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GRAM QUANTITY SYNTHESIS AND CHROMATOGRAPHIC ASSESSMENT OF 3,3′,4,4′-TETRACHLOROBIPHENYL

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SUMMARY

3,3′,4,4′-Tetrachlorobiphenyl (TCBP) was synthesized by diazotization of 3,3′-dichlorobenzidine followed by chlorination with cuprous chloride and hydrochloric acid. Purification of crude TCBP was conducted by alumina column chromatography and two recrystallizations from ethanol. The composition of the product during purification was assessed by gas–liquid chromatography with electron-capture detection and high-performance liquid chromatography with UV absorption detection. TCBP with greater than 99% purity (dioxin- and furan-free) was obtained with a yield of 40–44%. The identity of the major synthetic product was confirmed by gas chromatography–mass spectrometry (electron ionization) and nuclear magnetic resonance spectroscopy. The major impurity, a trichlorobiphenyl, constituted 0.37% of the final product. It is concluded that this synthetic product can be used to produce reliable results in toxicological studies.

INTRODUCTION

Industrial polychlorinated biphenyls (PCBs), which have been identified as important environmental hazards, are mixtures of several chlorinated biphenyl compounds that contain variable amounts of chlorinated dioxin and furan contaminants. In order to determine the mechanism of toxicity of each individual compound, it is necessary to prepare pure individual compounds for study. The syntheses of individual PCBs have been described[1–5]. However, it is recognized that many of the synthetic routes to PCBs result in the contamination of the final product with small amounts of material (*e.g.* chlorinated dioxins and furans), which may have a toxic potential several orders of magnitude greater than the PCB under study[6–9]. Thus, to assess the toxicity of individual PCB isomers, it is imperative to determine the purity of the compounds studied and to elucidate the chemical nature of impurities contained in the PCB sample. A review of the synthetic procedures employed for PCB synthesis gives insufficient information to allow careful assessment of the purity of the PCB synthesized and employed in toxicological studies. In this study, we have utilized a variety of current techniques to define the purity of the PCB during the course of its synthesis and purification on a preparative scale. This report is also intended to assist other investigators in the preparation of individual PCB isomers with respect to the purity, amount and economy required for large-scale *in vivo* toxicity studies.

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MATERIALS AND METHODS

Reagents and solvents

For use as a reference standard 3,3′,4,4′-tetrachlorobiphenyl (TCBP) was obtained with a purity of 99% and chlorinated dibenzo-*p*-dioxin (CDD) and dibenzofuran free from RFR (Hope, RI, U.S.A.). For the present synthesis, the starting material, 3,3′-dichlorobenzidine dihydrochloride, was purchased from Sigma (St. Louis, MO, U.S.A.). Cuprous chloride, analytical reagent, 97% pure, was obtained from BDH (Toronto, Canada). The following chemicals were purchased from Fisher (Whitby, Canada) as ACS grade: sodium nitrite crystals, 98.2%; A-950 alumina, neutral, Brockman activity 1, 80–200 mesh; anhydrous sodium carbonate; methylene chloride; carbon tetrachloride; hydrochloric acid; sulfuric acid; and glacial acetic acid. Hexane, ethyl acetate and acetone were obtained from Caledon (Georgetown, Canada) as HPLC grade. The water utilized in these studies was passed through a Barnstead Nanopure® apparatus (Canlab, Toronto, Canada). Melting points were determined on a Thomas Hoover Capillary melting point apparatus (A.H. Thomas, Philadelphia, PA, U.S.A.) and are uncorrected.

