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Determination of β-carbolines in foodstuffs by high-performance liquid chromatography and high-performance liquid chromatography–mass spectrometry

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ABSTRACT

A high-performance liquid chromatographic method combined with fluorimetric detection is described for the determination of β-carboline (norharman) and 1-methyl-β-carboline (harman). The analysis of foodstuffs for the identification of β-carbolines is facilitated by clean-up of samples using Bond Elut PRS cartridges. Recoveries were excellent. Further, a high-performance liquid chromatographic–mass spectrometric method was also developed for their identification. The concentrations of β-carboline among the foodstuffs and alcoholic beverages varied greatly. Also, norharman and harman were observed in uncooked foodstuffs, whereas acetaldehyde was found in most fermented food. The toxicological implication of β-carbolines in foodstuffs is discussed.

INTRODUCTION

β-Carboline (norharman) and 1-methyl-β-carboline (harman) have been prepared by the reaction of tryptophan with some aldehydes under an oxidative condition. It is well known that these β-carbolines inhibit monoamine oxidase [1] and benzodiazepine receptor binding [2]. In addition, β-carbolines, which are not mutagenic *per se*, have been reported to possess co-mutagenic activity [3,4] as carcinogens such as aniline and *o*-toluidine are mutagenic in the presence of β-carbolines. β-Carbolines have been found to be present in tryptophan pyrolysate, cigarette smoke condensate [5], cooked foods [6] and mushrooms [7]. As the concentrations of β-carbolines are so low that their extraction is extremely difficult, an analytical procedure for foodstuffs has not yet been established.

This paper describes a simple solid-phase method for the extraction of

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norharman and harman from foodstuffs and alcoholic beverages followed by analysis by high-performance liquid chromatography (HPLC) and high-performance liquid chromatography–mass spectrometry (LC–MS).

EXPERIMENTAL

Materials

L-Tryptophan, propionaldehyde, norharman and harman were purchased from Sigma. 1-Ethyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid, which was readily prepared from L-tryptophan and propionaldehyde as described by Brossi *et al.* [8], was heated at 240°C in the presence of 10% palladium–carbon without solvent for 30 min and yielded 1-ethyl-β-carboline, to be used as an internal standard (I.S.). The methanol used was of HPLC grade (Wako). Perchloric acid was obtained from Koso Chemicals. All other chemicals were of analytical-reagent grade.

Foodstuffs and alcoholic beverages were purchased from local groceries. Propylsulphonic acid (PRS) solid-phase extraction cartridge and the vacuum manifold used (Vac-Elut) were purchased from Analytichem International.

Sample preparation

For alcoholic beverages, a 10–20-ml aliquot was diluted to 30–50 ml with distilled water, and for vinegar and soy sauce a 1.0-ml aliquot was similarly diluted to 10 ml. Amounts of 3–5 g of solid samples (all powder except for miso, which is a soybean paste) were homogenized with 0.6 *M* perchloric acid followed by centrifugation, the supernatant being used for analysis. Each sample was poured into a beaker containing 60 ng of the internal standard, mixed and adjusted to pH 1.5 by the addition of 2 *M* hydrochloric acid. Solid-phase extraction cartridges (Bond Elut) of 3-ml capacity, containing propylsulphonic acid-derivatized silica (PRS) packing material, were positioned in a ten-cartridge-capacity Vac Elut system. The pressure was adjusted to 5–10 mmHg and each cartridge was conditioned by washing with 2 × 3 ml of methanol followed by 2 × 3 ml of 0.1 *M* hydrochloric acid. Before complete desiccation of the cartridge, the acidified sample was applied to the cartridge and drawn through, then washed with 6 ml of water. The cartridge was rinsed with 3 ml of 0.4 *M* phosphate buffer (pH 9.1). Up to this point, all the washings and unadsorbed portions of the samples were discarded. Clean 8-ml glass collection tubes were inserted into the vacuum manifold and β-carbolines were eluted with 3 ml of methanol–0.2 *M* phosphate buffer (pH 8.8) (1:1). A 5-μl volume of the eluate was injected directly into the HPLC system.

