

Chemical Studies on Tobacco Smoke

XI. Dibenzofurans in Cigarette Smoke*

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Dibenzofuran (DBF) is long known to be formed upon pyrolysis of phenol (4). Recently it was reported that DBF is also pyrosynthesized in a relatively high yield from nitrobenzene. In this case it appears that a phenoxy-radical is formed as a probable intermediate after a nitro-nitrate rearrangement (3). Since tobacco smoke contains volatile phenols (9, 14) and nitrobenzenes (10), it appears likely that tobacco smoke contains the so far unreported dibenzofuran (12, 14). This concept is supported by the tentative identification of DBF and several methyl-dibenzofurans (MDBF) in fraction BI of cigarette smoke. The neutral subfraction BI, which amounts to about 0.6% of the whole dry particulate matter, is the most carcinogenic fraction so far isolated from tobacco smoke (6, 14).

Dibenzofuran and its alkyl derivatives are weakly polar and therefore not easily separated from the higher alkanes, a major group of tobacco "tar" constituents (9, 14). Therefore, we developed a method which separates DBF from the tobacco paraffins. The technique is based on a distribution of the hexane extract of tobacco "tar" between hexane and dimethylformamide. The bulk of the tobacco paraffins remain in hexane while the dibenzofurans are enriched in dimethylformamide. Water is added to this layer, which is then extracted with hexane. The residue of the upper layers is chromatographed on alumina and subsequently on Sephadex LH-20. In the final step the dibenzofurans are isolated by gas chromatography and identified by ultraviolet absorption spectra and mass spectra. For the quantitative analysis DBF-¹⁴C was synthesized and used as internal standard.

MATERIALS AND METHODS

Apparatus: A Perkin-Elmer gas chromatograph Model 800 with flame ionization detector and 1 mV recorder was used for the analysis. The β -radiation was counted with a Nuclear Chicago Scintillation System 720. Ultra-

violet absorbance measurements were made in cyclohexane and 10 cm cells with a Cary Model 11 recording spectrophotometer. The mass spectra were obtained with a Hitachi-Perkin-Elmer RMU-6D by the Morgan Schaffer Corporation (Montreal, Canada). The energy of the bombarding electrons was kept at 70 eV.

For the analysis we smoked the cigarettes with a 30-channel automatic smoker with a vibrating liquid trap (11). Between vacuum source and trap we installed a dry-ice acetone cold trap and a Cambridge filter assembly. The Cambridge filter was replaced after each smoking of 60 cigarettes.

Reagents: All solvents were spectrograde. The purity of the phenols for the synthesis of dibenzofurans was checked by an earlier published method (5), the purity of 2-bromocyclohexanones by gas chromatography at 150° C on a 2 m column filled with 5% OV-1 on Gas Chrom P. Alumina Woelm neutral (activity II) was obtained from Alupharm Chemicals, Sephadex LH-20 from Pharmacia Fine Chemicals, Gas Chrom P (80–100 mesh), OV-1, OV-225, DC-200 and QF-1 from Applied Science Laboratories.

Synthesis of Dibenzofuran-1,2,3,4,4a,9b-¹⁴C₆: The method by Trippet for DBF (13) was modified to a micro-scale. An excess of 2-bromocyclohexanone was used to improve the utilization of phenol during the synthesis.

2-Phenoxy-cyclohexanone-¹⁴C: A mixture of 0.54 mg of phenol-¹⁴C (U.L.) (specific activity 25.9 mCi/mMol; Amersham-Searle) and 3.8 mg of unlabelled phenol was neutralized in a 5 ml round bottom flask with 0.46 ml of 0.1 N sodium hydroxide and dried for two days in a vacuum desiccator over phosphorous pentoxide. To the dry sodium phenolate at room temperature were added 2 ml of dry benzene and 32.5 mg of purified 2-bromocyclohexanone (200% excess). The mixture was stirred magnetically for five days at 25–30° C. An aliquot was taken, gas chromatographed, and showed a maximum yield of 71% (6.2 mg). The crude product was purified on 10 g of alumina (Woelm, neutral, activity II). The radioactive fractions were combined and yielded pure 2-phenoxy-cyclohexanone.