Synthesis of 3,3′,4,4′-tetrachlorobiphenyl

3,3′,4,4′-TCBP was prepared by the method of Cain[10] as modified by Van Roosmalen[3]. 3,3′-Dichlorobenzidine was converted into the corresponding diazonium salt, and two chlorine atoms were introduced by treatment with cuprous chloride and concentrated HCl. 3,3′-Dichlorobenzidine dihydrochloride (0.01 mole; 3.26 g) was suspended in concentrated HCl (0.06 mole; 5 ml) to which was added 5 ml of water, and the reaction flask was kept on ice. An aqueous solution of sodium nitrite (0.028 mole; 8.3 ml) was added dropwise to the mixture, which was stirred vigorously under nitrogen. To verify the presence of excess nitrous acid at the completion of the reaction, the reaction mixture was tested with potassium iodide-starch paper. Cuprous chloride (0.024 mole; 2.38 g) was dissolved in concentrated HCl (12 ml), and 10 ml of this solution were added dropwise to the stirred reaction mixture over a period of *ca.* 40 min. A frothy slurry resulted due to the liberation of free nitrogen gas. After 1 h at room temperature, a yellowish crude complex was obtained by filtration and dried overnight in a vacuum dessicator (yield of crude product was 4.3 g). For large batches, the amount of the starting material and reagents was increased 10-fold.

Alumina column chromatography purification

The purification of 3,3′,4,4′-TCBP was based on the procedures of Goldstein *et al.*[5], Parkinson *et al.*[11] and Kamops *et al.*[12]. Individual 1-g portions of the yellowish, crude TCBP dissolved in 100 ml of acetone were added to 6 g of alumina which had been activated overnight at 130°C, and the contents were stirred for several hours. The solvent was then removed by evaporation using a stream of nitrogen. The alumina containing the TCBP was then loaded onto a glass column (67 cm × 1.1 cm I.D.) which was dry-packed with 60 g of alumina. The column had a medium-porosity glass frit and a teflon stopcock. The TCBP was eluted with hexane, and 100-ml fractions were collected. The progress of the elution of the TCBP was monitored by gas–liquid chromatography with electron-capture detection (GLC–ECD). The frac-

tions were subsequently pooled and after removal of solvent on a rotary evaporator under reduced pressure, a white crystalline material remained.

Recrystallization

The white crystalline product was recrystallized twice from ethanol; *ca.* 400 ml of ethanol were required for 1 g of the TCBP.

Gas chromatography–mass spectrometry (GC–MS)

The identification of the synthetic product and the characterization of the minor contaminants were conducted using a Hewlett-Packard Model 5985B gas chromatograph–mass spectrometer. The apparatus was interfaced with a HP Model 2648A graphics terminal and data station. The GC conditions were: coiled glass column (1.8 m × 2 mm I.D.), containing 3% OV-101 on 80–100 mesh Chromosorb W HP (Chromatographic Specialties, Brockville, Canada); helium flow-rate, 30 ml/min; injection port temperature, 250°C; column temperature, 220°C. The mass spectrometer was operated in the electron ionization mode (70 eV); ion source temperature, 250°C; mass range, 40–500 a.m.u.

High-resolution MS was done on a CEC Model 21-110B mass spectrometer.

Nuclear magnetic resonance spectroscopy (NMR)

The samples were prepared by dissolving 40 mg of TCBP in 1 ml of C^2HCl_3. Proton spectra were recorded at 60 and 200 MHz on a Brucker Model instrument. All chemical shifts are reported as δ values with respect to the internal standard, tetramethylsilane.

Gas–liquid chromatography

Electron-capture detection. The chemical purity of the TCBP was assessed by GLC–ECD using a Hewlett-Packard Model 5730A gas chromatograph equipped with a ^{63}Ni electron capture detector, a 1 mV potentiometric recorder and Hewlett-Packard Model 3390A electronic integrator. A coiled glass column (1.8 m × 2 mm I.D.) was washed in sequence with distilled water, methanol, acetone, methylene chloride and hexane, dried with a stream of nitrogen and silanized with 10% Surfasil® (Chromatographic Specialties) in toluene for 1 h. The column was heated at 100°C for 30 min, cooled, rinsed with methanol and dried with a stream of nitrogen. The silanized column was packed with 3% OV-1 on 80–100 mesh Chromosorb 750 (Chromatographic Specialties), and conditioned for at least 15 h at 250°C using argon–methane (95:5) carrier gas at a flow-rate of 5 ml/min. The operating conditions were: injection port temperature, 250°C; column temperature, 220°C; detector temperature, 300°C; carrier gas flow-rate, 22 ml/min.

