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Analysis of tetrahydro-β-carboline-3-carboxylic acids in foods by solid-phase extraction and reversed-phase high-performance liquid chromatography combined with fluorescence detection

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Abstract

The presence and analysis of two tetrahydro-β-carboline-3-carboxylic acids in foods are studied. Sample preparation with benzenesulfonic acid strong cation-exchange columns followed by RP-HPLC–fluorescence allowed a reliable analysis and spectral characterization of 1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (THCA) and 1-methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (MTCA). Experimental data showed that upon oxidation tetrahydro-β-carboline-3-carboxylic acids gave rise to β-carbolines (norharman and harman) that were also chromatographically separated and their fluorescent profile monitored. This approach was useful to confirm identification of tetrahydro-β-carboline-3-carboxylic acids in foods. Several foods and beverages contained THCA and MTCA in varying proportions. Their occurrence in foods implies that diet is a source of these compounds in humans.

Keywords: Food analysis; Beverages; Tetrahydrocarboline; Tetrahydrocarbolinecarboxylic acids; Carbolines; Organic acids

1. Introduction

1,2,3,4-Tetrahydro-β-carbolines (THβCs) are naturally occurring tricyclic indole derivatives produced from indoleethylamines and aldehydes and/or α-ketoacids through Pictet–Spengler condensation [1]. 1,2,3,4-Tetrahydro-β-carboline-3-carboxylic acids (THβC-3-COOHs) are produced similarly by reaction of tryptophan and aldehydes (Fig. 1). This reaction readily occurs under mild conditions and is temperature and pH dependent [2]. Research in the last decades has pointed out the occurrence of THβCs and β-carbolines under physiological conditions in biological tissues and fluids [3–6]. Their possible role as neuromodulators [3,7], implications in alcoholism [4,5,8,9] and inhibition of benzodiazepine receptor [10] have been studied. Others have proposed and investigated THβCs as endogenous neurotoxins [11–13], or have described them as precursors of mutagenic *N*-nitroso compounds [14]. In the last few years, we and others have reported the widespread occurrence of THβC-3-COOHs in commercial foods and beverages [2,6,15–19,23]. Obviously, this suggests the ingestion of these compounds during food consumption that could be partially responsible for their further endogenous presence in tissues and physiological fluids.

In the analysis of tetrahydro-β-carboline-carboxylic acids several approaches have been used, including gas chromatography–mass spectrometry (GC–MS) [20–22,25], high-performance liquid chromatography (HPLC) [2,6,15,16], and HPLC–MS [6,23,24]. Reversed-phase (RP) HPLC with fluorescence detection is a sensitive and selective method with the advantage of avoiding sample derivatization

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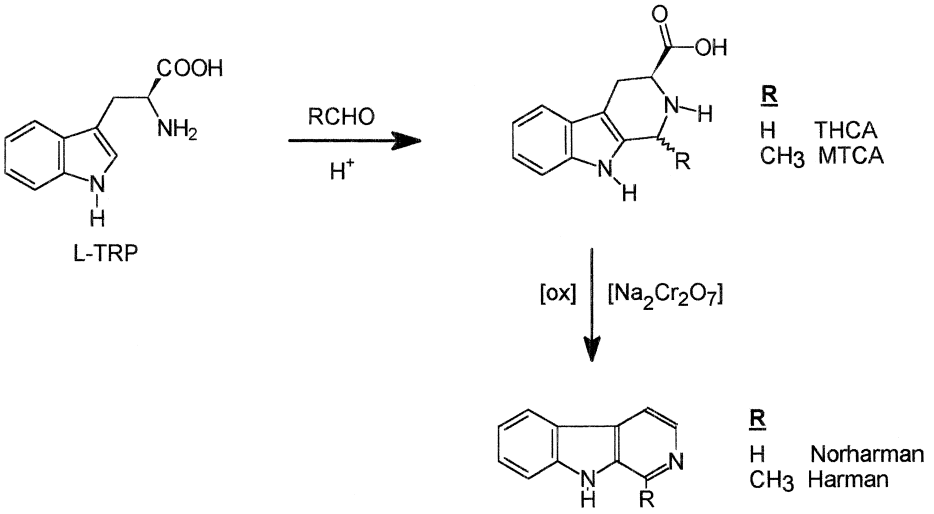

