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Analysis of α- and β-carbolines in mainstream smoke of reference cigarettes by gas chromatography–mass spectrometry

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Abstract

The levels of 2-amino-9H-pyrido[2,3-*b*]indole (AαC or 2-amino-α-carboline), 2-amino-3-methyl-9H-pyrido[2,3-*b*]indole (MeAαC or 2-amino-3-methyl-α-carboline), 9H-pyrido[3,4-*b*]indole (norharman), and 1-methyl-pyrido[3,4-*b*]indole (harman) have been determined in the mainstream smoke condensate from three reference cigarettes, namely Kentucky reference 1R5F, Kentucky reference 2R4F, and CORESTA CM4. The amino-α-carbolines, and norharman and harman (β-carbolines) can be classified as heterocyclic aromatic amines (HAAs) and are listed as biologically active agents in the mainstream smoke of cigarettes. For the analysis, the mainstream smoke condensate from cigarettes was collected on a filter pad, the analytes were isolated using solid-phase extraction (SPE), and quantified without derivatization on a GC–MS. Total amounts of carbolines found in the condensate increased from ultralight 1R5F to full-flavor CM4 cigarettes. The level of harman was about 250 ng/cigarette for the 1R5F cigarette and about 1025 ng/cigarette for the CM4 cigarette. Norharman was typically three times more abundant than harman. The AαC was found at about 10 times lower level compared to harman, and MeAαC was about 50 times lower than harman. The use of reference cigarettes can provide a common measure for laboratories to assess carboline amounts among cigarette brands.
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1. Introduction

The interest in the analysis of heterocyclic amines started with the finding that charred parts of grilled beef and fish as well as the smoke produced from broiling sardines have a high level of mutagenicity [1]. Some of the compounds responsible for this mutagenic activity were identified as heterocyclic aromatic amines (HAAs) [2,3]. Following these findings, considerable effort has been dedicated for the identification of HAAs in food, the measurement of their level, and of their mutagenic potential [4–9]. Except for harman and norharman, which are not mutagenic, about 20 HAAs identified in food are potent mutagens [4]. Harman and norharman were found only to enhance the mutagenicity of the other HAAs [10].

Cigarette smoke contains many thousands of chemicals, which are the subject of considerable scientific research to understand relationships between materials in the tobacco section, chemical production and transfer into smoke, and ultimately to biological effects in humans. The interest in measuring HAAs in cigarette smoke was high since harman and norharman, both β-carbolines, were already reported in cigarette smoke in 1962 by Poindexter and Carpenter [11] and were further analyzed by Snook and Chortyk [12] and by Totsuka et al. [13]. In 1998 Hoffmann and Hoffman published an updated list of biologically active agents in the mainstream smoke of nonfilter cigarettes [14]. While not claimed to be complete, this list has served as a place to start searching for the evaluation of levels of undesired compounds in cigarettes. The inclusion of HAAs in "Hoffmann's list" resulted from research initiated eighteen years earlier by Yoshida and Matsumoto [15,16].

The HAAs identified in cigarette smoke condensate by Yoshida and Matsumoto were amino-α-carboline (AαC) and

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methyl-amino-α-carboline (MeAαC). When they analyzed grilled beef and mainstream smoke condensate for these two HAAs by HPLC with fluorescence detection, they found that 9 g of grilled beef produced similar amounts as 23 cigarettes [16]. They also noted that not all of the HAAs from smoking would be absorbed in the body since part of the particulate matter drawn into the lungs is exhaled. Since then other research groups have added to the list of HAAs found in smoke.

In a communication by Yamashita et al. in 1986, the researchers used treated blue cotton to extract HAA from smoke condensate and analyzed samples by HPLC with electrochemical detection [17]. They found a level of 0.26 ng per cigarette of 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) for a Japanese brand of filtered cigarettes. Additional HAAs, 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline (MeIQ), 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx), 2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline (4,8-DiMeIQx), and 2-amino-3,7,8-trimethylimidazo[4,5-*f*]quinoxaline (7,8-DiMeIQx), were not detected, thus establishing an upper limit of 0.03 ng per cigarette for these analytes. Manabe and coworkers published reports in the early 1990s on the determination of amino-α-carbolines, amino-γ-carbolines, and 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) in cigarette smoke condensate [18–21]. With preparative and analytical HPLC separations, Manabe found a mean level of 16.4 ng per cigarette of PhIP from mainstream smoke condensate of filter tipped cigarettes. Also Manabe reported 0.38 ng per cigarette of 3-amino-1,4-dimethyl-5H-pyrido[4,3-*b*]indole (Trp-P-1), 0.95 ng per cigarette of 3-amino-1-methyl-5H-pyrido[4,3-*b*]indole (Trp-P-2), 42.03 ng per cigarette of AαC, and 2.50 ng per cigarette of MeAαC [18]. During the next decade, Kataoka et al. [22] and Sasaki et al. [23] published determinations of HAAs in smoke condensate. The β-carbolines harman and norharman received less attention, probably because their biological activity from cigarette smoke was not considered to be high.

