Isolation and Identification of *N'*-Acylalkaloids of Cigarette Smoke *

by M. E. Snook, O.T. Chortyk and R.F. Arrendale

Tobacco Safety Research Unit, Agricultural Research Service,
United States Department of Agriculture, Athens, Georgia, U.S.A.

SUMMARY

Methods have been developed for the analyses of the minor high-boiling bases of smoke. Fused silica glass capillary columns coated with Superox-4 were prepared and used successfully for the GC analyses of the basic fraction of smoke, containing compounds covering a wide range of volatility and polarity (nicotine to norharman). The Superox-4 capillary column also produced an excellent separation of the numerous *N'*-acylalkaloids, which have been reported in tobacco and tobacco smoke. Several new *N'*-acylalkaloids are reported. The described methodology should be a great asset in future isolation and identification studies of these and other, as yet to be characterized, minor bases of smoke and leaf.

ZUSAMMENFASSUNG

Zur Untersuchung der in geringen Mengen vorkommenden hochsiedenden Basen des Zigarettenrauches wurden Verfahren entwickelt. Mit Superox-4 beschichtete Quarzglas kapillarsäulen wurden erfolgreich zur gaschromatographischen Analyse der basischen Rauchfraktion eingesetzt, die Verbindungen einer weiten Spannbreite an Flüchtigkeit und Polarität enthält

(Nicotin bis Norharman). Auch konnte mit den Glaskapillarsäulen (Superox-4) die große Zahl der in Tabak und Tabakrauch bekannten *N'*-Acylalkaloide sehr gut getrennt werden. Es wurden auch mehrere neue *N'*-Acylalkaloide identifiziert. Die beschriebene Methodik könnte in der Zukunft bei der Isolierung und Identifizierung dieser und anderer noch zu bestimmender Basen in Blattabak und Rauch von großem Nutzen sein.

RÉSUMÉ

Des procédés ont été mis au point pour l'analyse des bases à point d'ébullition élevé qui sont présentes en faible quantité dans la fumée de cigarette. L'utilisation de colonnes capillaires de verre quartzéux revêtues de Superox-4 permet d'analyser avec succès par chromatographie en phase gazeuse la fraction basique de la fumée, laquelle contient des composés dont la volatilité et la polarité sont très diverses (de la nicotine au norharman). Les colonnes capillaires (Superox-4) permirent également de séparer dans d'excellentes conditions les nombreux *N'*-acylalkaloïdes dont on connaît l'existence dans le tabac et la fumée, ainsi que d'identifier de nouveaux composés de ce type. La méthode employée pourrait s'avérer très utile, à l'avenir, pour la séparation et l'identification de bases dont la présence dans le tabac en feuilles et la fumée est connue ou non.

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Table 1.
Acylalkaloids of tobacco and tobacco smoke.

	R	Molecular weight	References	
			leaf	smoke
<i>Acylornicotines:</i>				
Formyl	H-	176	(9, 23)	(2)
Acetyl	CH ₃ -	190	(9)	(2)
Butanoyl	CH ₃ (CH ₂) ₂ -	218	(22)	(3)
Hexanoyl	CH ₃ (CH ₂) ₄ -	246	(8)	(3)
Heptanoyl	CH ₃ (CH ₂) ₅ -	260		*
Branched heptanoyl	(C ₈)	260		*
Octanoyl	CH ₃ (CH ₂) ₆ -	274	(8)	(3)
Branched octanoyl	(C ₇)	274		*
Octenoyl	(C _{7:1})	272		*
6- + 7-Hydroxyoctanoyl	(C ₇ OH)-	290	(21)	
Carbomethoxy	CH ₃ O-	206	(10)	
Carboethoxy	CH ₃ CH ₂ O-	220	(22)	
4-Dimethylaminobutanoyl	(CH ₃) ₂ N(CH ₂) ₃ -	261	(22)	
<i>Other acylalkaloids:</i>				
N'-Formylanatabine		188	(24)	(2)
N'-Formylanabasine		190	(24, 25)	
N'-Acetylanatabine		202		(2)
N'-Valerylanabasine		246		(3)

* identified in this work.

Figure 1.
Scheme I: Fractionation of cigarette smoke condensate for bases (ether extraction).