Fig. 1. Pictet–Spengler reaction between L-tryptophan and acetaldehyde or formaldehyde to give 1,2,3,4-tetrahydro-β-carboline-3-carboxylic acids (MTCA and THCA). Chemical oxidation of MTCA and THCA gives rise to β-carbolines norharman and harman.

and solvent extraction. This paper summarizes the presence of THβC-3-COOHs in several foods and beverages through sample preparation with strong cation-exchange (SCX) columns followed by HPLC–fluorescence detection, and provides original data on the fluorescent spectra of the corresponding HPLC peaks (both reduced and oxidized compounds) as a selective monitoring of THβC-3-COOHs in foods.

2. Experimental

2.1. Reference compounds and food samples

1-Methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (MTCA) was purchased from Sigma (St. Louis, MO, USA). It mainly contains the diastereoisomer (−)-(1*S*,3*S*)-1-methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (1*S*,3*S*-MTCA) [6,16,25]. The diastereosimeric mixture of MTCA, (1*S*,3*S*)-1-methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (1*S*,3*S*-MTCA), and (−)-(1*R*,3*S*)-1-methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (1*R*,3*S*-MTCA) was also synthesized from L-tryptophan (Sigma) and acetaldehyde (Aldrich) [26]. 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 or propionaldehyde, respectively [26]. Nuclear magnetic resonance (NMR), MS and GC–MS (trifluoroacetyl and methoxycarbonyl methyl ester derivatives) data were consistent with the structures of the synthesized compounds [21,22,25].

A few commercial samples of foods (see Table 1) were purchased in a local supermarket and analyzed for THβC-3-COOHs as indicated below.

2.2. Solid-phase extraction (SCX) of THβC-3-COOHs

THβC-3-COOHs in foods and drinks were isolated using SCX solid-phase extraction [2,6,16,17]. Aliquots of 5–10 ml of wine, wine vinegar, beer, centrifuged juices, sauces and diluted soy sauce (1:15) were spiked with 0.5 ml ETCA solution (5 mg/l) used as internal standard (I.S.), and semicarbazide (Sigma) added at 1 mg/ml to prevent artifactual formation [20]. Similarly, 5–10 ml aliquots of centrifuged smoked fish or toasted bread homogenates in 0.6 *M* $HClO_4$ containing 1 mg/ml of semicarbazide were spiked with ETCA (5 mg/l). Those samples with the highest pH (such as wine, beer, juices or sauces) were acidified to pH 2 with 1 *M* HCl. The samples were loaded onto benzenesul-

Table 1
THCA and MTCA (1*S*,3*S*; 1*R*,3*S*) in foods[a] by solid-phase extraction and HPLC–fluorescence detection

Sample	THCA (mg/l)	*SS*-MTCA (mg/l)	*RS*-MTCA (mg/l)
Wine a	0.024	3.59	1.10
Wine b	0.02	3.96	1.17
Beer a	0.24	1.54	0.48
Beer b	0.08	0.41	0.12
Wine vinegar a	0.03	7.0	2.05
Wine vinegar b	0.04	6.5	1.78
Orange juice a	0.06	2.46	0.72
Orange juice b	0.06	2.73	0.87
Grape must a	0.02	0.56	0.175
Grape must b	0.02	1.2	0.34
Soy sauce	7.1	72	20
Sauce[b]	0.74	5.58	1.62
Toasted bread a[b]	0.8	0.06	–
Toasted bread b[b]	0.46	0.08	–
Smoked fish[b]	0.4	–	–

[a] Extensive studies on occurrence and origin are reported elsewhere [15–17].
[b] Amounts in μg/g.

fonic acid-derivatized silica SCX columns (Bond Elut, 3 ml size, Varian, Harbor City, CA, USA) previously conditioned with 6 ml of methanol and 6 ml of 0.1 *M* HCl, and passed at a flow-rate of 1 ml/min using a vacuum manifold. Then, the columns were immediately washed with 6 ml of 0.1 *M* HCl, 2 ml of methanol, and 6 ml of HPLC water and rinsed carefully with 2 ml of 0.4 *M* phosphate buffer, pH 9.1. THβC-3-COOHs were eluted with a 4 ml mixture containing methanol–0.4 *M* phosphate buffer, pH 9.1 (1:1). The eluates were injected into the HPLC system.

