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IDENTIFICATION AND DETERMINATION OF IMIDAZOLE DERIVATIVES IN CIGARETTE SMOKE

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SUMMARY

The identification of imidazole derivatives in cigarette smoke was performed by gas chromatography–mass spectrometry (GC–MS) on a fused-silica capillary column coated with Carbowax 20M. A fraction, enriched in imidazoles, was prepared from the smoke condensate by liquid chromatography on Sephadex LH 20 prior to the GC–MS analysis. Ten alkylated imidazoles were identified in cigarette smoke, 4(5)-methylimidazole and imidazole being the most abundant.

The determination of these compounds was achieved by high-performance liquid chromatography on LiChrosorb Si 60 after chemical derivatization with 4-chloro-7-nitro-benzo-2-oxa-1,3-diazole (NBD-Cl). This method is very selective since no clean-up procedure is necessary. The levels of 4(5)-methylimidazole and imidazole in cigarette smoke condensate vary between 100 and 500 ppm.

INTRODUCTION

Imidazole derivatives were first detected in tobacco smoke by Schumacher *et al.*[1], in the water-soluble fraction of the condensate. They were identified again by us[2] during the analysis of the basic fraction of cigarette smoke.

These compounds, particularly 4(5)-methylimidazole, attracted attention due to their possible toxicity, and the World Health Organization has fixed a specification for this compound in caramel colours which are widely used as food and drug additives.

At the present time, there is no information available about the concentration of imidazoles in tobacco smoke, and the purpose of this paper is to determine it.

This study is divided into two parts. First, we describe a procedure for the isolation and qualitative identification of imidazoles in cigarette smoke by liquid chromatography and gas chromatography–mass spectrometry (GC–MS), then a method for their determination by chemical derivatization and high-performance liquid chromatography (HPLC). Ring-nitrogen substituted imidazoles are excluded of this study.

The concentration of these compounds in cigarette smoke is rather low and the matrix is extremely complex, since several thousand different compounds are present in tobacco smoke. It is necessary, therefore, to use a purification procedure

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in order to achieve the GC–MS analysis of imidazoles. In his study of the effect of solvent change on the separation processes on Sephadex LH 20, Streuli[3] demonstrated that imidazoles have greater retention volumes than most organic compounds when eluted with certain polar organic solvents. We used this consideration to obtain a fraction of cigarette smoke condensate which contains mainly imidazoles. The GC–MS analysis was performed on this fraction.

Several methods have been used in order to determine 4(5)-methylimidazole in caramel colours. Recently, Thomsen and Willumsen[4] developed a method involving a quantitative ion pair extraction of 4(5)-methylimidazole, and a determination by reversed-phase ion-pair liquid chromatography with spectrophotometric detection at 215 nm. This method does not seem to be specific enough to be used with a complex mixture like cigarette smoke condensate. To solve this problem, we chose to use a chemical derivatization. We show in this study that 7-chloro-4-nitro-benzo-2-oxa-1,3-diazole (NBD-Cl), which is known to form coloured and fluorescent derivatives with secondary amines[5] and amino acids[6,7], also reacts with imidazoles. The reaction products (referred to as NBD-imidazoles) are coloured, non-fluorescent and very stable, and their separation can be achieved by HPLC on LiChrosorb Si 60. 4(5)-Methylimidazole and imidazole have been determined in cigarette smoke by this method. The advantage of the method described is its selectivity, since no clean-up procedure is necessary.

EXPERIMENTAL

Preparation of the condensate and fractionation

Ten filter-tipped, black-tobacco cigarettes were smoked according to international Coresta standards on a Filtrona 302 smoking machine. The particulate phase was trapped on a Cambridge fibre-glass filter which retains particles larger than 0.2 μm. The collected condensate was then dissolved in acetone. The mixture was concentrated to a volume of 1 ml and injected on a chromatographic column of Sephadex LH 20 (Pharmacia, Uppsala, Sweden). The column, 30 cm × 1 cm I.D., was slurry-packed with 8 g of Sephadex LH 20 in acetone. The elution was performed with acetone at a flow-rate of 1 ml/min and 10 fractions of 5.5 ml were obtained. The solvent was then eliminated, each fraction was redissolved in 100 μl of acetone and was then ready for GC–MS analysis.

Gas chromatography–mass spectrometry

GC–MS analysis was performed on a Nermag R 10-10 B instrument equipped with a Sidar 3D data system. A capillary (25 m × 0.22 mm I.D.) fused-silica column (Chrompack, Middelburg, The Netherlands) coated with Carbowax 20M was used. The temperature of the oven was kept at 120°C for 2 min and then increased to 190°C at the rate of 2°C/min. Helium was the carrier gas and its pressure at the head of the column was 400 g/cm^2. The splitting ratio was 1:10.

