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CAPILLARY GAS CHROMATOGRAPHY OF CARBOLINES

APPLICATION TO CIGARETTE SMOKE

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SUMMARY

A method was developed for the gas chromatographic analysis of carbolines (pyridoindoles) on fused-silica glass capillary columns, coated with Superox™-4. Separation characteristics are presented for all four possible isomers of carboline and several methyl-, methoxy- and dihydro-derivatives of β-carboline (norharman). These gas chromatographic techniques were utilized in the development of a rapid method for the analysis of norharman and harman (1-methyl-β-carboline) in cigarette smoke condensate. Levels of norharman and harman found in the smoke of a research reference cigarette were 11.2 and 3.6 μg/cigarette, respectively. Commercial non-filter cigarettes gave similar values, while filtered brands gave lower values. The identification of α-carboline in cigarette smoke is reported.

INTRODUCTION

Alkaloids with the pyridoindole structure (Fig. 1) are widespread in both plants and animals. Important members of this group of compounds are the carbolines and azacarbolines, of which β-carboline (norharman) and its derivatives are found most frequently[1]. Carbolines are also formed on thermal decompositions of foods[2], amino acids (especially tryptophan)[3], and proteins[4]. Recently, it has been shown that pyrolyzates from these materials were highly mutagenic in the Ames test[2–4]. The active principles in these pyrolyzates were identified as amino-α- and -γ-carbolines[5–7]. Thus, for example, tryptophan pyrolyzates contained large amounts of norharman and harman (1-methyl-β-carboline) and only trace amounts of the aminocarbolines[5]. Although norharman and harman are not mutagenic, Nagao *et al.*[8] showed that the mutagenic activity of the aminocarbolines was greatly increased by the presence of the norharmans. They also reported that norharman and harman were co-mutagens for several mutagens, including the polynuclear aromatic hydrocarbon benzo[*a*]pyrene (B*a*P). However, a contradictory result showed that norharman and harman inhibit B*a*P mutagenicity[9].

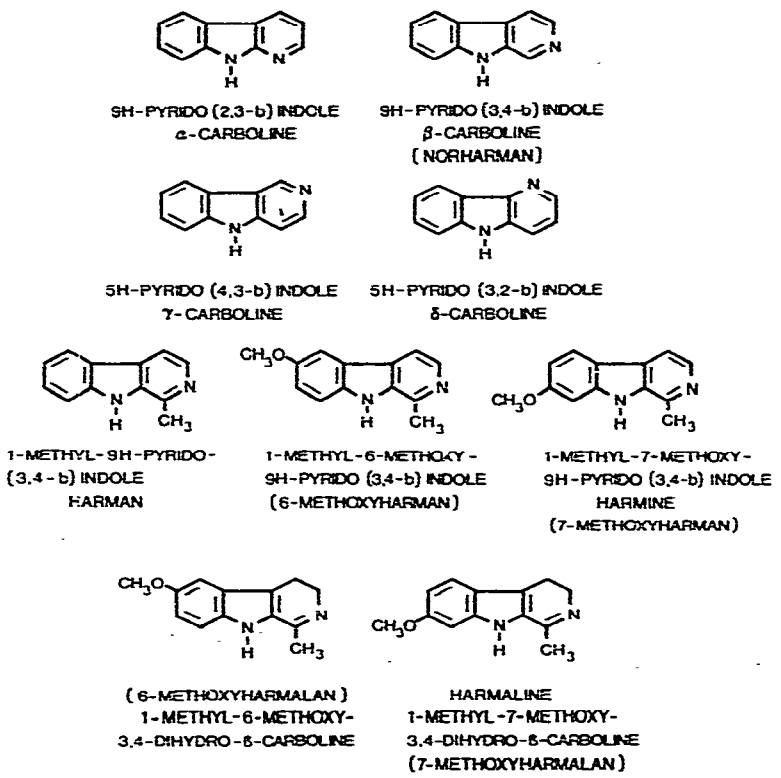

Fig. 1. Structures of some carbolines (pyridoindoles).