2.3. Chromatographic and spectral analysis of THβC-3-COOHs

The analysis of THβC-3-COOHs by RP-HPLC and fluorescence detection was performed using a 150 mm×3.9 mm, 5 μm, Nova-Pak C_{18} column (Waters, Milford, MA, USA) in a 1050 high-performance liquid chromatograph coupled to a 1046 variable-wavelength fluorescence detector and a 3365-Series II HP Chemstation. Chromatographic conditions were as follows: 50 m*M* ammonium phosphate buffer adjusted to pH 3 (buffer A) and 20% of buffer A in acetonitrile (buffer B); 0–32% buffer B in 8 min, then 90% B at 18 min; flow-rate 1 ml/min; injection volume 20 μl, and column temperature 40°C. Fluorescent detection wavelengths were excitation 270 nm and emission 343 nm.

Calibration curves used for quantitation were constructed with THCA and MTCA standards of known concentration that were carried out through the entire clean-up procedure. Blanks and control samples did not give artifacts during isolation and analysis. Confirmation of the identity of isolated THβC-3-COOHs was established by HPLC retention times and coelution with authentic standards. Also, fluorescence spectra of the HPLC peaks were compared with those of reference compounds. For this, eluting peaks corresponding to THβC-3-COOHs were trapped into the flow cell of the fluorescence detector by stopping the solvent pump, and the excitation and emission spectra monitored.

2.4. Oxidation of THβC-3-COOHs and chromatographic analysis

The samples isolated from SCX as above were injected into the HPLC system and the peaks corresponding to THβC-3-COOHs collected. Those collected peaks were treated with a $Na_2Cr_2O_7$ solution at 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 [27]. The organic solvent was evaporated under He stream, redissolved in 0.1 *M* HCl and injected into the RP-HPLC system 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.

3. Results and discussion

3.1. Chromatographic and spectral characterization of THβC-3-COOH in foods

Sample preparation by solid-phase extraction with SCX columns made possible the isolation of THβC-3-COOHs from many foods, and usually provided

clean HPLC chromatograms with few or no interferences, as reported elsewhere [2,6,15,16]. Chromatograms of THβC-3-COOHs (fluorescence detection) isolated during this research from samples of wine and orange juice are given Fig. 2. Spectral characterization of SCX-isolated THβC-3-COOHs from foods was done by trapping the HPLC eluting peaks into the flow cell of the fluorescence detector and then, monitoring excitation and emission profiles. Fig. 3 shows the experimental data of the fluorescent spectra obtained from MTCA chromatographic peaks in wine and orange juice. *SS*-MTCA and *RS*-MTCA isolated from those samples gave an emission pattern (a, b) matching with those of reference standards having a maximum around 345 nm when excited at 270 nm. The excitation spectra (c, d) (emission set at 343 nm) was also similar. Chemical identification based on retention times along with fluorescent pattern was in good agreement with identification provided by mass spectra using GC–MS [21,22,25], confirming the presence of those compounds in foods. Moreover, by measuring fluorescence spectra in addition to RP-HPLC of SCX-isolated samples ensured that quantified peaks in food samples correspond to 1,2,3,4-tetrahydro-β-carbolines.

3.2. THβC-3-COOH analysis in food samples

Table 1 summarizes the level of THCA and MTCA found in several single samples of foods and beverages analyzed by solid-phase extraction and RP-HPLC coupled to fluorescence detection (excitation 270 nm, emission 343 nm). Extensive studies on the occurrence and origin of those compounds in a larger number of commercial samples than those analyzed here have been reported elsewhere [15–17]. THβC-3-COOHs occurred in varying amount in all the samples analyzed reaching the highest concentration in soy sauce. MTCA presented as two diastereoisomers (1*S*,3*S* and 1*R*,3*S*) was generally the major β-carboline; nevertheless toasted bread and smoked fish contained more THCA in agreement with previous results [16]. As far as the analytical method is concerned, the solid-phase extraction using SCX columns gave recoveries of tetrahydro-β-carbolines in excess of 95%. The reproducibility of the method was fairly good giving RSDs ($n=4$) of 5.8 and 1.2% for THCA and MTCA, respectively.