To prepare a standard curve, known amounts of pure 3,3′,4,4′-TCBP were prepared in hexane (10 pg–1000 ng in 1–5 μl) and analyzed by GLC–ECD. The absolute peak area of the chromatographic signal was measured by electronic integration and plotted against TCBP content.

Nitrogen–phosphorus detection. In order to check for the presence of nitrogen-containing contaminants in the TCBP synthetic product, the above analysis was repeated using a Hewlett-Packard Model 5710A gas chromatograph equipped with a nitrogen–phosphorus detector (NPD). All chromatographic conditions were as de-

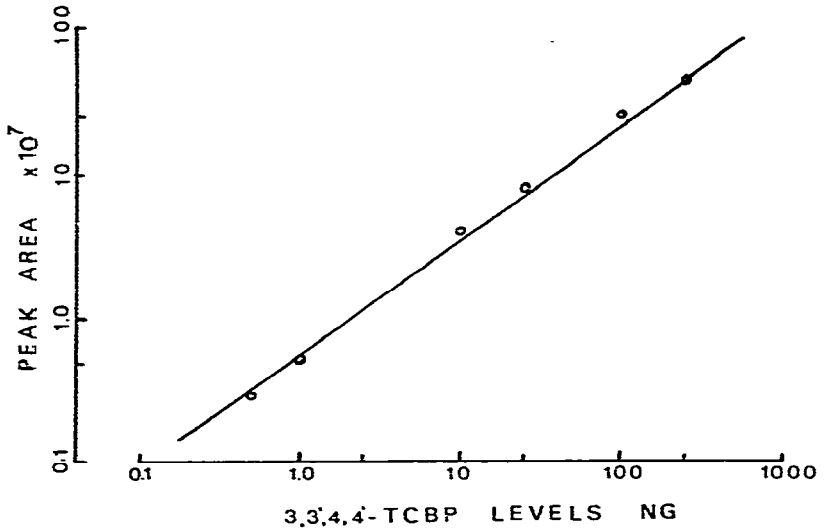

Fig. 1. GLC–ECD standard curve for 3,3',4,4'-TCBP. Note linearity over three orders of magnitude.

scribed above, except that the carrier gas flow-rate was 30 ml/min; hydrogen and air flow-rates were 3 and 50 ml/min, respectively.

High-performance liquid chromatography–ultraviolet absorption detection (HPLC–UV)

The chemical purity of the synthesized TCBP was assessed by HPLC–UV. The liquid chromatographic system consisted of the following components: solvent me-

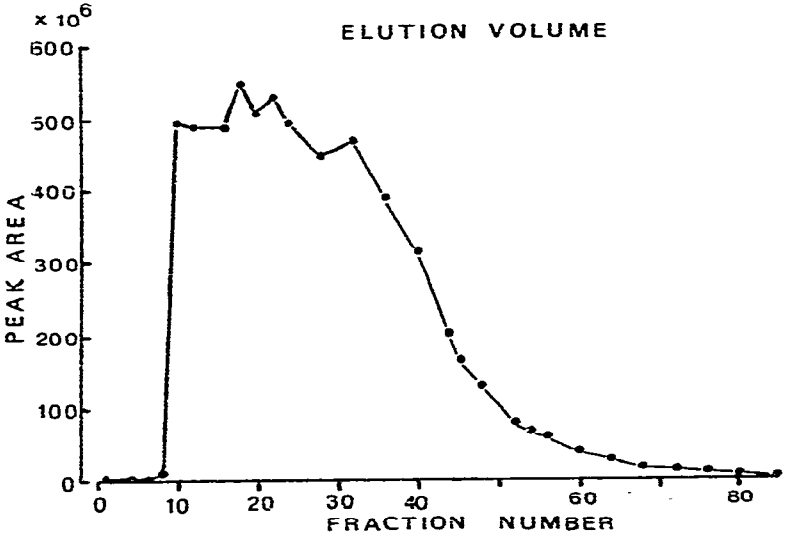

Fig. 2. Hexane elution profile of 10 g of TCBP on an alumina column. A 10-g portion of crude product was adsorbed onto 60 g of alumina and subsequently applied to a large glass column (100 cm × 24 cm I.D.) dry-packed with 150 g of alumina. Hexane elution fractions (100 ml) were collected and a 1-μl sample of each fraction was analyzed by GLC–ECD. The peak areas of the chromatograms were determined by electronic integration.

