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Review

Analysis of the bioactive alkaloids tetrahydro-β-carboline and β-carboline in food

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Abstract

Simple tetrahydro-β-carbolines (THβCs) and β-carbolines (βCs) are naturally occurring alkaloids in foods and food processing. This paper reviews the methods employed for their analysis. Procedures for THβC and βC isolation and clean-up to remove interfering compounds are carried out by liquid–liquid extraction, and/or better solid-phase extraction under both reversed-phase (C_{18}) and cation-exchange mechanisms. Chemical derivatizations of THβCs with methyl chloroformate, or anhydrides are accomplished before GC–MS. Quantitative analysis of THβCs and βCs is made by RP-HPLC (C_{18}) with fluorescence detection providing good selectivity and sensitivity. For the same reasons, HPLC–MS is increasingly applied to these compounds. Electrospray and atmospheric pressure chemical ionization easily produce protonated molecules $(M+H)^+$ of both THβCs and βCs. Fragmentation by collision induced dissociation or tandem mass spectrometry helps to complete trace identification. The occurrence of biologically relevant THβCs and βCs in foods highlights the interest of accomplishing their analysis. Foods containing those compounds represent a source of possible THβCs and βCs in humans.

Keywords: Food analysis; Alkaloids; Tetrahydrocarbolines; Carbolines; Amines, heterocyclic

Contents

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1. Introduction

Tetrahydro-β-carbolines (THβCs) and β-carbolines (βCs) are a group of naturally occurring alkaloids that possess a common tricyclic pyrido[3,4-*b*]indole ring structure. Compounds of this family have been reported in plant systems [1], cigarette smoke, roasted foodstuffs and mammalian tissues. In the last few years, we have shown the occurrence of THβCs in many commercial foods, and suggested that it may contribute to their ultimate presence in human biological tissues and fluids [2–4]. This also means that β-carbolines are naturally occurring substances in foods chemically produced during food production, processing and storage.

Research done in the last decades has pointed out the occurrence of THβCs and βCs under physiological conditions in biological tissues and fluids [5–9]. THβCs and βCs might function as neuromodulators via effects on monoamine oxidase, monoamine uptake and benzodiazepine receptors binding [5,10]. Simultaneously, THβCs and βCs have been increasingly studied in relation to alcoholism [7,11–14]. Collins and co-workers have reported that *N*-methylated THβCs and βCs are endogenous neurotoxins [15,16]. 1-Methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (MTCA), a tryptophan–acetaldehyde condensation THβC, is a precursor of mutagenic *N*-nitroso compounds [17], shows cytogenetic effects [18], and may cause neuronal cell death in vitro [19]. Furthermore, βCs produced during food cooking may exhibit comutagenic and genotoxic potential [20]. Taking all those reports together, we should conclude that a full delineation of the biological activity and possible toxicity of THβCs and βCs is desirable and still needed.

The analysis of THβCs and βCs in complex matrices involves their isolation and further identification prior to quantitative analysis. Given the relatively low concentration of these substances in most foods and biological samples, along with the very complex matrices involved, it is often necessary a previous clean-up for purification and trace enrichment. Analytical techniques with high efficiency and selectivity, mainly based on capillary gas chromatography (GC) and high-performance liquid chromatography (HPLC), are required to avoid coeluting and interfering peaks. Although UV absorption, and electrochemical detection systems are applicable, fluorescence and mass spectrometry are preferred for selective and sensitive detection. For identification purposes, mass spectrometry (MS) coupled with high-performance chromatographic techniques is the best on-line system due to its high selectivity and specificity. These aromatic heterocycles provide good mass spectra for detection following GC or HPLC.

2. Chemistry of tetrahydro-β-carbolines and β-carbolines

Those tricyclic compounds share a common name of 9H-pyrido[3,4-*b*]indole. Their chemical nomenclature, however, is sometimes confusing. It is adopted to call compounds with aromatic pyrido rings βCs and compounds with a reduced pyrido ring THβCs (see Fig. 1). βCs have a pyrido nitrogen in position 2 of the ring (N-2) compared to α- or γ-carbolines which have the nitrogen in positions 1 and 3, respectively. Those compounds usually contain several substituents both in the pyrido ring and/or the indole ring. The so-called Pictet–Spengler reaction of indoleethylamines with aldehydes or α-keto acids has proven to be the most efficient route for the synthesis of THβCs (Fig. 1). We have shown that this reaction depends upon temperature and pH, and easily occurs in food environments releasing tetrahydro-β-carboline-3-carboxylic acids (THβC-3-COOHs) through a reaction involving L-tryptophan and formaldehyde or acetaldehyde [2,4,21]. As shown in Fig. 1, the Pictet–Spengler reaction proceeds through the formation of an iminium cation intermediate (Schiff base) that under acid catalyzed cyclization gives rise to THβCs. Aldehydes other than formaldehyde, provide THβCs with a chiral carbon C-1 with two enantiomers. However, two diastereoisomers occur from L-tryptophan-derived tetrahydro-β-carboline-3-carboxylic acid (1*S*,3*S* and 1*R*,3*S*). The oxidation of the THβC pyrido ring produces the fully aromatic β-carbolines.

3. Sample preparation and clean-up

Separation of THβCs and βCs by chromatograph-

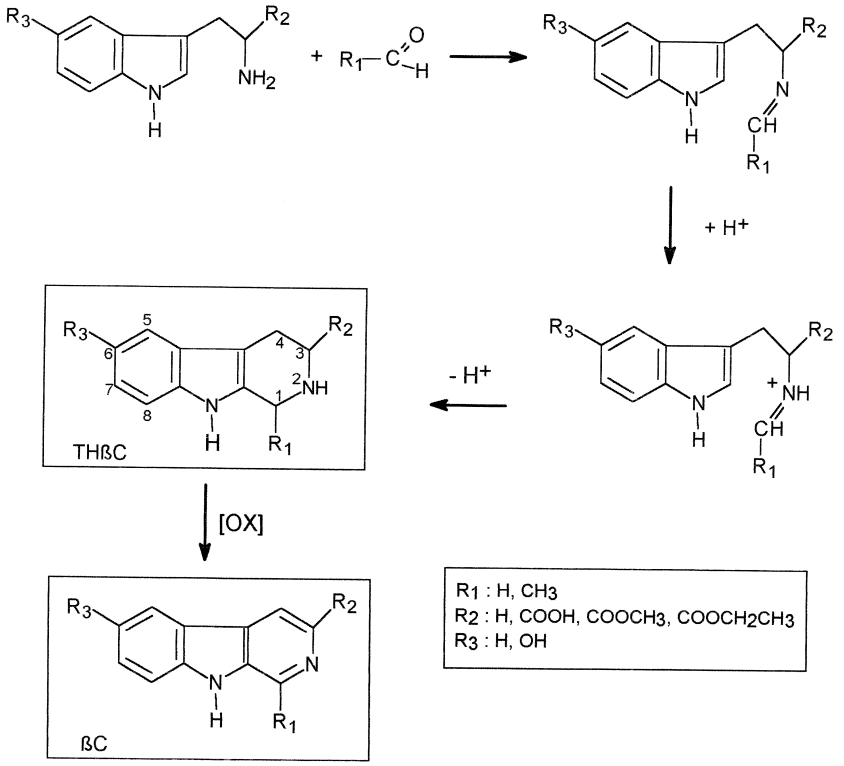

Fig. 1. Pictet–Spengler condensation between indoleamines and acetaldehyde or formaldehyde to give simple tetrahydro-β-carboline (THβC) alkaloids. Oxidation of THβCs provides β-carbolines (βCs).

ic means is generally preceded by clean-up procedures in order to isolate them from complex foods and biological matrices. Those include liquid–liquid extraction, and/or solid-phase extraction (SPE) by predominantly using ionic exchange and/or reversed-phase mechanisms. Very often, there is a chemical derivatization step prior to chromatographic analysis.

