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THE ISOLATION OF HARMANE AND NORHARMANE FROM TOBACCO AND CIGARETTE SMOKE

E. H. POINDEXTER, JR. and R. D. CARPENTER
Philip Morris Incorporated, Richmond, Virginia, U.S.A.

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Abstract—Harmane and norharmane were isolated from cured tobacco and its smoke. This establishes the presence of indole alkaloids in tobacco. Quantitative studies showed that cigarette smoke contained between 15 and 20 μg of combined harmane and norharmane per gram of tobacco smoked. The tobacco itself contained only about 1 per cent of the amount found in smoke. Thus, the pyrolytic formation of the harmane alkaloids was strongly indicated. The addition of radioactive tryptophan to the tobacco resulted in the isolation of radioactive harmane alkaloids in the smoke. Further studies indicated that enough tryptophan was present in the tobacco to account for the quantities of the harmane alkaloids found in the smoke.

INTRODUCTION

DURING a study of the basic fraction of cigarette smoke (Fig. 4) two strongly fluorescent compounds were observed on paper chromatograms. These were subsequently identified as harmane (I) and norharmane (II) by ultraviolet and infrared spectrophotometry. No reference to these compounds was found in recent reviews of tobacco chemistry.[1,2]

The isolation of these alkaloids was achieved by the acidic extraction of smoke, followed by cellulose column chromatography of the free bases and subsequent resolution by paper chromatography. The isolated compounds gave identical ultraviolet, infrared, and fluorescence spectra with those given by authentic harmane and norharmane.

RESULTS AND DISCUSSION

The alkaline fraction of smoke contained considerable blue fluorescent material, most of which remained in the steam pot during steam distillation. Examination of this pot residue in numerous paper chromatographic systems showed that 15% wt./vol. aqueous NaCl gave good separation of the slow moving fluorescent material from the faster running colored pigments and the nicotine alkaloids. The non-steam-volatile basic fraction was therefore separated on a cellulose column using 15% NaCl to elute most of the latter more mobile compounds; the blue fluorescent material was then readily eluted from the column with methanol. A *tert*-amyl alcohol–acetate buffer system resolved the fluorescent fraction into several zones, two of which were intensely blue in fluorescence. The elution and rechromatography of the two blue zones resulted in chromatographically pure alkaloids for analysis.

The R_f values of the zones isolated from smoke were shown to agree with those of the known reference compounds, harmane and norharmane, in several solvent systems as shown in Table 1. The infrared spectra of the compounds isolated from smoke were determined and shown to agree very closely to the spectra of authentic reference alkaloids, harmane and norharmane (Fig. 1). The ultraviolet spectra in both 0·1 *N* HCl and diethyl

[1] R. A. W. JOHNSTONE, and J. R. PLIMMER, *Chem. Rev.* **59**, 885 (1959).
[2] H. R. BENTLEY and E. G. N. BERRY, *The Constituents of Tobacco Smoke:* An Annotated Bibliography. (Research Paper No. 3.) The Tobacco Manufacturers Standing Committee (1959).

ether of the compounds also showed close agreement with the published spectra of the known alkaloids.[3-7]

The fluorescence spectra of the zones isolated from smoke also agreed with the spectra of authentic harmane and norharmane. The fluorescence excitation spectra of the zones isolated from smoke agreed with the ultraviolet absorption spectra in all peak positions.

TABLE 1. PAPER CHROMATOGRAPHIC COMPARISON OF ZONES ISOLATED FROM SMOKE WITH REFERENCE HARMANE AND NORHARMANE

	Rf Values		
Solvent system	I	II	III
Authentic norharmane	0·73	0·10	0·91
First zone from smoke	0·74	0·11	0·91
Authentic harmane	0·60	0·10	0·94
Second zone from smoke	0·60	0·10	0·94

I. *Tert*-amyl alcohol saturated with pH 5·7 acetate buffer. The paper is pretreated with 0·2*M* ammonium tartrate buffer.
II. 15% NaCl water solution
III. *n*-butanol–HCl–water (10 : 2 : 3)

A quantitative method was developed to determine the concentration of the harmane alkaloids in smoke and in tobacco. The results obtained for the amounts of harmane alkaloids in the smoke of several types of cigarettes and in leaf are given in Table 2. Smoke from burley tobacco was shown to contain more of the harmane alkaloids than the smoke from bright tobacco; the alkaloid concentration in smoke from the standard commercial blend closely resembled that of the smoke from the bright tobacco sample. Only traces of the harmane alkaloids were found to be present in the uncured leaf. Their concentration in smoke was approximately one hundred times greater than that found in the leaf, and thus, apparently, they were formed during the burning of the cigarette.

