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Determination and occurrence of various tetrahydro-β-carboline-3-carboxylic acids and the corresponding *N*-nitroso compounds in foods and alcoholic beverages

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An HPLC-fluorometric detection method and an HPLC-mass spectrometric (HPLC-MS) confirmation method were developed for the determination of 1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (THβCCA), 1-methyl-THβCCA (MTHβCCA), and 1-(hydroxymethyl)-THβCCA (HMTHβCCA) in foods and alcoholic beverages. The occurrence of these THβCCAs in various foods and beverages is of considerable interest because of their role as neuromodulators, and as precursors of *N*-nitroso-THβCCAs. Of various samples (nine soya sauce, 12 wines, 14 beers and ales, and 19 cured and smoked meats) analysed, the soya sauces and the wines contained more MTHβCCA (0·1–473 μg/g and 0·4–5 μg/g, respectively) than THβCCA (0–9 μg/g and 0·01–0·05 μg/g, respectively). On the other hand, the cured smoked meats contained more THβCCA (0–7·6 μg/g) than MTHβCCA (0–0·24 μg/g).The beer samples contained low concentrations (0·04–0·82 μg/g) of both. Appreciable concentrations of HMTHβCCA were also detected in soya sauce (0·1–5·1 μg/g) but the smoked meats contained only traces (0–0·24). Five smoked meat samples also contained some (13–340 ng/g) *N*-nitroso-THβCCA, but no detectable amounts of *N*-nitroso-MTHβCCA. The other samples were not tested for these compounds. The study suggests for the first time that the presence of THβCCAs in nitrite-preserved foods (e.g. cured smoked meats) may lead to the formation of the corresponding *N*-nitroso-THβCCAs.

INTRODUCTION

Tetrahydro-β-carbolines, also known as tetrahydro-1*H*-pyrido-[3,4-*b*]indoles (IUPAC nomenclature), are heterocyclic compounds formed by the condensation of indole ethylamine type compounds with various aldehydes. The chemical structures of these compounds can vary widely depending on the nature of the indole amine and that of the aldehyde involved in the reaction. A general scheme of such a condensation, known as Pictet Spengler reaction (Whaley & Govindachari, 1951), leading to the formation of a tetrahydro-β-carboline is shown in Fig. 1. Thus, *l*-tryptophan upon reaction with formaldehyde (R = H) or acetaldehyde (R = CH_3) produces 1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (THβCCA) and 1-methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (MTHβCCA), respectively. A similar reaction with glycolaldehyde (R = CH_2OH), on the other hand, forms 1-hydroxymethyl-THβCCA (HMTHβCCA). Most of the work, in the present study, relates to these three THβCCAs.

Although these compounds had been known for several decades and the possibility of their formation in foods had been predicted, it was not until the early 1980s that scientists began any systematic investigation of their occurrence and formation in foods. This apparent change in the interest in these compounds occurred mainly because of their reported properties as neuromodulators (Buckholtz, 1980) and as inducers of alcoholism in laboratory animals (Tuomisto *et al.*, 1982). Research during the past 10 years has shown that variable but appreciable levels (low to high ppm levels) of both THβCCA and MTHβCCA occur in wines (Bosin *et al.*, 1986; Herraiz *et al.*, 1993), beers and ales (Bosin *et al.*, 1986), soya sauce (Wakabayashi *et al.*, 1983; Adachi *et al.*, 1991), and in various smoked foods such

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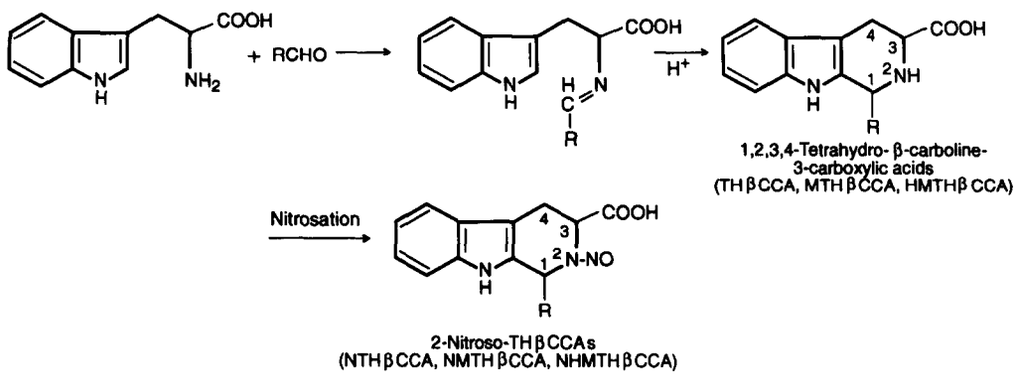

Fig. 1. The Pictet–Spengler reaction between L-tryptophan and aldehydes leading to the formation of various THβCCAs which upon nitrosation produce the respective *N*-nitroso-THβCCAs. R = H, CH_3 or CH_2OH corresponding to formaldehyde, acetaldehyde, or glycolaldehyde.

as cheeses, fish and cured meats (Papavergou & Clifford, 1992). In addition, the occurrence of 6-hydroxy-1-methyl-1,2,3,4-tetrahydro-β-carboline in both beer and wine (Beck *et al.*, 1983), 1-methyl-1,2,3,4-tetrahydro-β-carboline in various alcoholic beverages (Beck & Holmstedt,. 1981), HMTHβCCA in miscellaneous smoked foods (Papavergou & Clifford, 1992), and of 1-hydroxymethylfuryl-β-carboline in soya sauce (Oshita *et al.*, 1991) has also been reported.

Despite the above-mentioned progress, there is a need for further research in this area for the following reasons. First, although considerable data are available on the concentrations of THβCCA and MTHβCCA in beer and wine, such data for other foods (e.g. cured meats, soya sauce, infant formulas) are either lacking or very limited. Secondly, some of the THβCCAs, especially THβCCA and MTHβCCA, upon nitrosation, have been shown to produce highly mutagenic derivatives (Wakabayashi *et al.*, 1983; Valin *et al.*, 1985). But, very little research has been carried out to investigate the possible presence of the corresponding *N*-nitroso derivatives in foods, especially in nitrite preserved products (e.g. cured smoked meats) in which such compounds are most likely to be formed (Sen *et al.*, 1991). Thirdly, there also appears to be a need for improvement of analytical methodologies for the determination (e.g. mass spectrometric confirmation) of these compounds in various foods and beverages. The present study was undertaken to develop such analytical methods, and use them to determine the concentration of THβBCCA, MTHβCCA in beers and wines, soya sauces, and cured smoked meats, and of the corresponding *N*-nitroso compounds in the last-mentioned items.