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1,2,3,4-Tetrahydrodibenzofuran-¹⁴C: 1.7 mg of 2-phenoxycyclohexanone in a microtube was reacted at 100° C for 20 minutes with 200 µl of a mixture of five parts of H₃PO₄ and one part of P₂O₅. The reaction mixture was dissolved in ether and water and the aqueous phase was extracted three times with ether. The ether layer was washed with sodium bicarbonate and saturated sodium chloride solution and dried (Na₂SO₄). The dry solution was evaporated to 2 ml and analyzed. Gas chromatography showed a yield of 1.5 mg (97%) of 1,2,3,4-tetrahydrodibenzofuran-¹⁴C.

Dibenzofuran-¹⁴C: The tetrahydro-DBF was gradually transferred with ether into a tube with an indentation (inside diameter 4 mm) which separated a 0.5 ml volume of the lower part of the tube from the rest of the vessel. The residue of tetrahydro-DBF was mixed with 10 mg of 5% Pd/C. The vial, which was connected via a ground glass joint and side-tube to a flask containing n-hexane, was heated for 20 minutes to 200° C. After cooling, the reaction product was washed with ether into the lower part of the tube. The residue was twice more treated by heating with the catalyst. Afterwards the gas chromatographic analysis did not show any tetrahydro-DBF. Benzene/methanol (4:1; 0.5 ml) was added and the suspension was transferred into a folded micro-filter and exhaustively washed with benzene/methanol. The extract was concentrated under reduced pressure to about 10 ml, while the rest of the solvent was removed by freeze drying. The residue was chromatographed on alumina with n-hexane. The radioactive fractions were combined and resulted in 0.6 mg of dibenzofuran-1,2,3,4,4a,9b-¹⁴C₆ (41%); specific activity of 3.2 mCi/mMol. The radiochemical purity of the ¹⁴C-labelled product was ascertained by a second chromatography, which resulted in DBF-¹⁴C with the same specific activity.

Reference Compounds

Dibenzofuran: DBF was purchased from Chemical Procurement Laboratories, College Point, New York, purified on alumina and recrystallized from ethanol, m. p. 85–86° C. Its u. v. spectrum was identical with published data (8, 13).

1-Methyldibenzofuran: 1-MDBF was synthesized in three steps according to Chatterjea and Ray on a five gram scale with dihydroresorcinol and 2-chlorocyclohexanone as starting materials (1). The overall yield after purification by column chromatography on alumina was 40%; n_D²⁴ 1.6389. The spectrum was identical with the literature data (8, 13).

2-, 3- and 4-Methyldibenzofurans: These methyl derivatives of DBF were synthesized in three steps according to Trippett with phenols and 2-bromocyclohexanones as starting materials (13). The overall yields after column chromatography varied between 50–60%. 2-MDBF m. p. 43°; 3-MDBF m. p. 59–60°; 4-MDBF 40° C. The u. v. spectra were identical with reported data (8, 13).

Gas Chromatography: Preliminary data indicated that the enrichment of DBF from tobacco "tar" leads also to an enrichment of acenaphthylene and fluorene. Therefore, we developed two gas chromatography systems for complete separations.

- I. A 6 m stainless steel column, diameter 3 mm, filled with 5% OV-225 on Gas Chrom P and operated isothermally at 200° C; injection port 260° C; detector temperature 240° C (retention time for fluorene 25.4 minutes).
- II. A 6 m stainless steel column, diameter 3 mm, filled with 7.5% DC-200 and 5% QF-1 on Gas Chrom P, isothermal at 200° C; injection port 260° C; detector 240° C (fluorene 19.4 min.).

The retention times for the dibenzofurans are given in Table 1. The 2- and 3-MDBF could not be separated by the two described columns nor by several other tested columns. With an attenuation of 200 1 µg of DBF gave a full scale response with a 1 mV recorder.