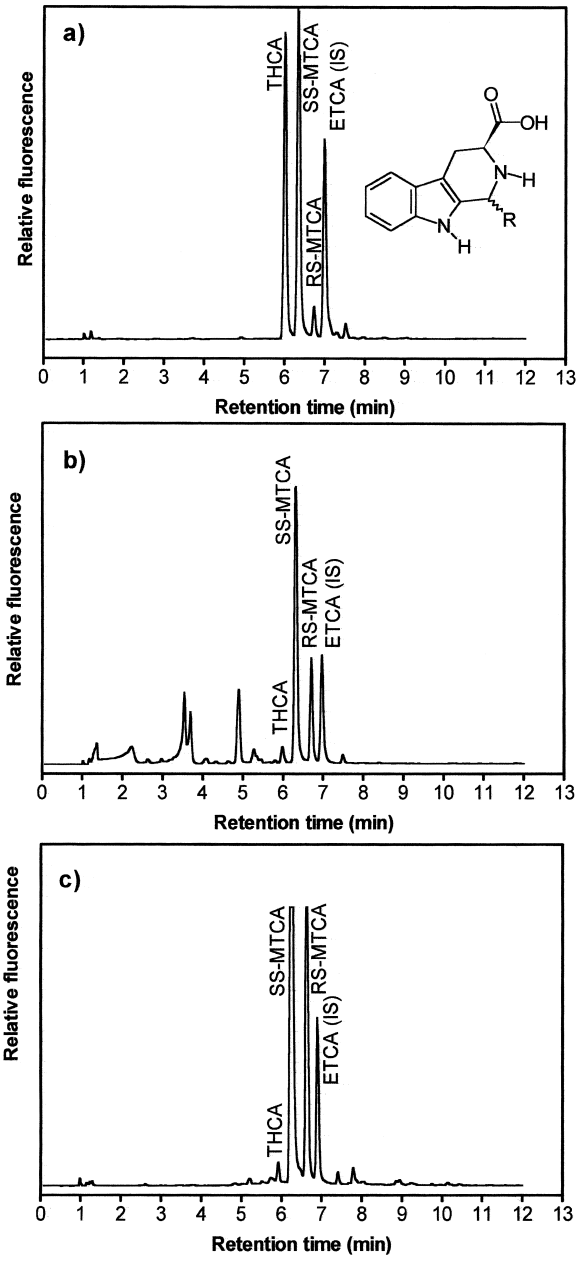

Fig. 2. THβC-3-COOH HPLC chromatograms (fluorescence detection: $\lambda_{excitation}$ 270 nm, $\lambda_{emission}$ 343 nm) of SCX solid-phase extracted samples of standards compounds (a), orange juice (b), and wine (c). Chromatographic conditions are described in Experimental. THCA: 1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid, MTCA: 1-methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (two diastereoisomers 1*S*,3*S* and 1*R*,3*S*) and ETCA: 1-ethyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid.

