ELSEVIER

Journal of Chromatography A, 765 (1997) 265–277

JOURNAL OF CHROMATOGRAPHY A

Presence of tetrahydro-β-carboline-3-carboxylic acids in foods by gas chromatography–mass spectrometry as their N-methoxycarbonyl methyl ester derivatives

T. Herraiz[a,*], F. Sanchez[b]

[a]*Instituto de Fermentaciones Industriales, CSIC, Juan de la Cierva 3, 28006 Madrid, Spain*
[b]*Instituto de Química Orgánica, CSIC, Juan de la Cierva 3, 28006 Madrid, Spain*

Received 10 September 1996; revised 23 October 1996; accepted 23 October 1996

Abstract

Various tetrahydro-β-carboline-3-carboxylic acids (THβC-3-COOH) are identified in commercial foods and drinks by GC–MS. Positive identification of 1-methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (MTCA) is demonstrated in soy and tabasco sauces, wine, beer, wine vinegar, cider, orange juice, toasted bread, blue cheese and yoghurt. 1,2,3,4-Tetrahydro-β-carboline-3-carboxylic acid (THCA) occurs in toasted bread, beer, cider, wine vinegar, soy and tabasco sauce, orange juice and blue cheese. MTCA and THCA are reported for the first time in several of these products. MTCA appears as a mixture of two diastereoisomers with the same mass spectra. MTCA is the major THβC-3-COOH in foodstuffs except for toasted bread that contains more THCA. GC–MS analysis of N-methoxycarbonyl methyl ester derivatives of THβC-3-COOHs was used for chemical identification. Those derivatives were synthesized in a qualitatively using methyl chloroformate or methyl chloroformate and diazomethane reagents. Electron impact mass spectra of N-methoxycarbonyl-THβC-3-COOH methyl esters are reported and fragmentation assigned and discussed. These results prove the presence of THβC-3-COOHs in commercial foodstuffs suggesting their uptake during the daily consumption of foods.

Keywords: Food analysis; Derivatization, GC; Sample preparation; Tetrahydrocarbolinecarboxylic acids; Carboxylic acids

1. Introduction

1,2,3,4-Tetrahydro-β-carbolines (THβC) (2,3,4,9-tetrahydro-1H-pyrido[3,4-*b*]indole) are formed from indole ethylamines and aldehydes through Pictet–Spengler condensation. In a parallel way, 1,2,3,4-tetrahydro-β-carboline-3-carboxylic acids (THβC-3-COOHs) are produced by reaction of tryptophan and aldehydes. This reaction readily even occurs under mild conditions and the rate is temperature and pH dependent [1].

Research in the last decade has pointed out the occurrence of THβC and β-carbolines under physiological conditions in biological tissues and fluids [2–5]. These compounds can function as neurotransmitters or neuromodulators via their effect on the monoamine oxidase and have been reported to form endogenously in the brain [2,6]. Furthermore, they have been increasingly implicated in alcoholism [2,7–11]. Administration of THβC to rats has been reported to significatively alter alcohol consumption [12]. Tetrahydro-β-carbolines can also be postulated as possible precursors of β-carboline-3-carboxylate, a potent inhibitor of benzodiazepine receptor, that

*Corresponding author.

PII S0021-9673(96)00940-5

may be a kind of endogenous ligand for this receptor, exhibiting pharmacological effects [13].

1-Methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (MTCA) has been described as a precursor of mutagenic N-nitroso compounds when tested in the Ames test using *Salmonella typhimurium* [14,15]. THβC could react with nitrite already in foods, in the mouth, or in the stomach, giving rise to mutagenic compounds [16]. Salvi and Choughuley [17] and Sen et al. [18,19] reported the nitrosation of some food-related THβC-3-COOHs.

MTCA may be involved in the aetiology of the eosinophilia–myalgia syndrome (EMS) associated with the ingestion of impure L-tryptophan which occurred in the USA in 1989 [20,21]. Initially, one impurity identified as 1,1′-ethylidene-bis-tryptophan (EBT) was suggested to cause the disease [22,23]. However, EBT is converted to MTCA in acid solution such as gastric fluid [20,24]. Recently, Brenneman et al. [25] have reported that the diastereoisomer 1*S*,3*S*-1-methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (1*S*,3*S*-MTCA), may cause neuronal death playing a role in the aetiology of some of the neurophatic features of L-Trp-EMS. Their results show that 1*S*,3*S*-MTCA exhibits a pharmacological action on neuronal survival. In this regard full delineation of the biological activities and toxicity of tetrahydro-β-carboline compounds is important [25].

The analytical assessment of THβC-3-COOHs is now accomplished by solid-phase extraction and RP-HPLC with fluorescent detection [4,26]. Thus, the content of THβC-3-COOHs in several foodstuffs has recently been studied [27]. Previously, they were described in soy sauce [4,14], smoked foods [19,28], beer and wine [1,4,10,26]. However, there is very little and partial research dealing with the positive chemical identification of these compounds by mass spectrometry. Indeed, additional research is currently needed for absolute confirmation of the presence of these compounds in common foods and drinks of the human diet.

By using GC–MS, this paper demonstrates the qualitative presence of THβC-3-COOHs in soy sauce, blue cheese, yoghurt, cider, tabasco sauce, orange juice, wine vinegar and in some alcoholic beverages such as wine, cider and beer. Some of those products have not been tested before for these compounds. As GC–MS needs a previous formation of GC volatile derivatives, the N-methoxycarbonyl methyl ester derivatives of THβC-3-COOHs were obtained prior to GC–MS. Those analytical procedures employed for isolation and chemical derivatization of THβC-3-COOHs might be useful for further application in this field. The possible significance of these compounds in foodstuffs is discussed.

2. Experimental

2.1. Reference compounds

MTCA (containing mainly the diastereoisomer 1*S*,3*S*-MTCA) was purchased from Sigma (St. Louis, MO, USA). The mixture of (−)-(1*S*,3*S*)-MTCA) (main component) and (−)-(*1R,3S*)-MTCA) (minor component) was prepared from L-tryptophan and acetaldehyde [29]. Synthesis of MTCA gave a mixture of two diastereoisomers: a major compound corresponding to (1*S*,3*S*) configuration and a minor one corresponding to (1*R*,3*S*) configuration [4,29]. The diastereo-selectivity of this type of reaction has been studied [30]. In the same manner, 1-ethyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (ETCA) and 1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (THCA) were prepared from L-tryptophan and propionaldehyde or formaldehyde, respectively [26,29]. Confirmation of the structures of the synthesized compounds was previously carried out by ^{1}H NMR, ^{13}C NMR, MS, GC–MS and RP-HPLC [31].

Syntheses of standard N-methoxycarbonyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid methyl ester (N-MC-THCA-ME), N-methoxycarbonyl-1-methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid methyl ester (N-MC-MTCA-ME) and N-methoxycarbonyl-1-ethyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid methyl ester (N-MC-ETCA-ME), were accomplished from standard THCA, MTCA and ETCA following the methods described below.