Distribution: Preliminary tests indicated that it was important to separate in an early step DBF and its methyl derivatives from the bulk of the tobacco "tar" paraffins. This separation could be best achieved by distributions between three solvent systems:

1. Methanol/water (4:1) and n-hexane,
2. n-Hexane and dimethylformamide (DMF),
3. Water/DMF (4:1) and n-hexane.

Table 1 Dibenzofurans: partition coefficients and retention times

| Partition coefficients (20° C) | | | Retention times (min.) | |
|-----------------------------------|--------------------------------|--------------------------------|---------------------------|-----------------|
| C _H /C _M | C _D /C _H | C _H /C _W | I ^a | II ^b |
| 10.9 | 4.6 | 2.3 | Dibenzofuran | 19.8 |
| 37.8 | 4.0 | 2.3 | 1-Methyldibenzofuran | 23.0 |
| 42.2 | 5.7 | 2.1 | 2-Methyldibenzofuran | 23.0 |
| 28.0 | 6.1 | 2.2 | 3-Methyldibenzofuran | 23.0 |
| 20.2 | 2.5 | 3.2 | 4-Methyldibenzofuran | 25.4 |

C_H = Concentration in n-hexane

C_M = Concentration in methanol/water (4:1)

C_D = Concentration in dimethylformamide

C_W = Concentration in water/dimethylformamide (4:1)

^aCondition I: 3 mm by 6 m stainless steel, 5% OV-225, 200° C, isothermal

^bCondition II: 3 mm × 6 m stainless steel, 7.5% DC-200, 5% QF-1, 200° C, isothermal

The second step leads to a separation of DBF from the tobacco paraffins. The route was essential, since large amounts of paraffins inhibit the column chromatographic enrichment of dibenzofurans. The distribution coefficients were determined for system 1 by u. v. spectra and for systems 2 and 3 by gas chromatography (Table 1).

Procedures

Isolation of Dibenzofurans: 7,000 85 mm nonfilter cigarettes were smoked with a multiple-unit automatic smoking machine (14) at a rate of one puff per minute of 2 seconds duration and an average puff volume of 35 ml; butt length was 23 mm. The trapped material was combined and resulted in 180 g of dry "tar". The "tar" was suspended in 250 ml of n-hexane and 250 ml of methanol/water (4:1). The lower layer was once more extracted with n-hexane. The combined hexane layers were extracted three times with 650 ml DMF. The DMF layers were concentrated under reduced pressure to 110 ml (3 mm Hg). To this concentrate 440 ml of water were added and the water/DMF layer was extracted three times with 550 ml of n-hexane. The upper layers were dried (Na_2SO_4) and evaporated under reduced pressure (3 mm Hg) to dryness (21.4 g).

The residue was dissolved in 50 ml of n-hexane and chromatographed on a 5×100 cm column filled with deactivated alumina. After a 2,000 ml n-hexane forerun, we collected in an automatic fraction collector 16 ml n-hexane fractions. Fractions 10 to 55 were indicated by gas chromatographic analysis and u. v. spectra to contain dibenzofurans. These fractions were combined and evaporated under reduced pressure. The residue

(160 mg) was dissolved and chromatographed at 32°C with 2-propanol on a 1.5×125 cm column filled with Sephadex LH-20. Each hour one 2.5 ml fraction was collected. Fractions 43–48 were combined and resulted in a light-yellow residue of 60 mg (Figure 1). This material was dissolved in about 0.5 ml of n-hexane and used for the isolation of individual components with a gas chromatograph with a 4:1 splitter. The column effluents with the retention times of the major components were collected, rechromatographed and analyzed by u. v. spectra and mass spectra. For the isolation of components with the retention times of DBF, 2- and 3-MDBF and acenaphthylene, we used column I; for the isolation of 4-MDBF and fluorene, column II.