INTRODUCTION

Nicotine and related alkaloids are by far the major components in the basic fraction of both tobacco and tobacco smoke. In addition, cigarette smoke also contains large quantities of simple pyridines and pyrazines and their alkyl and vinyl derivatives (1). Among the minor bases, found in appreciable quantities in smoke, are *N*-acyl derivatives of nornicotine, anabasine and anatabine (2, 3) (Table 1) and the carboline bases, harman (1-methyl-9*H*-pyrido[3,4-*b*]indole) and norharman (9*H*-pyrido[3,4-*b*]indole) first reported by *Poindexter* and *Carpenter* (4, 5). For analyses of leaf and smoke compounds, we have developed fused silica capillary columns coated with the thermally stable polar phase Superox-4 (6). Use of these columns has, for the first time, allowed the efficient separation of not only the important bases harman and norharman, but also the resolution of several *N*-acylnornicotines and acylanatabines from the total free bases fraction of smoke. We will also detail our newly developed isolation procedures for the above compounds, which have resulted in the identification of several new *N*-acylnornicotines in cigarette smoke condensate (CSC).

EXPERIMENTAL*

Preparation and Fractionation of Cigarette Smoke Condensate

All solvents were Burdick and Jackson "distilled-in-glass" solvents. Cigarette smoke condensate was prepared under standard smoking conditions from commercially available cigarettes, as previously described (7).

Scheme I (see Figure 1)

Fractionation of 1 kg of cigarette smoke condensate to obtain the basic subfractions by this procedure has been described before (7). Fraction F-D contained the compounds of interest.

Scheme II (see Figure 2)

Twenty grams of cigarette smoke condensate were dissolved in 600 ml of 30 % methanol/chloroform (MeOH/CHCl₃) and extracted with 150 ml of 2 N HCl followed by 4 × 100 ml 2 N HCl. Each aqueous HCl extract was cross extracted with 50 ml CHCl₃ and the CHCl₃ extracts were added to the original MeOH/

* Reference to a company or product name does not imply approval or recommendation by the United States Department of Agriculture.

Figure 2.
Scheme II: Fractionation of cigarette smoke condensate for bases (CHCl_3 extraction).

CHCl_3 solution. The combined aqueous HCl extracts were adjusted (with ice-cooling) to pH 12, with 15 N aqueous NaOH, saturated with NaCl and extracted with CHCl_3 (3×100 ml). Evaporation gave 3.05 g (15.25 %) of a free bases fraction. The bases were re-dissolved in diethyl ether (ether or E) and deposited onto 20 g of silicic acid (SA), the ether was removed on a rotary evaporator and the SA-sample mixture was placed on top of a column of SA (100 g), packed in petroleum ether, and eluted with the following solvents: F-A, 1 l benzene (B) / E (1 : 1); F-B, 0.5 l E; F-C, 0.5 l E; F-D, 1 l acetone; F-E, 1 l MeOH. The fractions were evaporated on a rotary evaporator and the yields in grams and percentage composition of cigarette smoke condensate were: F-A, 0.63 g, 3.15 %; F-B+C, 0.21 g, 1.05 %; F-D, 1.34 g, 6.70 %; F-E, 1.06 g, 5.30 %.

Adsorption Gel Chromatography

The gel chromatographic system consisted of four columns connected in series and packed with Bio-Beads S-X12 as described previously (12). Fraction F-D from Scheme I (acetone eluant) was dissolved in 50 ml of

benzene and aliquots were placed on the first gel column with a 1.0 ml loop injection valve. Six 1 ml aliquots of F-D were individually chromatographed through the gel columns. Benzene was pumped (Altex 110 pump) at a flow rate of 120 ml/h and 8 ml fractions were collected. Eluted gel fractions (GF) from all the runs with the same number were combined. Column eluant was monitored at 280 nm with a Chromatronix Model 230 detector. In a similar manner, fraction F-E (MeOH eluant) from Scheme II was dissolved in 2 ml of benzene and 1 ml of the solution was chromatographed on the gel system.

Gas Chromatography (GC)

The glass capillary GC columns used were fused silica columns (10 m or 22 m $\times$ 0.3 mm inside diameter) statically coated with Superox®-4 (2 mg / ml in methylene chloride), after pre-treatment and deactivation with Superox-4 (6). In addition, an SE-54 capillary column (30 m $\times$ 0.3 mm inside diameter) was used for the separation of *N*-acetylnornicotines. GC analyses were performed on a Hewlett-Packard 5830 gas chromatograph. (The columns were operated at 8 p.s.i. and 34 cm / s he-

lium linear velocity; split ratio of about 100 : 1; injector, 260 °C; FID, 310 °C; oven program, 120-250 °C at 4°/min.)

Identification of Compounds

Standards: A convenient source of nornicotine, anabasine and anatabine was the end fraction of the slow fractional distillation of commercial technical grade nicotine. Treatment of this fraction with acid halides produced the desired *N*-acyl derivatives. The formyl derivatives were prepared by treating the alkaloids with formic acid (8) or *N*-formylimidazole (9). The carbomethoxy derivatives were prepared by the method of Hecht et al. (10) by reaction of the alkaloids with methylchloroformate.