Harmane has been prepared by reacting tryptophan and acetaldehyde under oxidative conditions.[8] Since tryptophan and acetaldehyde are present in the cigarette during burning, it seemed likely that tryptophan could be a precursor of the harmane alkaloids. Radioactive tryptophan was therefore added to tobacco leaf, from which cigarettes were subsequently

TABLE 2. CONCENTRATION OF HARMANE ALKALOIDS IN THE SMOKE AND LEAF OF SEVERAL TYPES OF CIGARETTES

	Concentration (μg/g)	
Samples	Harmane	Norharmane
Bright cigarette smoke	3·6*	12·6*
Burley cigarette smoke	5·8*	14·1*
A standard commercial blend	3·3*	12·3*
Bright leaf	0·02	0·20
Burley leaf	0·02	0·18

* based on wt. of cigarette = 1·0 g

[3] RAYMOND-HAMET, *Compt. Rend.*, 232, 507 (1951).
[4] G. R. CLEO and D. G. I. FELTON, *J. Chem. Soc.*, 1658 (1952).
[5] B. WIKTOP, *J. Am. Chem. Soc.*, 75, 3361 (1953).
[6] J. D. SPENSER, *J. Chem. Soc.*, 3659 (1956).
[7] N. SCHMID, A. EBNOTHER and P. KARRER, *Helv. Chim. Acta.*, 33, 1486 (1950).
[8] R. H. F. MANSKE and H. L. HOLMES, *The Alkaloids*, Vol. II, p. 396, 1st Ed. Academic Press, New York (1952).

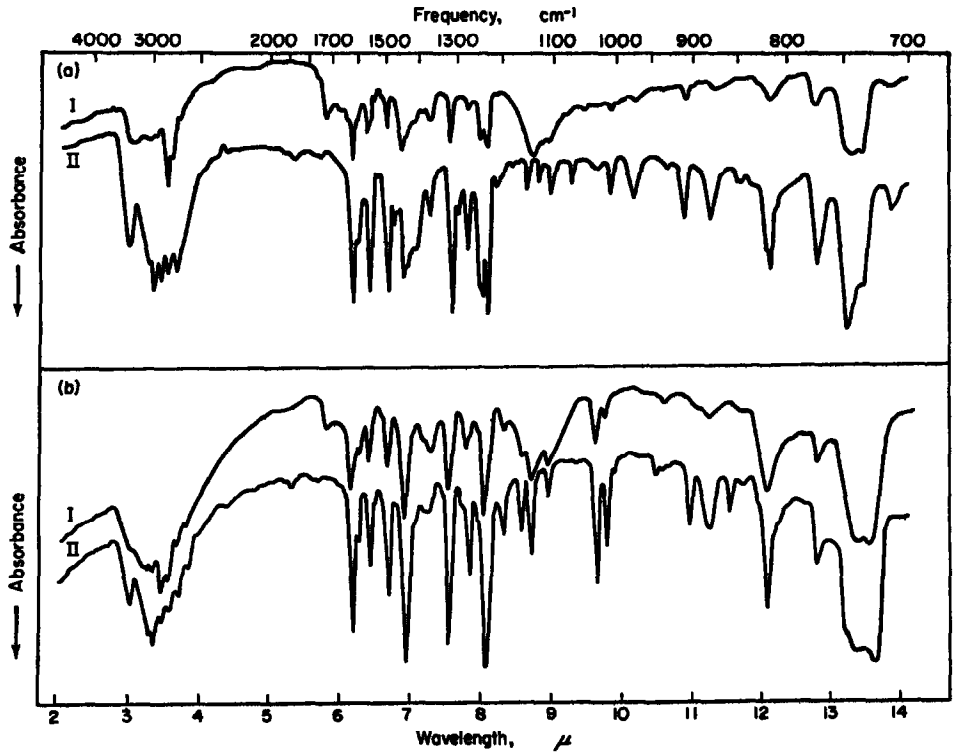


FIG. 1. INFRARED SPECTRA OF REFERENCE ALKALOIDS, HARMANE AND NORHARMANE AND THE COMPOUNDS ISOLATED FROM SMOKE

A–I. Compound isolated from smoke.
A–II. Harmane.
B–I. Compound isolated from smoke.
B–II. Norharmane.

made and smoked. The harmane alkaloids isolated from this smoke were found to be radioactive. By knowing the amount of radioactivity originally added to the leaf, and by measuring the amount present in the harmane alkaloids isolated from the smoke, the percent conversion of tryptophan to harmane alkaloids during burning was calculated. Another study was conducted by adding both untagged and radioactive tryptophan to the tobacco leaf and measuring the total increase of the harmane alkaloids in the smoke. The results of both experiments are shown in Table 3. From these data it is seen that the increase found in the harmane alkaloids in smoke due to the addition of tryptophan to the leaf agrees quite well with the increase calculated from the radiochemical conversion.