MATERIALS AND METHODS

Reagents and chemicals

All chemicals were of analytical reagent grade, and the organic solvents were of glass distilled variety purchased from either Caledon Laboratories (Georgetown, Ontario, Canada) or Burdick and Jackson (Muskegon, MI, USA). Semicarbazide hydrochloride was purchased from BDH Chemicals (Poole, Dorset, UK) and was dissolved (1% solution) in water or other solvents before use. Various sample preparation cartridges such as Bond-elut® SCX (3 ml), LC-18 (3 ml), and SAX (3 ml) were purchased from Varian (Harbor City, CA, USA); Waters (Milford, MA, USA) and Supelco Canada (Oakville, Ontario, Canada), respectively.

THβCCA, MTHβCCA, HMTHβCCA, and 1-phenyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (PTHβCCA) standards were received as gifts from various laboratories as mentioned under acknowledgements. The two *N*-nitroso derivatives investigated, namely, 2-nitroso-THβCCA (NTHβCCA) and 2-nitroso-MTHβCCA (NMTHβCCA) were synthesized as described previously (Sen *et al.*, 1991).

Samples

All samples of cured meats, soya sauce, and alcoholic beverages were purchased locally, but many of them were imported products from other countries. The meat samples were homogenized using a blender and then stored in sealed jars in a refrigerator until analysis which was usually carried out within 2 weeks. The soya sauces were stored at room temperature, whereas the wines and the beers, especially after opening, were refrigerated. These samples were also analysed within 2 weeks.

Determination of THβCCA, MTHβCCA, and HMTHβCCA

Wines

Two different methods were used to clean-up samples prior to analysis by high-performance liquid chromatography (HPLC) with fluorescence detection. The first one was designed to work as a rapid screening method whereas the other technique, although slightly longer and more time consuming, resulted in a better clean-up. The details are listed below.

Method 1. A 10 g aliquot of a wine sample was mixed with 2 ml of aqueous semicarbazide solution (added to

prevent artifactual formation of THβCCAs) (Papavergou & Clifford, 1992) and the mixture was evaporated to dryness under vacuum using a rotary evaporator (water bath, 40–45°C). The residue was dissolved in 2 ml water and the solution was passed through a strong anion exchange Supelco SAX SPE cartridge that had just been conditioned by washing with a 4 ml aliquot of 25% methanol in water. The loaded cartridge was washed with an additional 3 ml 25% methanol. Both the effluent and the wash were collected and evaporated to dryness as above (THβCCAs were not retained on the cartridge). The residue was redissolved in 2 ml of aqueous semicarbazide solution. The above solution was then passed through a Varian LC-18 sample preparation cartridge (preconditioned by washing with 6 ml each of methanol and water). After washing the cartridge with 2 ml of water, the adsorbed THβCCAs were eluted with 5 ml of 25% methanol containing 1% dissolved semicarbazide. The eluate was made up to 10·0 ml with water.

Method 2. This is a modified version of the procedure described by Herraiz *et al.* (1993). In this method, a 5 g aliquot of a sample was first mixed with 1 ml semicarbazide solution (1% in water) and the pH of the mixture was adjusted to ~2 with the addition of four to five drops of 1 M HCl. The solution was then passed through a Varian Bond-Elut (3 ml) SCX cartridge that had just been conditioned by washing successively with 6 ml 0·1 M HCl, 2 ml methanol, and 6 ml of water. All the washings were discarded. The adsorbed THβCCAs, if present, were eluted from the cartridge with 5 ml of dilute NH_4OH solution (1 part concentrated NH_4OH + 4 parts of water). The eluate, collected in a 50 ml round-bottomed flask, was evaporated under vacuum to ~2 ml using a rotary evaporator. Any residual ammonia was neutralized by dropwise addition of 1 M HCl (usually needed only two to three drops), the solution quantitatively transferred into a graduated test tube using a Pasteur pipette, and the volume was made up to 5·0 ml with water (a part of it was used to rinse the evaporation flask).

Beers and ales

These samples (10 g aliquots) were analysed by method 1, as described above for wines, with some minor modifications. After initial evaporation of the sample the residue was dissolved in 2 ml of water, and the solution was directly passed through a Varian LC-18 cartridge omitting the preliminary clean-up on the Supelco SAX cartridge.

Soya sauce

A 1 g aliquot of the sample was diluted with 3 ml of water and then mixed with 1 ml semicarbazide solution (1%). After acidifying (to pH ~2) the mixture with the addition of four to five drops of 1 M HCl, the sample was taken through a combination of the Bond-Elut SCX (method 2) and LC-18 (second half of method 1) clean-ups in the order mentioned.

Cured and smoked meats

The samples were first extracted and purified using a Celite column procedure similar to that used earlier by Pensabene *et al.* (1982) for the determination of *N*-nitrosamines in fried bacon. An aliquot of the semipurified extract was further cleaned up using the SCX Bond-Elut cartridge as described above in method 2. The details are as follows.

A 10 g aliquot of a well-homogenized meat sample was mixed (using a spatula) thoroughly in a beaker with 15 g acid-washed Celite (Fisher Scientific Co.) and 1 ml of semicarbazide solution, and the mixture was packed lightly in a glass chromatography column (40 cm × 3 cm, i.d.) which already had a small glass wool plug inserted at the bottom. After washing the packed column with a 100 ml mixture of semicarbazide-treated 20% dichloromethane in *n*-pentane (to remove fats and lipids), the THβCCAs were eluted with 100 ml methanol, and the eluate was evaporated to dryness using a rotary evaporator. The residue was dissolved in about 5 ml of water, the solution extracted with 10 ml of ethyl acetate, and the aqueous layer was again concentrated to about 1 ml using a rotary evaporator. The concentrated solution was transferred into a 15 ml graduated test tube using about 5–8 ml of water for rinsing (Pasteur pipette). The pH of the solution was adjusted to ~3·5 by the addition of eight to 12 drops of 1 M HCl, the volume made up to 10·0 ml with water, and a 2 ml aliquot was further cleaned up using the SCX Bond-Elut cartridge (method 2) as described above for the wines.