3.1. Sample preparation for tetrahydro-β-carbolines

Traditionally, an always possible problem of artifact formation has been of concern for high sensitivity assays of THβCs. Indeed, as aqueous condensation products, THβCs could form artificially during the sample preparation itself from traces of free aldehydes and indoleamines. Also, it seems troublesome to remove aldehydes from reagents and solvents employed during work-up. Various approaches have been taken to solve this problem. Among them, the use of semicarbazide as an aldehyde-trapping reagent [22] has been the most popular to prevent artifact formation. Also, using fluorescamine [23], or methyl chloroformate [24] to trap possible indoleamine precursors that are, then, no longer free for reaction. Furthermore, since the condensation to form THβCs is pH dependent as we know [21]; sample homogenization at neutral or slightly basic pH, could reduce or avoid artifact formation. Comparative work-ups at different pH values might be a good test to rule out possible artifacts. Others approaches are: minimize sample work-ups as much as possible, use direct sample introduction into the chromatograph, use various comparable analytical methods, and carry out blanks, and controls even with labeled precursors.

3.1.1. Solid-phase extraction procedures for THβCs

The development of SPE has improved and facilitated the isolation of THβCs from complex matrices like foods and biological samples. SPE based on reversed-phase mechanism has proven very useful. Already in 1982, THβCs from rat brain were isolated in C_{18} cartridges eluted with acetonitrile prior to GC–MS [25]. Given the rapidness of the extraction, this was a good method to avoid artifacts. Similarly, THβCs were extracted and concentrated from biological samples into C_{18} cartridges eluted with methanol providing recoveries from 75% to 94% [26]. The use of polymeric sorbents like Amberlite XAD-4 or XAD-7 eluted with methanol provided good recoveries for the isolation of THβC-3-COOHs from soy sauces [17] and smoked meats [27]. A minimum sample preparation procedure based on C_{18}-SPE has been employed for the analysis of tetrahydro-β-carboline-carboxylic acids prior to HPLC–MS [28]. In this procedure, aliquots diluted with distilled water were adjusted to pH 1, and loaded onto C_{18} cartridge. The cartridge washed with acidic water, and the compounds eluted with methanol containing 1% (v/v) trifluoroacetic acid (TFA). After evaporation, the extract redissolved in aqueous acetonitrile was applied to HPLC–electrospray ionization (ESI) MS–MS.

Based on ion-exchange SPE, a fast and reliable procedure for the extraction of food THβC-3-COOHs is accomplished on strong cation-exchange (SCX) columns [2,4,21,29]. In this procedure, acidic samples containing semicarbazide are loaded onto SCX-benzenesulfonic acid cartridges, washed with 0.1 *M* HCl, methanol, water, and rinsed with 0.4 *M* phosphate buffer (pH 9.1). THβC-3-COOHs are recovered with a mixture of 0.4 *M* phosphate buffer–methanol (1:1) (pH 10.1) that disrupts both ionic and hydrophobic interactions between those analytes and the sorbent. This method is advantageous to remove interfering compounds compared to C_{18}-SPE. By using this clean-up step followed by reversed-phase (RP) HPLC–fluorescence detection, we have obtained clean chromatograms of THβC-3-COOHs from many commercial foods with few or no interfering substances [2]. The recoveries from spiked samples of six foodstuffs (wine, beer, wine vinegar, cider vinegar, orange juice and yoghurt) were higher than 92% [2]. Good results with this procedure slightly modified in the elution protocol by using NH_4OH, were also reported [30].

For chemical identification purposes, a high concentration (enrichment) and purification of the sample is often needed. Both SCX and C_{18}-SPE cartridges can then be used in combination. An example is the work-up for chemical identification of THβC-3-COOHs in foods by GC–MS [3]. Food THβC-3-COOHs isolated and eluted from SCX cartridges as above, were retained onto C_{18} cartridges, and eluted with adequate mixtures of acidic water and acetonitrile. Another approach was used by Sen et al. [30] who isolated THβC-3-COOHs by using strong anion-exchange (SAX) and C_{18}-SPE cartridges. SAX removed interferences while C_{18}-SPE retained the compounds of interest that were eluted with methanol.

3.1.2. Chemical derivatization and extraction for THβCs

The sample preparation of THβCs very often includes a chemical derivatization stage. As shown in Fig. 2, chemical derivatization is accomplished with acylating reagents such as methyl chloroformate (MCF), trifluoroacetic anhydride (TFAA), pentafluoropropionic anhydride (PFPA) or heptafluorobutyric anhydride (HFBA), reacting with the N-2 pyrido nitrogen and hydroxy substituents. Less common is the reaction with indolic nitrogen [23]. Moreover, formation of methyl ester derivatives is convenient to handle 3-carboxylic compounds. Subsequent liquid–liquid extraction and organic solvent concentration provides the required isolation and enrichment of those derivatives for chromatography.

Aqueous derivatization of THβCs with methyl chloroformate produces carbamates that can be further analyzed by HPLC and GC–MS [2,3,24]. For that, the sample in phosphate buffer (pH 7.2) is treated with methyl chloroformate to form the *N*-methoxycarbonyl derivatives. Then, the pH is increased with saturated sodium carbonate solution, and treated again, with methyl chloroformate for complete reaction. Extraction of the sample with dichloromethane yields basic THβCs. Acidification of the remaining aqueous phase and extraction with dichloromethane yields acidic THβCs. This procedure provides quantitative derivatization and high

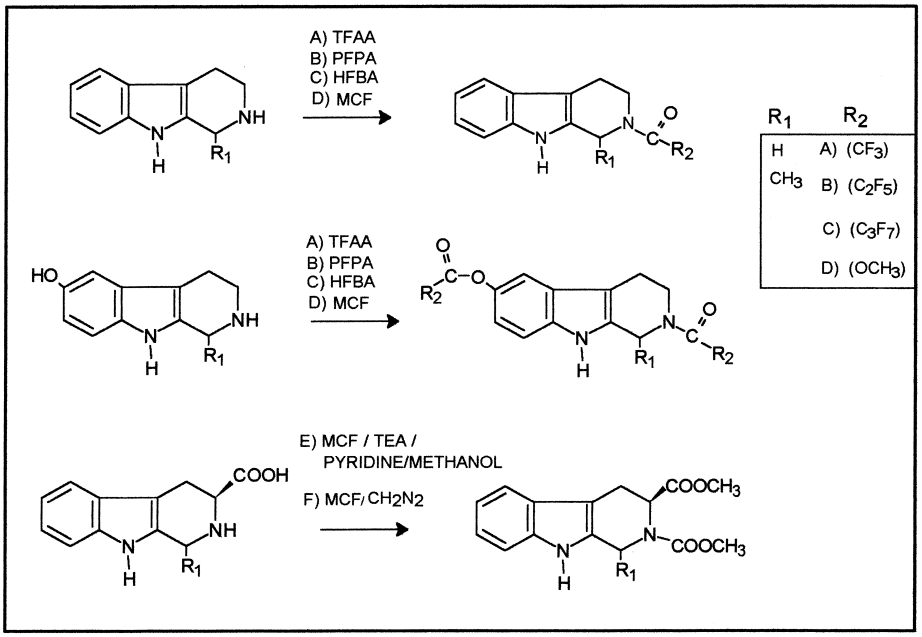

Fig. 2. Chemical derivatization reactions employed for the analysis of tetrahydro-β-carbolines. Chemical names are: TFAA, trifluoroacetic anhydride; PFPA, pentafluoropropionic anhydride; HFBA, heptafluorobutyric anhydride; MCF, methyl chloroformate; TEA, triethylamine; CH_2N_2, diazomethane.

recoveries [31], and it has been used as an additional confirmation method of the presence of THβCs in foods [2]. A similar derivatization procedure was applied to identify low amounts of formaldehyde-derived THβCs in human urine by GC–MS [32]. In this case, the extraction of *N*-methoxycarbonyl derivatives was carried out first into Chem-Elut, and then into C_{18} columns that were eluted with methanol prior to GC–MS. Besides facilitating the isolation of these compounds, reaction with methyl chloroformate helps to reduce the potential of artifact formation of THβCs during the work-up procedure since it reacts with available free indoleamines.