Synthesis of NBD-imidazoles

The following compounds (500 mg each): imidazole, 2-methylimidazole, 2-ethylimidazole, 4(5)-methylimidazole, benzimidazole, were dissolved in a solution of NBD-Cl (3%) in butyl acetate. The mixture was then heated to 90°C for 5 h. The

reaction products were purified by preparative liquid chromatography on silica gel (Silica gel 60, Merck, Darmstadt, F.R.G.) with ethyl acetate as eluent. The purity of the compounds was checked by thin-layer chromatography.

Reaction of cigarette smoke condensate with NBD-Cl

Three cigarettes were smoked mechanically according to international standards and the particulate phase was collected on a Cambridge fibre-glass filter. This filter was then introduced into a flask containing 5 ml of a solution of NBD-Cl (3%) in butyl acetate. The NBD-Cl was previously purified by recrystallization from water and alcohol mixed solvents. The filter was crushed and the mixture was put into an ultrasonic bath in order to obtain a good dissolution of the condensate. One ml of the solution was placed in a reaction vial and, after addition of 10 μl of a solution of triethylamine 0.1 *M* in butylacetate as catalyst, the vial was sealed and heated at 90°C for 2 h.

High-performance liquid chromatography

HPLC was performed on a Varian 8500 instrument equipped with a spectrophotometric detector (Spectromonitor III, LDC). The column (20 cm × 4.6 mm I.D.) was packed with LiChrosorb Si 60 (5 μm) (Merck). Two eluents can be used: methylene chloride–butanol (94:6) and butyl acetate–tetrahydrofuran (95:5). The solvents were dried on molecular sieves and 0.04% of water was added to the first system and 0.3% to the second one. In both cases the flow-rate of the eluent was 100 ml/h. The detector was set at 360 nm for the analyses of the derivatives and at 390 nm for the determination of imidazole and 4(5)-methylimidazole.

RESULTS AND DISCUSSION

Preparative liquid chromatography on Sephadex LH 20

According to Streuli[3], the best eluent to retain imidazoles on Sephadex LH 20 would be acetonitrile. We chose acetone for several reasons. If we consider the classification of the solvents established by Snyder[8], acetone and acetonitrile belong to the same group and can be expected to have very similar properties. Acetone is a more powerful solvent of smoke condensate and it is less toxic. In fact, the separation of imidazoles from the other organic compounds of the condensate obtained with acetone was more efficient than with acetonitrile.

Characterization of imidazoles by GC–MS

The analysis of imidazoles by GC, particularly when these compounds are not substituted on ring-nitrogen, seems to present some difficulties. Some authors have solved this problem by chemical derivatization. More often, ring-nitrogen acetylation has been proposed[9,10]. Wilks and co-workers[11,12] performed the GC of imidazoles without derivatization on a packed column coated with an alkali-treated phase (Carbowax 20M + KOH). Yamaguchi *et al.*[13] performed this analysis on a glass capillary column coated with Carbowax 20M. These authors do not mention whether any treatments were applied to their column and the chromatograms displayed in their paper show a large tailing on the peaks of imidazoles. We also observed this large tailing on imidazole peaks and it seems that the column inertness is very important