Chromatography

HPLC was performed on a Yanaco, L-4000W instrument using a stainless-steel column (150 × 4.6 mm I.D.) packed with Cosmosil C_{18}-P (5 μm) (Nacalai Tesque). The flow-rate was kept constant at 0.6 ml/min at ambient temperature. The compounds were detected with a fluorescence monitor (Shimadzu RF 535) equipped with a xenon lamp. The excitation wavelength was 300 nm (band width 13 nm) and the emission wavelength was 433 nm (band width 15 nm). The mobile phase was methanol–0.1 *M* potassium phosphate buffer (0.1 *M* KH_2PO_4 adjusted with H_3PO_4) (pH 3.0) (32:68, v/v).

Quantitation

Calibration graphs for norharman and harman were constructed by plotting peak-height ratios (norharman/I.S. and harman/I.S.) against concentration. The standard solutions consisted of 5–50 ng/ml of norharman, 10–100 ng/ml of harman and 20 ng/ml of I.S. The calibration graphs were linear over the concentration range studied. The concentrations of norharman and harman were determined from the peak-height ratio of each sample by reference to the calibration graphs. Their recoveries from the sample extracts were determined by comparison of the peak heights obtained after injection of a sample extract spiked with a known concentration. The recoveries of norharman and harman varied from 90 to 100%.

LC–MS

A liquid chromatograph–tandem quadrupole mass spectrometer (Shimadzu LC–MS-QP1000) equipped with a Vestec thermospray (TSP) interface was used for recording mass spectra and selected ion monitoring. The mobile phase, methanol–(0.1 *M* ammonium formate + 0.1 *M* formic acid) (pH 3.4) (23:77, v/v), was delivered by a syringe pump (Shimadzu LC-6A) at a flow-rate of 1.0 ml/min. Samples were injected with a Rheodyne Model 7125 injector fitted with a 100-μl loop. The exit temperature of the vaporizer was 140°C and the block temperature of the ionization chamber was 250°C. Positive-ion TSP mass spectra were obtained. Typical conditions for TSP-MS were scan range *m/z* 150–400 in 2 s, electron multiplier voltage 2450 V and preamplifier gain $7 \cdot 10^7$ V/A. The lower scan range limit of *m/z* 150 was used to avoid any background interference from ammonium formate.

Determination of ethanol and acetaldehyde concentrations

A 1-ml volume of each alcoholic beverage was diluted to 100 ml with distilled water and a 1.0-ml aliquot of this, 0.5 ml of vinegar and soy sauce and 0.5 g of miso were used as samples for ethanol determination. A 1.0-ml aliquot of each liquid sample and 1.0 g of miso were used as samples for acetaldehyde determination. A 6-ml volume of 0.6 *M* perchloric acid and each sample were mixed thoroughly and the mixtures were centrifuged at 1665 *g* for 10 min at 4°C. Volumes of 2 ml of the clear acidic supernatants were placed in vials and the headspace gas in each vial was analysed using a Perkin-Elmer F45 headspace analyser according to an earlier method [9].

RESULTS

Extraction

Four sorbents (C_8, C_{18}, SCX and PRS) of solid-phase extraction cartridges were evaluated for extraction of norharman and harman from applied samples. PRS yielded the cleanest and highest recoveries, whereas using a C_8, C_{18} or SCX cartridge many co-eluting peaks from beer samples appeared in the chromatogram.

Chromatography

Fig. 1 shows the HPLC of norharman, harman and I.S. in standards and a brandy sample. Two different columns packed with TSK gel ODS-80Tm (250 × 4.6 mm I.D.) and Cosmosil $5C_{18}$-P were evaluated for the separation of norharman and harman. With the TSK gel ODS-80Tm, the analysis of each sample took about

30 min, whereas only 10 min were necessary to achieve a good separation among the compounds analysed using $5C_{18}$-P, as shown in Fig. 1. The limit of detection of each β-carboline was 2 pg. The reproducibility of sake analyses ($n = 5$) was 2% (relative standard deviation) for norharman and 1.5% for harman.