Quantitative Analysis: For each analysis 300 U. S. cigarettes without filter tips and of 85 mm length were smoked individually under standard conditions with a 30-channel automatic smoker (11, 14). The mainstream smoke was led through acetone cooled from the outside with dry-ice acetone, a 250 ml gas wash bottle filled with n-hexane and finally through a Cambridge filter. The latter was changed after each smoking of 60 cigarettes. The smoke-acetone suspension in the collection vessel, the n-hexane from the gas wash bottle, and the five loaded Cambridge filters were combined. To this suspension we added as internal standard $5.2 \mu\text{g}$ DBF- ^{14}C (230,000 dpm). The filtrate of the suspension and the filter washings were evaporated under reduced pressure and the "tar" dried in a desiccator. The residue (average 8.3 g) was dissolved in 50 ml of methanol/water (4:1) and 50 ml of n-hexane. The lower layer was once more extracted with 50 ml of n-hexane and the combined hexane layers (100 ml) were now extracted three times with 100 ml of DMF. The 300 ml of DMF were concentrated under reduced pressure (3 mm Hg) to 40 ml and diluted with 160 ml of water. The suspension was three times extracted with 200 ml of n-hexane.

The dry residue resulting from the distributions amounted to about 1.0 g and was chromatographed on 100 g of deactivated alumina. After a 200 ml forerun we detected the first radioactivity, which continued to occur in the next 100 ml of n-hexane. The residue of this 100 ml of solution (average 9 mg) was dissolved in 0.2 ml of 2-propanol and chromatographed at 32°C on 5 g of Sephadex LH-20. Fractions numbers 20–25 contained the β -activity. The fractions were combined and concentrated under reduced pressure (3 mm of Hg) to about 2.0 ml and subsequently evaporated to dryness by freeze-drying. The residue (about 3 mg) was dissolved in 200 μl of n-hexane. From this colorless to very light yellow solution $3 \times 2 \mu\text{l}$ aliquots were employed for gas chromatographic analysis and $3 \times 20 \mu\text{l}$ for the counting of the β -activity.

RESULTS AND DISCUSSION

Figures 2 and 3 compare the mass spectra of synthetic and isolated dibenzofuran and 4-methyldibenzofuran.