2.2. Synthesis of N-methoxycarbonyl-THβC-3-COOH methyl esters (N-MC-THβC-3-COOH-ME) from THβC-3-COOHs

Samples containing standard or food-isolated THβC-3-COOHs were derivatized and subsequently

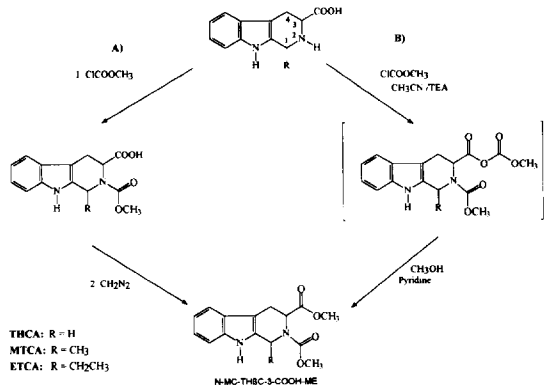

Fig. 1. Chemical synthesis of N-methoxycarbonyl-tetrahydro-β-carboline-3-carboxylic acid methyl esters (N-MC-THβC-3-COOH-ME) from tetrahydro-β-carboline-3-carboxylic acids (THβC-3-COOH).

analyzed by GC–MS. Formation of N-methoxycarbonyl-tetrahydro-β-carboline-3-carboxylic acid methyl esters was carried out as in Fig. 1. Details are as follows:

(A) Standard THβC-3-COOHs (approx. 0.1–0.5 mg) and those THβC-3-COOHs isolated from foodstuffs were derivatized with methyl chloroformate following basically the procedure reported by Bosin and Jarvis [32]. The amounts of reagents and solvents were adjusted to the volume of sample derivatized. Thus, one volume of sample (5–15 ml) in 0.1 *M* HCl–acetonitrile (70:30) plus semicarbazide (Sigma) at 1 mg/ml, was treated with one volume (5–15 ml) of 1 *M* phosphate buffer (pH 7), 1/10 vol. (0.5–1.5 ml) of methyl chloroformate reagent (Aldrich), vortexed and allowed to stand for 5 min. The pH of the reaction mixture was increased with 0.5 vol. (2.5–7.5 ml) of saturated Na_2CO_3, and 1/10 vol. (0.5–1.5 ml) of methyl chloroformate was again added, vortexed and allowed to stand for more than 10 min. The sample was adjusted to pH 8–9 with saturated Na_2CO_3 and extracted with 50–100 ml of CH_2Cl_2. The organic phase was discharged, and the aqueous phase was carefully acidified with conc. HCl, extracted three times with CH_2Cl_2 (50 ml), the organic phase concentrated in a rotoevaporator at 30°C and finally evaporated at room temperature under a He stream. The residue was redissolved in methanol and the isolated N-methoxycarbonyl-THβC-3-COOHs were esterified with 2 ml of a recently prepared solution of diazomethane (CH_2N_2) in diethyl ether to obtain the N-MC-THβC-3-COOH-ME. Then the samples were concentrated at room temperature under a He stream to remove the excess of ether and extracted three times with ethyl acetate following addition of Milli-Q water. The N-MC-THβC-3-COOH-MEs in ethyl acetate were concentrated at room temperature under a He stream up to aprrox. 0.1 ml and finally injected into GC–MS.

(B) Standard THβC-3-COOHs (<0.2 mg) and those THβC-3-COOHs isolated from foodstuffs reacted with methyl chloroformate (Aldrich) to achieve derivatization of both the carboxylic acid (COOH) and the amino (NH) groups. This method is used for the first time to derivatize these compounds and it is based on previous studies [33,34]. Compounds dissolved in (3–5) ml of 0.1 *M* HCl–acetonitrile (70:30) containing semicarbazide (1 mg/ml), adjusted to pH 4–5, were added with triethylamine (TEA) (0.5 ml) and methyl chloroformate (0.5 ml), vortexed for 1 min, and allowed to stand for 5 min. The addition of TEA (0.5 ml) and methyl chloroformate (0.5 ml) was repeated twice and the sample allowed to stand for 10–20 min at 0°C. One ml of methanol and 1 ml of pyridine were slowly added to the reaction sample placed in ice, mixed, and left to stand for 10 min at 0°C. Then, with 0.5 ml of pyridine and 0.5 ml of methyl chloroformate added, stirred for 10 min at 0°C, diluted 1:2 with Milli-Q water, and extracted three times with 15 ml of ethyl acetate. The organic phase was dried (Na_2SO_4), concentrated in rotoevaporator and under a He stream (room temperature) up to approx. 0.1 ml, and finally injected into the GC–MS system.

2.3. Isolation of THβC-3-COOHs from foodstuffs

THβC-3-COOHs were isolated from samples of foods and drinks purchased in local supermarkets and chromatographed by GC–MS following chemical derivatization as indicated above. The sample preparation was as follows:

2.3.1. Wine, wine vinegar, beer and cider samples

Various samples of red table wine (200 ml), wine vinegar (200 ml), beer (100 ml) and cider (400 ml) with semicarbazide added at 1 mg/ml, were concentrated separately in a rotoevaporator (40°C) to less than 30 ml. Concentrated samples of wine (10 ml), vinegar (4 ml), beer (18 ml) and cider (15 ml) were

diluted (1:3) with 0.6 *M* $HClO_4$ and loaded separately onto four C_{18} Sep-Pak plus cartridges (Waters, Milford, MA, USA), previously conditioned with methanol (2 ml) and 0.1 *M* HCl (2 ml). After loading the sample, each cartridge was washed with 0.1 *M* HCl (2 ml) and 0.1 *M* HCl–acetonitrile (90:10) (2 ml) and THβC-3-COOHs eluted with a mixture of 0.1 *M* HCl–acetonitrile (70:30) (4 ml). The eluates corresponding to each sample were mixed and derivatized using methods A and B.

2.3.2. Soy sauce and tabasco sauce

Samples of commercially available soy sauce (10 ml) were diluted three folds with 0.6 *M* $HClO_4$, semicarbazide was added (1 mg/ml), and loaded onto two C_{18} Sep-Pak plus cartridges. Samples of commercial tabasco sauce (10 ml) were centrifuged at 5300 *g* (20 min, 0°C), the supernatant diluted two fold with 0.6 *M* $HClO_4$, added with semicarbazide at 1 mg/ml, and loaded onto three C_{18} Sep-Pak plus cartridges. The clean-up procedure was carried out as above and the eluates derivatized using method A and B.

2.3.3. Orange juice (made from concentrated orange juice)

(a) Samples of commercially available orange juice (500 ml), with semicarbazide added at 1 mg/ml, were evaporated in a rotoevaporator (40°C) to reach 40 ml, centrifuged (5300 *g*, 20 min, 0°C), and the supernatant derivatized following method A. Alternatively, samples of orange juice (500 ml) were lyophilized, the residue redissolved in 0.1 *M* HCl, centrifuged and derivatized following method A. (b) Samples of orange juice (approx. 200 ml) or alternatively lyophilized orange juice redissolved in 0.1 *M* HCl (200 ml), were added with semicarbazide (1 mg/ml), adjusted to pH 2, passed through four C_{18} Sep-Pak plus cartridges conditioned, washed and eluted as in Section 2.3.1. The elution fractions were derivatized following methods A and B.