Our laboratory has applied the chemical derivatization with methyl chloroformate alone, and methyl chloroformate plus diazomethane to get the *N*-methoxycarbonyl methyl ester derivatives, allowing identification of THβC-3-COOHs in many foods by GC–MS [3]. Interestingly, as summarized in Fig. 2, under certain conditions, methyl chloroformate reacted in one derivatization step with both the pyrido nitrogen and the carboxylic acid providing the *N*-methoxycarbonyl methyl ester derivatives. This reaction based on the formation of mixed anhydrides [33] has been used for GC analysis of amino acids [34]. To achieve that, THβC-3-COOHs dissolved in 0.1 *M* HCl–acetonitrile (70:30) containing semicarbazide, and added with triethylamine, reacted with methyl chloroformate to give *N*-methoxycarbonyl derivatives and mixed anhydrides. The formation of methyl esters was achieved by adding methanol and pyridine at 0°C [3]. The compounds were extracted with ethyl acetate or dichloromethane and analyzed by GC–MS.

Several acylating reagents such as TFAA, PFPA, HFBA, and *N*-bis-trifluoroacetamide have proven their usefulness for analyzing THβCs (Fig. 2). Thus, 1,2,3,4-tetrahydro-β-carbolines were studied by GC–MS in alcoholic beverages and foods following derivatization with PFPA [22,35,36]. The samples, spiked with semicarbazide, were extracted either with 3% isoamyl alcohol in toluene, or dichloromethane, in basic medium. Then, the organic layers evaporated and derivatized with PFPA (60°C, 20 min). The recoveries reached a 70–80% with derivatization yields higher than 90%. In a similar

procedure, THβCs from biological samples and various foods were extracted and derivatized with HFBA (70°C, 30 min) [37]. TFAA was employed to get the 1-methyl-1,2,3,4-tetrahydro-β-carboline *N*-trifluoroacetyl derivative by reacting with TFAA–acetonitrile (1:1), following pretreatment with fluorescamine and extraction with ethyl acetate [23]. TFAA has been also successfully employed to obtain the *N*-trifluoroacetyl derivatives of THβC-3-COOHs (as methyl ester) from wines for subsequent GC–MS [38]. The chemical derivatization with the reagents of Fig. 2 improves not only the extractability by liquid–liquid extraction but also the GC properties of THβCs. However, it may lead to low recoveries, and quantitation problems. Furthermore, artifact formation by THβCs is more likely to occur owing to possible traces of aldehydes in derivatization reagents and solvents.

Most of the sample preparation protocols employ semicarbazide as an aldehyde-trapping reagent to avoid artifacts. However, semicarbazide may not completely remove aldehydes such as formaldehyde. Instead, we can employ fluorescamine [23,39] to block primary amines that could potentially condensate with free aldehydes to give artifact formation of THβCs during work-up. Tsuchiya et al. [39] analyzed 1,2,3,4-tetrahydro-β-carbolines in alcoholic beverages as follows: after treatment with fluorescamine, the reaction mixture was extracted with ethyl acetate in basic medium, back extracted to 0.2 *M* HCl, and then re-extracted with diethyl ether in basic medium for subsequent RP-HPLC after evaporation and redissolution. The recoveries obtained from spiking experiments were higher than 97.5% in beer, wine and urine.

Combination of liquid–liquid extraction, with or without sample derivatization, and SPE can afford the necessary isolation, concentration and clean-up of THβCs. In this regard, we have employed SPE (C_{18}, and SCX cartridges) and liquid–liquid extraction besides sample derivatization for identifying THβC-3-COOHs in commercial foods [3]. A new β-carboline ester: 1-methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid ethyl ester, has been recently isolated from alcoholic beverages by using both, dichloromethane extraction (pH 9–10), and SCX prior to HPLC and GC–MS [40]. Recoveries were higher than 90%. Derivatization with methyl chloroformate was accomplished prior to GC–MS for identification purposes. In an interesting work, *N*-nitrosotetrahydro-β-carbolines were isolated from smoked foods and fermented products following an scheme based on liquid–liquid extraction, and clean-up with alumina. The compounds were determined by post-column (HPLC)–chemical denitrosation–thermal energy analysis (TEA) (rapid screening test) [41]. If a positive test, then the samples were cleaned-up on C_{18} cartridges and further analyzed by HPLC–MS or GC–MS.

3.2. Sample preparation for β-carbolines

As for THβCs, both SPE and liquid–liquid extraction are applied for sample preparation of βCs. Although liquid–liquid extraction is effective, SPE is simple, rapid and usually gives good recoveries without interferences. The artifact production of βCs (norharman and harman) during work-up is not considered a complicating factor as it is for THβCs. This assumption, however, should be taken with caution owing to the possible formation of βCs from tetrahydro-β-carboline-carboxylic acids by oxidative decarboxylation.

A method based upon liquid–liquid extraction of βCs from food and biological samples was reported by Bosin and Faull [42,43]. For that, the samples plus semicarbazide, adjusted to pH 10, were extracted with ethyl acetate. After evaporating to dryness and redissolving in 0.01 *M* $HClO_4$, they were analyzed by HPLC. On the other hand, those extracts were reacted with pentafluorobenzyl bromide in tetrahydrofuran and sodium hydride to derivatize the βC harman that was extracted with hexane before GC–MS. An SPE-based procedure for the analysis of βCs in foodstuffs followed by HPLC and HPLC–MS was reported by Adachi et al. [44]. Samples were cleaned-up using Bond-Elut PRS (propylsulfonic acid-derivatized silica) cartridges with excellent recoveries. The acidic samples were loaded onto PRS; the cartridges washed with water, rinsed with 0.4 *M* phosphate buffer (pH 9.1), and β-carbolines eluted with methanol–0.2 *M* phosphate buffer (pH 8.8) (1:1). The recoveries of βCs (norharman and harman) were very good varying from 90 to 100%. This method has the advantage of yielding extracts more simple and faster than liquid–

liquid extraction. A method based on SCX-benzenesulfonic columns, is currently being used in our laboratory with recoveries higher than 90% of both norharman and harman (Herraiz, unpublished results).

Two non-polar heterocyclic amines having a βC structure (e.g., norharman and harman) are frequently isolated from well cooked meat and fish by using SPE procedures. For this purpose, they are firstly extracted with organic solvents (e.g., dichloromethane, toluene, ethyl acetate) on diatomaceous earth (Extrelut), and subsequently purified on PRS SPE cartridges, that removes most of the unwanted co-extracted interfering peaks [45,46]. In some cases, concentration of these eluates is conveniently achieved on C_{18} silica cartridges, and βCs finally eluted with a methanol–ammonia solution mixture. An average recovery of 66% for norharman and 57% for harman was reported in spiked meat samples [47], with detection limits in the ng/g level [46]. This method was applied to the analysis of heterocyclic amines in pan-fried, oven-cooked and barbecued fish [45]. For very complicated samples, a weak cation-exchange material (TSK CM650 resin) is used as a sorbent with different selectivity in which interfering peaks of extracts prepared by diatomaceous earth–PRS extraction are significantly lowered [46].

4. Chromatographic and spectral analysis

HPLC in combination with fluorometry and/or MS, and GC in combination with MS are the techniques predominantly used for separation and quantitation of THβCs and βCs. Some disadvantages of GC, such as the need of chemical derivatization to make volatile β-carbolines, possible artifact formation during derivatization or, even, difficulty in trace analysis are currently being solved by HPLC and mainly HPLC–MS of underivatized β-carbolines.

4.1. Chromatographic analysis of tetrahydro-β-carbolines

The use of HPLC with fluorescence or MS [ESI or atmospheric pressure chemical ionization (APCI) techniques] detection along with GC–MS, has overcome earlier limitations of THβC analysis, such as the need of large sample sizes, extensive sample preparation, and the lack of required specificity and selectivity. Also, tandem mass spectrometry (MS–MS) is a current powerful tool due to its sensitivity and inherent selectivity.