HPLC-fluorescence determination of THβCCAs

A 10–20 μl aliquot of the purified extracts or of the appropriate standards (0·1–1 μg/ml in water) was analysed by HPLC-fluorescence. The HPLC system consisted of a solvent delivery system (Model 9600, Eldex Scientific Instruments, NAPA, CA, USA) with built-in solvent programmer and a Rheodyne injector (Model 7125; sample loop 20 or 50 μl). The THβCCAs were separated using a stainless steel column (25 cm × 4·6 mm, i.d.) packed with Supelcosil LC-18 phase (5 μm) and a LC-18 guard column. The mobile phases and the programming conditions were as follows. Initially, 10% acetonitrile and 90% ammonium phosphate buffer, 50 mM, pH 3·0; then programmed over a period of 50 min to 80% acetonitrile and 20% of the above buffer. The mobile phase flow rate was 0·5 ml/min. The fluorescence detector (Model 2070, Varian) was set at 270 nm for excitation and 340 nm for emission.

HPLC-MS confirmation of THβCCAs

A TAGA 6000E (Perkin-Elmer SCIEX Incorp., Toronto, Ontario, Canada) mass spectrometer equipped with an atmospheric pressure chemical ionization (APCI) source was used in the HPLC-MS confirmation of THβCCAs. The MS system was operated in single quadrupole mode and controlled by a DEC PDP-11/23-based data system. Data acquisition and data processing were also performed on the same

data system. Effluent from the LC system was directed to a heated nebulizer interface SCIEX Incorp.) using dried air as the nebulizer gas (552 kPa) and nitrogen (high purity grade) as the auxillary gas (flow: 1 litre/min). Although the operating temperature of the heated nebulizer interface appears to be high (480°C), the actual temperature at the centre of the nebulizer was ~120–150°C because of the cooling effect from the solvent evaporation, thus minimizing the possibility of thermal breakdown of the analyte. This interface can also accommodate flow rates compatible to standard HPLC systems (up to 1 ml/min). Evaporized solvent(s) served as the source of reagent ions in the Corona discharge APCI source. Analytes were generally ionized by proton transfer reactions from reagent ions to produce $[M + H]^+$ ions. The MS was operated either in the repetitive scanning mode or selective ion monitoring (SIM) mode, and the resolution was set at the baseline separation of 1–1·2 mass units. Other MS conditions were similar to those reported by Lau *et al.* (1993).

The LC system consisted of two Shimadzu pumps (Model 600) and a Rheodyne injector (Model 8125). It was connected to a reversed phase Supelcosil LC-18-T column (Supelco Inc., 15 cm × 4·6 mm i.d., particle size 3 μm). The mobile phases were: (A) 0·5% formic acid, 9·95% acetonitrile and 90% 20 mM ammonium acetate buffer (pH of the mixture, 2·7); (B) 0·5% formic acid, 39·75% acetonitrile, and 59·75% of above buffer (pH of the mixture, 3·2). The mobile phase flow rate was set at 0·7 ml/min with the following gradient programming: 25% (B) for 12 min, then increased to 38% (B) over a period of 3 min. After each run, the column was returned to the initial condition, equilibrated for 10–15 min, and the analysis was resumed as above. The sample size injected was 20 μl in all cases.

Determination and GC-MS confirmation of N-*nitroso-THβCCAs*

Since the possibility of formation of the *N*-nitroso-THβCCAs is the greatest in nitrite-treated foods, only the cured and smoked meats were analysed for these compounds in the present study. The samples were analysed for NTHβCCA and NMTHβCCA by the method of Sen *et al.*, 1991) with some modifications. Briefly, the method consists of extraction of the sample with methanol in the presence of sulphamic acid (inhibitor of artifactual formation), removal of fats and lipids by partitioning with *n*-hexane, neutralization of the acid with dilute NH_4OH solution (to ~pH 5) and removal of methanol using a rotary evaporator, extraction of the aqueous residue (saturated with NaCl) with ethyl acetate, clean-up of the ethyl acetate extract (must not be dried over anhydrous Na_2SO_4) on an acidic alumina sample preparation cartridge, and final analysis using an HPLC-Nitrolite detection technique (Conboy & Hotchkiss, 1989). The Nitrolite detector works on the principle of denitrosation by UV irradiation followed by detection of the liberated nitric oxide by thermal energy analyser (Fine *et al.*, 1975). The column used for the HPLC separation was a Supelco LC-ABZ (5 μm) column (15 cm × 4·6 mm, i.d.) with a LC-ABZ guard column. The mobile phase consisted of 10% acetonitrile in 10 mM trifluoroacetic acid initially (no hold), and then changed to 50% acetonitrile over 40 min using a linear programming (flow rate, 1 ml/min). Such a slow programming was needed to achieve a complete resolution of the two compounds. Under these conditions *N*-nitrosoproline (a reference compound), NTHβCCA, and NMTHβCCA eluted after 5·8 min, 33·6 min, and 35·4 min, respectively.

Prior to GC-MS confirmation, the extracts giving a positive result for either NTHβCCA or NMTHβCCA were cleaned up on a LC-18 sample preparation cartridge, the purified extract treated with diazomethane, and the methyl ester derivatives of the above two compounds were analysed by GC-MS as described previously (Sen *et al.*, 1991). The MS confirmation was carried out in SIM mode at a resolution of either 1000 or 5000. The ions monitored were: *m/z* 259·0957 ($M^{+\cdot}$) and 229·0977 $(M\text{-}NO)^+$ for NTHβCCA-methyl ester, and *m/z* 273·1113 ($M^{+\cdot}$) and 243·1134 $(M\text{-}NO)^+$ for NMTH-βCCA-methyl ester. A perfluorokerosene peak at *m/z* 230·9856 was used as reference mass. GC conditions: 15 m DB-5 (0·25 μm film thickness; J & W Scientific Inc., Folsom, CA, USA) fused silica capillary column with a head pressure of 69 kPa He carrier gas. The GC oven was programmed from 60°C (held for 1 min) to 180°C at 50°C/min, then ramped to 250°C at 5°C/min.