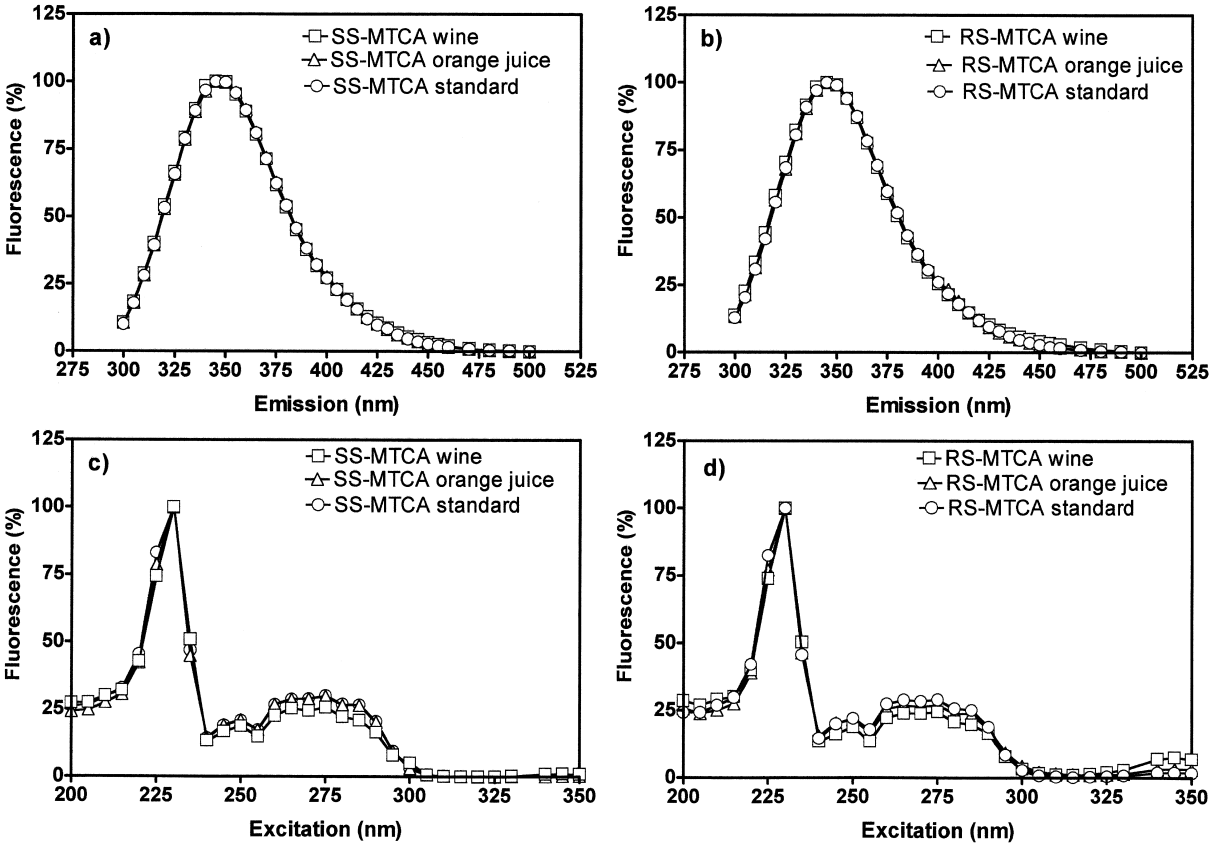

Fig. 3. Fluorescence emission (a, b) (excitation at 270 nm), and excitation (c, d) (emission at 343 nm) spectra of MTCA chromatographic peaks (1*S*,3*S* and 1*R*,3*S* diastereoisomers) from orange juice, wine and MTCA standards.

Research on the occurrence of those compounds in some foods of Table 1 such as wine, beer, soy sauces and smoked foods, has also been conducted by others [6,19,20,23,24] providing similar levels by using HPLC–fluorescence and/or HPLC–MS.

3.3. Oxidation of THβC-3-COOHs to β-carbolines

A second spectral and chromatographic characterization was done by analyzing the β-carbolines produced from THβC-3-COOHs oxidized with $Na_2Cr_2O_7$ (Fig. 1). Peaks of THβC-3-COOHs from SCX-isolated samples (both reference samples and foods) were collected at the end of the detector, and treated with $Na_2Cr_2O_7$ solution, as mentioned in Experimental to form the corresponding β-carbolines, that were then rechromatographically analyzed. Chromatographic and spectral data (Figs. 4 and 5) showed the formation of β-carbolines through decarboxylation and oxidation from the corresponding tetrahydro-β-carboline-3-carboxylic acid. The oxidized compounds were detected at different wavelengths (excitation 245 nm, emission 445 nm), and their retention time increased compared to tetrahydro-β-carboline-3-carboxylic acids. Under these chromatographic conditions, harman (oxidation product from *SS*-MTCA and *RS*-MTCA, Fig. 4) gave a fluorescence emission maximum around 430 nm (excitation 245 nm), and excitation maxima at 230, 245 and 300 nm (emission at 445 nm). Norharman (oxidation product from THCA) gave a fluorescent peak at 445 nm (excitation at 245 nm) and excitation peaks at 230, 245 and 300 nm (emission at 445 nm). Similar results to those reported for orange juice