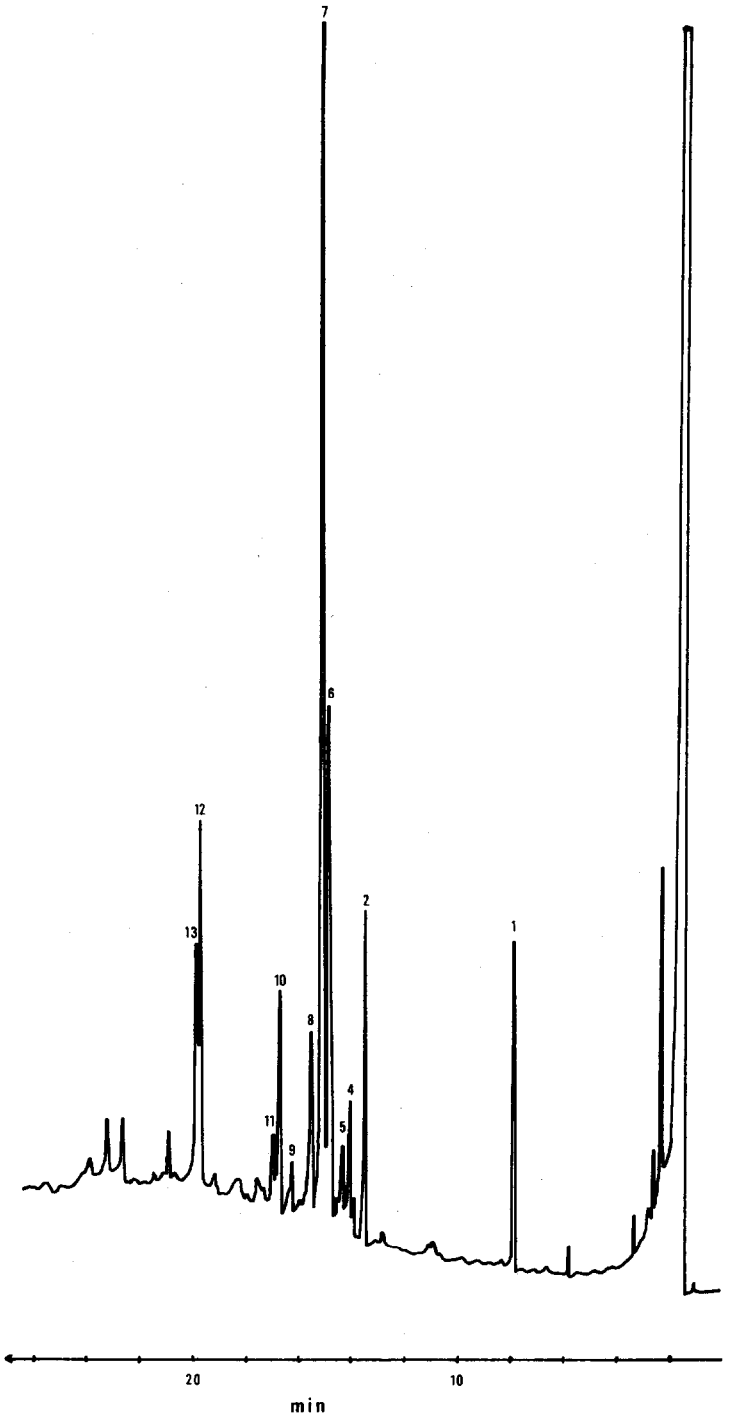

Fig. 1. Chromatogram of subfraction 9 of smoke condensate obtained on Sephadex LH 20 with acetone as eluent. Peak numbers are identified in Table I. Fused-silica capillary column (25 m × 0.22 mm I.D.) coated with Carbowax 20M.

TABLE I

COMPOUNDS IDENTIFIED IN THE IMIDAZOLE FRACTION OF CIGARETTE SMOKE

Peak No.	*Compound*
1	Nicotine
2	2-Methylimidazole
3	2-Ethylimidazole
4	2,4(5)-Dimethylimidazole
5	C_3H_7-imidazole
6	Imidazole
7	4(5)-Methylimidazole
8	C_3H_7-imidazole
9	4(5)-Dimethylimidazole
10	Ethyl-4(5)-imidazole
11	C_3H_7-imidazole
12	3-Hydroxypyridine
13	Methyl-3-hydroxypyridine

for the GC of imidazoles. Recently, great improvements have been made in the deactivation of glass capillary columns, and various methods have been developed for that purpose[14,15]. We prepared glass capillary columns according to the methods proposed by these authors. Carbowax 20M was chosen as the stationary phase. With these columns we could observe a reduction of the peak tailings but the results were unsatisfactory. A fused-silica capillary column, coated with Carbowax 20M without any other treatment, enabled us to obtain a sharp reduction of the tailing of imidazole peaks, but we could not obtain completely symmetrical peaks. Nevertheless, the separation of the imidazoles of the smoke condensate obtained with this column is acceptable.

Ten fractions were obtained by preparative liquid chromatography on Sephadex LH 20 and these fractions were analysed by GC–MS. As expected, fractions 1–6 contain the greater part of the organic compounds of the condensate, and no imidazoles can be detected. Imidazoles begin to appear in fraction 7 and are the main compounds in fractions 8–10. Fig. 1 shows the chromatogram of fraction 9 as an example. We obtained a good single-step purification of imidazoles, since there are only few impurities: a trace of nicotine and two compounds, 3-hydroxypyridine and methyl-3-hydroxypyridine. Ten alkylated imidazoles were identified by mass

TABLE II

SPECTROPHOTOMETRIC PROPERTIES OF NBD-IMIDAZOLES MEASURED IN BUTYL ACETATE

Compound	λ_{max} *(nm)*	ε_{max} *(l mol^{-1} cm^{-1})*
NBD-imidazole	380	13,500
NBD-4(5)-methylimidazole	395	12,500
NBD-2-methylimidazole	344	11,500
NBD-2-ethylimidazole	344	9500
NBD-benzimidazole	402	9700