2.3.4. Toasted bread, blue cheese and yoghurt samples

Samples of commercial toasted bread (20–40 g) were homogenized for 2 min, in 0.6 *M* $HClO_4$ (120–240 ml) and semicarbazide at 1 mg/ml, using an Ultra-turrax (<15 000 l/min, Janke and Kunkel, Ika-Werk), centrifuged at 6500 *g* (20 min, 0°C) and filtered. The filtrate was treated in two ways: (a) it was passed through six SCX benzenesulfonic acid columns (Bond Elut, 3 ml size, Varian, Harbor City, CA, USA) following a previous procedure [1,4,26]. SCX columns were previously conditioned with methanol (6 ml) and 0.1 *M* HCl (6 ml), and after loading the samples, the columns were washed with 6 ml of 0.1 *M* HCl, 2 ml of methanol, 6 ml of water and 2 ml of 0.4 *M* phosphate buffer (pH 9.1), and eluted with 4 ml of methanol–0.4 *M* phosphate buffer (1:1) (pH 9.5). Elution fractions were concentrated in a rotoevaporator (40°C) to remove methanol in presence of semicarbazide (1 mg/ml), acidified (pH 1–2), and loaded onto four C_{18} Sep-Pak plus cartridges. Conditioning, washing and elution was accomplished as in Section 2.3.1 and the eluates derivatized as in methods A and B. (b) The filtrate was loaded onto six C_{18} Sep-Pak plus cartridges, conditioned, washed and eluted as in 2.3.1. The eluates (approx. 25 ml) of 0.1 *M* HCl–acetonitrile (70:30) were subjected to derivatization using methods A and B.

Samples of commercially available blue cheese (30–40 g) were homogenized (2 min) with 80 ml of 0.6 *M* $HClO_4$ plus semicarbazide (1 mg/ml) using an Ultra-turrax, centrifuged and filtered as for bread samples. The filtrate was treated in two ways: (a) it was loaded onto six SCX benzenesulfonic acid columns (3 ml, Varian) and subsequently onto three C_{18} Sep-Pak plus cartridges, using the same sample preparation clean-up as for toasted bread. Derivatization of the eluates was accomplished following method A and B. (b) On the other hand, the filtrate was loaded onto five C_{18} Sep-Pak plus cartridges, conditioned, washed and eluted as in Section 2.3.1, and derivatized following methods A and B.

Samples of commercially available stirred yoghurt (150 ml) were diluted (1:2) with 0.6 *M* $HClO_4$ in presence of semicarbazide (1 mg/ml), centrifuged (6500 *g*, 20 min, 0°C) and the supernatants passed through six C_{18} Sep-Pak plus cartridges conditioned, washed and eluted as in Section 2.3.1. Elution fractions were derivatized following method A. On the other hand, samples of stirred yoghurt (200 g) were added with 50 ml of 0.6 *M* $HClO_4$, semicarbazide at 1 mg/ml, centrifuged (6500 *g*, 20 min, 0°C) and approx. 100 ml of supernatant loaded onto

six SCX benzenesulfonic acid columns (3 ml size, Varian), conditioned, washed and eluted as for bread samples. Elution fractions (approx. 30 ml), added with semicarbazide at 1 mg/ml, were concentrated in a rotoevaporator (40°C) to remove methanol, and part of this sample was subjected to derivatization following method B. The rest was acidified with 0.1 *M* HCl, loaded onto four C_{18} Sep-Pak plus cartridges as in Section 2.3.1, and the eluates derivatized following method B.

The procedures of solid-phase extraction (SPE) based on SCX and C_{18} columns were previously tested with authentic standard samples of THβC-3-COOHs. Several C_{18} and SCX cartridges were used for sample preparation in foodstuffs to avoid sample overloading due to limited sorbent capacity. RP-HPLC analysis was also performed as detailed below to follow the isolation and derivatization of THβC-3-COOHs. The absence of artifactual formation during the derivatization procedures was checked with standard solutions of tryptophan.

2.4. RP-HPLC analysis

Chromatographic analysis was performed in a 1050 high-performance liquid chromatograph with a 1046A fluorescence detector and a 3365-Series II HP Chemstation (Hewlett-Packard, Santa Clara, CA, USA). A 150 mm×3.9 mm, five μm, Nova-Pak C_{18} column was used for HPLC separation of THβC-3-COOH and N-MC-THβC-3-COOH-ME. Fluorescence detection was carried out at 270 nm for excitation and 343 nm for emission. Chromatographic conditions were: 50 m*M* sodium phosphate buffer adjusted to pH 3 with phosphoric acid (Eluent A); 20% of A in acetonitrile (Eluent B). 0% B to 32% B in 8 min, then 90%B at 18 min, 100% B at 20 min. The column was equilibrated with solvent A before the next injection. The flow-rate was 1 ml/min, oven temperature 40°C and the injection volume 20 μl. THβC-3-COOH eluted within 10 min whereas N-MC-THβC-3-COOH-ME eluted around 14 min.

2.5. GG–MS analysis

GC–MS analysis was performed with an HP G1800A GCD system (GC–MS), consisting of a gas chromatograph, an electron ionization mass detector and a computer data system for obtaining and recording electron impact (EI) mass spectra. Two different capillary columns and GC operating conditions were used: (a) a 30 m×0.25 mm I.D. methyl silicone capillary column, oven temperature = 220°C (5 min), 4°C/min to 240°C (10 min) and 0.9 ml/min of helium flow-rate; (b) a 20 m×0.25 mm I.D. methyl silicone column, oven temperature = 160°C (2 min), 4°C/min to 245°C (10 min) and 0.6 ml/min of helium flow-rate. Injection was in the split mode (1:100); injector temperature: 260°C; transfer line: 280°C, and the ionization mode was EI at 70 eV scanning from *m/z* 10 to 425 with MS data acquisition starting at 5 min.

3. Results

3.1. Derivatization of THβC-3-COOH to form N-MC-THβC-3-COOH-ME

GC–MS of THβC-3-COOHs requires the formation of volatile derivatives. Fig. 1 shows how N-methoxycarbonyl methyl esters of THβC-3-COOHs were obtained. Method A consists in a reaction first with methyl chloroformate as previously [32], and secondly with diazomethane to esterify the carboxylic group. Method B derivatizes both, the amino and the carboxylic groups with methyl chloroformate. This procedure is based on the formation of a very reactive mixed anhydride and its evolution to the methyl ester in presence of methanol [33] along with the amidation to release N-methoxycarbonyl [32,34]. THβC-3-COOHs are derivatized using the latter method for the first time.