tering pump (Model 110A Beckman, Fullerton, CA, U.S.A.); injection valve with a 20-μl injection loop (Valco, Houston, TX, U.S.A.); UV detector with a 8-μl microflow cell and 254-nm filter (Model 153, Altex, Berkeley, CA, U.S.A.); strip chart recorder (Fisher). The stainless-steel column (20 cm × 4 mm I.D.) was packed with 5 μm silica gel (Partisil 5; Whatman, Clifton, NJ, U.S.A.). The eluting solvent was degassed hexane, and the flow-rate was 1.0 ml/min. Peak height measurements were used for quantitation.

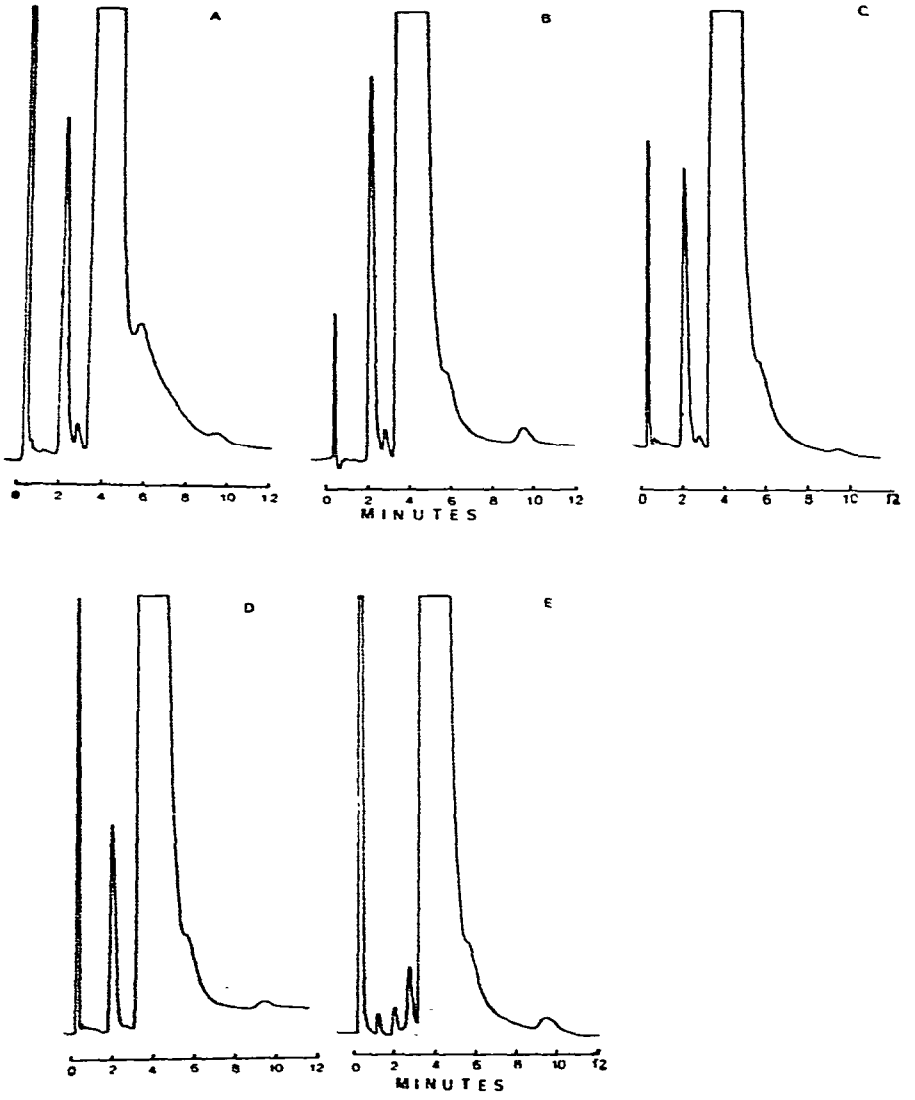

Fig. 3. GLC–ECD chromatograms of 3,3′,4,4′-TCBP in the course of purification. A, Crude product; B, post alumina; C, one ethanol crystallization; D, two ethanol crystallization; E, RFR 3,3′,4,4′-TCBP (99% CDD-free).