Figure 1 Separation of dibenzofurans from cigarette smoke condensate

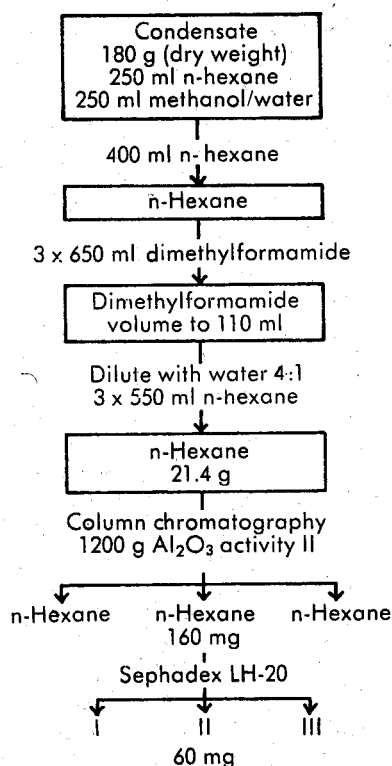


Figure 2 Mass spectra of dibenzofuran

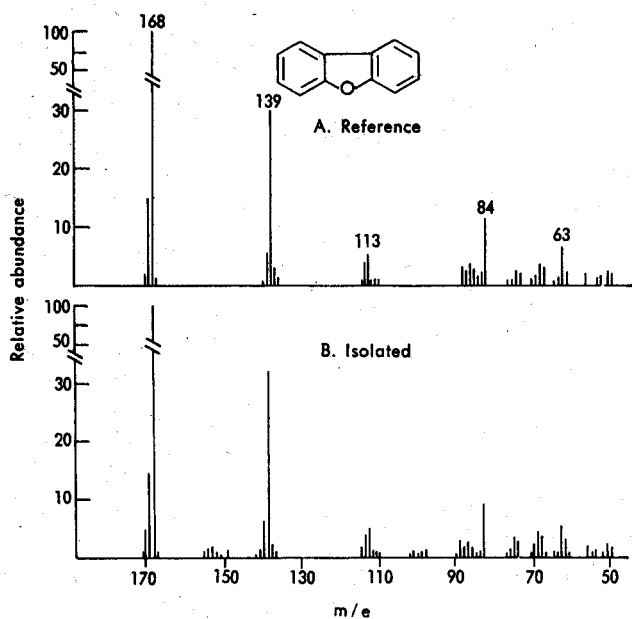
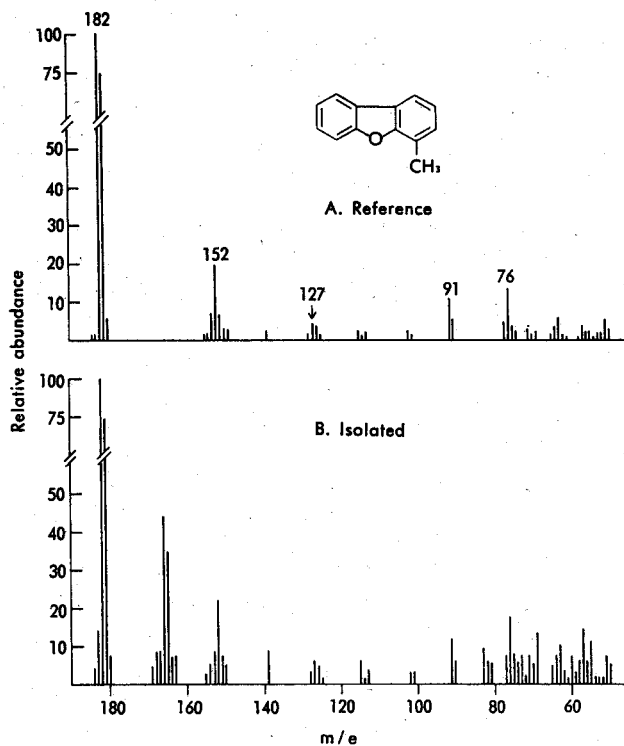


Figure 3 Mass spectra of 4-methylbenzofuran



The impurity in the mass spectrum of the isolated 4-MDBF with m/e 166, 165 and 163 was given by fluorene, which was not completely separated from 4-MDBF and which was identified by its u. v. spectrum. The mass spectra of 2- and 3-methylbenzofuran and the isolated mixture of 2- and 3-MDBF are shown in Figure 4 and the u. v. spectra in Figure 5. The latter indicated that these two isomers are present in cigarette smoke in about the same concentration. The differences in the fragmentation patterns and relative intensities in the mass spectra of the four monomethyl-DBF are only minor and thus do not suffice as a sole method for their differentiation. However, because the fragmentation patterns are characteristic of the methyl-DBF,

Figure 4 Mass spectra of 2- and 3-methylbenzofurans

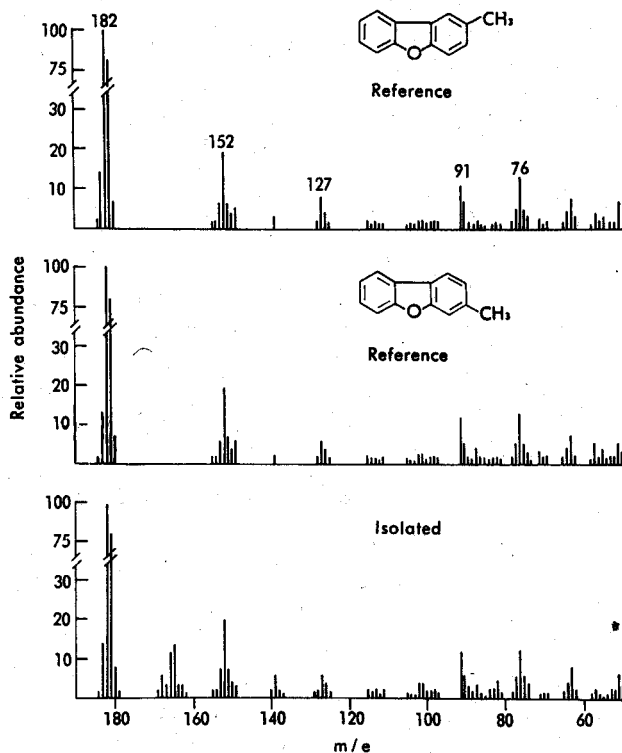
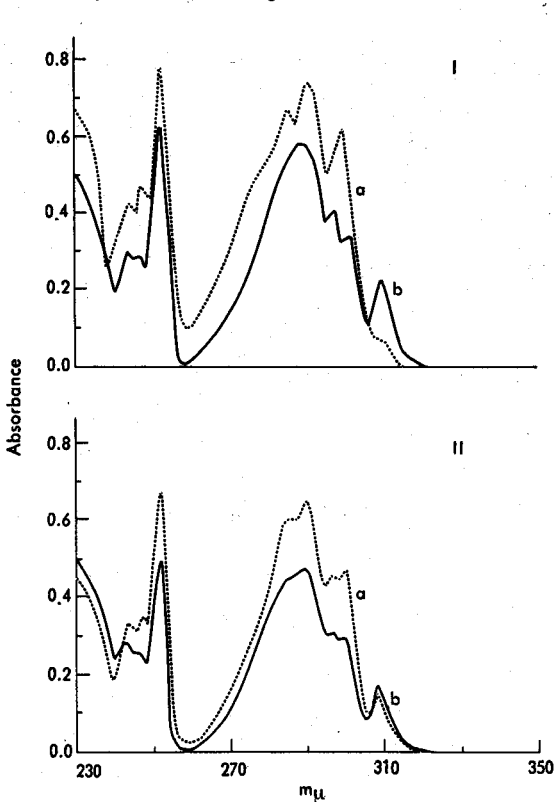