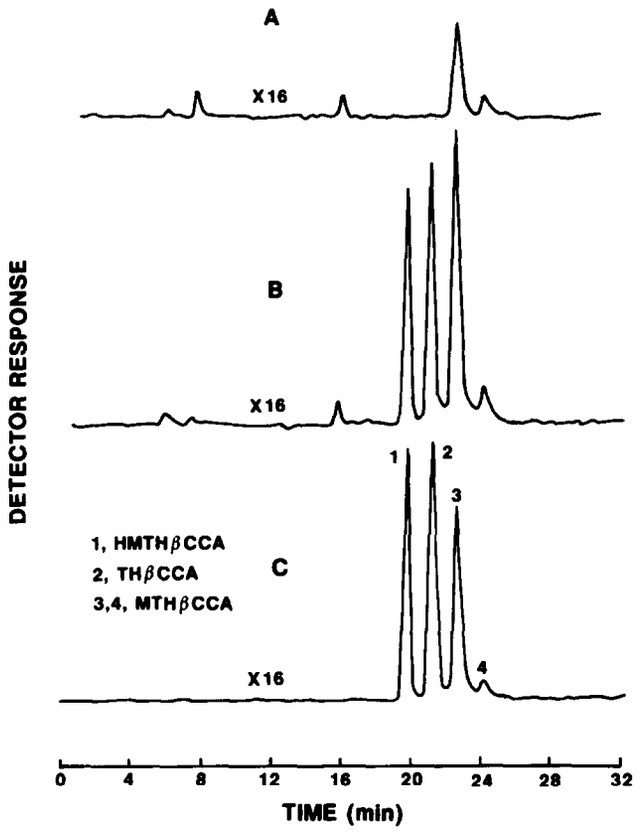

Fig. 2. Chromatograms from HPLC-fluorescence analyses: (A) 10μl/5 ml extract of a white wine, (B) the same after spiking with 0·66–0·9 μg/g levels of each THβCCAs, and (C) the spiking standards (10 ng of Nos 1 and 2 and 7 ng of No. 3).

Table 1. Recoveries of THβCCAs from various spiked samples

Item[a]	Spiking level (μg/g)	Method used	% Recoveries		
			THβCCA	MTHβCCA	HMTHβCCA
Beer (A)	0·2	1	102·3	90·2	83·9
Beer (B)	0·6–0·9	1	89·8	89·4	91·8
White wine (C)	0·6–0·9	1	84·5	147·5[b]	76·6
Red wine (D)	0·6–0·9	1	91·1	159·5[b]	92·1
White wine (C)	0·6–0·9	2	101·4	101·9	95·8
Red wine (D)	1–2	2	90·2	102·7	92·4
Soya sauce (E)	0·6–0·9	2	82·4	87·4	92·8
Soya sauce (F)	0·6–0·9	2	80·4	76·6	85·9
Cured smoked meats (G)	0·5	2[c]	68	76·2	72·9
Cured smoked meats (H)	0·5	2[c]	77·6	65·9	88·8
Cured smoked meats (I)	0·1	2[c]	92·2	97·1	74·5

[a]A, B, C, etc. denote different brands and correspond to those shown in Table 2.
[b]Higher values due to interference (inadequate clean-up).
[c]Following the Celite column extraction and preliminary clean-up.

RESULTS AND DISCUSSION

The analytical methods used in this study for the determination of THβCCAs in various substrates were mostly based on those previously reported by Adachi *et al.* (1991) and Herraiz *et al.* (1993). On HPLC analysis, the three THβCCAs resolved well from each other (Fig. 2), but MTHβCCA gave two peaks due to its two diastereoisomers, namely, (1*S*, 3*S*)-MTHβCCA and (1*R*, 3*S*)-MTHβCCA at a ratio of about 11:1. No attempt was made in this study to determine the concentrations of individual diastereoisomers. All results reported for MTHβCCA are the sum of the two isomers.

The clean-up method using Bond Elut SCX cartridges (method 2) as described previously by Herraiz *et al.* (1993) worked fairly well, but the recoveries of the three THβCCAs, especially that of THβCCA, were sometimes low and inconsistent. For example, with one batch of SCX cartridges the mean recovery of THβCCA from spiked wine (n = 4) was only 63·9% (range, 45·6–75%) whereas with an earlier batch of SCX cartridges the corresponding figure was >75%. It was later discovered that the eluent (a 1:1 mixture of methanol and 0·4 M phosphate buffer, pH 9·1) normally used in this technique (Herraiz *et al.*, 1993) did not quantitatively elute the THCCAs from the strong cation exchange resin present in such cartridges. Hence the eluent was changed to a mixture of concentrated NH_4OH and water (1:4) which gave excellent recoveries of all three THβCCAs (Table 1).

Because of the above-mentioned initial difficulties with the Bond Elut cleanup, we developed an alternative technique (method 1) that is based on passing of the sample through both a strongly acidic anion-exchange resin cartridge (SAX) and a LC18 reversed-phase

