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MODIFICATION OF A HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC–GAS CHROMATOGRAPHIC PROCEDURE FOR SEPARATION OF THE 22 TETRACHLORODIBENZO-*p*-DIOXIN ISOMERS

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SUMMARY

A published method for separation of the 22 tetrachlorodibenzo-*p*-dioxin (TCDD) isomers by high-performance liquid chromatography (HPLC)–gas chromatography (GC) was modified to improve the recovery and chromatographic resolution of individual isomers. For the first step, reversed-phase HPLC, minor changes were made in column temperature (50 to 40°C, to prolong column life) and in injection solvent (chloroform to benzene, to avoid photodecomposition). For the second step, normal-phase HPLC, the silica gel columns were used in a less active state, and 0.4% toluene was added to the hexane eluent as a polar organic modifier. Resolution of the 22 isomers was significantly improved by this change of solvent system, and recovery of subnanogram quantities of TCDDs from the silica gel columns was increased from 20 to 80%. For the final step a capillary GC column (OV-275) was used in place of a packed column.

INTRODUCTION

Polychlorinated dibenzo-*p*-dioxins (PCDDs) are a class of 75 positional isomers containing from one to eight chlorine atoms. A number of PCDDs have been identified in products derived from chlorinated phenols and in air emissions from various incineration processes[1]. There is evidence that PCDD isomers can vary considerably in their toxicity and biological activity[2,3]. Concern over environmental polution by PCDDs has centered on 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (2,3,7,8-TCDD), which is very toxic to certain animal species[2]. However, there are 22 TCDD isomers, and it is important that analytical procedures be able to distinguish between them.

Mass spectrometry (MS) is considered the most sensitive and specific method for identification of PCDDs. With conventional MS instruments organic compounds are converted to positively charged ions in the MS source by energetic electrons (electron-impact ionization). Since this technique does not distinguish between positional PCDD isomers, TCDD isomers must be separated before the sample enters the MS

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source. A method[4] recently developed for this purpose uses a combination of reversed-phase and normal-phase high-performance liquid chromatography (HPLC), followed by packed-column gas chromatography (GC)–low-resolution MS. The normal-phase HPLC step requires that the adsorbent, silica gel, be in a very active state and that a non-polar solvent, hexane, be used as an eluent. Under these conditions we have found that at least 10 ng of each TCDD isomer is required for analysis, since peaktailing on the silica gel column results in poor recovery at lower amounts.

In view of the need to determine, 2,3,7,8-TCDD at ppt (10^{12}) concentrations in many environmental samples, especially biological tissues, we have modified the normal-phase HPLC solvent system and adsorbent activity to improve the recovery and separation efficiency for low concentrations of TCDD isomers. The packed GC column described in the original procedure has adequate resolution to separate the few isomers which coelute through the two HPLC steps. We prefer a capillary column because we believe that future advances in capillary GC technology will allow for complete separation of all 22 TCDD isomers without recourse to HPLC. Indeed, since the work described in this paper was completed, a narrow-bore-polar (Silar 10C) capillary column has been reported to separate 2,3,7,8-TCDD from the 21 other TCDD isomers[5]. Complete separation of all 22 isomers by capillary GC has not yet been achieved.

EXPERIMENTAL

Precautions

In view of the toxic properties of PCDDs rigorous procedures should be adopted to minimize exposure of laboratory personnel. Laboratory waste should be disposed of either by high-temperature incineration ($>1000^{\circ}C$) or by burial in an approved chemical landfill.

TCDD isomers

Synthetic standards of 2,3,7,8-TCDD, [U-^{13}C]TCDD, [2,3,7,8-^{37}Cl]TCDD and 1,2,3,4-TCDD were obtained respectively from L. Shadoff, Dow Chemical, Midland, MI, U.S.A.; R. Mitchum, National Center for Toxicology, Jefferson, AK, U.S.A.; R. Baughman, Harvard University, Cambridge, MA, U.S.A.; and Analabs, New Haven, CT, U.S.A. Ten TCDD isomers (1,3,6,8; 1,3,7,9; 1,2,6,7; 1,2,8,9; 1,4,6,9; 1,2,6,9; 1,3,7,8; 1,2,7,8; 1,2,6,8; and 1,2,7,9) were provided as mixtures of two or more compounds by H. R. Buser, Swiss Federal Research Station, Wadenswill, Switzerland. These 10 isomers were prepared by pyrolysis of potassium chlorophenates, followed by cleanup with alumina columns[6]. Mixtures of the 10 remaining isomers were prepared by pyrolyzing potassium chlorophenates under a nitrogen stream inside a heated reactor tube[4], which contained silica gel to trap the isomers.