When taken together with the previous literature, the levels are scattered over orders of magnitude for any particular analyte determined from seemingly similar cigarettes. Differences in cigarette conditioning and smoking methods may be reasons for the contradictory results, but sample preparation and chromatographic performance cannot be excluded as contributors to the scatter in the data. Since standardized protocols such as those recommended by the US Federal Trade Commission (FTC) or the International Organization for Standardization (ISO) can be used for the conditioning and machine smoking of cigarettes [24–27], the variability in results due to smoking can be eliminated, or at least significantly reduced. The problem remains with sample preparation and analysis. A wide selection of readily available sample preparation materials and analytical instrumentation can be satisfactorily applied and cross validated with analyses on reference cigarettes. In the tobacco analysis field, interlaboratory studies have been reported on the analysis of reference cigarettes for a large number of chemicals on the Hoffmann list [28].

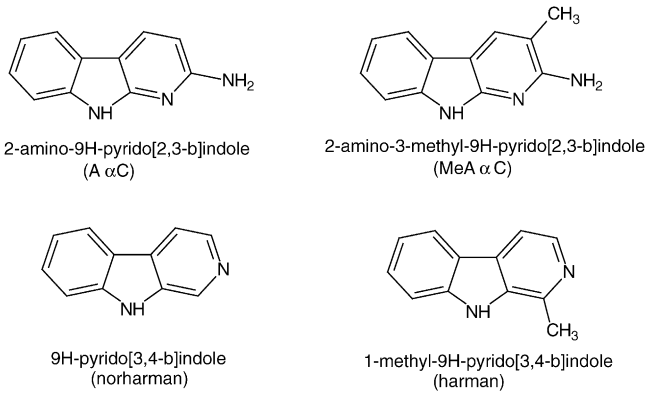

Fig. 1. Structures of 2-amino-9H-pyrido[2,3-*b*]indole (AαC or 2-amino-α-carboline), 2-amino-3-methyl-9H-pyrido[2,3-*b*]indole (MeAαC or 2-amino-3-methyl-α-carboline), 9H-pyrido[3,4-*b*]indole (norharman), and 1-methyl-pyrido[3,4-*b*]indole (harman).

The work presented herein covers the development and application of a straight-forward solid-phase extraction (SPE)–GC–MS method to quantitate harman, norharman, AαC and MeAαC in cigarette smoke condensate. The structures of these compounds are shown in Fig. 1. These four compounds represent the most prevalent carbolines found in cigarette smoke. The SPE was performed using a single cation-exchange cartridge, and the GC–MS was performed with the electron impact mode on a benchtop instrument. This method was applied to mainstream smoke condensate from three reference cigarettes which were conditioned and smoked in the FTC regime.

2. Experimental

2.1. Reagents and materials

Harman, norharman, AαC and MeAαC were purchased from Toronto Research Chemicals (North York, Canada), and all other reagents were purchased from Sigma (St. Louis, MO, USA). Concentrated ammonium hydroxide (28%) was purchased in 500 mL bottles and stored in a refrigerator. Standard solutions of the analytes were made in acetonitrile and diluted to the appropriate concentration. Solid-phase extraction cartridges containing 200 mg of MCX cation-exchange resin were purchased from Waters (Milford, MA, USA). Kentucky Reference 1R5F and 2R4F cigarettes were obtained from the Kentucky Tobacco Research & Development Center, University of Kentucky, Lexington, KY, USA. CORESTA monitor CM4 cigarette was obtained from Cerulean (Milton Keynes, UK). CM4 cigarette contained only flue-cured tobacco, while both 2R4F and 1R5F cigarettes were made with blended tobaccos. All three types of reference cigarettes had cellulose acetate filters. The cigarettes were conditioned at

60 ± 2.5% relative humidity and 22 ± 1 °C for 48 h prior to use.

2.2. Automated smoking conditions

A Borgwaldt RM 20 smoking machine (Hamburg, Germany) was used to collect particulate phase from mainstream smoke. Twenty cigarettes were smoked per run. Smoking was carried out under FTC recommendations. The FTC parameters include a puff volume of 35 mL, puff interval of 60 s, and puff duration of 2 s. Airflow around the cigarettes in the machine was 125 ± 30 mm/s to achieve the recommended Industry Monitoring "tar" level. The particulate phase from mainstream smoke was collected on a 92 mm Cambridge pad (Whatman, Maidstone, UK), and the pad placed in a glass jar and capped.