Based on the percent conversion of tryptophan to the harmane alkaloids during burning, approximately 3 mg of tryptophan is necessary in the tobacco leaf of one cigarette (i.e. 1·0 g) to form the amount of harmane alkaloids found in the smoke. The concentrations of both free tryptophan and protein-bound tryptophan were determined by a microbiological method,[9] and the results given in Table 4 show that the concentration of tryptophan in the bright leaf agreed well with the predicted amount necessary to produce the harmane alkaloids during burning. The concentration of the harmane alkaloids in burley smoke, on the other hand, was lower than expected from the concentration of the

[9] R. D. GREENE and A. BLACK, *J. Biol. Chem.* **155**, 1 (1944).

tryptophan found in the burley leaf. This difference may be due to different rates of conversion of protein-bound tryptophan, as the two types of tobacco are different in their chemical composition and their burning characteristics.

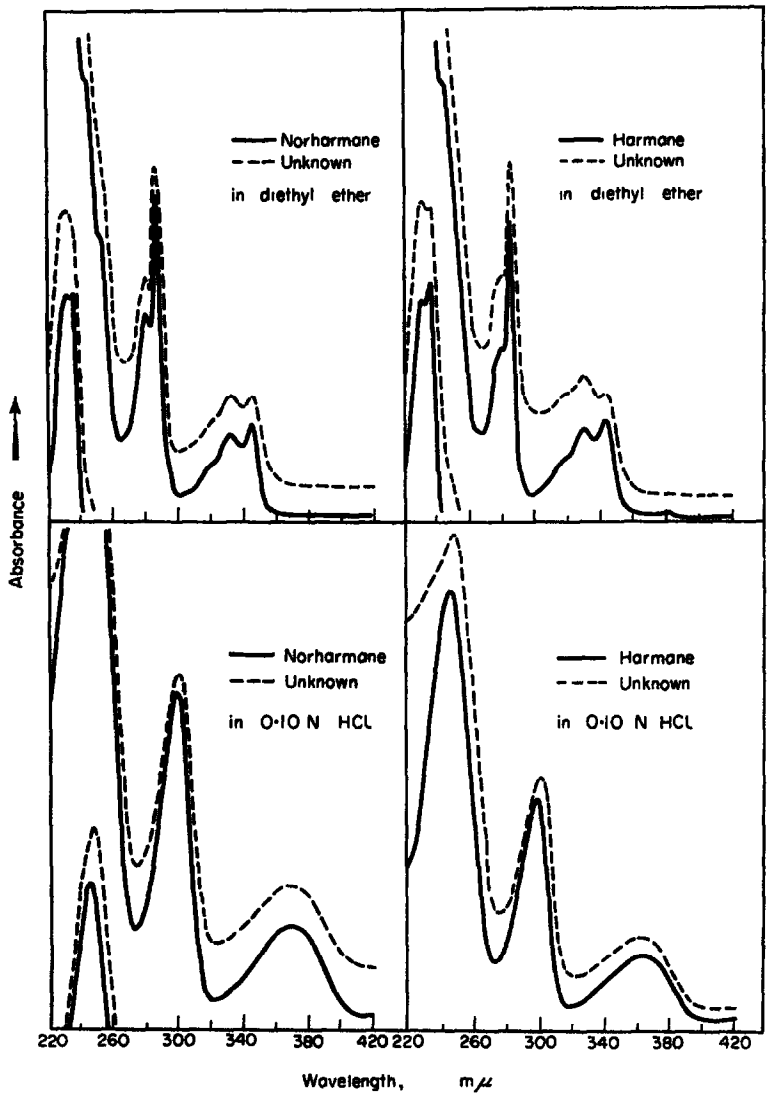


FIG. 2. ULTRAVIOLET SPECTRA OF REFERENCE ALKALOIDS, HARMANE AND NORHARMANE, AND THE COMPOUNDS ISOLATED FROM SMOKE

EXPERIMENTAL

Determination of i.r., u.v., and fluorescence spectra

The infrared analyses of the two isolated compounds were carried out using a Perkin–Elmer Model 221 double-beam spectrophotometer equipped with a sodium chloride prism. The samples were examined as micro potassium bromide pellets, 1½ mm in diameter, with the Perkin–Elmer 6X microsampling unit attached. Large blanks were observed initially,