Figure 5 Ultraviolet absorption spectra of 3-methylbenzofuran (Ia, 0.65 mg/l), 2-methylbenzofuran (Ib, 0.60 mg/l), a synthetic mixture of 2- and 3-methylbenzofurans (IIa, 0.30 mg/l each), and a mixture of 2- and 3-methylbenzofurans isolated from cigarette smoke (IIb)



they served for their identification in cigarette smoke together with the retention times from two gas chromatographic systems and their u. v. spectra. The isolated acenaphthylene and fluorene, which were already reported to occur in cigarette smoke (2, 12, 14),

Figure 6 Gas chromatogram of concentrates of dibenzofurans from cigarette smoke, column I

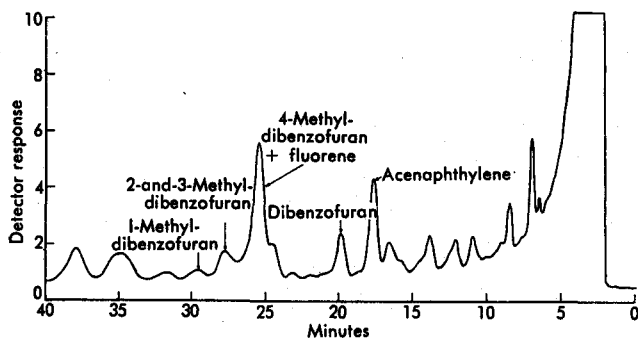
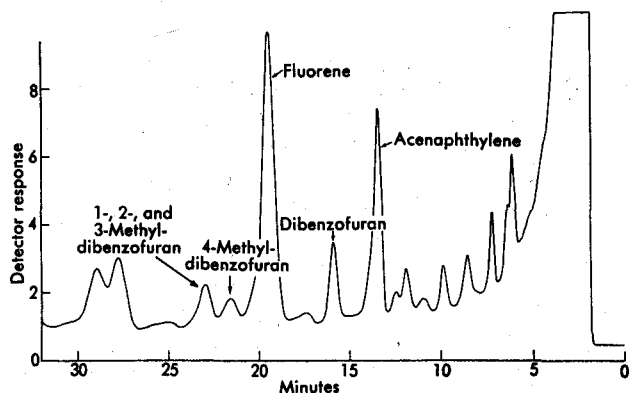


Figure 7 Gas chromatogram of concentrates of dibenzofurans from cigarette smoke, column II



were identified by the retention times in the two gas chromatographic systems and by their ultraviolet absorption spectra.