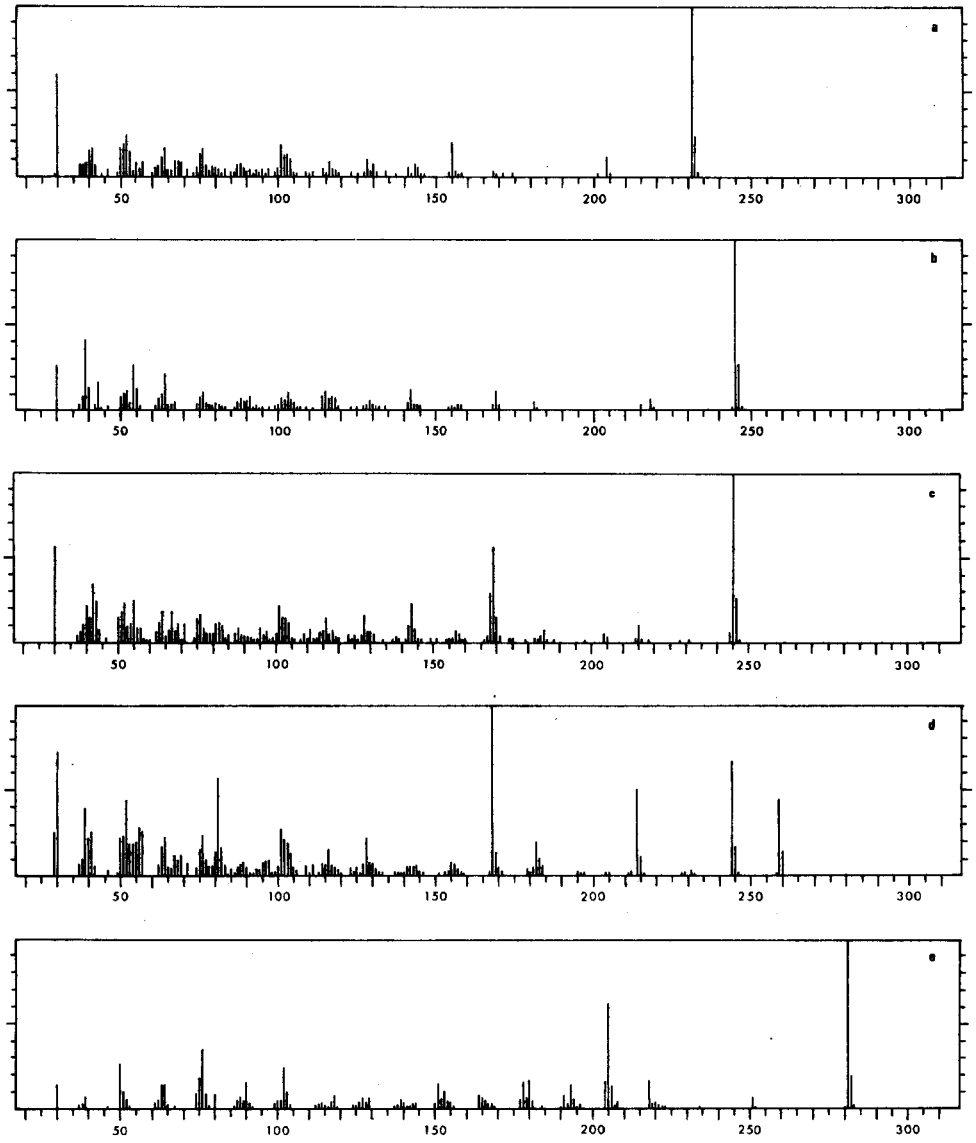

Fig. 2. Mass spectra of NBD-imidazoles. (a) NBD-imidazole; (b) NBD-4(5)-methylimidazole; (c) NBD-2-methylimidazole; (d) NBD-2-ethylimidazole; (e) NBD-benzimidazole.

spectrometry (Table I). 4(5)-Methylimidazole and imidazole are by far the most abundant imidazoles in cigarette smoke; 4(5)-substituted imidazoles are generally more abundant than 2-substituted imidazoles. The mass spectra of imidazoles were compared with those published in the literature[16,17] and with those obtained from synthetic compounds, where possible.