TABLE 3. THE CONVERSION OF TRYPTOPHAN IN TOBACCO TO HARMANE ALKALOIDS IN SMOKE

	I*
Activity added by way of tryptophan to filler, (mμc/cigt)	169
Activity found in norharmane in the smoke, (mμc/cigt)	0·80
Activity found in harmane in the smoke, (mμc/cigt)	0·26
Percent activity in norharmane	0·47
Percent activity in harmane	0·15

	Concentration found in smoke	
	Harmane	Norharmane
I. Only radioactive tryptophan added to the filler (2 μg/cigt)	4·3 μg/cigt	11·2 μg/cigt
II. Radioactive tryptophan (2μg/cigt)+untagged tryptophan (820 μg/cigt) added to the filler	5·9 μg/cigt	15·7 μg/cigt
Increase	1·6 μg/cigt	4·5 μg/cigt
Predicted increase from the radiochemical studies	1·2 μg/cigt	3·9 μg/cigt

* 2 μg/cigt of radioactive tryptophan and 820 μg/cigt of untagged tryptophan added to the filler.

and it was found important to clean all glassware by the procedure recommended by Perkin–Elmer when using the potassium bromide ultra-micro die.[10] All ether and distilled water were redistilled to eliminate solvent impurities.

The chromatographic paper was also found to contain impurities which were eliminated from the alkaloids by the following procedure: the acid eluant (containing the alkaloids from the chromatographic zones) was extracted with ether. The solution was then made basic and the alkaloids extracted into fresh ether. After drying over anhydrous sodium sulfate, the ether solution was concentrated to 0·5 ml just prior to preparing the potassium bromide micropellet.

The ultraviolet spectra were determined with a Cary Model 14 spectrophotometer. The fluorescence spectra were determined with an Aminco-Keirs spectrophosphorimeter, employing a 1P21 phototube detector. A 1 cm rectangular quartz cell was used to measure the fluorescence. The fluorescence spectra were recorded on an Electro Instruments Model 101 X–Y recorder.

Radioactivity measurements were made in a Liquid Scintillation Counter, model LSC-10B, manufactured by Tracerlab Inc.

TABLE 4. CONCENTRATION OF TRYPTOPHAN IN TOBACCO, AND A COMPARISON OF THE PREDICTED CONCENTRATION OF HARMANE ALKALOIDS IN SMOKE WITH THE CONCENTRATION FOUND

	Bright	Burley
Free tryptophan (mg/g)	0·07	0·45
Protein bound tryptophan (mg/g)	3·0	9·0
Predicted concentration of harmane (μg/cigt)*	4·6	14·2
Predicted concentration of norharmane (μg/cigt)†	14·4	44·4
Concentration of harmane found in smoke (μg/cigt)	3·6	5·8
Concentration of norharmane found in smoke (μg/cigt)	12·6	14·1

* Based on 0·15 per cent conversion of total tryptophan.
† Based on 0·47 per cent conversion of total tryptophan
Weight of cigarette = 1·0 g.

[10] Perkin–Elmer Corporation: Instruction Manual for KBr Ultra-Micro Die. 186–0007.

Chromatography

The chromatographic columns (16×1·5 cm) were packed by adding small amounts of Whatman cellulose powder at a time, and packing tightly with a tamping rod to prevent solvent channelling. The column was eluted with 15% wt./vol. aqueous NaCl, to remove