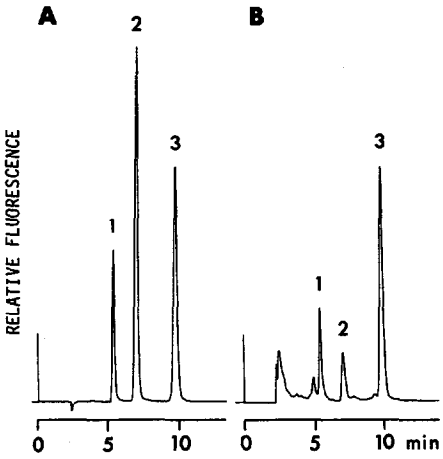

Fig. 1. High-performance liquid chromatograms of β-carbolines. (A) Standard mixture; (B) brandy sample. Peaks: 1 = norharman; 2 = harman; 3 = 1-ethyl-β-carboline (internal standard). A 5-μl volume of the eluate from the extraction cartridge was injected and the amounts injected in a brandy sample were 22 pg of norharman and 9 pg of harman.

LC–MS

The TSP ionization mass spectra of norharman and harman extracted from sake are displayed in Fig. 2. The mass spectra were identical with those obtained from standards. Formation of the MH^+ ion of either norharman and harman was observed as a base peak under TSP ionization conditions. The total ion chromatogram and mass chromatograms of norharman and harman obtained in the analysis of a standard mixture and an extract of sake are illustrated in Fig. 3. Peaks representing norharman as MH^+ were seen at m/z 169 at a retention time (R.T.) of 5.8 min and from harman as MH^+ at m/z 183 at an R.T. of 8.5 min. Hence LC–MS analysis provided a definitive structural identification. The amounts injected to obtain the data in Figs. 2 and 3 were calculated to be 0.27 μg for norharman and 2.3 μg for the harman.

Concentrations of β-carbolines

Table I gives the concentrations of norharman and harman in various alcoholic beverages. Generally, a small amount of β-carbolines was observed. The highest concentrations of both norharman and harman were obtained in sake A, which was brewed in a rural district. Sake and wine contained larger amounts of harman than beer and whisky. The norharman concentration was high in beer, whereas it was lower and similar in sake, wine and whisky. The concentration of harman was higher than that of norharman in sake and wine.

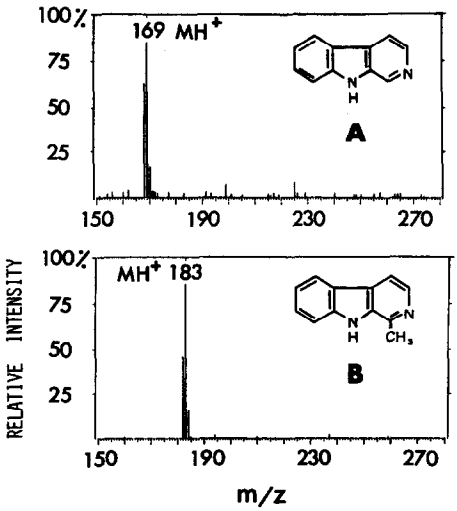

Fig. 2. Thermospray ionization mass spectra of β-carbolines from sake. (A) Norharman; (B) harman. A 5-μl volume of the eluate from the extraction cartridge was injected and the amounts injected were 0.27 μg of norharman and 2.3 μg of harman.

Concentrations of norharman and harman in various foodstuffs are given in Table II. Wide variations existed in the concentrations of β-carbolines among the foodstuffs. Both norharman and harman were highest in vinegar A (made from wheat), whereas norharman was lowest in vinegar D (made from corn) and harman