Mass Spectrometry (MS)

GC-MS analyses were performed on a Hewlett-Packard 5985B GC/MS system. Separations were made on a 45 m × 0.3 mm inside diameter fused silica capillary column coated with Superox-4 (20 p.s.i. helium column pressure; linear velocity: 30 cm/s at 100 °C; temperature program: 40 °C for 2 min, then 40 °C to 120 °C at 20 °/min followed by 120 °C to 250 °C at 4 °/min). Injection of the sample was made in the splitless mode and the GC effluent was led into the mass spectrometer through an open-split interface, constructed in our laboratory (11).

RESULTS AND DISCUSSION

GC Separation of Smoke Alkaloids

The GC liquid phase, Superox-4, is a 4×10^6 molecular weight polyethylene glycol. It is similar in polarity to Carbowax 20M, but more thermally stable. Our recent preparation of fused silica glass capillary columns, coated with Superox-4 (6), has allowed successful GC analyses of many high-boiling polar compounds of cigarette smoke condensate. We have applied this capillary GC technology to the development of methods of analysis for high-boiling compounds of the basic fraction of cigarette smoke condensate. The Superox-4 columns were found to give excellent peak shapes for not only the important bases, harman and norharman, but also allowed for the efficient separation of a number of *N*-acetylnornicotines, anabasines, and anatabines. The GC separation of a number of synthesized *N*-acylalkaloids on a Superox-4 column is shown in Figure 3. The chromatogram also includes some commonly found tobacco alkaloids: nicotine, nornicotine, myosmine, anabasine, nicotyrine, anatabine, 2,3'-dipyridyl, cotinine, and harman and norharman. As expected for the polar Superox-4 column, the *N*-acylalkaloids eluted before the more polar formyl derivatives.

Isolation of *N*-Acylalkaloids

We have developed procedures for the large scale (20-1000 g cigarette smoke condensate) fractionation of smoke bases. One of these procedures (Figure 1), based on our previously derived fractionation of cigarette smoke condensate for bioassays (7), involved partitioning of cigarette smoke condensate between aqueous NaOH and diethyl ether to remove acidic materials. Extraction of the diethyl ether solution with aqueous HCl separated the bases, which were subsequently extracted and fractionated by silicic acid chromatography. In a second procedure (Scheme II, Figure 2), cigarette smoke condensate was dissolved in 30 % CH₃OH / CHCl₃ and the bases were extracted into aqueous HCl. After adjustment of the acid extract to pH 12, the bases were extracted into CHCl₃. Extraction of a smaller quantity of cigarette smoke condensate (Scheme II) and without prior removal of an acid fraction allowed a more efficient extraction of basic material.

Preliminary examination of the total free bases fraction from the two methods showed that ether extraction (Figure 1) failed to extract the *N*-acetyl- and formylalkaloids, but did extract the *N*-hexanoyl- and octanoylnornicotines. This was in agreement with Schumacher et al. (2) who found *N*-acetyl- and formyl nornicotines and -anatabines in the water-soluble portion of smoke partitioned between water and ether. Also, Heckman and Best (3) found the *N*-butanoyl-, hexanoyl-, and octanoylnornicotines in an acid extract of the ether soluble portion of smoke partitioned between water and ether. In comparison, the gas chromatogram of the total base fraction from Scheme II (Figure 4) showed the successful extraction of the *N*-acetyl- and formylalkaloids, as well as of the higher homologs.

Both Schemes I and II employed silicic acid (SA) chromatography to separate and concentrate the *N*-acylalkaloids and norharman from the multitude of other bases in cigarette smoke condensate. The *N*-C₆- and C₈-acetylnornicotines as well as norharman eluted from the SA column with acetone. Gas chromatograms of the acetone eluant (fraction F-D) from Scheme I and Scheme II are given in Figures 5 and 6, respectively. The chromatograms also show the additional compounds identified in the fractions. *N*-Acetyl- and formyl nornicotine required the more polar solvent, methanol, to be eluted from SA (Scheme II). A gas chromatogram of the methanol fraction from Scheme II (Figure 7) also showed the presence of nicotine and cotinine in this fraction. Interestingly, *N*-acetyl- and formylanatabine were concentrated mainly in the acetone fraction (Figure 6).