Properties of NBD-imidazoles

The reaction products of NBD-Cl with imidazoles are coloured and show large

absorption bands in the visible range of the spectrum. Table II summarizes the principal spectrophotometric properties of NBD-imidazoles. When analysed by thin-layer chromatography, the spots of these compounds are yellow and they absorb when they are observed in UV-light at 365 nm. But after a few minutes exposure they fluoresce (green). All the synthetized compounds have this property, which makes it possible to identify them after separation in TLC.

The mass spectra of NBD-imidazoles are shown in Fig. 2. They were obtained by GC–MS (70 m × 0.3 mm I.D. glass capillary column coated with SE 54; tem-

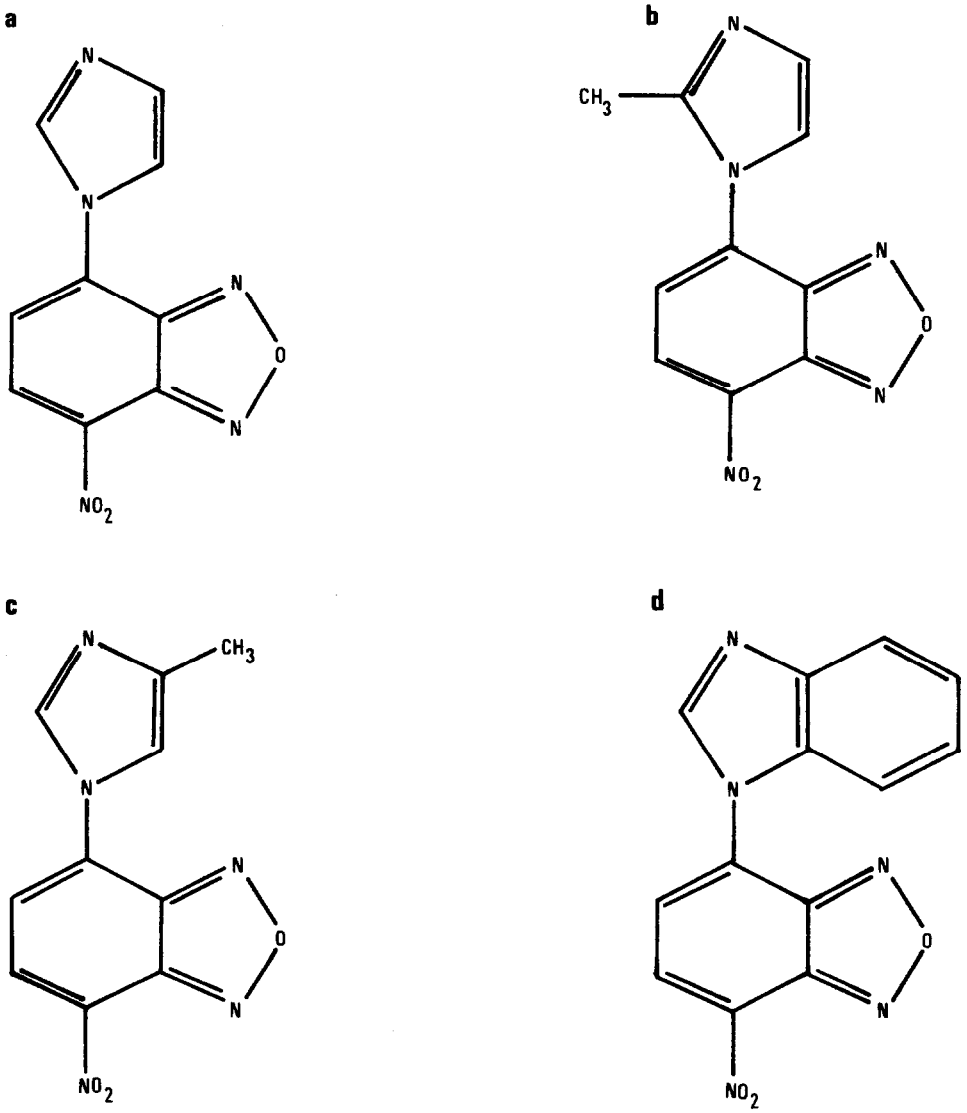

Fig. 3. Structure of NBD-imidazoles. (a) NBD-imidazole; (b) NBD-4(5)-methylimidazole; (c) NBD-2-methylimidazole; (d) NBD-benzimidazole.

