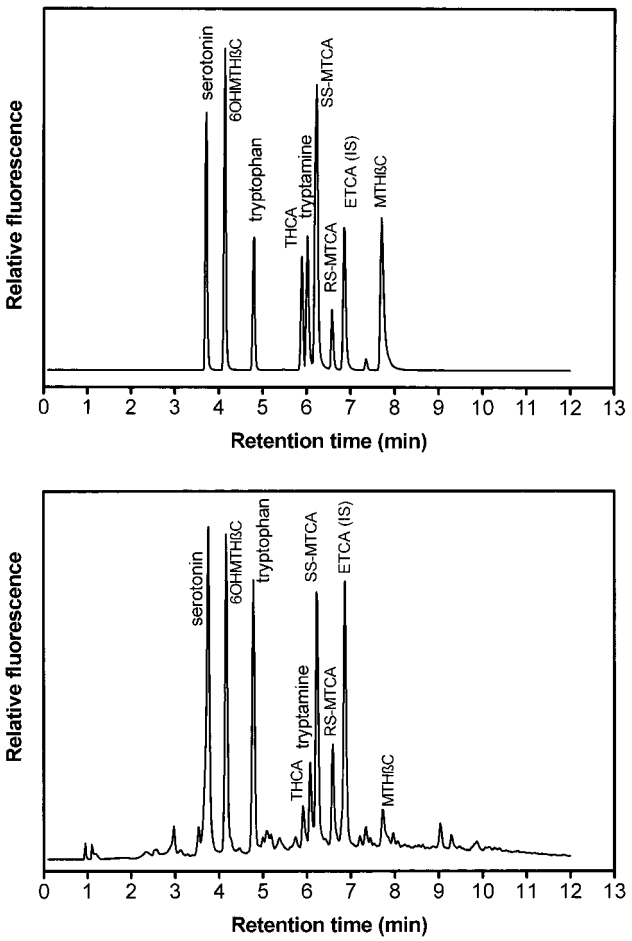

Figure 2. RP−HPLC−fluorescence analysis of tetrahydro-β-carbolines and biogenic amines. (a) (top) Reference compounds; (b) (bottom) SCX extract from dark chocolate. Chromatographic conditions: Novapak C18 column (Waters); eluent A, 50 mM phosphate buffer (pH 3); eluent B, 20% A in acetonitrile; 32% B in 8 min, and 90% B in 18 min; 40 °C. Fluorescence: 270 nm excitation and 343 nm emission.

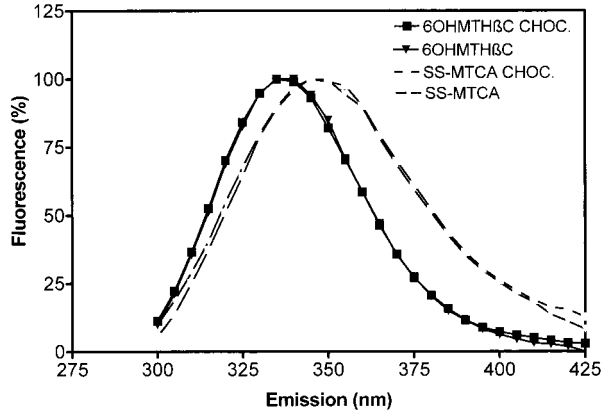

Figure 3. Pattern of fluorescence emission spectra (excitation, 270 nm) of THβC chromatographic peaks trapped into the flow-cell of the fluorescence detector. 6-Hydroxy-1-methyl-1,2,3,4-tetrahydro-β-carboline (6OHMTHβC) and 1*S*,3*S*-1-methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (SS-MTCA) both from reference compounds and those isolated from chocolate.

compounds were 6OHMTHβC and MTCA in both diastereoisomers (1*S*,3*S* and 1*R*,3*S*), with concentrations ranging from 0.16 to 3.92 μg/g (6OHMTHβC), 0.35 to 2 μg/g (*SS*-MTCA), and 0.14 to 0.88 μg/g (*RS*-MTCA) within the samples analyzed. THCA and MTHβC ranged from 0.01 to 0.85 μg/g and not detected to 0.21 μg/g, respectively. The content of THβCs (6OHMTHβC, MTCA, and MTHβC) was generally higher in dark chocolate than in commercial cocoa powders, milk chocolate, and cereals containing chocolate. This seemed to be correlated with the total content of cocoa in the samples. However, the content of THCA was generally higher in cereals containing chocolate than in the rest of the samples. Table 1 also lists the content of the two biogenic amines serotonin and tryptamine. Serotonin, which is the presumable precursor of 6OHMTHβC after condensation with acetaldehyde, averaged 2.9, 0.58, 1.25, and 0.095 μg/g for chocolates, milk chocolates, cocoas, and cereals containing chocolate. Tryptamine, a presumable precursor of MTHβC averaged 0.83, 0.16, 0.69, and 0.04 μg/g for the same products, respectively.