3.2. GC–MS of standard N-MC-THβC-3-COOH-ME

GC–MS analysis of standard THβC-3-COOHs as N-methoxycarbonyl methyl esters derivatives (N-MC-THβC-3-COOH-ME) provide chromatograms as in Fig. 2. N-MC-MTCA-ME and N-MC-ETCA-ME give rise to two peaks corresponding to (1*R*,3*S*)-diastereoisomer (peak 1) and to (1*S*,3*S*)-diastereoisomer (peak 2). To assign this configuration, the diastereoisomeric peaks of standard were repetitively separated by RP-HPLC, in which the configuration is

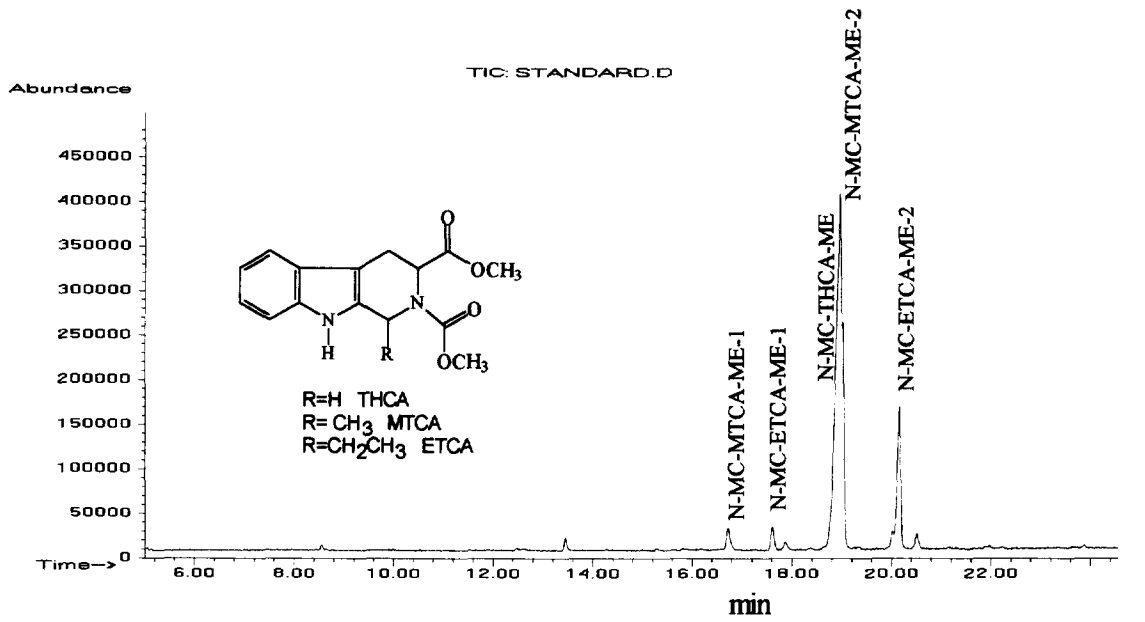

Fig. 2. GC–MS chromatogram of standard compounds of N-MC-THβC-3-COOH-MEs. MTCA and ETCA appears as two diastereoisomers (1*R*,3*S*) (peak 1) and (1*S*,3*S*) (peak 2). A 20 m×0.25 mm methyl silicone capillary column. 160°C (2 min), 4°C/min, 245°C (10 min). Injector and transfer line temperatures were 260 and 280°C, respectively. Flow-rate 0.6 ml/min.

already known [4,10,14,26,27], and collected peaks derivatized and further analyzed by GC–MS. Elution order in GC is opposite to that of RP-HPLC which confirms previous results [31]. The major peak of N-MC-MTCA-ME (1*S*,3*S*-isomer) was not resolved completely from N-MC-THCA-ME in any of the capillary columns and different chromatographic conditions.

Mass spectra of authentic THβC-3-COOHs derivatives are presented in Fig. 3a–e. The molecular ion (M^+) is abundant giving *m/z*: 288, 302 and 316 for THCA, MTCA and ETCA, respectively. N-MC-MTCA-ME and N-MC-ETCA-ME give *m/z* 287 ions corresponding to (M^+-CH_3) and ($M^+-(CH_2-CH_3)$). $M^+-(COOCH_3)$ produces 229, 243 and 257 for THCA, MTCA and ETCA, respectively. $M^+-(COOCH_3)_2$, plus the radical in position one accompanied by elimination of one proton could give *m/z* 169 and 168 for THCA and MTCA. Fragments at 143 and 157 for THCA and MTCA could derive from $M^+-(CH\text{-}COOCH_3+N\text{-}COOCH_3)$. Electron impact mass spectra are consistent with the structures of N-MC-THβC-3-COOH-ME. The fragmentation pattern is similar to that of N-trifluoroacetyl methyl ester derivatives used to identify THβC-3-COOHs in wines [31]. MTCA and ETCA diastereoisomers (*SS* and *RS*) provide two chromatographic peaks with practically the same mass spectra. Both methods of Fig. 1 provided a successful qualitative derivatization of authentic THβC-3-COOHs.

3.3. THβC-3-COOHs in foodstuffs by GC–MS

Food extracts were derivatized to give N-MC-THβC-3-COOH-MEs and subsequently injected into GC–MS. Fig. 4a–j shows the GC–MS chromatograms obtained from different food extracts. Electron impact mass spectra of peaks eluting with the same retention time as those standards of N-MC-THβC-3-COOH-MEs is evidence that both MTCA and THCA occur in foodstuffs. Indeed, mass spectra obtained from derivatized food extracts are in good agreement with those of standards (Fig. 5). The results are briefly reported in Table 1. Two isomers of MTCA (1*R*,3*S*, peak 1 and 1*S*,3*S*, peak 2) are identified in all foodstuffs studied. (1*R*,3*S*)-MTCA diastereoisomeric peak is smaller and elutes earlier than (1*S*,3*S*)-MTCA. The average ratio of (1*S*,3*S*)-MTCA to