2.3. Sample preparation

All steps in the sample preparation were completed within 7 h of sample collection. Each sample pad was extracted with 50 mL of 0.1 M HCl/20 mM ascorbic acid solution using a wrist-action shaker for 30 min. The extraction solution was identical to that used in the impingers by Kataoka et al. [22]. After shaking, 40 mL of the extract was used for SPE.

An automated RapidTrace workstation (Caliper Technologies, Hopkinton, MA, USA) was fitted with 3 mL MCX cartridges having 200 mg resin. The MCX stationary phase is a cation exchanger with some reversed-phase character (see e.g. [29]). Software to load large volumes with the RapidTrace was purchased from Analytical Associates, Miami Shores, FL, USA. The RapidTrace was programmed to perform the following: load 40 mL extract onto the unconditioned cartridge, rinse with 12 mL 0.1 M HCl, dry with nitrogen for 1 min, rinse with 12 mL methanol, rinse with 12 mL of methanol–0.5 M acetate buffer pH 5.5 (1:1, v/v), dry with nitrogen for 6 min, and then elute with 12 mL of acetonitrile–concentrated ammonium hydroxide (95:5, v/v). The acetonitrile–concentrated ammonium hydroxide solution was made daily to avoid any decrease in concentration. All solutions were applied to the SPE cartridge at a rate of 1.5 mL/min. MCX cartridges had been used by DeBruin et al. to extract PhIP from human breast milk [30], and the above SPE steps were developed specifically for the smoke condensate matrix.

The final eluent from the MCX cartridge was transferred to a silanized centrifuge tube and evaporated to dryness at 55 °C with nitrogen. Residue in the centrifuge tube was reconstituted with 150 μL acetonitrile and placed in a silanized autosampler vial with a tapered insert. The use of non-silanized glassware showed some losses of analyte.

2.4. Sample analysis

Samples were analyzed on an Agilent 6890/5973 GC–MS system. Sample injection volume was 1 μL. The GC system was equipped with a 30 m × 0.25 mm i.d. and 0.25 μm film thickness Phenomenex ZB-50 column (equivalent to DB-17). The liner was deactivated fused silica containing a glass wool plug, and the injector was operated at 310 °C with a split ratio of 1:20. The liner was changed every second day or after approximately 25 samples. Helium flow through the column was constant at 1 mL/min. The oven was programmed at an initial temperature of 50 °C for 2 min, heated to 190 °C at 30 °C/min with a hold time of 0.5 min, heated to 240 °C at 5 °C/min, then to 335 °C at 20 °C/min with a hold time of 4 min. The mass spectrometer was operated in the positive electron impact mode, with 70 eV electron energy, 250 °C temperature of the ion source, and scanning from 45 to 430 mass units.

Harman, norharman, AαC and MeAαC were quantitated using their molecular ion signal areas at 182, 168, 183 and 197 *m/z*. External calibration at eight levels was performed over the ranges expected in ultralight, light and full-flavor cigarettes. Analyte areas were plotted against the standard solutions (in ng/μL) or against nanograms per cigarette for a 20 cigarette sample. For harman and norharman, the calibrations covered 95 to 1350 ng/cigarette and 200–3100 ng/cigarette, respectively. For AαC and MeAαC the calibration ranges were 11–162 ng/cigarette and 5–75 ng/cigarette, respectively. The external calibration data were linear over the specified ranges, and the correlation coefficients were 0.99 or higher. Day-to-day variations in signal intensities were typically between 5 and 10%, thus a three-point calibration was performed along with a blank with each set of samples. If an unknown sample was above the calibration range, the sample was diluted with acetonitrile to 1/2 of the initial concentration and reinjected into the GC–MS. When a sample of smoke condensate was processed and analyzed, the extracted ion chromatograms indicated that the analytes were easily identified and resolved from matrix interferences. This has been achieved using both the characteristic ions for the analyte and the retention time obtained from standards. An overlay of the extracted ion data from a 2R4F cigarette sample is presented in Fig. 2.