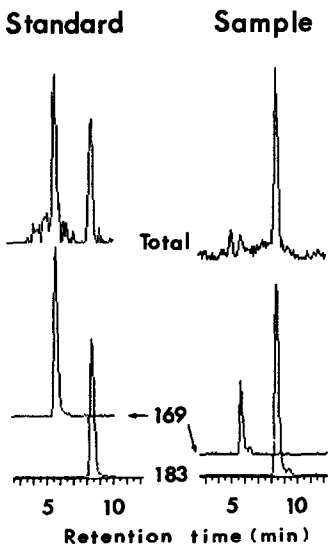

Fig. 3. Total ion chromatograms and mass chromatograms of β-carbolines from a standard mixture and sake sample obtained using thermospray LC–MS. The MH^+ ions of norharman (mol. wt. 168) and harman (mol. wt. 182) were obtained at m/z 169 and 183, respectively. A 5-μl volume of the eluate from the extraction cartridge was injected and the amounts injected in a sake sample were 0.27 μg of norharman and 2.3 μg of harman.

TABLE I

CONCENTRATIONS OF NORHARMAN AND HARMAN IN ALCOHOLIC BEVERAGES

Values are means ± S.D.

Sample[a]	*N*	Norharman (ng/ml)	Harman (ng/ml)
Sake	6	0.2 ± 0.2	4.1 ± 4.0
Wine	5	0.5 ± 0.2	8.5 ± 14
Beer	8	2.7 ± 0.7	1.7 ± 0.7
Whisky	5	1.2 ± 1.1	2.1 ± 2.6
Shochu	2	0.1 ± 0	0.1 ± 0
Brandy	1	0.5	0.2
Sake A	1	67	590
Liqueur	1	26	85

[a] Brand names were as follows. Sake: Hakutsuru, Kikumasamune, Umenishiki, Sawanotsuru, Yamatokotsuchi, Nadagiku. Wine: Kirin Wine Club White, Kirin Wine Club Red, Kirin Wine Club Rosé, Zeller Schwarze Katz, Piat d'Or. Beer: Kirin Lager, Asahi Super Dry, Kirin Light Beer, Suntory Malt's, Sapporo Hardy, Kirin Fine Pilsner, Coors, Budweiser. Whisky: Suntory Red, Nikka Pure Malt, Hi Nikka Whisky, Torys, Glenfiddich. Shochu: Iichiko, Kumesen. Brandy: Remy Martin VSOP. Sake A: Akitanishiki. Liqueur: Kurisake.

TABLE II

CONCENTRATIONS OF NORHARMAN, HARMAN, ETHANOL AND ACETALDEHYDE IN VARIOUS FOODSTUFFS

Each value represents a single determination.

Sample[a]	Source	Norharman (ng/ml or g)	Harman (ng/ml or g)	Ethanol (mg/ml or g)	Acetaldehyde (μg/ml or g)
Vinegar					
A	Wheat	96	730	0.57	15
B	Rice	22	56	2.1	3.1
C	Grape	5.6	35	13	70
D	Corn	1.9	15	1.2	1.9
Soy sauce					
A	Soybean	71	250	21	13
B	Soybean	15	130	24	14
Miso					
A	Soybean	8.2	35	0.11	8.3
B	Rice	15	0.9	2.8	13
C	Barley	45	9.6	4.4	6.9
Soybean protein	Soybean	3.0	0.8		
Soybean flour	Soybean	10	4.7		
Corn starch	Corn	3.6	0.9		
Rye flour	Rye	39	12		

[a] Brand names were as follows: vinegar A, Mitsukan; vinegar B, Mitsukan; vinegar C, Bodegas Reserva 25; vinegar D, Tamanoi Su; soy sauce A, Kikkoman; soy sauce B, Higashimaru; miso A, Oucho; miso B, Toyama Kouji miso; miso C, Ehime Inaka miso; soybean protein, Meiji protein powder; soybean flour, Fukumoto; corn starch, Nisshoku; rye flour, Mukai.

was lowest in corn starch. In general, fermented products from grain contained a relatively large amount of β-carbolines.

Concentrations of ethanol and acetaldehyde

Table II gives the concentrations of ethanol and acetaldehyde in foodstuffs. The highest level of ethanol was found in soy sauce B and the lowest in miso A. The highest level of acetaldehyde was found in vinegar C (made from grape) and the lowest in vinegar D (made from corn). Table III shows the amount of acetaldehyde contained in alcoholic beverages. The acetaldehyde concentration was highest in a liqueur and lowest in a shochu.