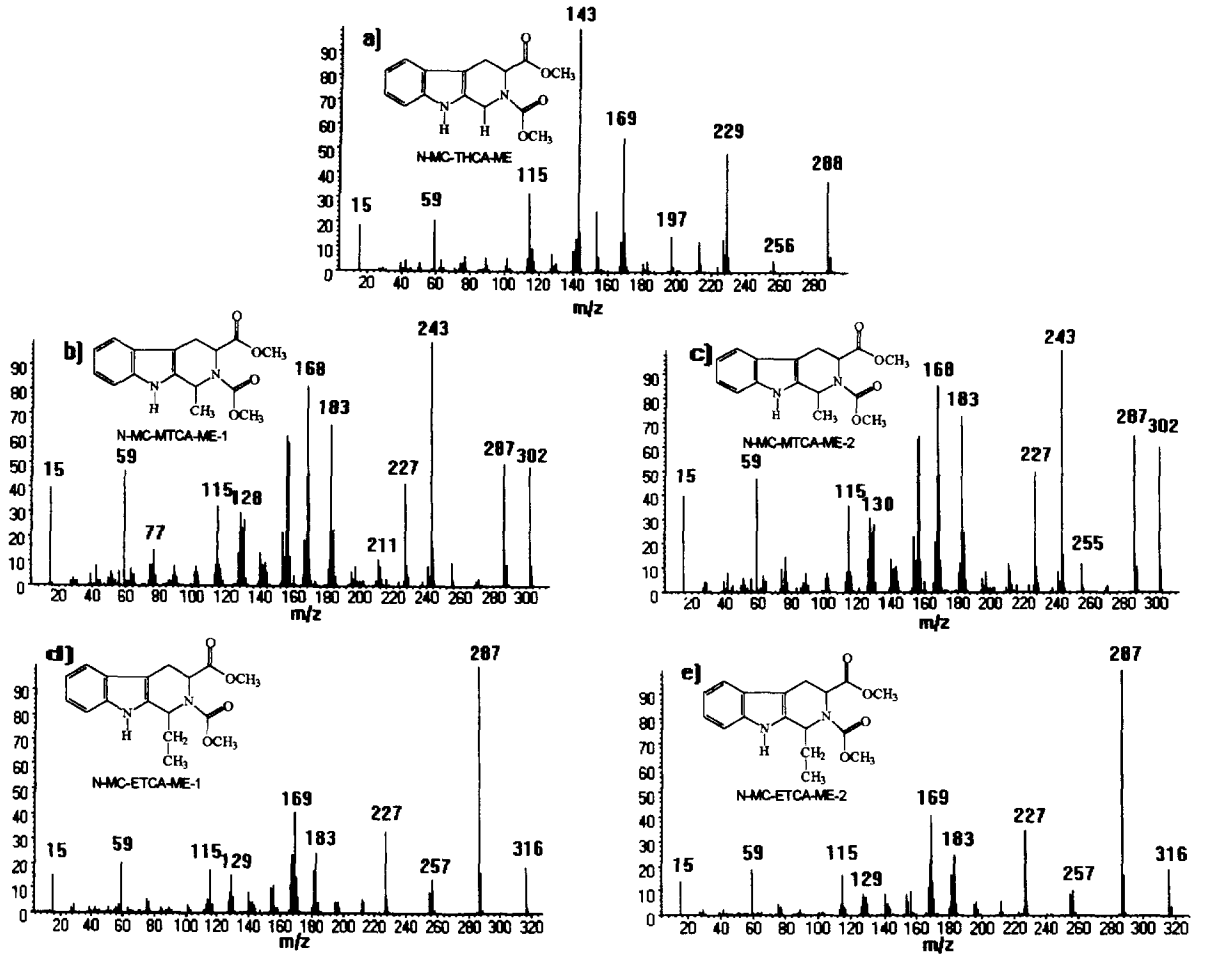

Fig. 3. Electron impact (EI) mass spectra obtained after GC–MS of standards N-MC-THβC-3-COOH-MEs.

(1*R*,3*S*)-MTCA in food extracts was 4.8 (measured as area ratio of *m/z* 302 ion). THCA appears in most foodstuffs as the minor THβC-3-COOH except for toasted bread, in which THCA was the major component. No identification of ETCA was achieved in any of the foodstuffs studied.

3.4. HPLC of standard and foods-isolated N-MC-THβC-3-COOH-MEs

Qualitative HPLC analysis was also carried out for standard and food-isolated N-MC-THβC-3-COOH-MEs. Fig. 6a and b show chromatograms of standard and soy sauce-isolated N-MC-THβC-3-COOH-MEs, respectively. THCA as its N-methoxycarbonyl methyl ester derivative is well separated from MTCA using RP-HPLC. MTCA and ETCA provide two diastereoisomeric peaks that are assigned to 1*S*,3*S* and 1*R*,3*S* [4,10,14,26,27]. RP-HPLC of food-isolated THβC-3-COOHs derivatives corroborated the presence of these compounds in foodstuffs. Additionally, HPLC was used to follow the extraction and derivatization steps.

4. Discussion

The results presented above clearly prove the presence of THCA and MTCA in foodstuffs. So far, the research focusing on the MS identification of

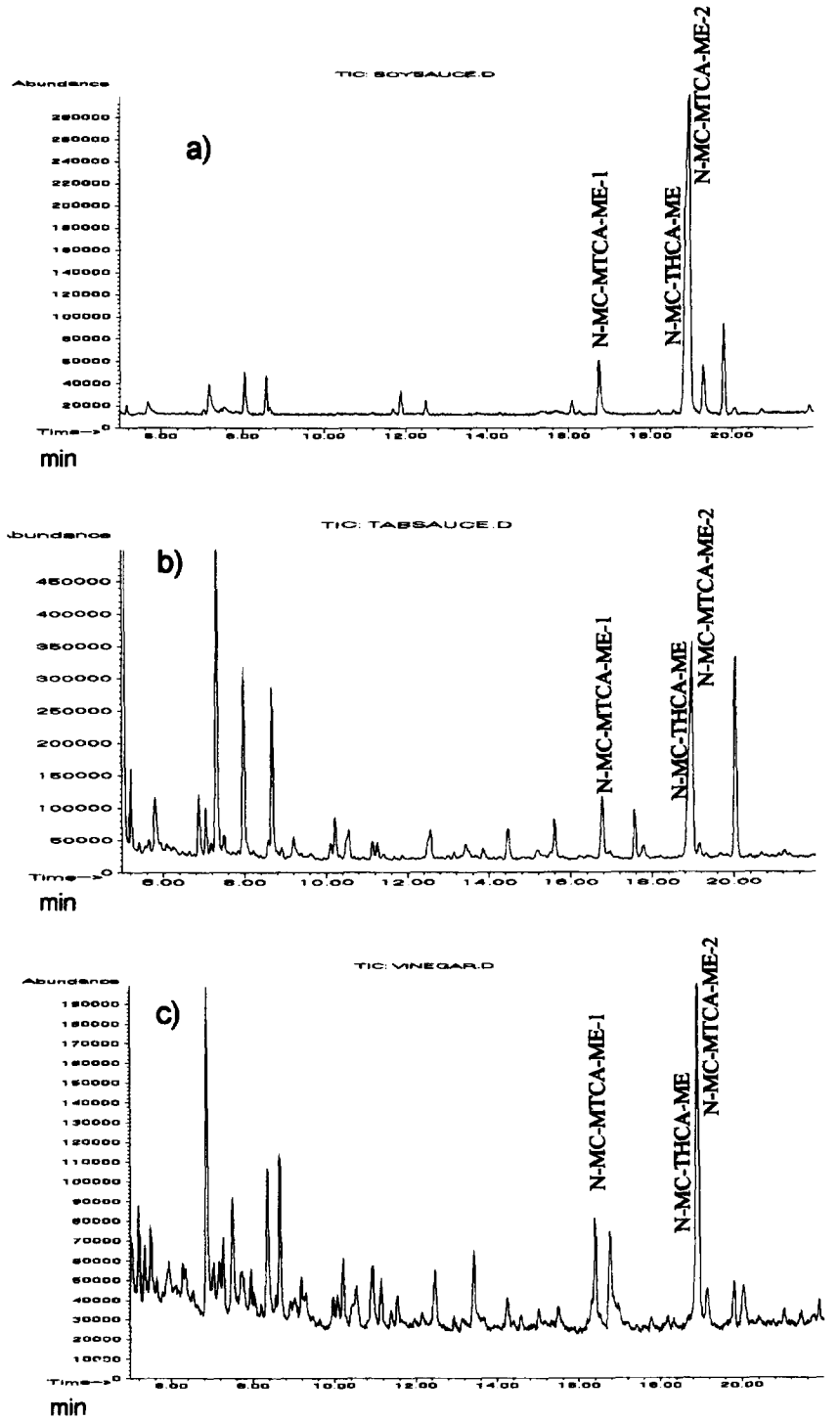

Fig. 4. GC–MS chromatograms of THβC-3-COOHs isolated from food extracts as their N-methoxycarbonyl methyl esters derivatives. The samples are: soy sauce (a), tabasco sauce (b), wine vinegar (c), wine (d), beer (e), cider (f), orange juice (g), toasted bread (h), blue cheese (i) and yoghurt (j).