Cigarette smoke condensate (CSC) has been shown to contain relatively large amounts of norharman and harman by Poindexter and Carpenter[10,11]. This information, coupled with the fact that cigarette smoke contains numerous tumorigenic polynuclear aromatic hydrocarbons, provided the impetus for our development of a rapid analysis for these biologically active compounds of CSC. To our knowledge, nothing has been done on the quantification of norharmans in smoke since the initial work, 20 years ago[10,11]. Previously reported methods[10–12] for the isolation and separation of carbolines included extensive column and thin-layer chromatographic steps, which preclude rapid quantification. More recently, Shoemaker *et al.*[1] applied gas chromatography (GC) to the separation of some β-carbolines on packed columns. Using our refined glass capillary GC methods with Superox™-4 coated fused-silica glass capillary columns[13–15], we developed GC conditions for the separation of the isomeric carbolines and several derivatives. The application of this GC methodology to an acid extract of smoke condensate has allowed a more rapid quantification of norharman and harman in the main-stream of cigarette smoke.

EXPERIMENTAL*

Solvents and standards

All solvents were from Burdick & Jackson (Muskegan, MI, U.S.A.), "distilled-in-glass" grade and were used as received. Chloroform contained 1% (v/v) of ethanol for stability. Norharman, harman (Aldrich, Milwaukee, WI, U.S.A.), triphenylene (K & K Labs., Plainview, NY, U.S.A.), 6-methoxy-harman, 6-methylharmalan (Regis, Morton Grove, IL, U.S.A.), harmine, harmaline (Fluka/Tridom, Hauppauge, NY, U.S.A.) were shown to be pure (>99%) by GC and were used without further purification.

Synthesis

γ-Carboline. γ-Carboline was prepared by the method of Dalton *et al.*[16] The product was recrystallized from methanol, m.p. 227–229°C [lit.[17] 225–227°C, 230–233°C[16]]. Mass spectra: 168(M^+, 100), 167(9.5), 141(9), 149(19), 115(5), 114(11), 113(8), 88(6), 84(13), 83(6.5), 73(6), 71(11), 70(13), 69(9), 63(6), 61(7), 57(13).

α-Carboline. α-Carboline was synthesized by the method of Stephenson and Warburton[18]. However, the final product was isolated from the reaction mixture by the method of Dalton *et al.*, as described for γ-carboline[16]. The reaction mixture was poured into water and the acidity was adjusted with aqueous ammonium hydroxide to pH 4. The precipitate that formed at this pH was α-carboline. It was filtered off and recrystallized from ethanol, m.p. 209–211°C [lit. 211°C[19], 219–220°C[18]]. Its ultraviolet spectrum matched that in the literature[18]. Mass spectra: 168(M^+, 100), 167(6.5), 141(17), 140(23), 115(6), 114(16), 113(9), 88(5), 87(5), 84(9), 63(6).

δ-Carboline. δ-Carboline was synthesized from 3-amino-2-phenylpyridine (prepared from phenyllithium and 3-aminopyridine[20]) by the method of Abramovitch *et al.*[21]. The product was purified by basic alumina chromatography (Bio-Rad Alumina, grade AG-10, 100–200 mesh). δ-Carboline was eluted from the column with benzene–diethyl ether (1:1, v/v). The product was recrystallized from benzene and was shown by GC to be 86% pure δ-carboline. The UV spectrum of this material matched that in the literature[21] and it was used without further purification. Mass spectra: 168(M^+, 100), 167(9), 142(5), 141(6), 140(12), 115(3), 114(7), 113(4), 89(3), 88(3), 87(3), 84(5), 76(3), 75(3), 70(4).

Cigarette smoke condensate preparation and fractionation

Cigarettes (90–150, depending on tar levels) were preconditioned at 60% relative humidity for 48 h and smoked under standard conditions: 2-sec puff, 1 puff/min, 35-ml draw/puff, 30-mm butt length. A Borgwaldt 30-port smoking machine was used and the main-stream smoke (smoke inhaled by smokers) was collected in dry ice-cooled traps. The traps were allowed to warm to room temperature, and the CSC was quantitatively removed from the traps and connecting tubes by alternate rinses with chloroform and methanol. A total of *ca.* 200 ml of chloroform and 100 ml of methanol was used. The CSC extracts were poured into a 500-ml separatory funnel and extracted three times with 2 *N* aqueous hydrochloric acid (2 × 100 ml, followed by 1