DISCUSSION

To the best of my knowledge this is the first report on THβC alkaloids in chocolate. Four compounds were identified and subsequently quantified: 6-hydroxy-1-methyl-1,2,3,4-tetrahydro-β-carboline (6OHMTHβC), 1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (THCA), 1-methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (1*S*,3*S* and 1*R*,3*S* diasteroisomers) (MTCA), and 1-methyl-1,2,3,4-tetrahydro-β-carboline (MTHβC). Chocolates, cocoas, and chocolate-containing cereals contained varying amounts of each compound ranging from undetectable to several μg/g. Within a report of an improved chromatographic method of carbolines, Tsuchiya et al. (1996a) analyzed MTHβC in a sample of cacao and reported a higher concentration than those found here. This discrepancy is probably due to differences in the samples. We have previously reported that several foods and fermented alcoholic beverages contain appreciable amounts of two of those THβCs found in chocolates, THCA and MTCA, reaching up to several mg/kg (Herraiz et al., 1993, Herraiz, 1996−2000). Interestingly, the concentration of THCA and MTCA in chocolate and cocoa is comparable to that of alcoholic beverages such as wine, beer, and liquor, which contain a relatively high amount of those compounds. The origin of these tetrahydro-β-carbolines is a reaction involving L-tryptophan and aldehydes that are present or otherwise released during the processing of foods and beverages. Its chemical formation depends on the amount of precursors, storage time, pH, temperature, and processing conditions (Herraiz and Ough, 1993; Herraiz, 1996). The same reaction is likely to occur in chocolates that suffer a fermentation from cacao beans and heating processes. Then, it is expected that serotonin, L-tryptophan, and tryptamine afford the corresponding THβCs (6OHMTHβC, MTCA, and MTHβC) through a Pictet−Spengler condensation with acetaldehyde (see Figure 1).

The biological significance of tetrahydro-β-carbolines and β-carbolines is related to their potential pharmacological actions on the nervous system, playing a role as neuromodulators via effects on monoamine oxidase (MAO), biogenic amine (serotonin) uptake/release, and benzodiazepine receptor binding. Then, these compounds exogenously supplied, or hypothetically produced in vivo, might become bioactive, exhibiting behavioral and/or toxicological implications. In this regard, it is very likely that part of the β-carbolines found in the human tissues and fluids have a dietary origin. Although the concentration of THβCs in foods is usually