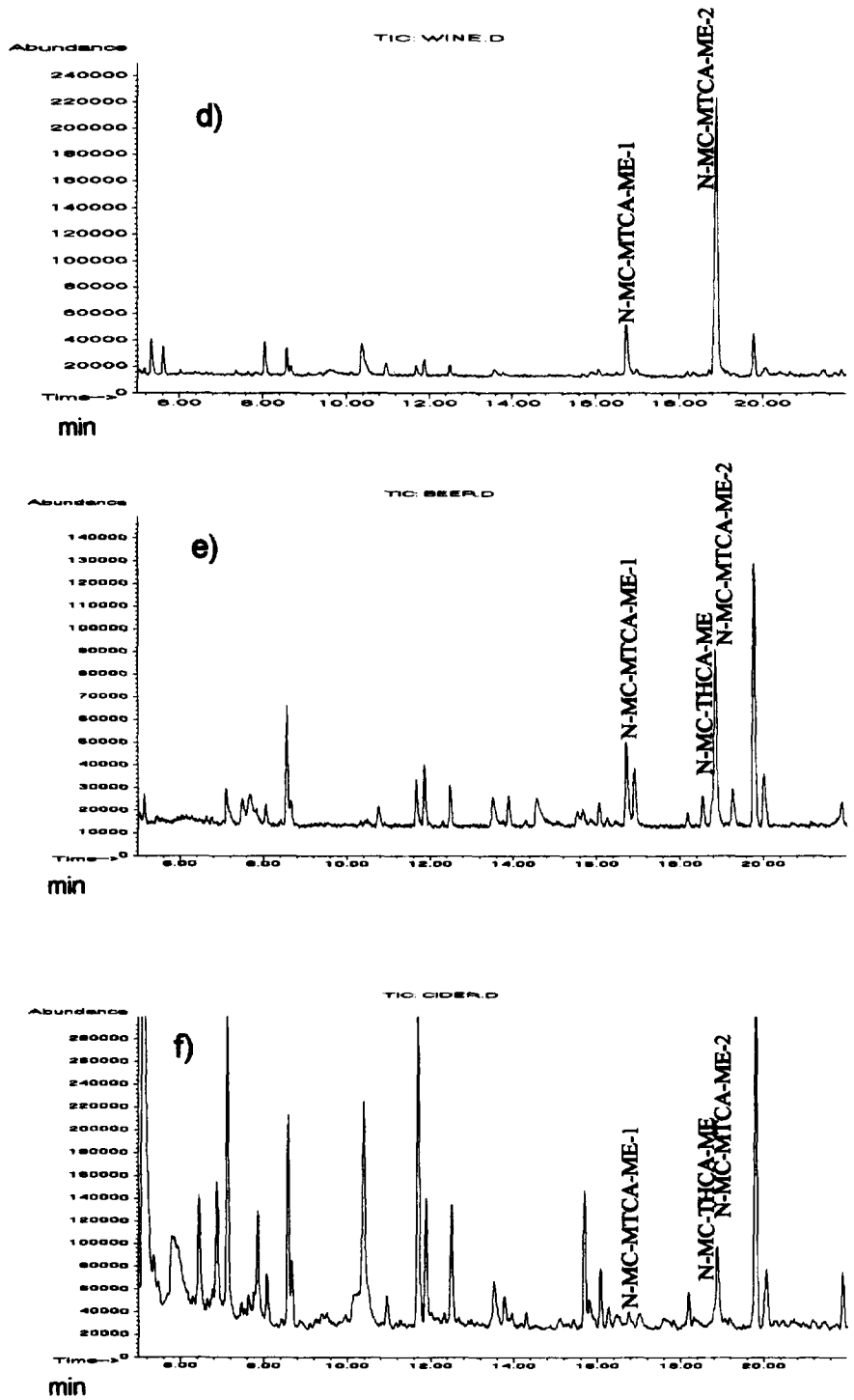

Fig. 4. (*continued*)

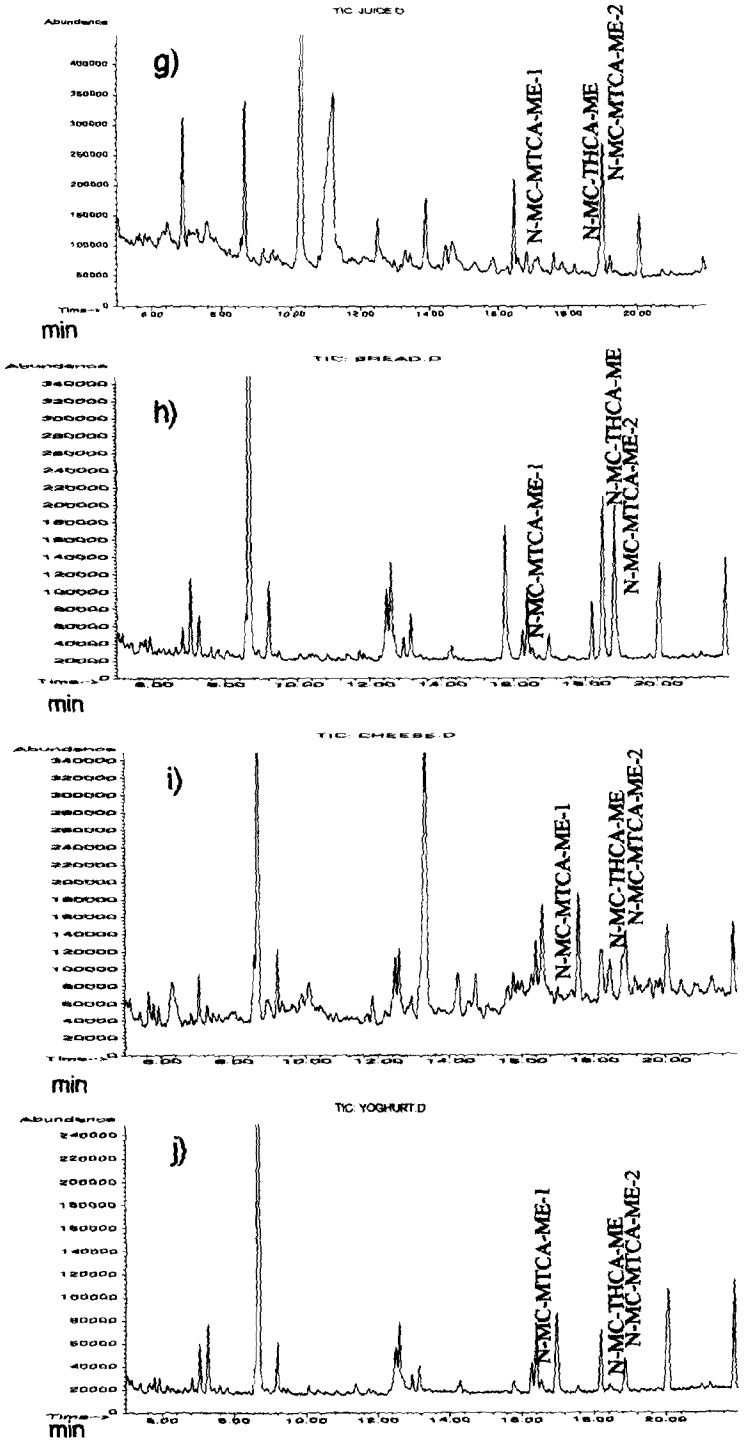

Fig. 4. (*continued*)