* Reference to a company or product name does not imply approval or recommendation by the USDA

× 50 ml). Each acid extract was cross-extracted with 50 ml of chloroform, and the chloroform solutions were added to the extracted organic CSC solution. The pooled hydrochloric acid extracts were adjusted to pH 12 (with ice-water cooling) with 15 *N* aqueous sodium hydroxide. This basic solution was then saturated with sodium chloride (magnetic stirring) and extracted with chloroform (3 × 100 ml). The chloroform (total bases) extract was combined in a 500-ml round-bottomed flask with 5 ml of the internal standard (triphenylene: *ca.* 1 mg/5 ml benzene) and the solution was almost completely evaporated on a rotary evaporator. The semi-liquid residue was redissolved in a small volume of 0.05 *M* methanolic potassium hydroxide, with the addition of a few drops of methylene chloride to ensure solubility of the triphenylene, and then concentrated to a volume of *ca.* 1 ml with a stream of dry nitrogen for subsequent GC analysis.

Gas chromatography

The glass capillary column was a fused-silica column (25 m × 0.3 mm I.D.), which was statically coated with Superox™-4 (3 mg/ml in methylene chloride), after pretreatment and deactivation with Superox™-4, by the method of Arrendale and coworkers[13–15]. GC analyses were performed on a Hewlett-Packard 5830 gas chromatograph. The column was operated at 8 p.s.i. and 34 cm/sec helium linear velocity; with split ratio of *ca.* 100:1; 180–250°C at 4°/min; injector, 260°C; flame ionization detector, 280°C. The chromatograph was modified for split control and contained glass injection-port liners. A hole was drilled in the middle of a 1/8 in. to 1/16 in. Swagelok reducing fitting and a 1/16 in. stainless-steel tube was silver-soldered to the fitting at this position, creating a T-joint. This fitting was connected to a Hewlett-Packard metal injection-port liner, which accepted glass "drop-in" liners and the capillary column. The attached 1/16 in. stainless-steel tube was coupled to a 1/4 in. × 6 in. stainless-steel buffer volume tube, which was connected outside the GC oven to a needle valve for split control, as described previously[22].

Compound identification

Harman and norharman were identified in the whole-bases extract by coinjection of known standards and by GC–mass spectrometry (MS). Norharman, harman, and α-carboline were also identified in a subfraction of the CSC bases by UV spectroscopy and GC–MS.

RESULTS AND DISCUSSION

We have had considerable success[14,15] in preparing glass capillary GC columns coated with Superox™-4, a 4,000,000 molecular weight polyethylene glycol similar in polarity to Carbowax 20M. The high thermal stability (*ca.* 300°C) of this phase has now made possible the preparation of fused-silica glass capillary columns which give excellent peak shapes for high-boiling basic compounds, such as the carbolines. After experimentation with GC conditions and various phase thicknesses on the wall-coated open-tubular columns, we developed a successful separation of the carbolines, as exemplified by the separation of a mixture of standards (Fig. 2). The GC order of elution of all four isomers of carboline is reported for the first time. α-Carboline has a much lower pK_a than the other isomers[21] and this was reflected in its early GC