Lipophilic adsorption gel chromatography proved to be an efficient method for obtaining relatively pure isolates of the norharman and *N*-acylalkaloids suitable for identification of minor components. We have successfully utilized the Bio-Beads S-X12 gel to isolate and purify many different classes of compounds from tobacco and tobacco smoke, such as polynuclear aromatic hydrocarbons (12), aromatic ketones (13), aro-

Figure 3.
Separation of alkaloid standard on Superox-4 fused silica capillary column (22 m × 0.3 mm
inside diameter, 120 °C to 250 °C at 2°/min).

Figure 4.
Gas chromatogram of total free bases from commercial non-filter cigarette smoke (Scheme II extraction) on a Superox-4 column (22 m × 0.3 mm inside diameter, 120 °C to 250 °C at 4°/min).

Figure 5.
Gas chromatogram of acetone fraction F-D from silicic acid chromatography of total bases
(Scheme I) on a Superox-4 column (10 m × 0.3 mm inside diameter, 130 °C to 250 °C at 4°/min).

Figure 6.
Gas chromatogram of acetone fraction F-D from silicic acid chromatography of total bases
(Scheme II) on a Superox-4 column (22 m $\times$ 0.3 mm inside diameter, 120 °C to 250 °C at 4°/min).

Figure 7.
Gas chromatogram of methanol fraction F-E from silicic acid chromatography of total bases (Scheme II) on a Superox-4 column (22 m $\times$ 0.3 mm inside diameter, 120 $^{\circ}$ C to 250 $^{\circ}$ C at 4 $^{\circ}$ /min).

Figure 8.

UV trace (280 nm) for elution of fraction F-D (Scheme I) for gel chromatography on Bio-Beads S-X12 (benzene).

Figure 9.

Gas chromatogram of selected gel fractions (Scheme I, fraction F-D) containing harman and norharman on a Superox-4 column (10 m $\times$ 0.3 mm inside diameter, 130 $^{\circ}$ C to 250 $^{\circ}$ C at 4 $^{\circ}$ /min).

matic nitriles (14), phenolics (15, 16), leaf lipids (17), and indoles and carbazoles (18). Aromatic compounds are retained by the gel to a greater extent than interfering aliphatic compounds and can be effectively concentrated and obtained in relatively pure form. The retention is based on a form of π - π electron interaction between solute and gel, as in the case of Bio-Beads (a styrene-divinylbenzene copolymer) in benzene. The adsorption effects are useful in separating different classes of compounds (i.e. aliphatic hydrocarbons, alcohols, aromatic compounds, phenols, etc.). However, the permeation properties of the gel operate within classes of aliphatic compounds and elutions are in order of decreasing molecular size (17).

These properties of the Bio-Beads S-X12 gel were used successfully to isolate the norharmans and *N*-

Figure 10.

Gas chromatogram of GF-39 from Bio-Beads S-X12 chromatography of fraction F-E (methanol, Scheme II) on a SE-54 fused silica capillary column (30 m $\times$ 0.3 mm inside diameter, 100 $^{\circ}$ C to 280 $^{\circ}$ C at 4 $^{\circ}$ /min).

acylalkaloids from the more abundant nicotine and dipyridyl compounds. Fraction F-D (Figure 1) was chromatographed on Bio-Beads S-X12 in benzene (Figure 8). Since the norharmans have greater π -electron densities than the other alkaloids, they were retained more by the gels. They eluted last from the gel systems in relatively pure form, as illustrated by the gas chromatogram of combined gel fractions 46-60 (Figure 9). The methyl derivative (harman) eluted before the parent (norharman) due to steric interruption of the π - π interaction by the methyl group.

Due to their greater molecular weight and overall molecular width, the *N*-C₆- and C₈-acylnornicotines eluted from Bio-Beads before nicotine. Their elution from the gels in gel fractions 34-38 indicated that these *N*-acylnornicotines have properties midway between aliphatic compounds (elution: GF 29-33) and aromatic nicotine and dipyridyl compounds (elution: GF 40+). This is due to the long alkyl chains in the molecules. In contrast, the short-chain *N*-C₁- and C₂-acylnornicotines were found to elute later (due to permeation effects) and co-eluted with nicotine. This is shown in Figure 10, a chromatogram of GF-39 from the Bio-Beads separation of fraction F-D (methanol, Figure 2), which contains both nicotine and the short-chain *N*-acylalkaloids. The chromatogram also shows that the *N*-formyl- and acetylnornicotines are cleanly separated on the SE-54 capillary column, in contrast to the Superox-4 column (Figure 7).*

Gas chromatographic analyses of the individual gel

* However, for the unrefined basic fractions, the SE-54 columns caused tailing of separated peaks and were not used.

Figure 12.