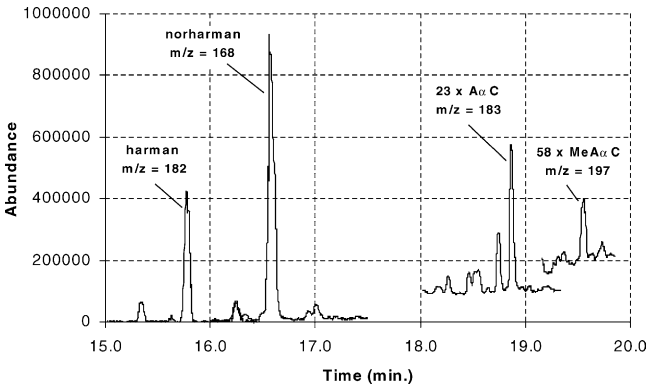

Fig. 2. Overlay of the extracted analyte ion signals from a 2R4F sample. Each signal is scaled for viewing.

Table 1
Average analyte recoveries (six replicates) and standard deviation (S.D.) in percent for the SPE–GC–MS method at two added analyte levels per pad

	0.6 μg/pad		15.0 μg/pad	
	Recovery (%)	S.D. (%)	Recovery (%)	S.D. (%)
Harman	95.6	6.7	93.8	5.0
Norharman	102.5	6.2	93.9	5.7
AαC	79.9	7.4	90.7	4.9
MeAαC	87.8	7.5	94.5	6.7

3. Results and discussion

3.1. Recovery and limits of detection

The recovery for each analyte was examined at two levels corresponding to 30 and 750 ng/cigarette (0.6 and 15.0 μg/pad, respectively). This procedure involved comparing spiked Cambridge pads without condensate processed through the sample preparation steps to equivalent analyte amounts prepared in acetonitrile. The data from six replicates are reported in Table 1, which summarizes the recovery findings. Both harman and norharman were recovered with high efficiency at both levels. When calculating unknown analyte amounts, the recovery values at 750 ng/cigarette were used for harman and norharman. For AαC and MeAαC there was an increase in the recovery at the higher level. Presumably, a small amount of loss may have occurred due to irreversible retention on the SPE cartridge or through active sites during the preparation steps. This loss was reflected to a lesser proportion at 750 ng/cigarette. However, most cigarettes have closer to 30 ng of AαC and MeAαC, and the recovery at this level was used in final calculations of unknowns for these two analytes. All analyte recoveries were monitored periodically for quality assurance.

Limits of detection (LODs) were determined by analyzing a diluted 1R5F sample seven times. For each analyte, the LOD was reported as three times the calculated standard deviation of the seven measurements. The LODs for harman and norharman were 33.1 and 84.7 ng/cigarette, respectively. The calculated LODs for AαC and MeAαC were 3.1 and 1.4 ng/cigarette, respectively. For some samples, such as those from a 1 mg tar cigarette, MeAαC be expected to be close or lower than the LOD. If needed, the split ratio of the GC injector may be changed to provide additional sensitivity. However, the LOD values were good enough to assure reliable measurement of the analytes from the ultralight cigarettes.

3.2. Analysis of reference cigarettes

For this work, three reference cigarette types were selected that were of different styles and produced total particulate matter (TPM) from the 'ultralight' to 'full-flavor' levels using the FTC smoking conditions. CM4 produced a TPM of 17 mg, and 2R4F cigarettes produced a TPM of 12 mg, making it a 'light' cigarette. With a TPM of 2.5 mg, 1R5F cigarettes fall into the 'ultralight' category. The TPM for each cigarette corresponds to the level indicated by the supplier, and also was measured by weighing the pads after the smoke condensate was collected.

The results of the determinations for the target analytes in the particulate phase of mainstream smoke from the reference cigarettes are presented in Table 2. When the 1R5F and 2R4F cigarettes are compared, all carboline levels increased from the ultralight to the light cigarette. This increase can be expected since these two cigarettes have similar tobacco blends. The CM4 cigarette with flue-cured tobacco produced the highest levels of harman and norharman, but CM4 smoke condensate had less AαC and about the same level of MeAαC compared to 2R4F cigarette. With the ultralight 1R5F and full-flavor CM4 data presented herein, the large change in TPM makes a meaningful comparison difficult: it can be concluded that the 1R5F carboline data are the lowest, and that data normalized by the TPM show a significant increase in HAA concentration for the 1R5F. The levels for TPM measured on the smoke condensate samples and the levels normalized by TPM for each analyte are given in Table 3.

A survey of the literature for data on cigarettes similar to 2R4F is summarized in Table 4. Earlier work by Yoshida et al. concluded that flue-cured cigarette condensate contained less AαC and MeAαC than burley cigarette condensate [15]. These results on AαC and MeAαC were confirmed by ours on the flue-cured CM4 and blended cigarettes. With the SPE–GC–MS method, the results for harman and norharman from 2R4F condensate were roughly similar to those of Totsuka on Japanese filter cigarettes. Using TPM to normalize the data from CM4 and 2R4F condensates in Table 4, harman and norharman appear to be generated in equal amounts from flue-cured and blended reference cigarettes. Snook and Chortyk found similar levels of harman and norharman in the condensate of flue-cured, burley, Maryland, and Turkish tobacco cigarettes [12].