perature 180–240°C), The mass peak of these compounds is very important and it is often the base peak of the spectrum. Two types of fragmentation can be observed. The first one corresponds to the loss of 27 a.m.u. from the molecular ion, which is observed in the mass spectra of NBD-imidazole and NBD-4(5)-methylimidazole. In the mass spectrum of NBD-2-methylimidazole, a loss of 41 a.m.u. is observed. This is a typical fragmentation of the imidazole ring which loses HCN under electron impact. In the case of NBD-2-methylimidazole the eliminated fragment is CH_3CN and this indicates that the fragmentation probably involves the 2 and 3 positions on the ring. This mechanism is specific for a ring-nitrogen substituted imidazole[18]. However, this type of fragmentation is not preponderant and the corresponding peaks are weak in the mass spectra. The NBD-benzimidazole spectrum does not show this fragmentation because of the stability of the benzimidazole ring. The second type of fragmentation is more important. It is characterized by a loss of 30 a.m.u. from the molecular ion, followed by a loss of 46 a.m.u. It corresponds to the elimination of NO and NO_2 and it is logical to relate this fragmentation to a nitrobenzooxadiazole ring although the real mechanism of this fragmentation is probably complex. The NBD-2-ethylimidazole spectrum shows a loss of 15 a.m.u. from the molecular ion which corresponds to the cleavage of the alkyl chain. It is followed by the fragmentation of the nitrobenzooxadiazole ring. These observations are in accordance with the structures shown in Fig. 3 for the derived compounds. Two derivatives are theoretically possible in the case of 4(5)-alkylated imidazoles. We were not able to distinguish two derivatization compounds with 4(5)-methylimidazole, since only one peak was observed in GC and in HPLC. This is in accordance with other studies on the chemical derivatization of imidazoles[19]: there is only one compound formed, *viz.* the one with no steric hindrance.

Study of the derivatization reaction

The formation of NBD-imidazoles depends strongly on the reaction solvent. Table III summarizes the NBD-imidazoles and NBD-4(5)-methylimidazole yields obtained in various solvents under the same conditions. In butylacetate, at 90°C after 2 h of reaction, we observed a 65% yield for 4(5)-methylimidazole and imidazole; 100% yields are obtained by addition of triethylamine as catalyst.

TABLE III

REACTION YIELDS OF THE NBD-IMIDAZOLES SYNTHESIS AT 90°C AFTER 2 h

Solvent	*Reaction yield (%)*	
	4(5)-Methyl-imidazole	*Imidazole*
Methanol	15	15
Isobutyl ether	42	23
Acetonitrile	43	46
Methyl isobutyl ketone	66	75
Chloroform	75	84
Butyl acetate	65	65
Butyl acetate + triethylamine 10^{-3} *M*	100	100
Butyl acetate + triethylamine 10^{-2} *M*	100	100