Mass spectra of reference *N*-*n*-heptanoylnicotine, GC-separated (isolated) *N*-*n*-heptanoylnicotine and *N*-*ant*/iso-heptanoylnicotine (* mass number indicates enhanced ion intensity).

Figure 11.
Gas chromatogram of GF-36 from Bio-Beads S-X12 chromatography of fraction F-D (acetone, Scheme I) on a Superox-4 column (10 m $\times$ 0.3 mm inside diameter, 130 $^{\circ}$ C to 250 $^{\circ}$ C at 4 $^{\circ}$ /min).

Peaks c and d ($C_{8:1}$) are described in the text.

fractions of the Bio-Beads separation of fraction F-D (acetone, Figure 1) (GF 34-38) showed that a relatively pure N' - C_6 - C_8 -acylnornicotine isolate had been obtained. The gas chromatogram of GF-36 is shown in Figure 11. As expected, N' -hexanoyl- and octanoylnornicotines were the major components present (the C_8 isomer eluted from the gels slightly before the C_6 isomer). In addition to the N' - C_6 - and C_8 -acylnornicotines, several other isomers were characterized in this subfraction, including N' -heptanoyl-, *anteiso*-heptanoyl-, *iso*-octanoyl- and four different isomers of octenoylnornicotines, which are being reported for the first time. MS data for these compounds are presented in Figures 12-14.

Major MS fragmentation pathways, as reported by Bolt (8), are given in Figure 15. Comparative analyses of the M^+-15 , M^+-29 and M^+-43 ion intensities in the isolated N' - n - C_7 -, n - C_8 -, *anteiso*- C_7 -, and *iso*- C_8 -acylnornicotines with the same ions in *n*-heptanoic acid and *anteiso*-heptanoic acid (19) and in previously identified *n*-, *anteiso*- and *iso*-hydrocarbons of tobacco (20) left no doubt as to the validity of the assigned structures. The detection of the N' -octenoylnornicotines in

smoke is very interesting, as Miyano et al. recently reported the presence of N' -(6-hydroxyoctanoyl)- and N' -(7-hydroxyoctanoyl)nornicotines (Table 1) in Japanese domestic tobacco (21). The N' -octenoylnornicotines were found in the smoke of American commercial non-filter cigarettes. Although N' -hydroxyoctanoylnornicotines have yet to be identified in American flue-cured tobacco, they probably exist and are converted by the proposed pyrolytic dehydration pathway (Figure 16) to the octenoyl compounds. This scheme indicates three possible isomers would be formed; however, MS-indicated four peaks in GF-36 (Figure 11) were N' -octenoylnornicotines. Migration of the double bond, which is known to occur in pyrolysis reactions, could produce a fourth isomer (N' -(4-octenoyl)nornicotine). The presence of an ion of m/z 203 (Figure 15) in the spectra of all four peaks precludes migration of the double bond closer to the carbonyl than the γ position, unless hydrogen transfer occurs. There is also the possibility of *cis/trans* isomers. Two peaks in GF-36 (Figure 11, marked c and d) were found to have greatly enhanced abundances of m/z 203 ions (Figure 14), relative to the usual base peak of m/z 189. It is postulated

Figure 13.
Mass spectra of reference *N*-*n*-octanoylnornicotine and GC-separated (isolated) *N*-iso-octanoylnornicotine (*mass numbers indicate enhanced ion intensities).

Figure 15.
Principal mass fragmentation pathways for *N'*-acylnornicotines according to Bolt (20).

Figure 16.
Postulated pyrolytic syntheses of *N'*-octenoylnornicotines.

this is due to cleavage of a stable allylic radical ($\cdot CH_2CH=CHCH_2CH_3$) from the side chain of the molecular ion (Figure 15) competing favorably with the usual β -cleavage and γ -hydrogen transfer (*McLafferty* rearrangement (8)). We could find no evidence for the presence of *N'*-carbomethoxy-, carboethoxy-, butanoyl-, nonanoyl- or decanoylnornicotines in the gel isolate of Scheme I. The butanoyl isomer was probably lost in the NaOH extraction of Scheme I as were the formyl and acetyl isomers. The C_9 and C_{10} isomers apparently are not found in smoke.

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Figure 14.

Mass spectra of GC-separated (isolated) *N*-octenyl/nornicotines. Upper spectrum refers to peaks a and b of Figure 11. Lower spectrum (postulated *N*-(5-octenyl)nornicotine isomers) refers to peaks c and d. (* mass number indicates enhanced ion intensity.)

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Authors' address:

*Tobacco Safety Research Unit,
Agricultural Research Service,
U.S. Department of Agriculture,
P. O. Box 5677,
Athens, Georgia, 30613, U.S.A.*