GC separation is greatly improved by chemical derivatization of THβCs (Fig. 2). Those analyses usually incorporate deuterated analogues as internal standards for more precise quantitation by GC–MS. Beck and co-workers [22,35,36] analyzed 1-methyl-1,2,3,4-tetrahydro-β-carboline (MTHβC), 6-hydroxy-1-methyl-1,2,3,4-tetrahydro-β-carboline (6-OH-MTHβC), and 6-hydroxy-1,2,3,4-tetrahydro-β-carboline (6-OH-THβC) in alcoholic beverages and foods as their *N*-pentafluoropropionyl derivatives by GC–MS (70 eV) using a SE-52 capillary column. The bis-(pentafluoropropionyl) derivatives of 6-OH-MTHβC and 6-OH-THβC exhibited high-molecular-mass ions ($M^{+\cdot}$ at m/z 494 and 480, respectively) that were used for quantitation. Analysis by GC–MS [37] and GC–MS–MS (positive and negative mode) [48] of THβC heptafluorobutyryl (HFB) derivatives has been reported with detection limits in the low pg range. Thus, the N-HFB derivative of 1-methyl-1,2,3,4-tetrahydro-β-carboline was chromatographed onto an SE-52 capillary column and the ions $M^{+\cdot}$ (m/z 382) and $M^{+\cdot}$-CH_3 (m/z 367) monitored [37]. Recently, Bringmann et al. [49] analyzed 1-trichloromethyl-1,2,3,4-tetrahydro-β-carboline (TaClo), a new chlorinated THβC in biological samples, as its HFB-derivative by GC–MS and selected ion monitoring (SIM). High sensitivity detection of TaClo was also possible using GC–electron-capture detection since halogens are excellent electron acceptors. When using GC–MS, negative ion chemical ionization (NICI) MS is a good sensitive and specific method for identifying THβCs. GC–NICI-MS was applied to determine 1-methyl-1,2,3,4-tetrahydro-β-carboline in urine and foodstuffs as its *N*-bis-trifluoroacetyl derivative [23,39]. This compound showed not only good ionization efficiency by providing a single ion (m/z 378) in the methane NICI mode (GC–NICI-MS), but also good GC properties. GC–MS was also employed to analyze *N*-nitroso-THβCs derivatives in nitrite treated foods by using a DB-5 column and monitoring the $(M\text{-}NO)^+$ and molecular ions ($M^{+\cdot}$) [30].

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) (in both diastereoisomers: 1*S*,3*S* and 1*R*,3*S*) were reported and analyzed by Herraiz and Ough in wines, as their *N*-trifluoroacetyl methyl ester derivatives [38]. Those compounds and their diastereoisomers were separated by GC–MS onto a SPB-5 fused-silica capillary column, and their EI-MS spectra and fragmentation provided and assigned [38]. Later, the same compounds were identified by GC–MS (EI) as their *N*-methoxycarbonyl methyl ester derivatives, and their occurrence reported in many commercial foods [3]. Those derivatives exhibited good GC properties when chromatographed onto a methylsilicone capillary column (Fig. 3). Their excellent profile from EI-MS spectra (Fig. 4) enabled identification of THCA and MTCA in food extracts [3]. We also studied EI-induced fragmentation of up to 40 THβCs and their precursors – tryptamine and tryptophan derivatives [50]. Fragmentation of THβCs is dominated by the Retro–Diels Alder rearrangement that provides the ions $C_{10}H_9N^+$ (*m*/*z* 143) for tetrahydro-β-carbolines and $C_{11}H_{11}N^+$ (*m*/*z* 157) for 1-methyl-tetrahydro-β-carbolines [50,56]. Both compounds exhibit abundant molecular ions ($M^{+\cdot}$). *N*-Trifluoroacetyl and *N*-methoxycarbonyl derivatives suffer the loss of derivatization groups $COCF_3$, and $COOCH_3$, respectively. Interestingly, 1,3-disubstituted 1,2,3,4-tetrahydro-β-carbolines contain two diastereoisomers (1*S*,3*S*; 1*R*,3*S*) which are resolved by GC–MS providing similar mass spectra (Fig. 5).

GC–MS analysis of derivatized THβCs provides good qualitative and quantitative results. Nevertheless, HPLC combined with fluorescence and MS detection is often the method of choice given its simplicity, good selectivity, and excellent sensitivity. The best commonly available method for quantitative analysis of THβCs is RP-HPLC with fluorescence detection under appropriate wavelengths for excitation and emission (e.g., 270 nm excitation and 343 nm emission). Indeed, this is a suitable procedure for THβC-3-COOHs following SCX sample preparation. As shown in Fig. 6, good and clean chromatograms with the compounds of interest are generally obtained when analyzing food extracts by RP-HPLC onto a C_{18} column, and eluting with a gradient of acetonitrile in phosphate buffer (pH 3) [2]. Furthermore, interesting and additional information on peak purity is given by profiling the fluorescent spectra from THβC chromatographic peaks [2,55,60]. Given the good sensitivity of the method (detection limits under ng/g), the direct injection of the sample without previous clean-up, is sometimes useful to rule out possible artifacts. RP-HPLC–fluorescence has been employed to analyze tetrahydro-β-carboline-3-carboxylic acid ethyl esters from alcoholic beverages with good results [40]. Another approach is the analysis of *N*-methoxycarbonyl derivatives of THβCs by RP-HPLC–fluorescence [2,24]. They keep the fluorescence properties, and are retained longer than the parent compounds under the same chromatographic conditions. RP-HPLC analysis of those *N*-methoxycarbonyl compounds is useful as an additional chromatographic method for confirming the absolute presence of free THβCs in foods [2].

A significant improvement for the analysis of THβCs is RP-HPLC coupled to MS detection. As shown in Fig. 7, HPLC–ESI-MS analysis of two major THβC-3-COOHs chromatographed onto a C_{18} column provides the protonated molecular ions $(M+H)^+$ easily used for identification and quantitation. HPLC–MS equipped with a thermospray (TSP) interface was employed to identify THβC-3-COOHs in alcoholic beverages (sake) [29]. Chromatographic separation was achieved on a TSK gel ODS by using 23% aqueous methanol and 0.1 *M* ammonium formate (pH 3.4). LC–TSP-MS has been employed to identify *N*-nitroso-tetrahydro-β-carbolines in foods [41]. LC–APCI-MS was used to identify THβC-3-COOHs in smoked meats and wines [30], by using a C_{18} column in a gradient containing mixtures of formic acid, ammonium acetate and acetonitrile. The authors acknowledged that HPLC–MS quantitation agreed well with HPLC–fluorescence. Recently, Gutsche and Herderich [28,51] used HPLC–ESI-MS–MS for identification of tetahydro-β-carboline-carboxylic acids. Chromatographic separation was performed on a C_{18} column by using a binary gradient of 0.05% TFA in water and acetonitrile. The ESI process ionized THβCs in the positive mode providing exclusively protonated molecules $(M+H)^+$. In addition, low-energy collision induced dissociation (CID) of those protonated molecules gave the neutral loss of the iminoacetic acid moiety ($C_2H_3NO_2$, 73 u) due to the retro-Diels–Alder