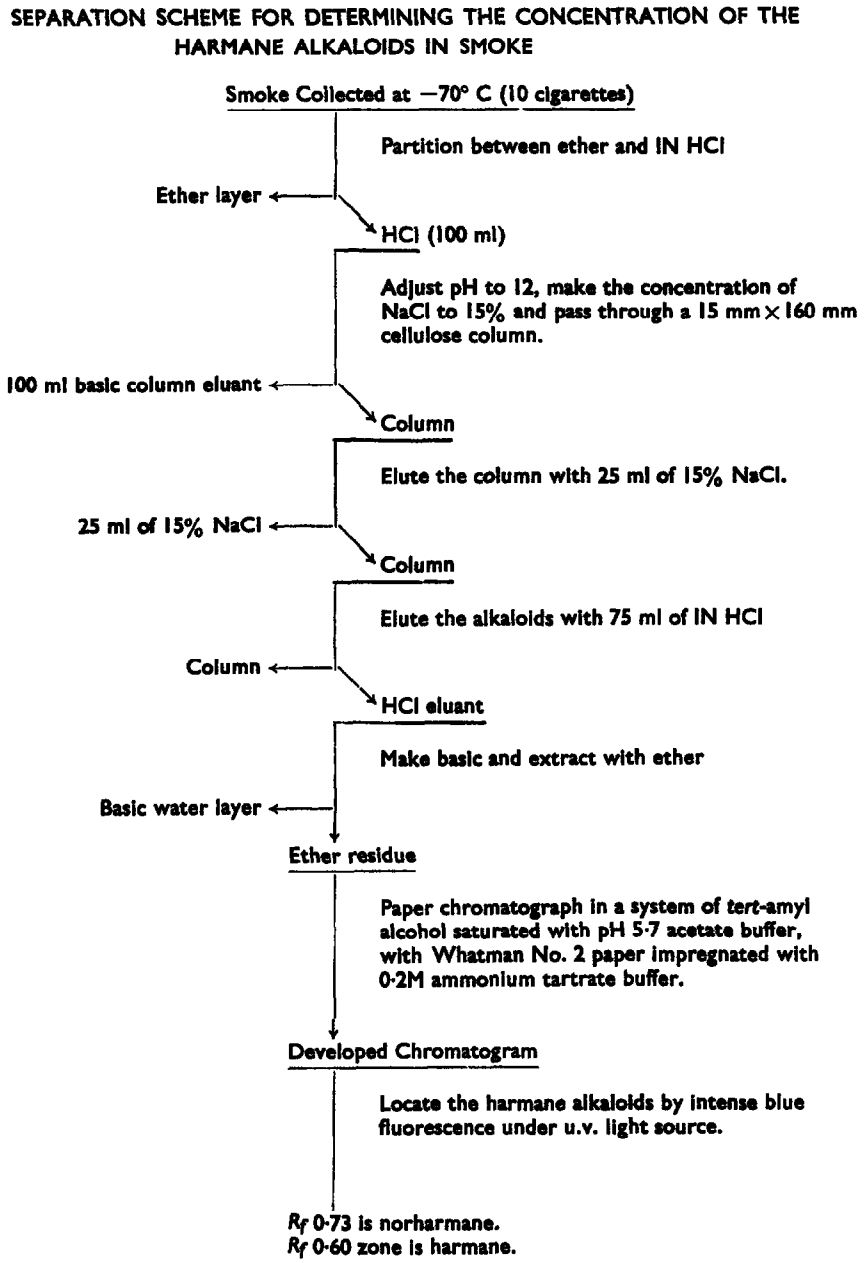
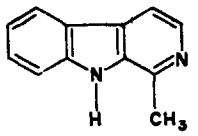


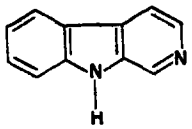
FIG. 3. SEPARATION SCHEME FOR DETERMINING THE CONCENTRATION OF THE HARMANE ALKALOIDS IN SMOKE

nicotine and the colored material present in the smoke, and the harmane alkaloids were displaced with methanol. The isolated harmane and norharmane fraction was resolved in a system consisting of *tert*-amyl alcohol saturated with pH 5·7 sodium acetate buffer



I Harmane

II Norharmane



STRUCTURAL FORMULAS OF HARMANE AND NORHARMANE

(0·2 *M*) on Whatman No. 2 paper pretreated with 0·2 *M* ammonium tartrate and dried before chromatography. The harmane alkaloids were located on the paper chromatogram by u.v. light.

Quantitative determination of the harmane alkaloids

The outline of the quantitative method for the harmane alkaloids in smoke is shown in Fig. 3. After final paper chromatographic development, the alkaloid zones were cut out and eluted with 1·0 *N* HCl. The HCl was extracted with ether to remove impurities, the aqueous phase was made alkaline to pH 12, and the alkaloids extracted quantitatively with one volume of ether. The alkaloids were then re-extracted into 1·0 *N* HCl and made up to a known volume prior to ultraviolet analysis. For harmane the peak at 365 mμ ($E^{1\%}_{1\,cm}$, 248) and for norharmane, the peak at 370 mμ ($E^{1\%}_{1\,cm}$, 237) was measured by using a baseline correction technique drawn from the minimum of each curve to the minimum at 399 mμ or 408 mμ respectively.

Experiments with known quantities of the harmane alkaloids showed that 81 per cent recovery was achieved with the method. This factor was used to find the correct concentration of the harmane alkaloids isolated from smoke.

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