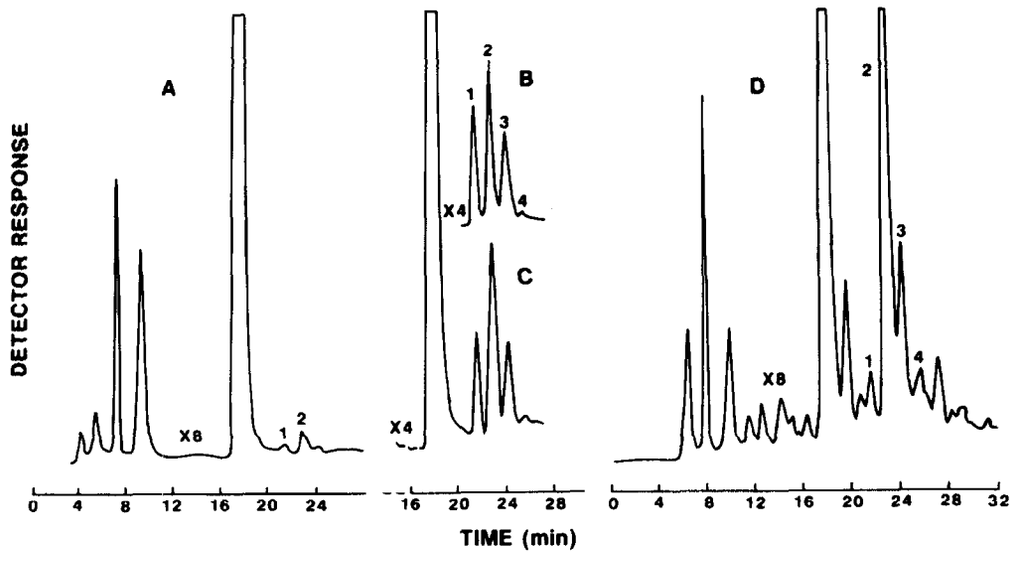

Fig. 3. HPLC-fluorescence analysis of: (A) 40 μl/5 ml extract of a smoked meat containing traces of HMTHβCCA and THβCCA, (B) spiking standards equivalent to 100 ng/g spiking level for each, (C) the above meat spiked at 100 ng/g levels (40 μl/5 ml extract injected), (D) 40 μl/5 ml extract of another smoked meat which contained 0·07 μg/g HMTHβCCA, 1·1 μg/g THβCCA, and 0·32 μg/g MTHβCCA. Note: in (B) and (C) the first 16 min of chromatograms are not shown.

Table 2. Concentrations of various tetrahydro-β-carboline-3-carboxylic acids detected in wines, beers and ales, and soya sauce

Sample	Country of origin	Concentrations of THβCCAs (μg/g)		
		THβCCA	MTHβCCA	HMTHβCCA
Red wines				
A	France	0·04	3·3[c]	N[a]
B	France	0·03	1·6	Traces[b]
C	France	0·03[c]	2·3[c]	N
D	Italy	0·04	2·0	N
E	Italy	0·01	1·3	N
F	USA	Traces	1·2	N
		av 0·03	av 1·95	
White wines				
A	USA	0·05[c]	5[c]	N
B	Germany	0·02	0·8	N
C	Canada	Traces	0·5	N
D	Canada	Traces	0·4	N
E	France	0·03	0·7	N
F	France	0·02[c]	1·1[c]	N
		av 0·02	av 1·42	
Beer and ale				
A	Mexico	0·6	0·08	0·04
B	Mexico	0·09	0·06	0·04
C	Mexico	0·04	0·03	Traces
D	Mexico	0·11	0·45	0·02
E	Mexico	0·09	0·03	0·02
F	Canada	0·12	0·27	Traces
G	Canada	0·1	0·42	N
H	Canada	0·1	0·12	N
I	Canada	0·09	0·27	N
G	Canada	0·05	0·08	0·07
H	Canada	0·16	0·82	0·07
I	Canada	0·08	0·22	0·04
J	Canada	0·33	0·07	N
K	Canada	0·04	0·10	N
		av 0·14	av 0·21	av 0·02
Soya sauce				
A	Singapore	4·0	N	2·5
B	China	4·4	N	1·7
C	China	0·9	3·9	1·2
D (naturally brewed)	USA	4·8[c]	371[c]	2·9[c]
E (naturally brewed)	USA	4·4[c]	473[c]	3·4[c]
F (naturally brewed)	USA	4·1[c]	451[c]	2·9[c]
G	Taiwan	9·0	204	5·1
H	Canada	N	0·1	0·1
I	Canada	N	0·1	0·2
		av 3·5	av 167	av 2·22

[a]N = negative (<0·01 ppm or <0·1 ppm for soya sauce).
[b]Traces (~0·01 ppm).
[c]Confirmed by HPLC-MS.

sample preparation tube. THβCCAs were eluted from the latter with 25% methanol in water. Method 1 was mainly used for the analysis of beer and ale. Later when the elution problem from Bond Elut SCX cartridge was resolved, all subsequent analyses were carried out using this technique. In general, Bond Elut clean-up resulted in a cleaner extract than that obtained by method 1.

An additional preliminary extraction and clean-up on a Celite column had to be developed for the analysis of the cured smoked meats. The procedure, although somewhat lengthy, worked fairly well. The overall recoveries of all three THβCCAs added to meats at 0·1–0·5 μg/g levels ranged between 65·9–97·1% (Table 1). In view of the lengthy overall procedure, these recovery values seemed reasonable. For the other samples (e.g. wines, beer and ale, soya sauce) the recoveries were much better and ranged between 67·6 and 102·7% (Table 1). The precision of the overall method was also fairly good. For example, the coefficient of variation of results for three replicate analyses of a beer was within ±10%, and those for three replicate analyses each of three wines were within ±5%. The minimum detection limit (>3 × noise) of the method varied depending on the sample size used for the analysis. It ranged from 0·01 μg/g for a sample size of 10 g (for wines, beers and ales, and meats) to 0·1 μg/g for soya sauce for which only a 1 g sample was used for the analysis. Examples of a few typical chromatograms obtained from the analysis of a sample of wine and a cured smoked meat are presented in Figs 2 and 3, respectively.

The levels of the three THβCCAs detected in various

Table 3. Concentrations of THβCCAs and the two *N*-nitroso-THβCCAs in cured and smoked meats

Sample	μg/g			ng/g	
	THβCCA	MTHβCCA	HMTHβCCA	NTHβCCA	NMTHβCCA
Smoked meat	0·03	0·01	0·02	N[a]	N
Summer sausage	1·1	0·32	0·07	N	N
Smoked pork	0·97	N	0·03	N	N
Naturally smoked Polish sausage	0·32	N	0·07	N	N
Fully cooked smoked ham	0·07	N	0·01	N	N
Summer sausage	0·77[b]	N	0·01	N	N
Smoked Black Forest ham	0·06	N	0·03	N	N
Pastrami	0·02	N	0·01	N	N
German sausage (Lot No. X)	7·6[b]	0·38	0·24[b]	340[c]	N
Smoked turkey breast	0·20	N	0·02	N	N
Polish Kielbasa sausage	0·60	N	0·03	N	N
Pepperoni	0·5	1·15	N	13[c]	N
Calabrese sausage	0·73	1·19	N	N	N
Teewurst sausage	0·26	N	N	N	N
German sausage (Lot No. Y)	5·91	N	N	39[c]	N
Speckwurst sausage	0·22	N	N	N	N
Summer sausage	1·91	0·48	N	14[c]	N
Smoked chicken breast	0·46	N	N	34[c]	N
Smoked meat	0·01	N	N	N	N