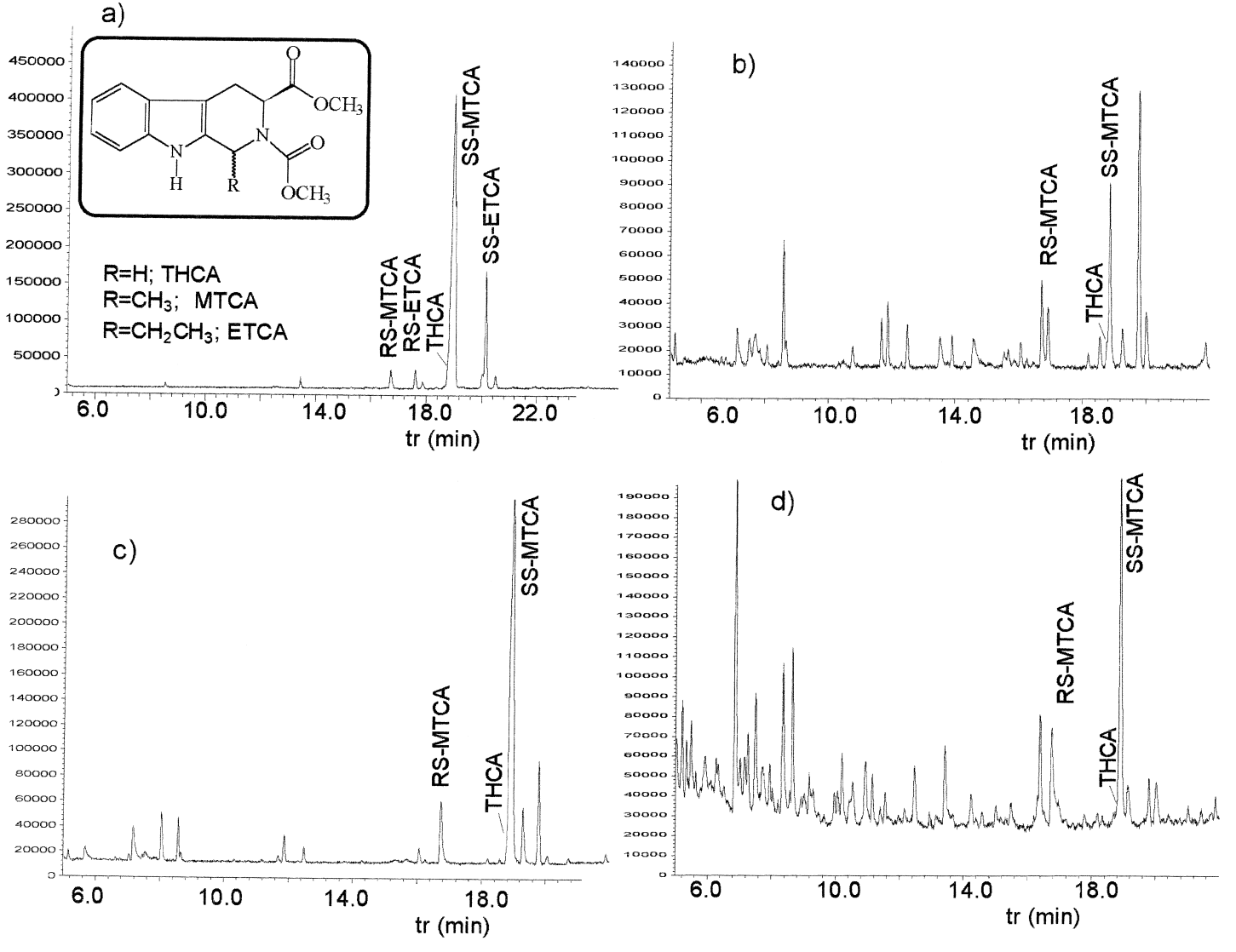

Fig. 3. GC–MS chromatograms of THβC-3-COOHs (THCA, MTCA – 1*S*,3*S* and 1*R*,3*S* diastereoisomers –, and ETCA – 1*S*,3*S* and 1*R*,3*S* diastereoisomers), as their *N*-methoxycarbonyl methyl ester derivatives from standard sample (a), and those isolated from beer (b), soy sauce (c) and wine vinegar (d). From Ref. [3] with permission.

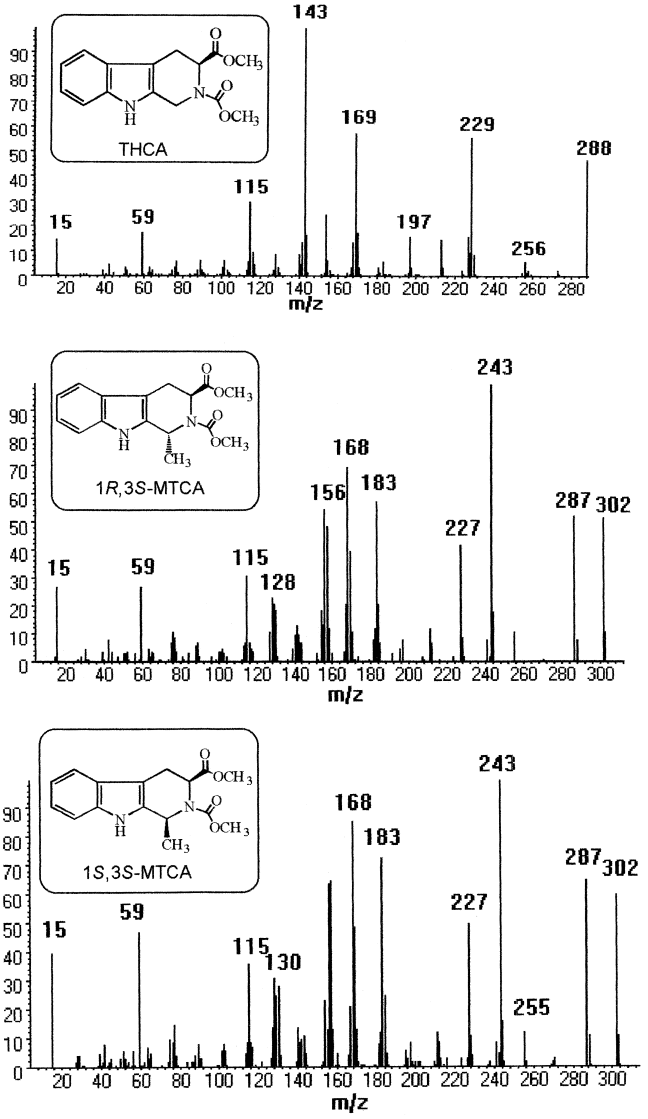

Fig. 4. Electron ionization mass spectra of two major THβC-3-COOHs found in food samples as their *N*-methoxycarbonyl methyl ester derivatives. From Ref. [3] with permission.

fragmentation [28], that led to substructure specific identification of THβCs in fermented beverages, sauces and yeast extracts. By using selected reaction monitoring (SRM), the limits of detection for various THβCs were established in the ng/ml range. Due to the excellent selectivity and sensitivity of SRM, no sample preparation step was required prior to analysis, excluding any artifact formation [51].

4.2. Chromatographic analysis of β-carbolines

BCs show very high native fluorescence and this facilitates their sensitive analysis by HPLC means. The use of HPLC with fluorescence detection in combination with GC–MS or better still HPLC–MS for identification, is overcoming former limitations in βC analysis. In an early GC method, the βC harman