Formation of carbolines from pyrolysis of plant and animal material is believed to require amino acids as a precursor.

Table 2
Average levels, and standard deviation (S.D.; $n = 5$) of harman, norharman, AαC and MeAαC in three reference cigarettes

	Harman ng/cigarette		Norharman ng/cigarette		AαC ng/cigarette		MeAαC ng/cigarette	
	Average	S.D.	Average	S.D.	Average	S.D.	Average	S.D.
1R5F	254	17.1	676	46.3	29.9	2.3	4.9	0.5
2R4F	668	33.7	1731	78.4	60.4	1.8	9.5	0.3
CM4	1026	38.9	2534	139.5	45.8	3.2	10.3	1.2

Five separate analyses for each cigarette were spread over two weeks. The values are given in ng/cigarette.

Table 3
Average levels for TPM in mg/cigarette and the levels of harman, norharman, AαC and MeAαC normalized by TPM in three reference cigarettes

	TPM (mg/cigarette)		ng harman/mg TPM	ng norharman/mg TPM	ng AαC/mg TPM	ng MeAαC/mg TPM
	Average	S.D.	Average	Average	Average	Average
1R5F	2.36	0.05	107.63	286.44	12.67	2.08
2R4F	10.62	0.27	62.90	162.99	5.69	0.89
CM4	17.04	0.21	60.21	148.71	2.69	0.60

The values are given in ng HAA/mg TPM.

Table 4
Literature survey of data on cigarettes similar to 2R4F

Reference	Cigarette type	Harman	Norharman	AαC	MeAαC
[15]	"Blended" w/filter			60–80	9–11
	Flue-cured			25–32	10–15
	Burley			110–134	20–22
[18]	US filter			42.6	2.01
[22]	Japan filter			1.96	
[13]	Japan filter (12.9 mg TPM)	1050	1900		
	Japan filter (16.7 mg TPM)	1520	3080		
This work	2R4F	668	1731	60.4	9.5

The values are given in ng/cigarette.

In a study of tobacco leaf, free amino acids were found to be higher in burley than in flue-cured tobacco [31]. However, the level of free amino acids in tobacco does not directly correlate with the amount of amino-α-carbolines in smoke condensate and does not explain the similar levels of β-carbolines in flue-cured and burley tobacco.

The mutagenic activity of smoke condensate from flue-cured and burley cigarettes was examined by Mizusaki et al. [32]. They found that flue-cured tobacco condensate produced significantly lower mutagenic activity than burley tobacco condensate and that the nitrate level in cigarettes was correlated to mutagenic activity in the smoke condensate. It could be possible that a major factor in the mutagenic activity correlation is the level of AαC and MeAαC generated in the smoke. The co-mutagens harman and norharman may play a role in mutagenicity of smoke condensate by enhancing the mutagenicity of HAAs [10]. For example, it has been established that norharman and aniline can be metabolically activated to form genotoxic aminophenylnorharman [33]. The data presented from this study can be used to establish relationships of HAA generation among cigarette brands and to gauge exposures from smoking to other dietary and environmental exposures. The levels of HAAs measured in this study show that the smoking of 20 2R4F cigarettes generates lower levels of harman and norharman than found in 100 g broiled beef [13], and significantly lower levels of AαC and MeAαC than found in grilled beef [16]. These results are illustrated in Fig. 3.

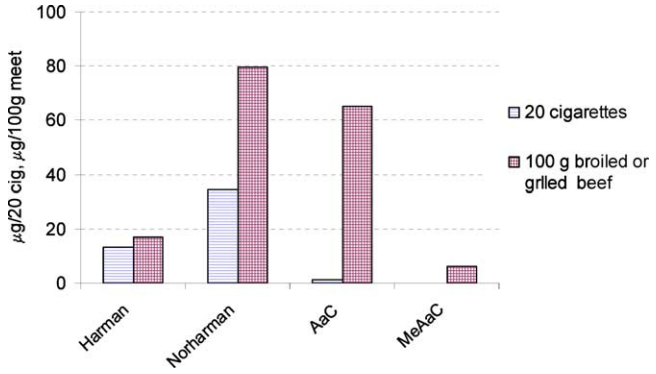

Fig. 3. Comparison in the levels in μg of harman, norharman, AαC, and MeAαC for 20 2R4F cigarettess (one pack) and for 100 g of broiled beef [13,16].

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