[a]N = Negative (detection limits, 0·01 μg/g all compounds analysed).
[b]Confirmed by LC-MS.
[c]Confirmed by GC-MS-SIM (resolution 1 K or 5K).

samples of wines, beers and ales, and soya sauces are presented in Table 2. Of these, the soya sauces seemed to contain the highest levels (up to 473 μg/g) of all three compounds. In an earlier study, Wakabayashi *et al.* (1983) observed that soya sauces produced by natural fermentation formed higher levels of MTHβCCA than those produced by acid hydrolysis. Apparently, most of the L-tryptophan is destroyed during the acid hydrolysis, and hence the lower yield of THβCCAs. The soya sauces containing lower levels of MTHβCCAs (samples A to C and H to I, Table 2) were most likely produced by the acid hydrolysis method. In both wines and soya sauces, the concentrations of MTHβCCA seem to be higher than those of THβCCA. Whereas in beers and ales, the levels are comparable. The average concentrations of THβCCA in wines, soya sauce, and beers were 0·025 μg/g, 3·5 μg/g, and 0·14 μg/g, respectively. The corresponding figures for MTHβCCA were 1·68 μg/g, 167 μg/g, and 0·21 μg/g.

On the other hand, the mean concentrations of THβCCA (1·14 μg/g) are much higher than those of MTHβCCA (0·186 μg/g) in most of the cured smoked meats (Table 3). In this respect, our results agree very well with those reported by Papavergou and Clifford (1992) who noted very high levels (summarized in Table 4) of THβBCCA in some slow cured (long maturation period) dried sausages and salami, especially in several Greek and Danish products. The data in the publication by Papavergou and Clifford (1992), however, had inadvertently been printed incorrectly. Those

Table 4. Comparison of data on the concentrations of THβCCAs in various foods and beverages as reported by investigators in different countries

Item analysed	*n*	Country	Average concentrations (μg/g or ml) detected			References
			THβCCA	MTHβCCA	HMTHβCCA	
Wines	7	USA	1·13(N[a]–1·7)	5·9 (1·3-9·1)	—	Bosin *et al.* 1986)
	16	USA	0·05(N-0·13)	2·12(0·53-4·9)	—	Herraiz *et al.* (1993)
	12	Canada	0·025(0·01-0·05)	1·62(0·4-5·0)	N	This study
	5	Japan	0·17(0·07-0·26)	4·9(3·71-6·09)	—	Adachi et al. (1991)
Beers and ales	10	USA	4·8(2·06-10·86)	1·67(0·32-4·24)	—	Bosin *et al.* (1986)
	8	Japan	0·19(0·13-0·25)	1·29(0·64-1·94)	—	Adachi *et al.* (1991)
	14	Canada	0·14(0·04-0·6)	0·21(0·03-0·82)	0·02(N-0·07)	This study
Soya sauce	2	Japan	15(5·9-24)	365(116-614)	—	Adachi et al. (1991)
	13	Japan	—	346(4-711)	—	Wakabayashi *et al.* (1983)
	9	Canada	3·5(N-9)	167(0·05-473)	2·22(0·2-5·1)	This study
Cured and smoked meats	14	UK	3·3(0·06-22·6	0·18(N-0·88)	0·095(N-0·44)	Papavergou & Clifford (1992)
	19	Canada	1·14(N–7·6)	0·18(N-1·2)	0·03(N-0·24	This study

[a]N = negative (detection limits, 0·005–0·01 μg/g).

reported for THβCCA should be for MTHβCCA, and vice versa. This became apparent to us when our results appeared exactly the reverse of that reported in the above publication. Papavergou and Clifford were informed about this discrepancy, and they confirmed the error. However, in the original PhD thesis of Papavergou (1990), which was made available to us later, the results had been reported correctly. In addition to these three THβCCAs, Papavergou and Clifford (1992) also detected high levels of 1-phenyl-THβCCA in 2 out of 14 (3816 μg/g and 10561 μg/g) and of 1-salicyl-THβCCA in one out of 14 (24772 μg/g) smoked meats. Because of unavailability of standards, these compounds were not included in the present study.

Although the reported data in the literature on the occurrence of THβCCAs in alcoholic beverages and soya sauce are limited (summarized in Table 4), our data agree fairly well with most except those reported by Bosin *et al.* (1986) for alcoholic beverages. Their data on these products seem also to be somewhat higher than those reported by other investigators. Although the exact reason for this is not clear, it might be attributable to the different nature of the products and to the differences in their processing conditions. Various factors such as concentration of the reactants, pH, temperature during processing, and relative reactivity of the aldehyde involved influence the formation of THβCCAs in the final products. This explains for such a wide variability in the levels of THβCCAs detected in various products.

The HPLC-MS technique used for confirmation of THβCCAs worked reasonably well. Figure 4 gives the APCI mass spectra of the three standards. As can be seen, all of them produced the respective $(M + H)^+$ ions which were used for selected ion monitoring (SIM) purposes. The spectra for THβCCA and MTHβCCA compare well with those obtained by thermospray ionization (Adachi *et al.*, 1991). However, the relative ratios of the ions at *m/z* 169 to that of $(M + H)^+$ seem to be higher in our studies than those observed by the above researchers. These workers used the HPLC–thermospray ionization–MS technique for the confirmation of THβCCA and MTHβCCA in Japanese sake, but did not look for the presence of HMTHβCCA.

Since the fragment ion at *m/z* 169 was produced by all three standards, SIM at *m/z* 169 gave positive responses for all of them at the respective retention times. An example of such monitoring of a soya sauce extract is presented in Fig. 5. In general, the HPLC-MS data agreed fairly well with those obtained by HPLC-fluorescence analysis (Table 5). The three cases where the two sets of results did not agree were: MTHβCCA values for the red wine 'A' and both MTHβCCA and HMTHβCCA values for the German sausage 'X'. The presence of other interfering compounds and/or quenching of the fluorescence most likely caused these discrepancies. Nevertheless, the technique appears to be useful for rapid confirmation of the compounds in cleaned-up extracts of foods and alcoholic beverages.