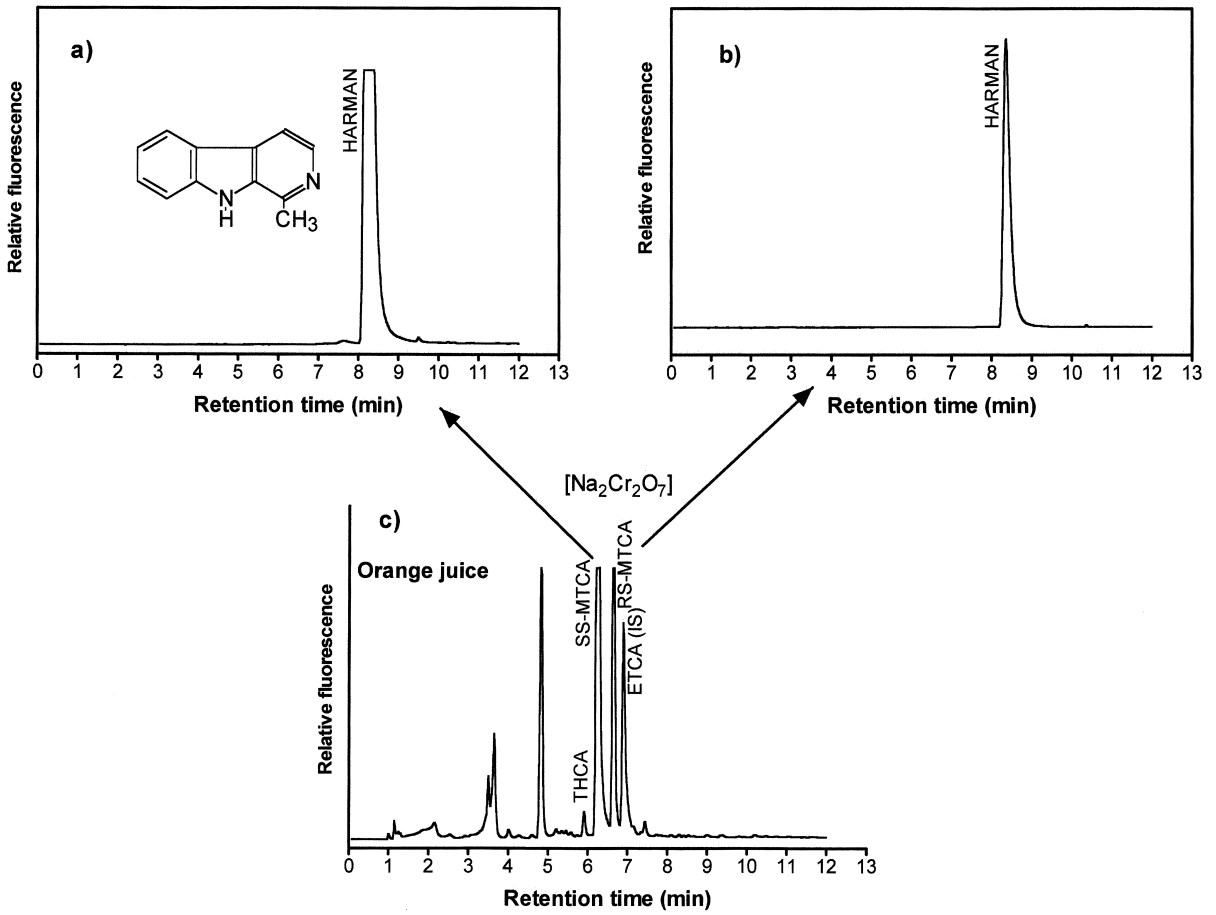

Fig. 4. RP-HPLC chromatograms of $Na_2Cr_2O_7$-oxidized MTCA isolated from orange juice (1*S*,3*S* and 1*R*,3*S* diastereoisomers) (a, b). MTCA peaks were collected, oxidized and rechromatographically analyzed (excitation: 245 nm, emission 445 nm). Chromatogram of THβC-3-COOH from SCX-isolated orange juice (excitation: 270 nm, emission: 343 nm) (c).

given in Figs. 4 and 5 were obtained for other foods in Table 1. The formation of harman and norharman from dichromate-oxidized MTCA and THCA evidenced the occurrence of the two latter compounds in foods. This approach can be used to confirm identification of THβC-3-COOH in foods.