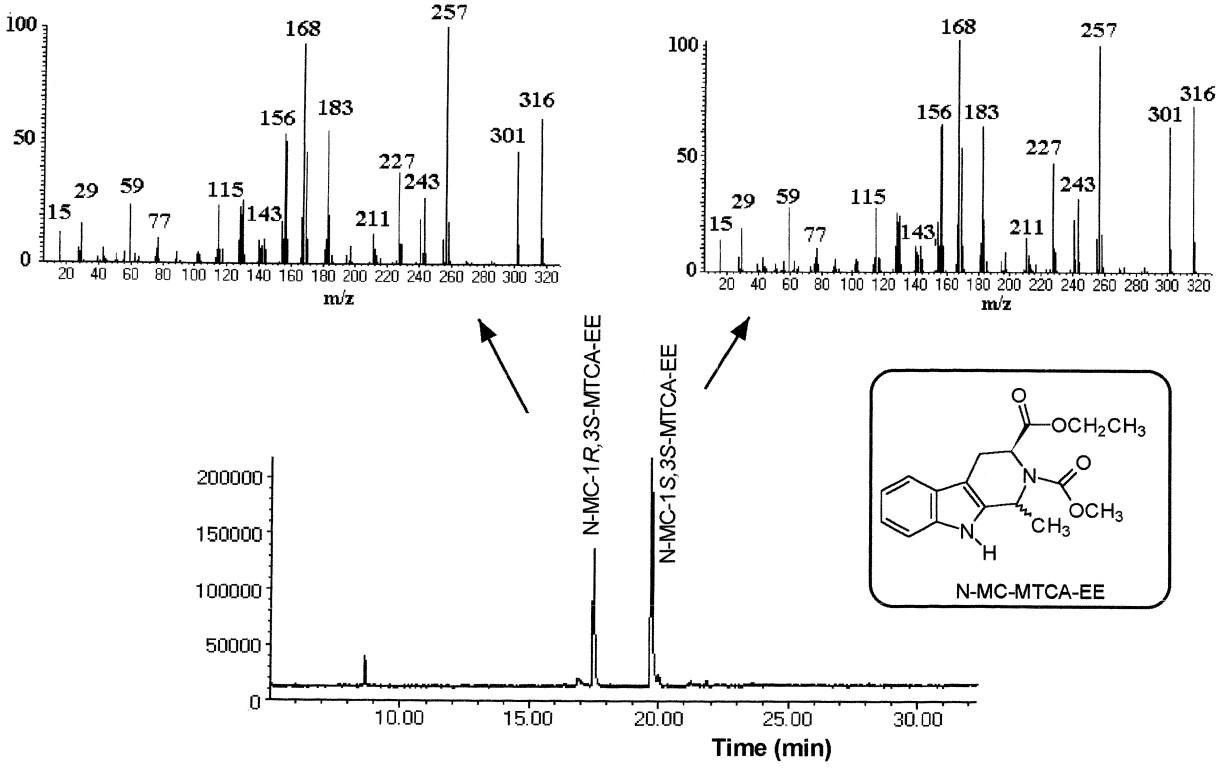

Fig. 5. Chromatographic separation and electron ionization mass spectra of 1-methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid ethyl ester (1*S*,3*S* and 1*R*,3*S* diastereoisomers) as *N*-methoxycarbonyl derivatives. This compound was reported in fermented alcoholic beverages [40]. From Ref. [50] with permission.

was derivatized with pentafluorobenzyl bromide [42,43]. The resulting 9-pentafluorobenzylharman improved the GC–MS properties, and gave a simple NICI mass spectrum with a single ion (*m*/*z* 181) corresponding to indole anion, that was used for structural determination of harman in alcoholic beverages and biological samples. A medium polarity fused-silica capillary column (DB-5) was used for GC separation. For quantitative purposes, those authors analyzed βCs by HPLC (C_{18}) with fluorescence detection by eluting with 0.5% triethylamine in methanol–water (60:40). βCs were detected using 252 nm for excitation and a 430 nm cut-off filter for emission [42,43]. A GC–MS method for the analysis of nonpolar heterocyclic amines (e.g., harman and norharman) in cooked meats, pan residues and meat extracts has been recently reported [52]. Its main advantage is that separation of β-carbolines is made without chemical derivatization. The gas chromatograph was equipped with a capillary column (Rtx-50, 50% phenyl–50% methylpolysiloxane) directly introduced into the ion source of MS system. The mass spectrometer was operated in the negative ion mode (70 eV) giving ions of 182 and 168 *m*/*z* for harman and norharman, respectively. The authors, however, acknowledged tentative quantitation due to varying degrees of recovery of these compounds.

HPLC–fluorescence is a suitable method for quantitative analysis of βCs. Furthermore, the fluorescence spectra provide relevant qualitative information upon the compounds involved, as we have recently shown while studying the oxidation of THβCs to give βCs [60]. Very often, however, βCs appear with interfering chromatographic peaks, and mass spectrometric detection is required. Because of their involatility, βCs are not generally amenable to

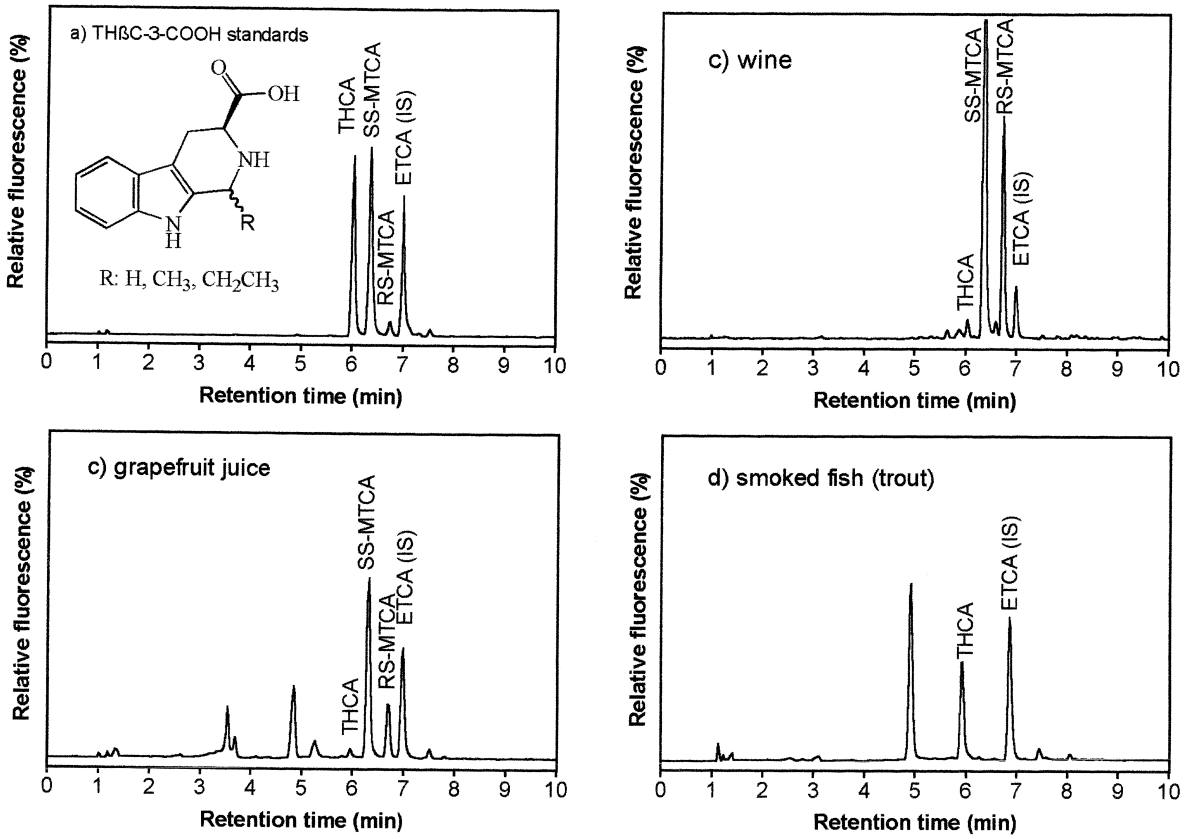

Fig. 6. RP-HPLC–fluorescence of 1,2,3,4-tetrahydro-β-carboline-3-carboxylic acids (THβC-3-COOHs) in: (a) standards, (b) wine, (c) grapefruit juice, (c) smoked fish. NovaPak C_{18} column; 0 to 20% of acetonitrile in buffer phosphate (pH 3) in 8 min. Excitation 270 nm, emission, 343 nm. THCA: 1,2,3,4-Tetrahydro-β-carboline-3-carboxylic acid; MTCA: 1-methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (1*S*,3*S* and 1*R*,3*S* diastereoisomers); ETCA: 1-ethyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (I.S., internal standard).

direct assay by GC–MS where chemical derivatization is usually needed. Currently, this is overcome by HPLC–MS that combines the efficiency of HPLC with the sensitivity and selectivity of mass spectrometry. Interferences from complex matrices, and the need of normally laborious and time-consuming isolation procedures are also reduced. HPLC–MS in the electrospray ionization mode gives a simple mass spectrum of βCs in which the main peaks are due to protonated molecular ions $(M+H)^+$ (m/z 169 for norharman, m/z 183 for harman and m/z 197 for ethylnorharman) (Fig. 8). βCs are stable toward the ionization process and do not undergo notable fragmentation. Thus, single-ion monitoring (SIM) of the $(M+H)^+$ ions from the respective βCs could then be employed for analysis. Thermospray-positive ion mode HPLC–MS was set up to identify βCs in some foodstuffs [44]. For that, the authors used a liquid chromatograph–tandem quadrupole mass spectrometer equipped with a thermospray interface. The mobile phase was methanol–(0.1 *M* ammonium formate+0.1 *M* formic acid) pH 3.4 (23:77, v/v). As in electrospray ionization (Fig. 8), positive-ion TSP mass spectra also gave the $(M+H)^+$ ions of either norharman (m/z 169) and harman (m/z 183) as base peaks. For quantitative purposes, βCs were analyzed in a C_{18} column with fluorescence detection (excitation 300 nm, emission 433 nm). In this case, the mobile phase was methanol–0.1 *M* KH_2PO_4 (pH 3) (32:68, v/v) [44].