As mentioned earlier, only the cured meat samples were analysed for *N*-nitroso-THβCCA and *N*-nitroso-MTHβCCA. Of 19 such samples analysed, five contained detectable levels (13–340 ng/g) of NTHβCCA (Table 3). The sample (a German sausage) containing the highest level of NTHβCCA also contained the highest level (7·6 μg/g) of THβCCA, the corresponding

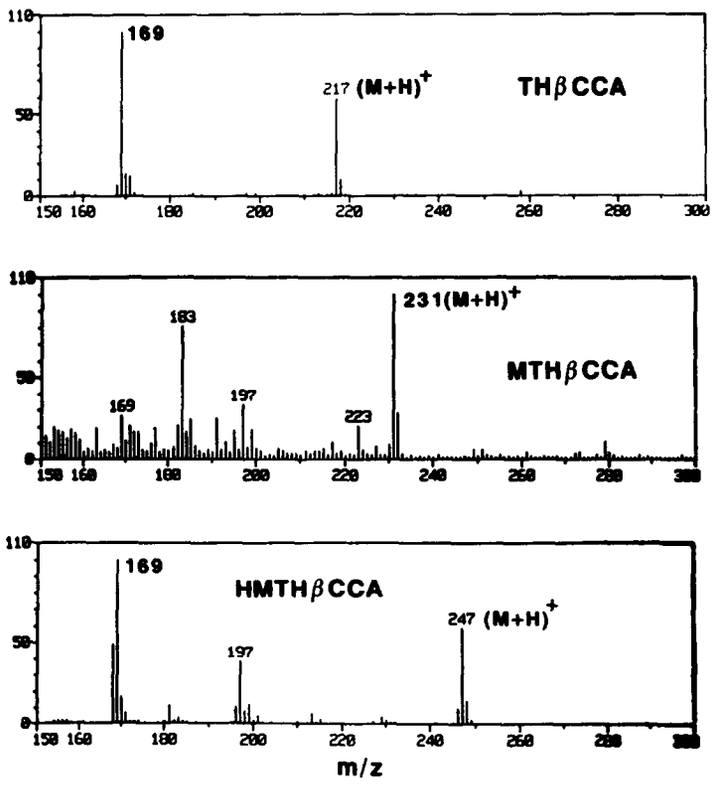

Fig. 4. APCI mass spectra of the three THβCCAs after HPLC-MS analysis.

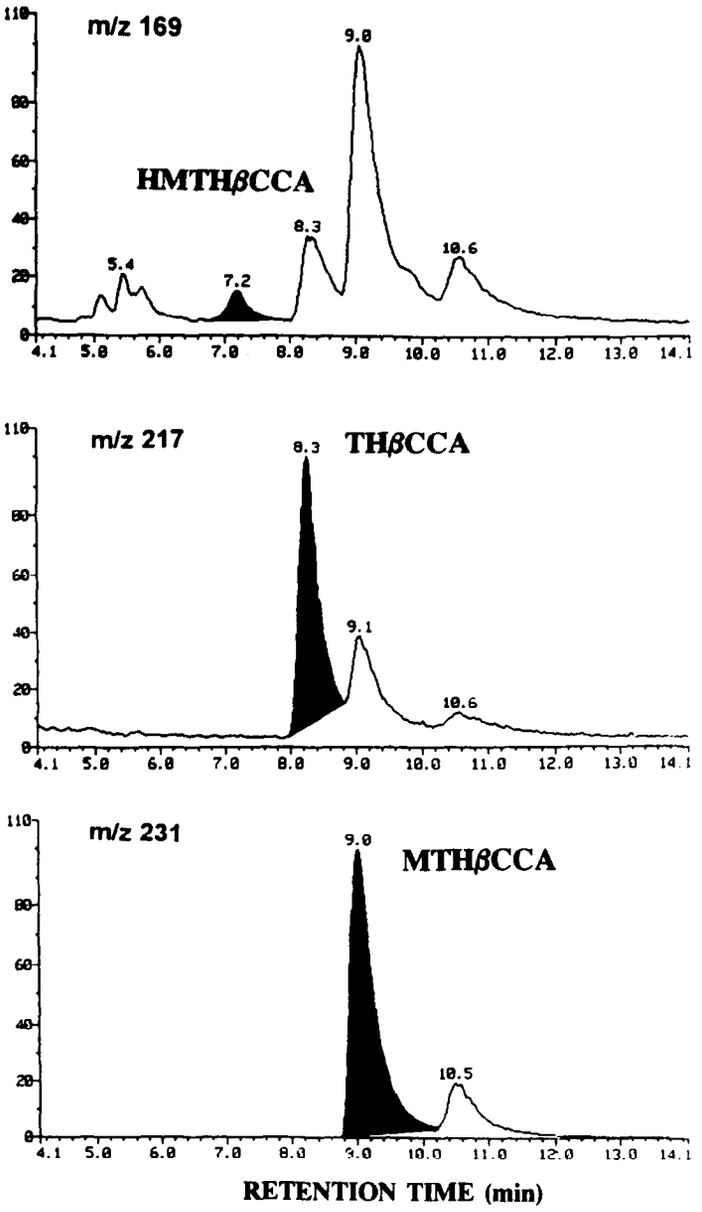

Fig. 5. HPLC-MS-SIM mass chromatograms for three ions of a cleaned-up soya sauce extract (sample D in Table 2) showing the presence of all three THβCCAs.

Table 5. Comparison of HPLC-MS and HPLC-fluorescence data for a few selected samples (ng/g)

Sample	HPLC-MS-SIM			HPLC-fluorescence		
	THβCCA	MTHβCCA	HMTHβCCA	THβCCA	MTHβCCA	HMTHβCCA
White wine (F)[a]	27	1136	N[b]	20	1100	N[c]
White wine (A)	36	4353	22	50	5000	N
Red wine (A)	N	848	N	40	3300	N
Red wine (C)	6	1314	N	30	2300	N
German sausage (X)	6508	N	1719	7600	380	240
Summer sausage	549	N	N	770	N	10
Soya sauce (D)	3010	318000	2800	4800	371000	2900
Soya sauce (E)	2900	367000	2830	4400	473000	3400
Soya sauce (F)	2610	339000	2530	4100	451000	2900

[a]The letters in the parentheses refer to brands as shown in Table 2.
[b,c]N = negative (detection limits 5 ng/g and 10 ng/g, respectively).