For the quantitative analysis, five times 300 cigarettes were smoked and dibenzofuran-¹⁴C was added as internal standard. From the final gas chromatograms (Figures 6 and 7) it was calculated that the mainstream smoke from an 85 mm U. S. blended cigarette without filter tip contains 106 ng of dibenzofuran, 40 ng of 1-methyl-dibenzofuran, 100 ng of 2- and 3-methyldibenzofurans, and 52 ng of 4-methyldibenzofuran (Table 2). The experimental deviation of the analysis varied between 5.7–11.2%. The recovery of dibenzofuran was better than 75%. An analysis showed that the carcinogenic subfraction BI contains about 30% of the dibenzofurans of the mainstream smoke. The isolation of dibenzofuran and 1-, 2-, 3- and 4-methyldibenzofurans from cigarette smoke represents their first isolation from a respiratory environment (7, 12, 14).

Added during proof reading: The two components indicated in the gas chromatogram-Figure 6 with the retention times of 34.5 and 37.5 minutes and in gas chromatogram-Figure 7 with the retention times of 27.5 and 29 minutes were identified as two methylfluorenes (not 9-methylfluorene).

SUMMARY

A method was developed for the isolation of dibenzofuran (DBF) and its four methyl derivatives (MDBF) from cigarette smoke. Since an excess of alkanes was

found to inhibit the column chromatographic separation of dibenzofurans, distribution systems had to be developed which, in the initial step of the analysis, would lead to an enrichment of DBF and MDBF with loss of the bulk of the tobacco smoke alkanes. The three distributions which lead to a preliminary concentration of these heterocyclics were the solvent pairs methanol/water (4:1) and n-hexane; n-hexane and dimethylformamide; and water/dimethylformamide (4:1) and n-hexane. The resulting tobacco "tar" extract was chromatographed on deactivated alumina and subsequently on Sephadex LH-20. The final concentrate of DBF was separated into individual components by gas chromatography. With the aid of this method we separated from cigarette smoke dibenzofuran and 1-, 2-, 3- and 4-methyldibenzofurans. These five dibenzofurans were identified by the retention times in two gas chromatographic systems, ultraviolet absorption spectra, and mass spectral analysis. The final DBF concentrate from cigarette smoke also contains, as major components, acenaphthylene and fluorene.

For the quantitative analysis we employed dibenzofuran-1,2,3,4,4a,9b-¹⁴C₆ as internal standard. This labelled compound was synthesized by the Trippett method on a microscale from phenol and 2-bromocyclohexanone.

The mainstream smoke of an 85 mm U.S. nonfilter cigarette contained 106 ng of dibenzofuran, 40 ng of 1-methyldibenzofuran, 100 ng of 2- and 3-methyldibenzofurans, and 52 ng of 4-methyldibenzofuran. The identification of DBF and its four methyl derivatives in cigarette smoke represents their first isolation from a respiratory environment.

Table 2 Dibenzofurans in cigarette smoke (a) (ng/cig.)

| Number of analysis | Dibenzofuran | 1-Methyldibenzofuran | 2- and 3-Methyl-dibenzofuran | 4-Methyldibenzofuran |
|-----------------------|--------------|----------------------|------------------------------|----------------------|
| I | 112 | 42 | 115 | 56 |
| II | 112 | 36 | 86 | 46 |
| III | 106 | 35 | 109 | 54 |
| IV | 101 | 40 | 92 | 58 |
| V | 99 | 45 | 100 | 48 |
| Average | 106 | 40 | 100 | 52 |
| Standard deviation | 6.0 | 4.2 | 11.9 | 5.2 |
| Deviation coefficient | 5.7% | 10.5% | 11.9% | 10.0% |

(a) Calculated with the isotope dilution method using dibenzofuran-¹⁴C as internal standard.