The identification and quantitation of heterocyclic amines of the βC type (norharman and harman) in

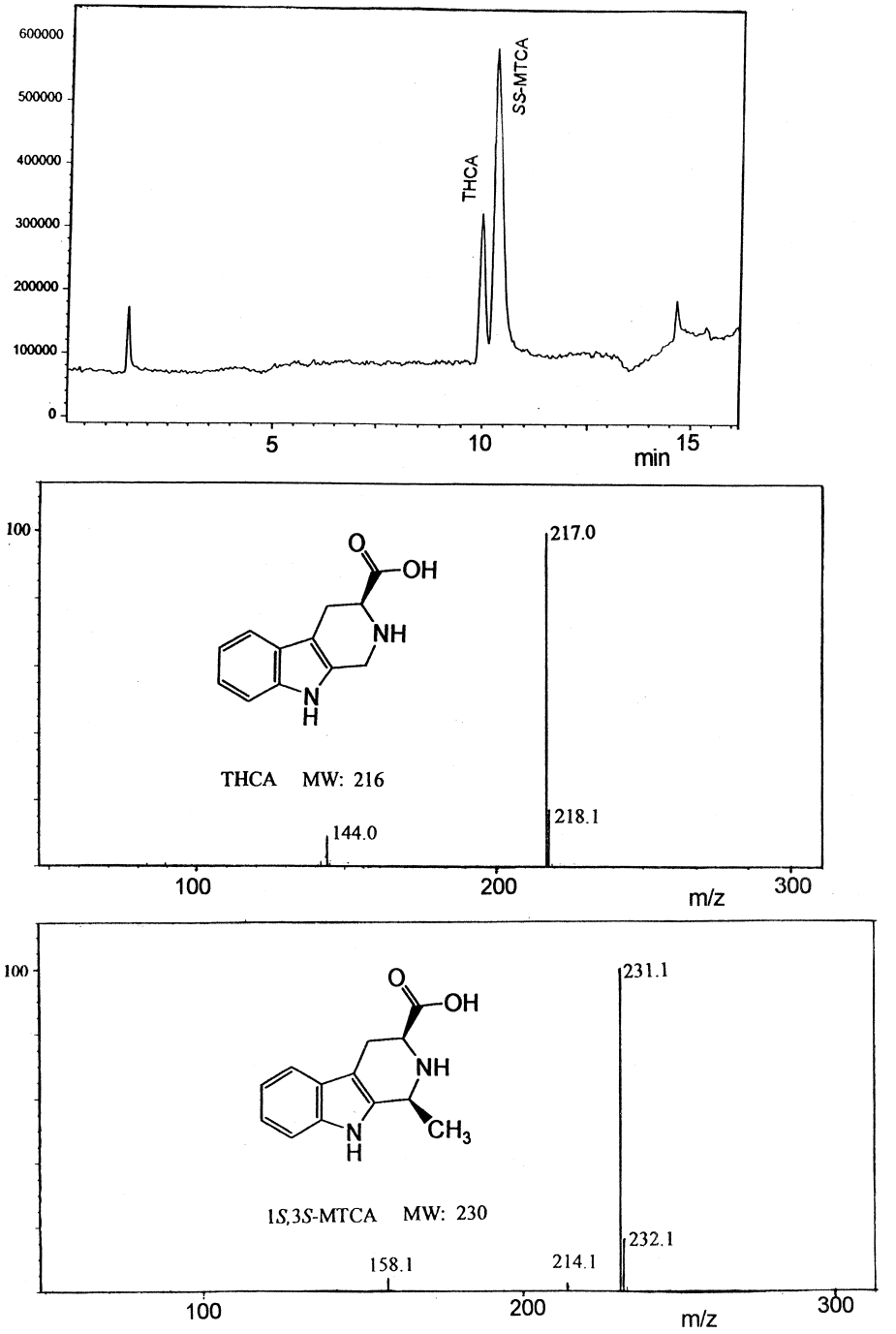

Fig. 7. HPLC–MS (electrospray-positive ion mode) of two major THβC-3-COOHs. Equipment: LC–mass-selective detector series HP 1100 (Hewlett-Packard). Column NovaPak C_{18} 150×3.9 mm (Waters). Eluents: (A) formic acid (1%), (B) formic acid (1%) in acetonitrile; 0 to 30% B in 15 min. Flow: 1 ml/min; *T* 40°C. Cone voltage: 40 V.

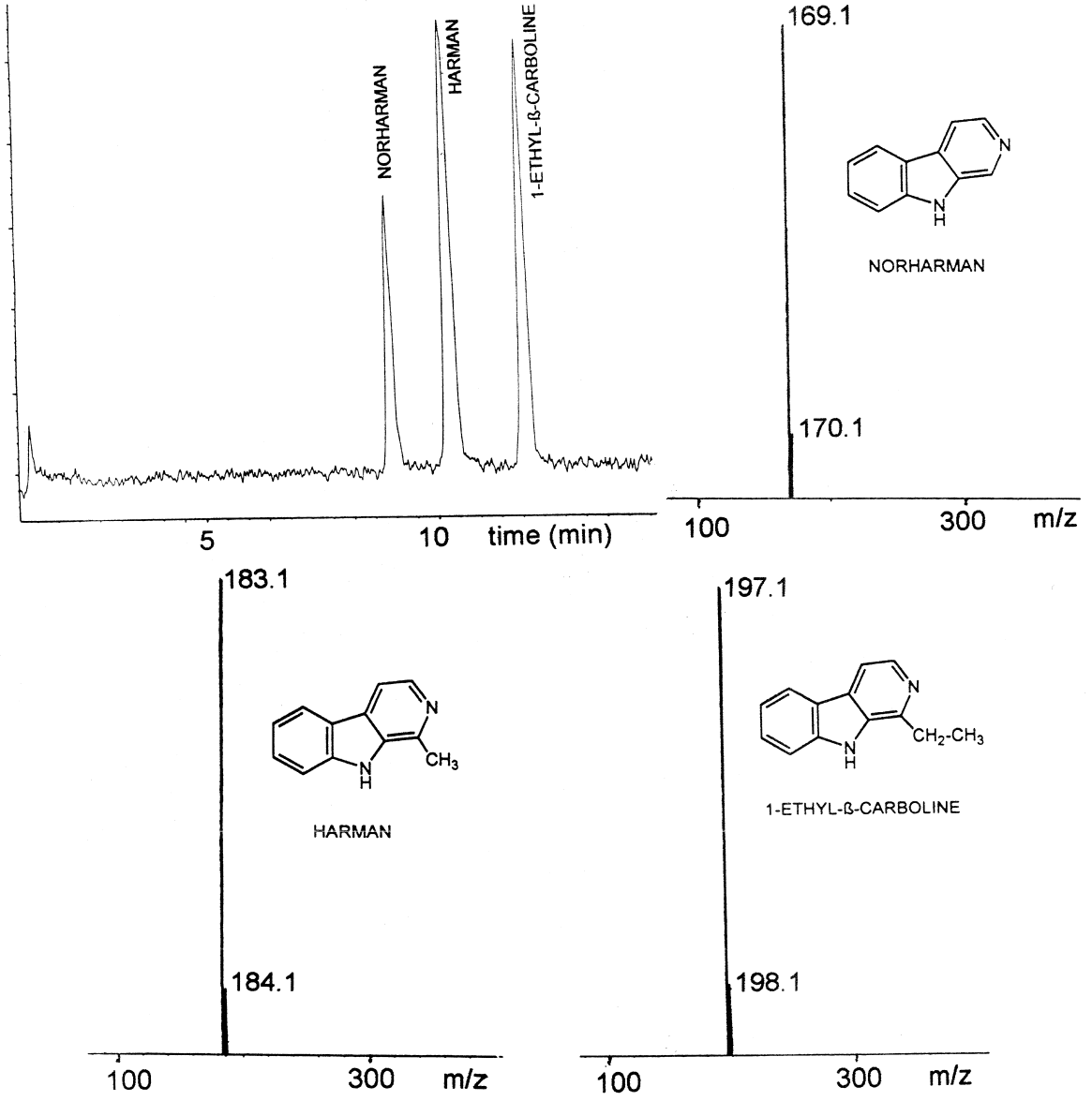

Fig. 8. HPLC–MS (electrospray-positive ion mode) of βCs (norharman, harman and 1-ethyl-β-carboline). Equipment: LC–mass-selective detector series HP 1100 (Hewlett-Packard). Column SB-C_{18} 5 cm×2.1 mm (Zorbax). Eluents: (A) formic acid (1%), (B) formic acid (1%) in acetonitrile; 0 to 20% B in 15 min. Flow: 1 ml/min; T 40°C. Cone voltage: 40 V.