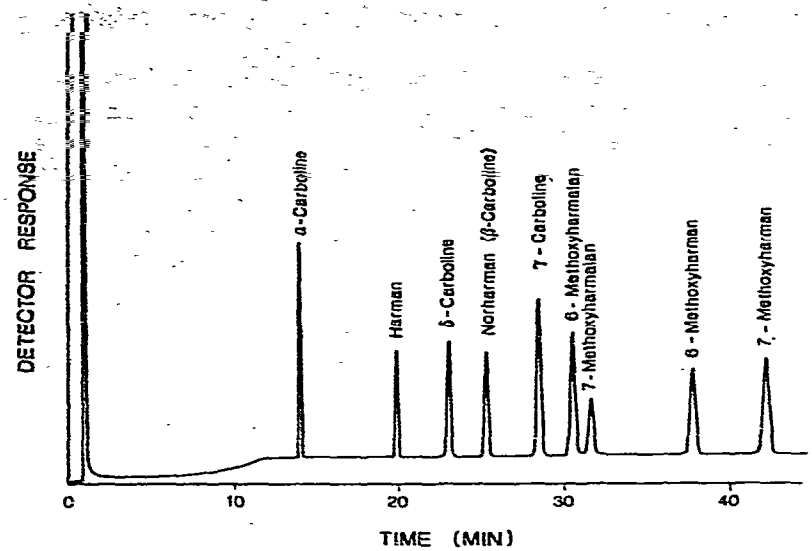

Fig. 2. Gas chromatogram of standard carbolines on a fused-silica capillary column coated with Superox™-4.

elution. The presence of a methyl group between the two nitrogen atoms in harman (Fig. 1) also lowers the polarity of the molecule and results in its early elution, relative to the parent norharman. This observation is in contrast to the activity of phases like OV-17 and Dexsil 300 GC, where separation is based mainly on boiling points and harman elutes after norharman[23]. Methoxy groups dramatically increased the po-

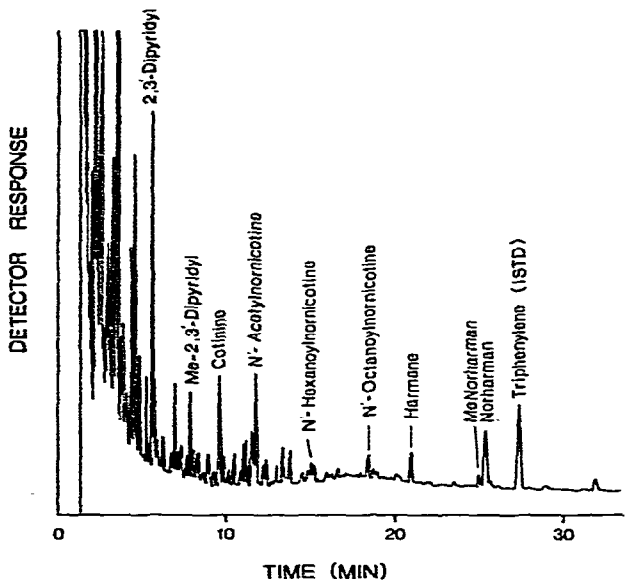

Fig. 3. Gas chromatogram of total basic fraction of cigarette smoke condensate from non-filter reference cigarettes on the Superox™-4 capillary column. ISTD = Internal standard; Me = methyl.

larity of harman and resulted in at least a doubling of retention time, relative to harman.

Since norharman and harman were found many years ago in CSC, we were interested in determining their concentration in smoke condensate from modern cigarettes. Using the successful analysis of carbolines on the Superox™-4 capillary column, we developed a rapid method for their quantification in relatively small amounts of CSC. The final method involved partition of the condensate bases from a methanol–chloroform solution of CSC into an aqueous acid solution, liberation of the free bases, and analysis by capillary GC on the Superox™-4 column. The resulting gas chromatograms were generally similar to the chromatogram of the whole base fraction from the CSC of research reference cigarettes shown in Fig. 3. Since we were only interested in the GC profile of the high-boiling carbolines, the oven temperature program was started at a high temperature (180°C). Consequently, many volatile bases, which occur in large amounts in CSC (*e.g.*, pyridines, pyrazines, nicotine and related alkaloids) eluted in the solvent front or shortly thereafter. Compounds in the GC profile were identified by coinjection of standards and by their GC–MS characteristics. One of the earliest resolved peaks was found to be 2,3′-dipyridyl. In contrast, previous investigators found this compound to be one of the last to be eluted from capillary columns coated with less thermally stable phases[24–26]. Besides harman and norharman, other compounds identified included methyl-2,3′-dipyridyl, cotinine, N′-acetylnornicotine, N′-hexanoylnornicotine, N′-octanoylnornicotine and another isomer of methylnorharman.