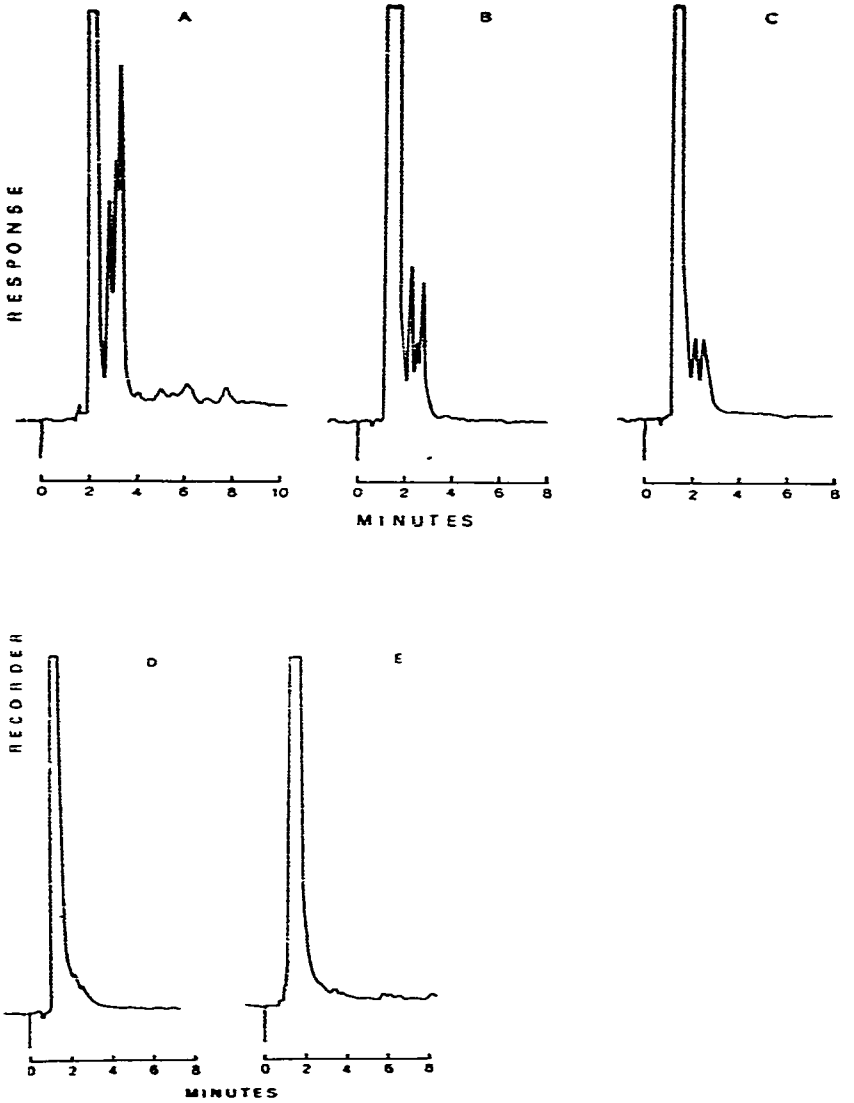

Fig. 4. HPLC–UV chromatograms of 3,3′,4,4′-TCBP in the course of purification. A–E as in Fig. 3.

RESULTS AND DISCUSSION

Data regarding the yield of product at each step were obtained by GLC–ECD with reference to the 3,3′,4,4′-TCBP standard curve (Fig. 1). The calibration curve was linear over the range from 500 pg to 500 ng of 3,3′,4,4′-TCBP, and the amounts injected for yield analysis were between 1 and 100 ng.