cooked meats is accomplished by HPLC followed by UV and fluorescence (300/440 nm) detection. A good peak symmetry and separation efficiency is reported with reversed-phase silica columns of the type TSK gel. Binary mobile phase gradients containing acidic buffers (between pH 3 and 4) and acetonitrile provides good peak shapes [46]. Regarding HPLC–MS, some authors [53,54] have identified norharman and harman besides the rest of mutagenic amines, in meat extracts by using ESI, and APCI HPLC–MS under positive ionization. The detection limit in the full scan and SIM modes were in the low ng and pg ranges, respectively. The separation was performed on a C_{18} column with a binary mobile

phase composed of ammonium acetate and acetonitrile in gradient mode. The $(M+H)^+$ ions of β-carbolines were used for analysis, and positively confirmed by using in-source fragmentation.

5. Tetrahydro-β-carbolines and β-carbolines in foods

As mentioned above, simple THβCs are alkaloids that occur naturally in foods as a chemical condensation between indoleamines and aldehydes or α-keto acids [2–4,21,28] (see Fig. 1). This reaction may occur during food production, processing and storage. In addition to the type of food involved, many chemical and technological factors, such as: amount of precursors available, pH, temperature, storage time, oxidants, antioxidants, preservatives, yeasts, processing conditions (e.g., fermentation, smoking, heating, cooking and ripening), will ultimately affect the level of those compounds in foods [2,4,21,55]. Table 1 summarizes the content of THβCs and βCs in foods. Among the THβCs encountered, those THβC-3-COOHs are the major compounds which can reach up to several mg/kg, and exceptionally a hundred of mg/kg. They had been reported in soy sauce, fermented alcoholic beverages, and smoked foods [2,4,17,27,29–31,38]. As we have shown [2,4], THβC-3-COOHs are widespread in many commercial foods and beverages, including many types of wines and beers, vinegar, liqueurs, seasonings, yoghurt, cheese, pickles, juices and soft drinks, bread, and fish. THβC-3-COOHs also occur in fruits, fruit processed products (fruit juices, and jams) and baby foods (fruit purees) [55,62]. Several 1,3-dicarboxylic THβCs, and 1-carboxylic-THβCs, not included in Table 1, have been identified in alcoholic beverages and sauces [28,57]. We have reported the first β-carboline ester: 1-methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid ethyl ester, that was

Table 1
Concentration ranges of THβCs and βCs found in foods[a]

	Concentration range	Ref.
THβCs		
THCA	Wine (0–0.65 mg/l); beer (nd–0.84 mg/l); wine vinegar (0.01–0.12 mg/l); sauce (2.1–69.6 mg/l); smoked meat (0.08–22 μg/g); bread (0.12–2.51 μg/g); yoghurt (0.007–0.02 μg/g); cheese (0–0.87 μg/g); fruit juices (0–1.45 μg/g); jams (0.06–0.42 μg/g), flour (0.13–2.5 μg/g)	[2,4,21,27,29–31,55,62]
MTCA	Wine (0–17.8 mg/l); beer (0.31–17.03 mg/l); wine vinegar (3.9–9.5 mg/l); sauce (0–711 mg/l); smoked meat (0–1.2 μg/g); bread (0.04–0.54 μg/g); yoghurt (0.05–0.13 μg/g); cheese (0–2.56 μg/g); fruit (0–8.4 μg/g); fruit juice (0.03–11.6 μg/g); jams (0.12–2.65 μg/g); soft drinks (0–0.35 mg/l)	[2,4,17,21,27,29–31,55,62]
OH-MTCA	Smoked meat (0–0.44 μg/g); soy sauce (0.1–5.1 μg/g)	[27,30]
THβC	Wine (0.27–1.05 μg/l); beer (0.95–11.8 μg/l)	[39]
MTHβC	Wine (0.8–101.9 μg/l); beer (1.6–91.7 μg/l); fruit (0–0.11 μg/g)	[36,37,39]
6OH-THβC	Beer (6.2–44.1 μg/l)	[22]
6OH-MTHβC	Wine (0–0.22 μg/l); beer (3.6–86.2 μg/l); fruits (0–0.36 μg/g)	[35]
MTCA-EE	Wines (3.4–534 μg/l); beer (0–10 μg/l), distillates (0–28 μg/l)	[40]
βCs		
Harman	Wine (0.8–11.7 μg/l); beer (7.3–140 μg/l), vinegar (0.015–0.73 μg/l); soy sauce (0.13–0.25 μg/g); cooked meats (0–20 ng/g); cooked fish (0–130 ng/g)	[43–45,61]
Norharman	Wine (0.3–0.7 μg/l); vinegar (0.0019–0.096 μg/l); cooked meats (0–30 ng/g), cooked fish (0–200 ng/g)	[44,45,61]

[a] THCA: 1,2,3,4-Tetrahydro-β-carboline-3-carboxylic acid; MTCA: 1-methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid; OH-MTCA: 1-hydroxymethyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid; THβC: 1,2,3,4-tetrahydro-β-carboline; MTHβC: 1-methyl-1,2,3,4-tetrahydro-β-carboline; 6OH-THβC: 6-hydroxy-1,2,3,4-tetrahydro-β-carboline; 6OH-MTHβC: 6-hydroxy-1-methyl-1,2,3,4-tetrahydro-β-carboline; MTCA-EE: 1-methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid ethyl ester; harman: 1-methyl-9H-pyrido-[3,4-*b*]indole; norharman: 9H-pyrido-[3,4-*b*]indole.

found in alcoholic beverages [40]. *N*-Nitroso THβCs were detected in nitrite treated foods [30,41], suggesting that foods rich in THβC-3-COOHs, if preserved in nitrite, could form the corresponding *N*-nitroso compounds. 1,2,3,4-Tetrahydro-β-carbolines and its 6-hydroxytetrahydro-β-carbolines were reported in some alcoholic beverages and fruits in minor amounts (usually at the ng/ml level, as shown in Table 1) [22,35–37,39]. A less simple THβC with a pyrrolidinethione moiety was found in salted radish roots [58]. Concerning βCs, it is known the occurrence of both norharman and harman heterocyclic amines in well cooked meat and fish at ng/g level [20,45,52,61]. Minor amounts of them were reported in some alcoholic beverages and foodstuffs [43,44]. Less simple β-carboline derivatives with a furan moiety were also identified in soy sauces [59].

6. Overview and conclusion

THβCs and BCs are naturally occurring alkaloids in foods produced by Pictet–Spengler chemical condensation. This suggests that foods are an exogenous source of possible THβCs and βCs ultimately present in human biological tissues and fluids. Since those compounds are biologically relevant, their analysis in foods should be recommended. Clean-up procedures include liquid–liquid extraction and/or SPE (using both C_{18} and cation-exchange mechanisms). Acylating reagents such as TFAA, HFBA, PFPA, MCF are widely employed for chemical derivatization of THβCs before GC–MS. For analysis, both GC–MS, and HPLC with fluorescence or mass detection are employed. HPLC–fluorescence is recommended for routine quantitative analysis of both THβCs and βCs owing to its high selectivity and sensitivity. HPLC–MS is adequate for quantitation and identification purposes since electrospray or APCI easily provides the protonated molecular ions $(M+H)^+$ of THβCs and βCs.

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