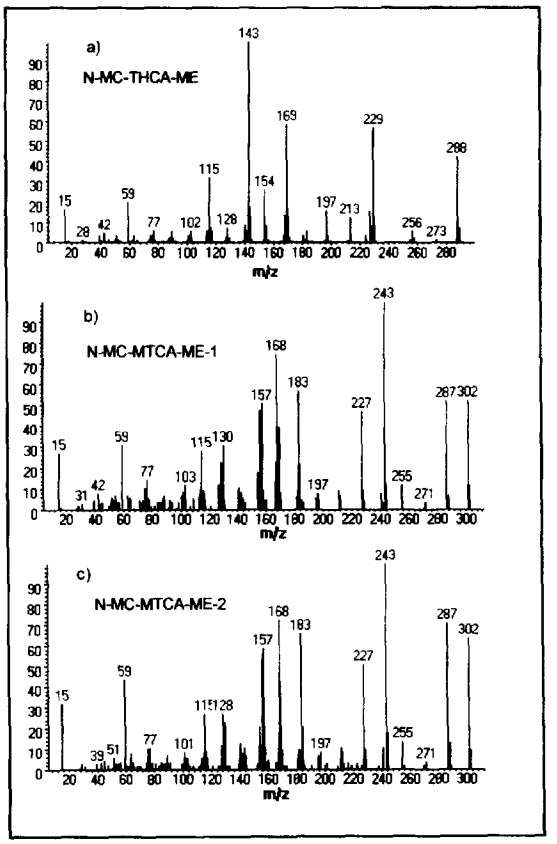

Fig. 5. Mass spectra of THCA and MTCA found in foodstuffs as their N-MC-THβC-3-COOH-ME derivatives by GC–MS. (a) Toasted bread; (b) and (c) wine vinegar. Similar mass spectra are obtained for the rest of foodstuffs containing these compounds (Table 1).

these compounds in food samples has been very limited [4,10,28,31]. Indeed, the presence of THβC-3-COOHs in tabasco sauce, toasted bread, orange juice, yoghurt, blue cheese, cider and wine vinegar is demonstrated here for the first time. This was accomplished by using GC–MS of N-MC-THβC-3-COOH-MEs volatile derivatives of THβC-3-COOHs. Those derivatives are suitable for GC–MS and were obtained by chemical derivatization using methyl chloroformate and methyl chloroformate and diazomethane, and are based on previous studies [32–34]. Although derivatization was only checked in a qualitative level, it was useful to investigate the presence of these compounds in food samples, which was the aim of this research. It should be pointed

Table 1
THβC-3-COOHs found in foodstuffs by GC–MS of N-methoxycarbonyl methyl ester derivatives (N-MC-THβC-3-COOH-ME)[a]

Foodstuff	THβC-3-COOH		
	R,S-MTCA	*S,S*- MTCA	THCA
Wine	+	+	–
Beer	+	+	+[b]
Cider	+[b]	+	+
Wine vinegar	+	+	+
Soy sauce	+	+	+
Tabasco sauce	+	+	+[c]
Orange juice	+	+	+[b]
Toasted bread	+[c]	+[c]	+
Blue cheese	+	+	+
Yoghurt	+	+	(+)

[a] +, Identification positive; –, identification negative; (+), identification tentative. N-MC-THβC-3-COOH-MEs were prepared both by methods (A) and (B) of Fig. 1, except when indicated otherwise.
[b] N-MC-THβC-3-COOH-MEs obtained by method (A).
[c] N-MC-THβC-3-COOH-MEs obtained by method (B). Chemical names are in the text.

out, however, that after a positive identification of these compounds by MS, any further quantitative analysis seemed easier and more sensitive using RP-HPLC-fluorescent detection of the free compounds [1,4,26,27]. Additionally, identification of these compounds may also be accomplished by HPLC–MS coupling [4].

The fact that most of the foodstuffs examined contain THβC-3-COOHs suggests that their formation throughout the Pictet–Spengler condensation between tryptophan and aldehydes may readily occur during food production, processing and storage. Previously it was reported that acetaldehyde and formaldehyde react with tryptophan even under mild conditions to provide THβC-3-COOHs [1,26]. These studies also showed that low pH and high temperature accelerate the reaction rate and longer storage time before consumption would also increase the amount of THβC-3-COOHs produced if tryptophan remains in the medium. The concentration of precursors, specially tryptophan, but also aldehydes, should also be a decisive factor in the formation of THβC-3-COOHs. Technological factors during elaboration, processing or storage, such as smoking, maturation and ripening, fermentation or heating and oxidative processes could enrich the concentration of these compounds in foodstuffs. In this regard several

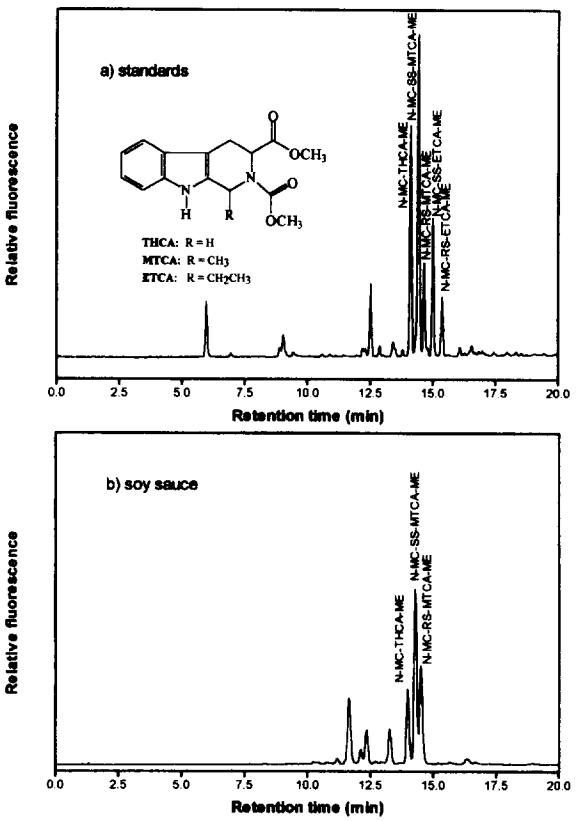

Fig. 6. RP-HPLC chromatograms of N—THβC-3-COOH-ME standards (a) and those obtained from extracted and derivatized soy sauce (b).

ways to reduce their content in alcoholic fermented products have been suggested [1].