The results given above evidence the usefulness of solid-phase extraction–RP-HPLC–fluorescence detection for the analysis and characterization of both tetrahydro-β-carbolines and β-carbolines. Also, summarizes the widespread presence of MTCA and THCA in commercial foods that we have extensively reported elsewhere [15–17]. THβC-3-COOHs should be considered as naturally occurring substances easily produced during food production, processing and storage. Those compounds readily occur by a condensation between free L-tryptophan and aldehydes favored in low pH and high temperature [2]. The presence of THβC-3-COOHs in foods suggests that the diet is a source of tetrahydro-β-carbolines in humans. Thus, the endogenous presence of these compounds in biological materials [4–7] could arise, at least in part, from food consumption besides certain endogenous formation itself. Although in the last few years, several studies have considered the possible mutagenicity, toxicity or neuroactivity of tetrahydro-β-carbolines and β-carbolines [3–5,7,11,13,14], a full delineation of

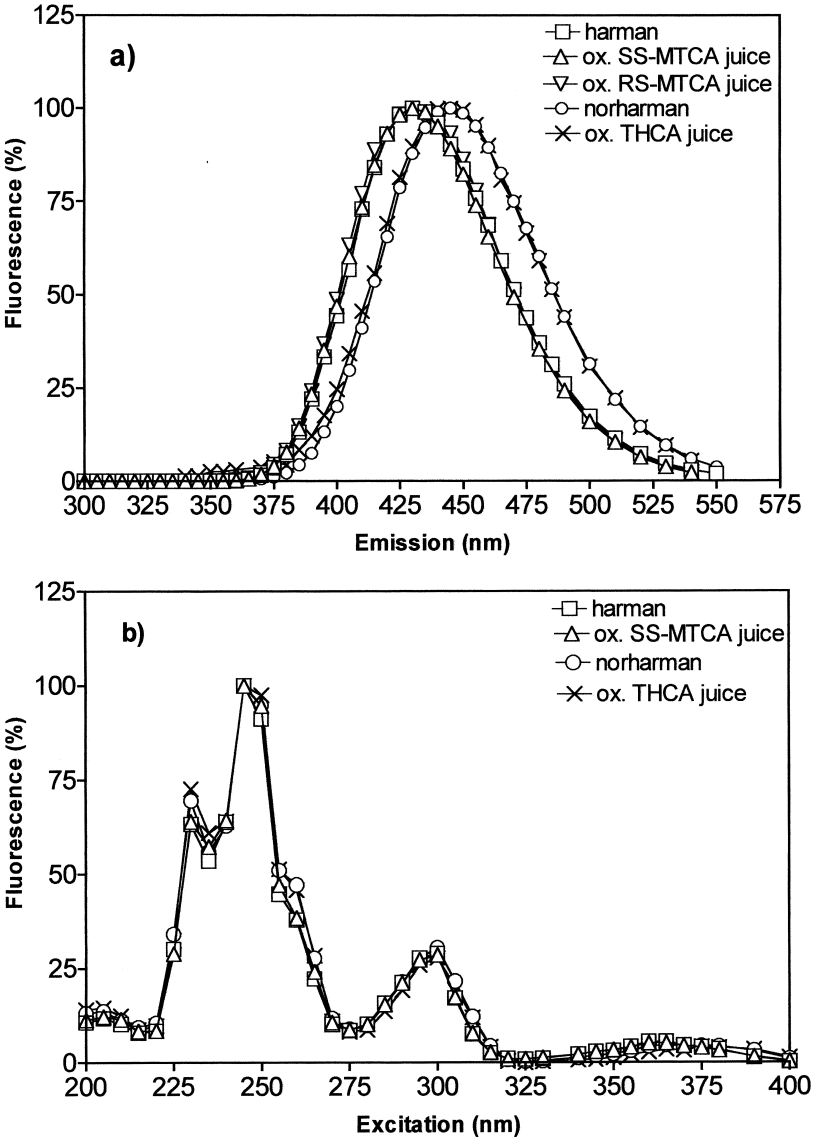

Fig. 5. Fluorescence emission (a) (excitation 245 nm), and excitation (b) (emission 445 nm) spectra of standard β-carbolines norharman and harman, and those obtained through $Na_2Cr_2O_7$-oxidation of THCA and MTCA HPLC peaks from SCX-extracted orange juice.

their biological activity and/or toxicity is still needed.

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