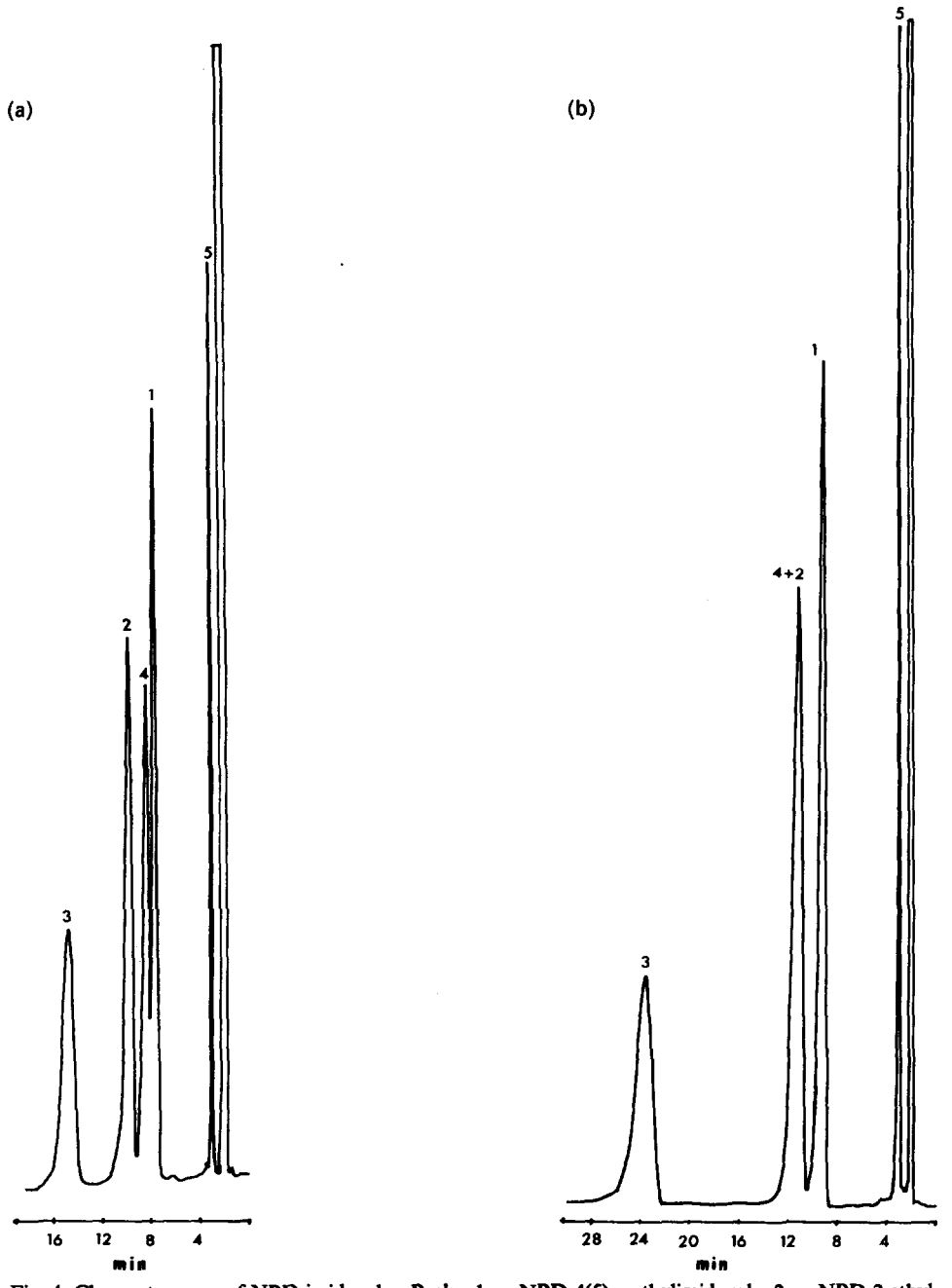

Fig. 4. Chromatograms of NBD-imidazoles. Peaks: 1 = NBD-4(5)-methylimidazole; 2 = NBD-2-ethylimidazole; 3 = NBD-imidazole; 4 = NBD-2-methylimidazole; 5 = NBD-benzimidazole. Column, Li-Chrosorb Si 60 (5 μm), 200 × 4.6 mm I.D.; flow-rate, 100 ml/h; detection at 360 nm. Mobile phase: chromatogram (a), methylene chloride–butanol (94:6); chromatogram (b), butyl acetate–tetrahydrofuran (95:5).