TABLE III

CONCENTRATIONS OF ETHANOL AND ACETALDEHYDE IN ALCOHOLIC BEVERAGES

Each value represents a single determination.

Sample[a]	Ethanol % (v/v)	Acetaldehyde (μg/ml)
Sake A	16	30
Sake B	16	13
Wine A	14	31
Beer A	4.5	13
Whisky A	43	24
Shochu A	25	5.5
Brandy A	40	51
Liqueur A	14	55

[a] Brand names were as follows: sake A, Akitanishiki; sake B, Sawanotsuru; wine A, Kirin Wine Club Red; beer A, Kirin Fine Pilsner; whisky A, Suntory Red; shochu A, Iichiko; brandy A, Remy Martin VSOP; liqueur A, Kurisake.

DISCUSSION

As β-carbolines containing an amino group are not easily volatilized, direct analysis by gas chromatography–MS is difficult and a derivatization reaction such as amidation is necessary [6,10]. We have reported here for the first time the analysis of norharman and harman directly and qualitatively using LC–MS. The MH^+ ion of either norharman or harman was demonstrated as a base peak. We identified the molecular weights of the compounds extracted from sake by the use of LC–MS. The HPLC system reported here gave a good separation of norharman and harman. No peak-interfering norharman or harman appeared in the liquid chromatogram (Fig. 1). Previously, the concentration of harman in alcoholic beverage was determined by HPLC [10], but the sensitivity was not reported, which precludes any comparison with the present method. The proposed method has the advantage of yielding extracts more simply and faster than liquid–liquid extraction [5,6,10], because the alkaline eluate of the solid-phase extraction cartridge through which food samples passed could be injected onto the HPLC column.

Although pancreatic or liver cancer is predominant in habitual drinkers and

alcoholic patients, it is not yet clear whether the direct action of alcohol or a very small amount of some substance contained in alcoholic beverages having mutagenic or carcinogenic activity could cause the cancer. The alcoholic beverages tested in this study were shown to contain β-carbolines, although the amount was very small. No relationship was found between the alcohol content and β-carboline concentration of alcoholic beverages. Our results for the concentration of harman in wine were in agreement with that obtained by Bosin and Faull [10], although the concentration in beer did not coincide with their result, which was much higher. As alcoholic patients consume large amounts of alcoholic beverages, it follows that relatively large amounts of β-carbolines would be ingested. In addition, acetaldehyde, a metabolite of ethanol, may have a possible role in forming β-carbolines endogenously in alcoholic patients. Accordingly, β-carbolines might also be involved in promoting cancer in alcoholic patients to some extent.

The content of norharman in the charred part of broiled bread, beef and sardines was reported to be 18–158 ng/g and that of harman 6–52 ng/g [6]. It was shown that cigarette smoke contained large amounts of norharman and harman, whereas tobacco leaf contained only 1% of the amount in smoke. The concentration of norharman in smoke was about 12 μg/g and that of harman was 4 μg/g [5]. In comparing the concentrations of β-carbolines between cigarette smoke and the present foodstuffs tested, the former was much greater than the latter. Norharman and harman must be formed during pyrolysis of tryptophan; however, even non-heated foodstuffs were observed to contain β-carbolines in the present study. This is the first report showing the existence of norharman in uncooked food. In addition, we showed that acetyldehyde, possibly formed from ethanol added to a condiment as a preservative, was present in most foodstuffs. However, no relationship between concentrations of ethanol and acetaldehyde or acetaldehyde and β-carbolines can be derived. As the involvement of not only acetaldehyde, but also tryptophan, in the formation of β-carbolines was considered, foodstuffs with high tryptophan contents should contain larger amounts of β-carbolines than alcoholic beverages.

In conclusion, this study has established a simple and sensitive method for determining norharman and harman, which have a co-mutagenic activity to produce human cancer, and found them not only in alcoholic beverages but also in some uncooked foods.

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