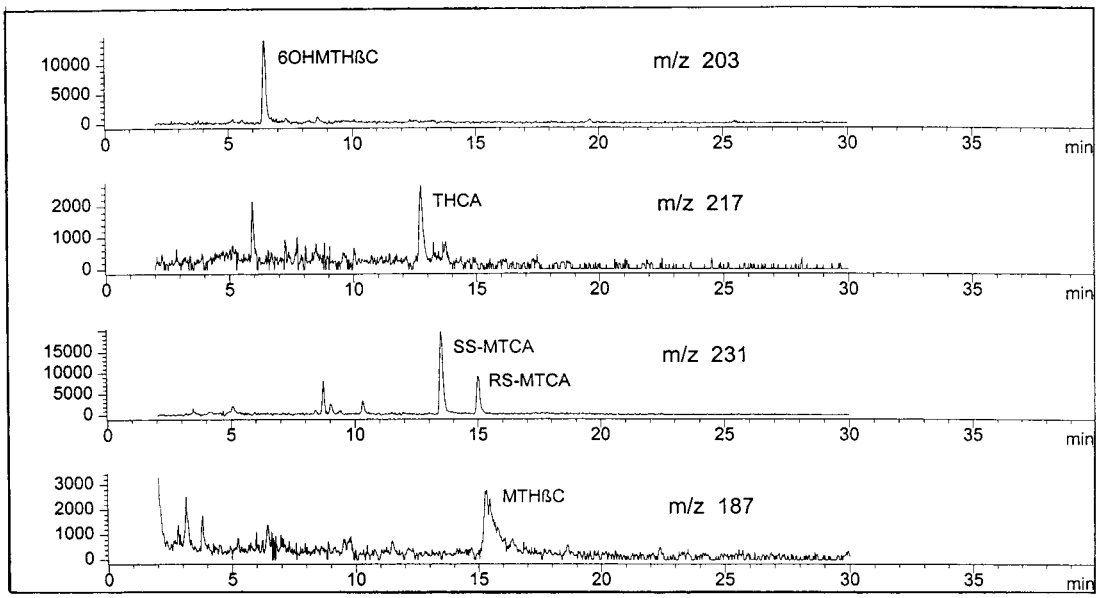

Figure 4. Reconstructed ion chromatograms of an extract of chocolate analyzed by HPLC−MS (electrospray). Chromatographic conditions: Novapak C18 column; eluent A, 0.5% HCOOH; eluent B, 0.5% HCOOH in acetonitrile; 60 min 60% B; 40 °C.

Table 1. Concentration (μg/g) of Serotonin, Tryptamine, and Tetrahydro-β-carboline Alkaloids in Chocolates, Cocoas, and Chocolate-Containing Cereal Products

	dark chocolate ($n = 10$)			milk chocolate ($n = 4$)			cocoa powder ($n = 8$)			chocolate cereals ($n = 6$)		
	x	SD	range	x	SD	range	x	SD	range	x	SD	range
serotonin	2.90	1.55	1.37−5.08	0.586	0.34	0.13−0.87	1.25	1.02	0.4−3.3	0.095	0.06	nd-0.18
tryptamine	0.83	0.33	0.2−1.16	0.16	0.08	0.05−0.23	0.69	0.45	0.068−1.33	0.04	0.018	nd-0.07
6OHMTHβC	2.64	0.88	1.46−3.92	0.54	0.10	0.43−0.68	1.42	0.57	0.72−2.3	0.28	0.095	0.16−0.39
THCA	0.34	0.12	0.23−0.68	0.10	0.027	0.07−0.13	0.18	0.10	0.012−0.38	0.48	0.31	0.06−0.85
SS-MTCA	1.74	0.24	1.37−2.0	0.512	0.05	0.47−0.59	1.16	0.30	0.76−1.55	0.46	0.12	0.35−0.65
RS-MTCA	0.69	0.11	0.53−0.88	0.195	0.03	0.18−0.25	0.46	0.13	0.3−0.66	0.22	0.07	0.14−0.27
MTHβC	0.13	0.064	0.05−0.21	0.056	0.036	nd-0.10	0.071	0.02	0.05−0.11	0.014	0.016	nd-0.03

not excessively high, the successive ingestion of these compounds during the diet would surely increase the THβCs in the body.

Chocolate is usually described as a craved food, and although the hedonic appeal of chocolate (fat, sugar, texture, and aroma) is likely to be the predominant factor, it also has been pointed out that it may possibly contain potential pharmacologically active substances responsible for the craving (Bruinsma and Taren, 1999). In this regard, several compounds have been considered, such as phenylethylamine, methylxantins, and the recently reported anandamide (Di Tomaso et al., 1996). THβCs are a novel group of potential pharmacologically active substances in chocolate. Despite their supposed relative low concentration (i.e. an average of 30 g/person/day consumption of dark chocolate would account for an ingestion of up to 0.21 mg/person/day of total THβCs), the presence of THβCs exhibiting potential bioactive or neuroactive properties could play a role in craving, and this hypothesis deserves further attention. However, against this assumption are both the relatively low levels of these alkaloids in chocolate, and also their presence in many other foods including fruit products (Herraiz, 1996, 1998). Also, because THβCs and related β-carbolines are mild inhibitors of MAO, then, they could hypothetically potentiate the effect of amines in chocolate (among them phenylethylamine, tryptamine, serotonin, and others) (Baker et al., 1987). On the other hand, the existence of a relationship between alcohol consumption and tetrahydro-β-carbolines has deserved much attention in the past (Myers, 1989). Lankford and Myers (1994) reported that the consumption of alcohol by rats is substantially lowered by chocolate drinks. Interestingly, this paper reports now the same tetahydro-β-carbolines in chocolate as in some alcoholic beverages (Herraiz et al., 1993, Herraiz, 1996).

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