The crude synthetic product was yellowish brown and had a melting point of 152–155°C. The first step in the purification was alumina column chromatography, which was based on available procedures[5,11,12]. The recovery of compound from the

TABLE I

PURITY OF 3,3',4,4'-TETRACHLOROBIPHENYL BY GLC–ECD FOLLOWING ALUMINA COLUMN CHROMATOGRAPHY AND REPEATED ETHANOL RECRYSTALLIZATION

Peak areas are expressed as percentage of total area of the chromatogram as determined by electronic integration

Retention time (min)	*Peak area (%)*				
	Crude product (A)	*Post column (B)*	*First ethanol recrystallization (C)*	*Second ethanol recrystallization (D)*	*Commercial reference standard (E)*
1.24	0.008	0.003	0.002	0.002	0.027
2.15	1.086	0.972	0.526	0.368	0.053
2.97	0.109	0.088	0.079	*	0.155
3.85	98.553	98.836	99.296	99.550	99.632
6.28	0.202	*	*	*	*
10.50	0.043	0.102	0.097	0.080	0.132

* Signal present but non-measurable by electronic integration.

column after application of 1–10 g amounts ranged from 78 to 87%. Elution through the column removed all the coloured impurity and upon removal of the eluent, a white residue, m.p. 173–176°C, was obtained. A typical profile for a 10-g load of crude material on a alumina column with elution by hexane is shown in Fig. 2. Preliminary studies revealed that florisil or silica gel column chromatography did not provide satisfactory purification. However, the use of alumina or alumina/Florisil column chromatography resulted in improved purification. Since the adsorptivity of florisil was reported to differ from lot to lot[12,13], alumina alone was added as the matrix. Comparison by GLC–ECD of the product before and after alumina chromatography (Figs. 3A and B, Table I) and by HPLC–UV (Figs. 4A and B) illustrates the reduction in the amount of contamination.

The second step in the purification procedure was recrystallization from ethanol, which produced a white crystalline solid. The overall yield of purified material ranged from 40 to 44% (4.0–4.4 mmole). Considerable purification was achieved by this means, and the melting points were 176–177°C after one recrystallization and 177–177.5°C after two recrystallizations. However, the melting point for 3,3',4,4'-TCBP has been reported to be 177–178°C[2], 172°C[3,10] and 173°C[1]. In a separate experiment, recrystallization from glacial acetic acid produced a brown-coloured crystalline solid, m.p. 171–173°C. Thus the discrepancy amongst the reported melting points may be related to the mode of recrystallization and/or the presence of a chromophore. Analysis by GLC–ECD (Figs. 2C and D, Table I) and HPLC–UV (Figs. 4C and 1D) of the ethanol-recrystallized product illustrates the successive purification with each recrystallization. The final product had a purity of greater than 99.5% by GLC–ECD, which compares favourably with the commercially available 3,3',4,4'-TCBP reference standard (Fig. 3E, Table I). Analysis by HPLC–UV gave comparable values. The identity of the final product is supported by retention times (t_R) from GLC–ECD (Fig. 3) and HPLC–UV (Fig. 4), mass spectra and NMR spectra.