Several experiments were subsequently performed in order to substantiate the quantitative aspects of the total method. The reproducibility of the hydrochloric acid extraction procedure was determined and the results are shown in Table I. A small

TABLE I

REPRODUCIBILITY OF HYDROCHLORIC ACID EXTRACTION PROCEDURE

Compound	*Sample 1**	*Sample 2*	*Sample 3*	*Average*	*R.S.D. (%)***
Harman (mg/g CSC)	0.142	0.143	0.155	0.147	4.92
Norharman (mg/g CSC)	0.319	0.337	0.344	0.333	3.87

* 3-g samples of CSC.

** Relative standard deviation.

TABLE II

RECOVERY OF HARMAN AND NORHARMAN BY HYDROCHLORIC ACID EXTRACTION PROCEDURE

	*Recovery (%)**	
	Harman	*Norharman*
Sample 1	102	101
Sample 2	105	110
Sample 3	100	100

* Milligrams found minus milligrams originally in CSC divided by milligrams spiked multiplied by 100.

TABLE III

HARMAN AND NORHARMAN LEVELS IN CSC*

Cigarette	*Harman (μg/cigarette)*	*Norharman (μg/cigarette)*	*Tar (mg/cigarette)*
Kentucky reference 2R1	3.6	11.2	34.4
Commercial			
Non-filter	3.0	10.4	24.0
Filter brand A	1.8	5.6	17.0
Filter brand B	0.7	2.0	8.0

* Cigarettes smoked to a 30-mm butt length.

amount of old CSC was dissolved in methanol–chloroform and the solution was separated into three equal portions. Each portion was extracted and analyzed for harman and norharman. The data showed that the percent relative standard deviation for the extraction was less than 5%. Subsequently, the recovery of harman and norharman was determined by adding to CSC amounts of harman and norharman comparable to the levels already present. The percentage recoveries (after subtraction of original amounts) showed that the method was quantitative (Table II).

The total basic fraction from CSC is a highly complex mixture. Since large amounts of material were being injected in each analysis, considerable amounts of non-volatile material accumulated in the injection port and on the front of the column. Therefore, to ensure reproducible quantitative results, the injection-port glass liner had to be changed after about five analyses. Also, it was observed that capillary columns with bores smaller than 0.3 mm tend to deteriorate more rapidly.

The developed methodology was subsequently applied to the analyses of smoke from different commercial cigarettes. The levels of harman and norharman from the non-filter reference cigarettes (University of Kentucky 2R1) and from commercial filter and non-filter cigarettes are compared in Table III. The levels of harman and norharman in the reference and non-filter cigarettes compared favorably with previous values[11]. The filter cigarettes gave lower values, but it was apparent that harman and norharman levels correlated well with tar levels of the different cigarettes.

Other than norharman, none of the other carboline isomers could be identified or quantitated in the complex whole-bases fraction of CSC. α-Carboline was cochromatographed with the total basic fraction, and inspection of the chromatogram indicated that its concentration in CSC was less than one-tenth that of norharman. However, we had previously isolated α-carboline and a methyl derivative from the basic fraction of CSC by a combination of silicic acid and gel adsorption chromatography[27]. Erroneous reports of the UV-absorption spectra of α-carboline in the literature[28,29] had led us to conclude initially that the isolated compound was probably 5,6-benzo-7-azaindole, an isomer of α-carboline. However, the use of the correct UV spectrum of α-carboline (λ_{max} 296[18]), coupled with other data on the synthesized compound reported here, confirmed that the earlier compound was in fact α-carboline. The α-carboline was found in a benzene–diethyl ether (1:1, v/v) fraction from the chromatography of CSC bases on silicic acid[27], whereas norharman requires a

more polar solvent such as acetone or chloroform to elute from this support. This is in agreement with the much lower pK_i of α-carboline relative to norharman. α-Carboline has been reported in smoke; however, inconsistencies in the reported UV spectra raised questions as to its identification[30]. We can now report the identification of α-carboline in smoke. The presence of α-carboline is interesting from the standpoint that 2-amino-α-carboline and 2-amino-3-methyl-α-carboline, potent mutagens in the Ames test, have recently been identified and quantitated in CSC[2]. Alkyl-α-carbolines have also recently been reported in smoke by Heckman and Best[31]. Close examination of the silicic acid subfraction of the bases, containing norharman, failed to reveal any trace of γ- or δ-carbolines.

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