MTCA occurs in foodstuffs as a mixture of two diastereoisomers easily separated by GC and HPLC, and it is usually found as the major THβC-3-COOH compared to THCA. This fact must be certainly due to the higher amount of acetaldehyde compared to formaldehyde in most foodstuffs [35]. An exception to this is toasted bread in which THCA occurs as the main component.

Since THβC-3-COOHs are present in foods, an exogenous intake of these compounds occurs during food ingestion. In the last few years, endogenous presence of these compounds in biological tissues and fluids has been pointed out [2,3,5]. So, although endogenous formation should not be ruled out, current results suggest that THβC-3-COOHs present in the body, and which are later excreted into the urine may arise from food uptake. The present findings together with those reported in the last few years are evidence that this is an important field for investigation by nutritionists and food scientists alike since THβC-3-COOHs may have health consequences [36].

5. Conclusion

By GC–MS it is shown that MTCA and THCA occur in commercial foodstuffs such as soy sauce, tabasco sauce, wine, beer, wine vinegar, orange juice, cider, blue cheese, yoghurt and toasted bread. MTCA generally occurs as the main THβC-3-COOH, except for toasted bread, containing mainly THCA. As far as we know, the presence of THβC-3-COOHs in tabasco sauce, toasted bread, orange juice, yoghurt, blue cheese, cider and wine vinegar is proved for the first time. GC–MS was accomplished after synthesizing the N-methoxycarbonyl methyl ester derivatives of THβC-3-COOHs following reaction with methyl chloroformate or methyl chloroformate and diazomethane. Since THβC-3-COOHs occur in commercial foodstuffs as common constituents, an exogenous intake of these compounds is expected during food ingestion.

Acknowledgments

The authors are grateful to the DGICYT (Spanish Government), projects ALI940737 and MAT94-0359-C02-02 and Comunidad Autonoma de Madrid, project COR-0035-94, which made this research possible. We thank also Dr. Christophe Fontenas for helping in the diazomethane preparation.

References

[1] T. Herraiz and C.S. Ough, J. Agric. Food Chem., 41 (1993) 959.
[2] R.D. Myers, Experientia, 45 (1989) 436.
[3] H. Rommelspacher, T. May and R. Susilo, Planta Med., 57 (1991) S85.
[4] J. Adachi, Y. Mizoi, T. Naito, Y. Ogawa, Y. Uetani and I. Ninomiya, J. Nutr., 121 (1991) 646.

[5] A. Brossi, in G.A. Cordell (Editor), The Alkaloids – Chemistry and Pharmacology, Vol. 43, Academic Press, San Diego, CA, 1993, p. 119.
[6] N.S. Buckholtz, Life Sci., 27 (1980) 893.
[7] G. Cohen and M. Collins, Science, 167 (1970) 1749.
[8] O. Beck, T.R. Bosin, B. Holmstedt and A. Lundman, in, Beta-carbolines and tetrahydroisoquinolines, Proc. of a Workshop held at The Salk Institute, La Jolla, CA, 1981, Alan R. Liss, New York, NY, 1982, p. 29.
[9] H. Rommelspacher and L. Schmidt, Pharmacopsychiatry, 18 (1985) 153.
[10] T.R. Bosin, S. Krogh and D. Mais, J. Agric. Food Chem., 34 (1986) 843.
[11] H. Rommelspacher, H. Damm, S. Lutter, L.G. Schmidt, M. Otto, N. Sachs-Ericsson and G. Schmidt, Alcohol, 7 (1990) 27.
[12] L. Tuomisto, M.M. Airaksinen, P. Peura and C.J.P. Eriksson, Pharmac. Biochem. Behav., 17 (1982) 831.
[13] C. Braestrup, M. Nielsen and C.E. Olsen, Proc. Natl. Acad. Sci. USA, 77 (1980) 2288.
[14] K. Wakabayashi, M. Ochiai, H. Saitô, M. Tsuda, Y. Suwa, M. Nagao and T. Sugimura, Proc. Natl. Acad. Sci. USA, 80 (1983) 2912.
[15] M. Higashimoto, T. Yamamoto, T. Kinouchi, H. Matsumoto and Y. Ohnishi, Mut. Res., 367 (1996) 43.
[16] N. Valin, D. Haybron, L. Groves and H.F. Mower, Mut. Res., 158 (1985) 159.
[17] N.A. Salvi and A.S.U. Choughuley, J. Sci. Food Agric., 52 (1990) 537.
[18] N.P. Sen, S.W. Seaman, P.A. Baddoo, D. Weber and G. Malis, Food Add. Cont., 8 (1991) 275.
[19] N.P. Sen, S.W. Seaman, B.P-Y. Lau, D. Weber and D. Lewis, Food Chem., 54 (1995) 327.
[20] K. Sakimoto, N. Engl. J. Med., 323 (1990) 992.
[21] J. Adachi, K. Yamamoto, Y. Ogawa, Y. Ueno, Y. Mizoi and Y. Tatsuno, Arch. Toxicol., 65 (1991) 505.
[22] A.N. Mayeno, F. Lin, C.S. Foote, D.A. Loegering, M.M. Ames, C.W. Hedberg and G.J. Gleich, Science, 250 (1990) 1707.
[23] E.A. Belongia, C.W. Hedberg, G.J. Gleich, K.E. White, A.N. Mayeno, D.A. Loegering, S.L. Dunnette, P.L. Pirie, K.L. MacDonald and M.T. Osterholm, N. Engl. J. Med., 323 (1990) 357.
[24] J. Ito, Y. Hosaki, Y. Torigoe and K. Sakimoto, Food Chem. Toxicol., 30 (1992) 71.
[25] D.E. Brenneman, S.W. Page, M. Schultzberg, F.S. Thomas, P. Zelazowski, P. Burnet, R. Avidor and E.M. Sternberg, J. Pharm. Exper. Ther., 266 (1993) 1029.
[26] T. Herraiz, Z. Huang and C.S. Ough, J. Agric. Food Chem., 41 (1993) 455.
[27] T. Herraiz, J. Agric. Food Chem., 44 (1996) 3057.
[28] E. Papavergou and M.N. Clifford, Food Addit. Cont., 9 (1992) 83.
[29] A. Brossi, A. Focella and S. Teitel, J. Med. Chem., 16 (1973) 418.
[30] P.D. Bailey, S.P. Hollinshead, N.R. McLay, K. Morgan, S.J. Palmer, S.N. Prince, C.D. Reynolds and S.D. Wood, J. Chem. Soc., Perkin Trans. 1 (1993) 431.
[31] T. Herraiz and C.S. Ough, Am. J. Enol. Vitic., 45 (1994) 92.
[32] T.R. Bosin and C.A. Jarvis, J. Chromatogr., 341 (1985) 287.
[33] S. Kim, J.I. Lee and Y.C. Kim, J. Org. Chem., 50 (1985) 560.
[34] P. Hušek, J. Chromatogr., 552 (1991) 289.
[35] V.J. Feron, H.P. Til, F. de Vrijer, R.A. Woutersen, F.R. Cassee and P.J. van Bladeren, Mutation Res., 259 (1991) 363.
[36] Anonymous, J. Nutr., 49 (1991) 367.