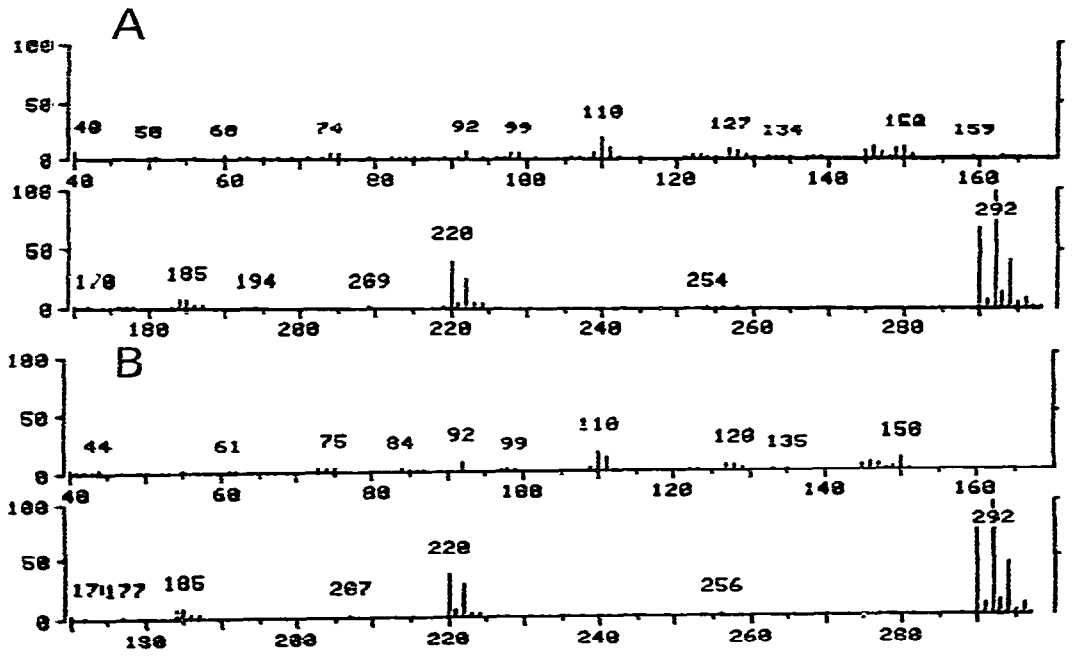

Fig. 5. Mass spectra of commercial 99% pure and CDD-free, 3,3',4,4'-TCBP, reference standard (A) and the purified TCBP (B).

The mass spectrum of the synthetic product (GLC t_R 3.85 min) was indistinguishable from that for the commercial reference product (Figs. 5A and B). Electron ionization produced an abundant molecular ion at *m/z* 290 (relative intensity of 75% compared with the base ion, 292 *m/z*) with characteristic chlorine isotope peaks. The fragmentation pattern (Fig. 5) is consistent with TCBP and is identical with the pattern reported by the Environmental Protection Agency (Washington, DC, U.S.A.) for 3,3',4,4'-TCBP[14]. The molecular nature of the three minor impurities, corresponding to GLC–ECD signals at t_R 2.15, 2.97 and 10.5 min, was tentatively identified (Fig. 6). The mass spectrum of the major contaminant (t_R 2.15) had a molecular ion at *m/z* 256 (Fig. 6a) with the characteristic trichloro-isotopic fragmentation pattern and is consistent with 3,4,4'-trichlorobiphenyl. The GLC–ECD signal at t_R 2.97 min was tentatively identified as being due to a tetrachlorobiphenyl (possibly 2,3',4,4'-tetrachlorobiphenyl) (Fig. 6b). The unidentified material at t_R 10.5 min is *not* chlorinated (Fig. 6c). A further search for dioxins and furans by GC–MS indicated no detectable amounts of either potential contaminant. Moreover, GLC–NPD analysis failed to detect the presence of nitrogen-containing components. Our 3,3',4,4'-TCBP product had more contamination with the trichlorobiphenyl (0.37%) but considerably less contamination with the tetrachlorobiphenyl (0.05%) when compared with the commercially available reference standard (0.05 and 0.155%, respectively). High-resolution MS indicated that the mass of the molecular ion was 289.9204, which compares well with the theoretical mass of 289.9224 for $C_{12}H_6Cl_4$ (TCBP).

NMR data of the purified 3,3'4,4'-TCBP were in good agreement with those reported by Hutzinger *et al.*[13]. There was a multiplet, δ 7.06–7.60, at 60 MHz and a