ZUSAMMENFASSUNG

Zur Bestimmung von Dibenzofuran (DBF) und seinen Methyl-Derivaten (MDBF) im Cigarettenrauch wurde eine analytische Methode entwickelt. Da die Gegenwart höherer Alkane die säulenchromatographische Trennung von Dibenzofuranen erheblich verringert, wurde eine neue Methode entwickelt, die zu Beginn der Analyse DBF von der Hauptmenge der Tabakrauch-Alkane trennt. Diese Bedingung wurde erreicht durch Verteilung des „Teeres“ zwischen drei Lösungsmittelpaaren. Dies waren Methanol/Wasser (4:1) und n-Hexan, n-Hexan und Dimethylformamid und Wasser/Dimethylformamid (4:1) und n-Hexan. Der Extrakt des Tabakrauchkondensates, der nach den drei Verteilungsschritten gewonnen wurde, wurde an deaktiviertem Aluminiumoxid und anschließend an Sephadex LH-20 chromatographiert. Das Konzentrat, das nach dieser Auftrennung gewonnen wurde, konnte durch Gaschromatographie in einzelne Komponenten zerlegt werden. Mit Hilfe dieser Methode wurde aus dem Kondensat von Cigarettenrauch isoliert: Dibenzofuran, 1-, 2-, 3- und 4-Methyldibenzofuran. Diese fünf Dibenzofurane wurden identifiziert durch Retentionszeiten in zwei gaschromatographischen Systemen, Ultraviolett-Spektren und massenspektrographische Analyse. Das endgültige Konzentrat enthält neben den fünf DBF auch noch als Hauptkomponenten Acenaphthylen und Fluoren.

Für die quantitative Analyse wurde Dibenzofuran-1,2,3,4,4a,9b-¹⁴C₈ als innerer Standard gewählt. Diese ¹⁴C-markierte Verbindung wurde im Mikromaßstab nach Trippett aus Phenol und 2-Bromcyclohexanon synthetisiert.

Der Hauptstrom einer filterlosen Cigarette des amerikanischen Marktes von 85 mm Länge enthält 106 ng Dibenzofuran, 40 ng Methyldibenzofuran, 100 ng 2- und 3-Methyldibenzofuran und 52 ng 4-Methyldibenzofuran. DBF und 1-, 2-, 3- und 4-MDBF wurden bisher als Bestandteile der Luftverunreinigung nicht gefunden.

RÉSUMÉ

Nous avons développé une méthode de séparation du dibenzofurane (DBF) et de ses 4 dérivés méthylés (MDBF) de la fumée de cigarette. Nous avons trouvé qu'un excès d'alcane inhibait la séparation des dibenzofuranes par chromatographie sur colonne. Nous avons donc dû développer une méthode permettant d'enrichir l'extrait en DBF et MDBF en éliminant la plus grande partie des alcanes présents dans la fumée de tabac. L'enrichissement préliminaire en hétérocycliques a été obtenu par trois extractions consécutives dans les paires de solvants suivants: méthanol/eau (4:1) et n-hexane; n-hexane et diméthylformamide; et eau/diméthylformamide (4:1) et n-hexane. L'extrait de «goudron» de tabac a été séparé par chromatographie sur alumine désactivée, ensuite sur Sephadex LH-20. Le composé final enrichi en DBF a été séparé en ses différents composés par chromatographie en phase gazeuse. Grâce à cette méthode nous avons

isolé le dibenzofurane et les 1-, 2-, 3- et 4-méthyldibenzofuranes dans la fumée de cigarette. Nous avons identifié ces cinq dibenzofuranes par le temps de rétention dans deux systèmes de chromatographie en phase gazeuse, le spectre d'absorption ultraviolet, et l'analyse par spectrométrie de masse. Le concentré final de DBF dans la fumée de cigarette contient aussi de l'acenaphthène et du fluorène comme composés majeurs.

Pour l'analyse quantitative nous avons employé le dibenzofurane-1,2,3,4,4a,9b-¹⁴C₈ comme étalon interne. Ce composé marqué a été obtenu par la synthèse Trippett en microquantités à partir du phénol et du 2-bromocyclohexanone.

La fumée principale d'une cigarette U.S. 85 mm sans filtre contient 106 ng de dibenzofurane, 40 ng de 1-méthyldibenzofurane, 100 ng de 2- et 3-méthyldibenzofuranes, et 52 ng de 4-méthyldibenzofurane. L'identification de DBF et des 4 dérivés méthylés dans la fumée de cigarettes représente leur première isolation dans des conditions respiratoires.

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