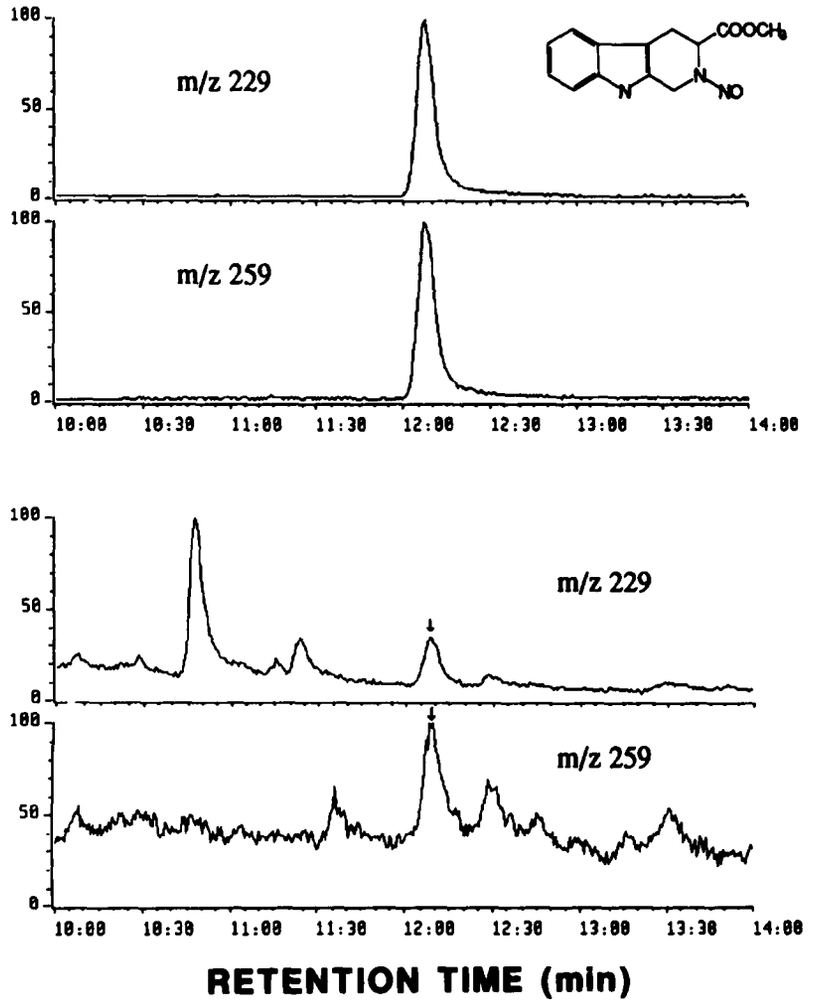

Fig. 6. GC-MS-SIM mass chromatograms at a resolution of 5000: top two for NTHβCCA-methyl ester standard; bottom two for a cleaned-up methylated extract of the German sausage (sample X in Table 3).

precursor amine. Figure 6 shows GC-MS-SIM mass chromatograms obtained from the analysis of this sample. To our knowledge this appears to be the first reported occurrence of NTHβCCA in a food and the first evidence of an association between the occurrence of THβCCA (the precursor amine) and the formation of the corresponding *N*-nitroso compound. These findings suggest that foods rich in THβCCAs if preserved with nitrite, could form the corresponding *N*-nitroso compounds. In a previous study (Sen *et al.*, 1991), we demonstrated formation of both NTHβCCA and NMTHβCCA in nitrosated samples of soya sauce, some fermented Japanese and Chinese vegetables, and cured meats. Therefore, there is also a possibility of formation of *N*-nitroso-THβCCAs *in vivo* in the human stomach due to the interaction of salivary nitrite and ingested THβCCAs. Further research is, however, needed to demonstrate if this actually occurs, and, if so, to what extent.

Of the products analysed thus far, soya sauce seems to be the major source of THβCCAs in the diet, especially for people in East Asia (e.g. Japan, China, Korea). According to one estimate, the per capita annual consumption of soya sauce in Japan is about 10 litres (Wakabayashi *et al.*, 1983). On the basis of Japanese and our own data (Table 4), this would amount to per capita daily ingestion of, on the average, about 410 μg THβCCA (assuming an average concentration of 15 μg/ml), 9480 μg MTHβCCA (assuming an average concentration of 346 μg/ml) and 60 μg HMTHβCCA (assuming an average concentration of 2·2 μg/ml). The picture is, however, quite different for people on a Western type diet. In the latter case, alcoholic beverages and cured smoked meats are probably the major sources of these compounds, although consumption of soya sauce is gradually increasing due to the introduction of various ethnic foods in the Western countries. Since only a few items have been analysed thus far, the actual intake of THβCCAs through diet is likely to be much higher because other foods may also contain these compounds. Preliminary studies by Adachi *et al.* (1991) suggest that many other foods such as wheat, rye, soya bean flours, vinegar, cow's milk, and milk formula contain varying amounts of these compounds.

In summary, our study confirms previously reported observations that certain alcoholic beverages (mainly wines and beers and ales), soya sauce, and some cured smoked meats contain low ng/g to fairly high μg/g concentrations of THβCCA and MTHβCCA. In addition,

the present study indicates the presence of similar concentrations of HMTHβCCA, which was not analysed in the majority of the previous studies, and of NTHβCCA (low ng/g) in some cured smoked meats. The toxicological significance of the findings, however, is not clear. Although many *N*-nitroso compounds are potent carcinogens in laboratory animals (Preussmann & Stewart, 1984), nothing is known about the carcinogenicity of NTHβCCA or any other *N*-nitroso-THβCCAs. According to Wakabayashi *et al.* (1983), nitrosation of MTHβCCA produces highly mutagenic products which are yet to be characterized. Therefore, the possibility of formation of these mutagenic compounds in cured smoked meats, or *in vivo* in the human stomach, cannot be ruled out. Since THβCCAs may occur in foods in fairly high concentrations, further research along these lines is highly desirable.

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