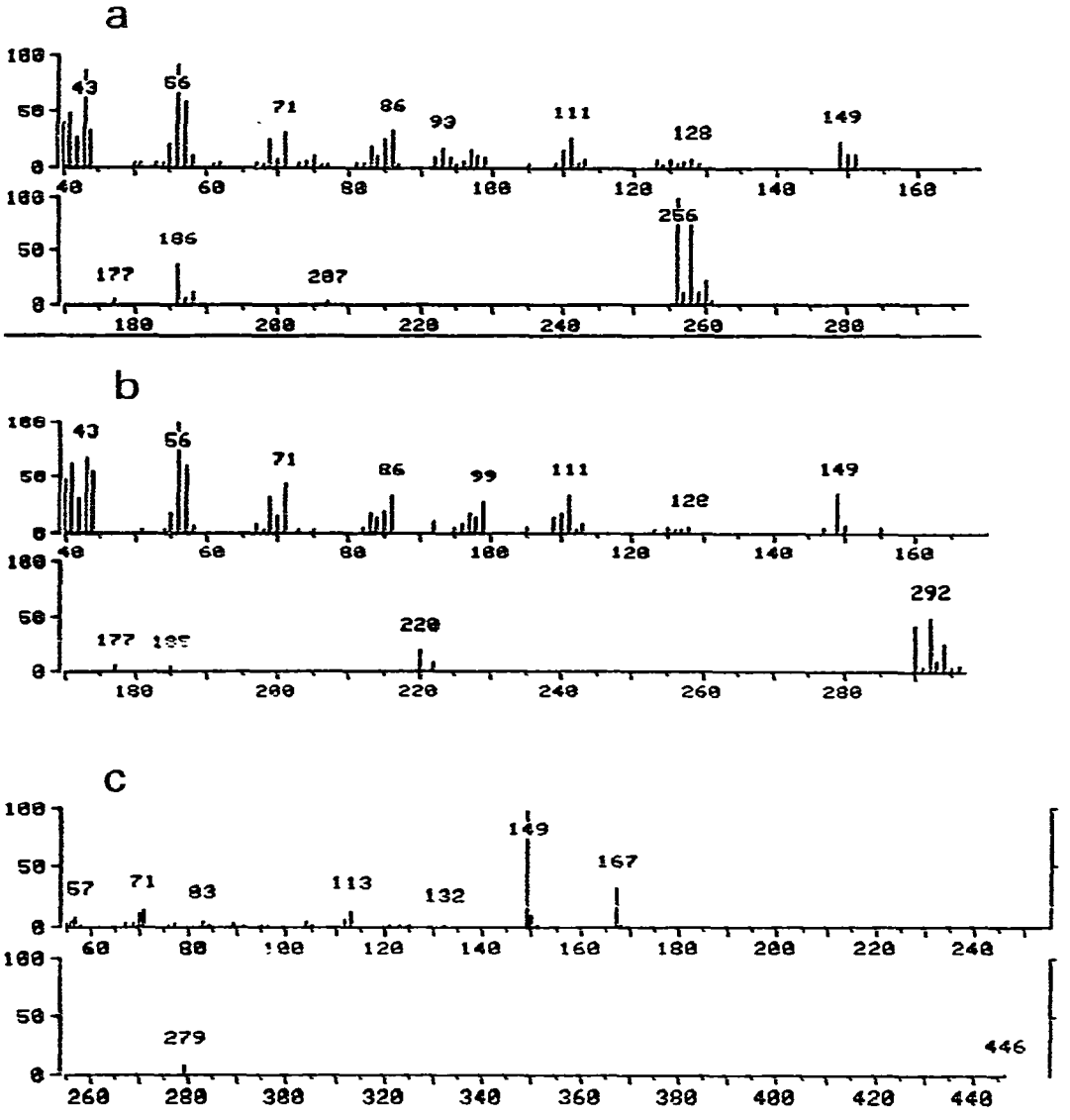

Fig. 6. Mass spectra of minor contaminants of the 3,3′,4,4′-TCBP synthetic product. The spectra were obtained for contaminants with GLC t_R 2.15 min (a), 2.97 min (b) and 10.5 min (c).

multiplet, δ6.90–7.56, at 200 MHZ, relative to the internal standard, tetramethylsilane, when dissolved in C^2HCl_3.

Thus, we have shown that 3,3′,4,4′-TCBP can be synthesized in large amounts and purified by one alumina chromatographic step followed by two recrystallizations from ethanol. The product, which is greater than 99.5% pure, is dioxin- and furan-free and contains a trichlorobiphenyl contaminant as the major impurity (0.37%). We believe that this product can be used to produce reliable results in toxicological studies.

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