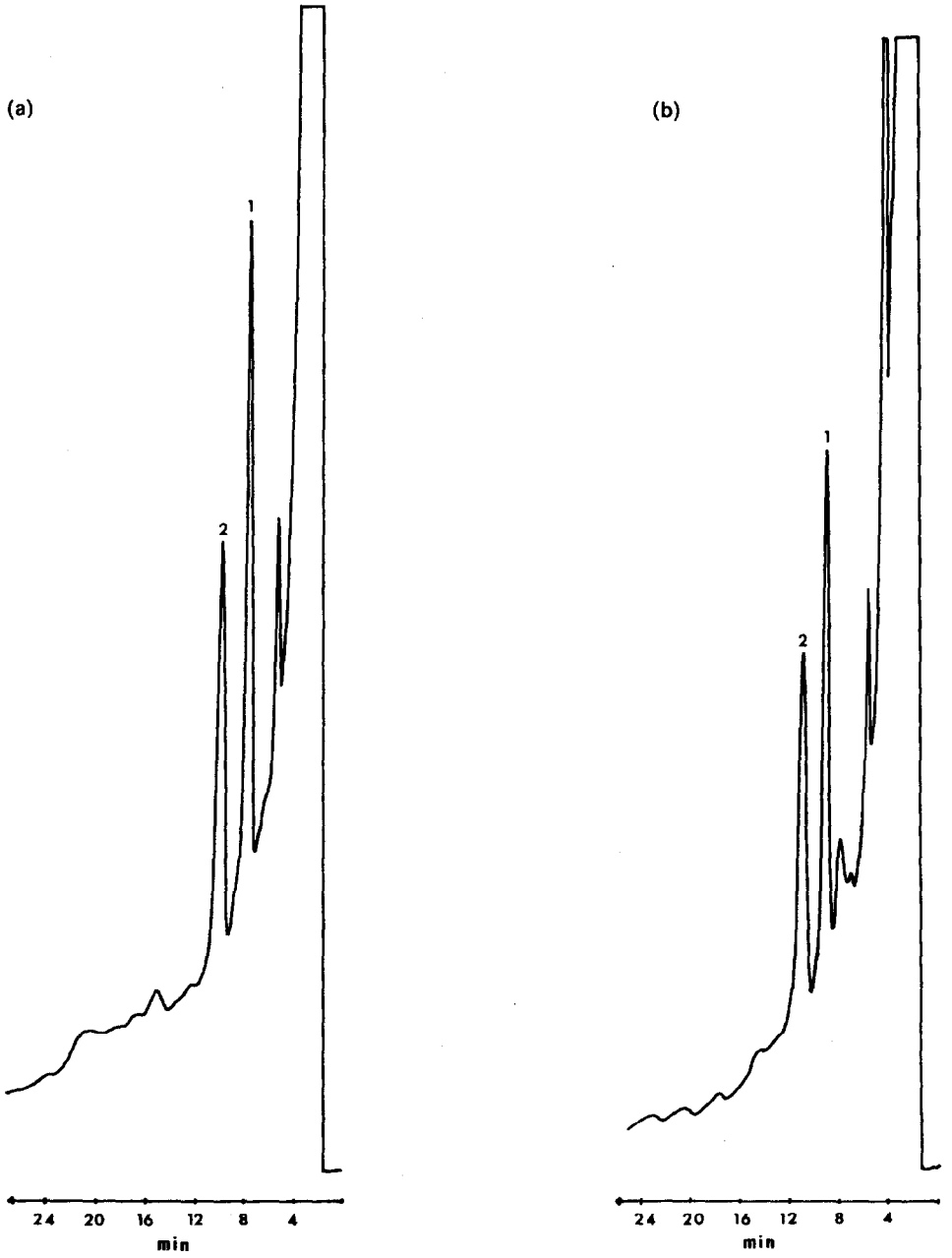

Fig. 5. Chromatograms of NBD-imidazoles in cigarette smoke condensate. Peaks: 1 = NBD-4(5)-methylimidazole; 2 = NBD-imidazole. Chromatographic conditions as in Fig. 4 except detection at 390 nm.

TABLE IV

LEVELS OF IMIDAZOLES FOUND IN THE SMOKE OF VARIOUS KIND OF COMMERCIAL BRANDS

Cigarette	*4(5)-Methylimidazole*		*Imidazole*	
	Level (μg/cig)	*Concentration tar (ppm)*	*Level (μg/cig.)*	*Concentration tar (ppm)*
Dark air-cured tobacco				
filter	6.8	522	4.1	316
non-filter	15.0	555	8.8	327
low tar	2.3	408	1.6	278
American blend	5.5	325	3.3	196
Virginia tobacco	2.3	125	1.0	103

The kinetics of NBD-imidazole formation in the smoke condensate have been studied. In all the cases, the reaction is complete within 2 h, and the reaction products are stable since no change is detected after 24 h. The study of the reaction shows that the concentration of smoke condensate in the reagent should not go beyond the limit of 10 mg/ml. Above this limit the reaction is not longer quantitative.

High-performance liquid chromatography of NBD-imidazoles

NBD-imidazoles were separated by HPLC on LiChrosorb Si 60. Two solvent systems were studied as the mobile phase: methylene chloride–butanol (94:6) and butyl acetate–tetrahydrofuran (95:5). Fig. 4 shows the chromatograms of the synthetic NBD-imidazoles in both systems. In the first system, the five NBD-imidazoles are separated, which is not the case in the second one, where NBD-imidazoles and NBD-2-ethylimidazole have the same retention time. Fig. 5 shows the separation obtained with the condensate. Since NBD-2-ethylimidazole and NBD-imidazole are not separated in the second system, it seems that the first should be chosen. In fact, the level of 2-ethylimidazole in cigarette smoke is very low compared with imidazole, as was demonstrated in the first part of this study. Therefore the systematic error made in the determination of imidazole in the second system is negligible. Both systems are convenient for the determination of the two principal imidazoles of cigarette smoke.

Results

Table IV gives the levels of imidazole and 4(5)-methylimidazole determined in various kinds of commercial brands.

Formation of imidazoles in cigarette smoke

Different pathways may be considered to explain the formation of imidazoles in cigarette smoke. But two mechanisms seem to be predominant: (a) thermal degradation of histidine. This amino acid is a 4-substituted imidazole and is present in tobacco. Its pyrolysis can lead to alkylated imidazoles. (b) A synthesis similar to Radziszewski's: α-dicarbonyl compounds like glyoxal, methylglyoxal and diacetyl react with ammonia and an aldehyde like formaldehyde or acetaldehyde to give im-

idazoles. All these compounds are synthetized during the pyrolysis of tobacco and this is therefore a possible pathway for the formation of imidazoles in tobacco smoke.

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