Solvents

All solvents were distilled-in-glass quality purchased from Burdick and Jackson, Muskegon, MI, U.S.A. Water saturation of hexane and toluene was accomplished by passing the solvents through columns containing 60 g of silica gel mixed with 18 g of water.

Procedure

Step 1: Reversed-phase HPLC. The reversed-phase HPLC system consisted of a Waters ALC/GPC-204 liquid chromatograph with two series-connected 250 × 6.2 mm Zorbax ODS (DuPont Instruments Division, Wilmington, DE, U.S.A.) columns contained in a Perkin-Elmer 1220 oven. The operating conditions were: isocratic eluent, methanol at 2.0 ml/min; column temperature, 40°C; UV detector, 0.005 to 0.1 absorbance units full scale (a.u.f.s.) at 313 nm.

The system was calibrated daily with an authentic standard of 2,3,7,8-TCDD, which had a retention time of approximately 15.5 min under these conditions. TCDD isomers which were being analyzed through the entire three-stage chromatographic procedure were collected in 25-ml round-bottomed flasks from peaks eluting at between 12 and 18 min. For injection 10 µl of 2-propanol was drawn up into the syringe, followed by the standard or isomer mixture in 30 µl of benzene and then an additional 10 µl of 2-propanol. Between analyses the injector and sample loop were cleaned with two 5-ml injections of methanol.

Step 2: Normal-phase (silica) HPLC. Cyclohexane (10 ml) was added to each fraction collected in Step 1, and the contents of a 25-ml round-bottomed flask were evaporated to 1 ml in a boiling-water bath. After transfer to a 6-mm-I.D., tapered reflux concentration tube a sample extract was concentrated to 30 µl by a combination of nitrogen evaporation and refluxing[7]. The sample was then ready for analysis by normal-phase HPLC.

Chromatographic separations were accomplished on two 250 × 6.2 mm Zorbax-Sil (DuPont) columns. The HPLC instrumentation was similar to that for reversed-phase HPLC, except that no oven was required (analysis was at ambient temperature) and the standard 2-ml injector loop was replaced by a 150-µl loop. Additional operating conditions were: eluent, 0.4% toluene (water-saturated) in hexane at a flow-rate of 2 ml/min; UV detector, 0.005 to 0.1 a.u.f.s. at 313 nm.

New columns were washed with 250 ml of methylene chloride at a flow-rate of 3 to 4 ml/min; then 60 ml of a mixture of 2,2-dimethoxypropane–acetic acid–methylene chloride (2:2:96) was passed through at a flow-rate of 2 ml/min. Activation was completed by washing the columns with 375 ml of methylene chloride at a flow-rate of 3 to 4 ml/min. The columns were then equilibrated at a flow-rate of 3 ml/min with 300 ml of hexane (water-saturated), followed by 300 ml of 0.4% toluene (water-saturated) in hexane, the solvent system for TCDD isomer separations. At this state of column activity an authentic standard of 2,3,7,8-TCDD had a retention time of 13 to 14 min. When the retention time decreased to less than 12 min, the columns were reactivated by treatment with 300 ml of methylene chloride. Peaks from the analysis of reversed-phase HPLC fractions were again collected in 25-ml round-bottomed flasks. Benzene (10 ml) was added, and the contents were evaporated to 100 µl in two stages as described above.

For final concentration the sample extract was placed in a 100 × 2 mm capillary tube. The closed end was inserted in a flask of carbon dioxide–acetone, and the open end was drawn out in an oxygen-enriched flame to a capillary constriction < 1 mm. The constricted tube was placed in a vacuum apparatus attached to a water aspirator, and the benzene was removed under vacuum until the liquid level almost completely disappeared[7]. The tube was then cut, and the interior wall was washed with 10 µl of benzene for analysis by GC–MS.

Step 3: Capillary GC–high-resolution MS. A Carlo Erba Fractovap Series 4160 gas chromatograph with an on-column injector was coupled to a Kratos MS50 mass spectrometer via a jet separator. For optimum transfer of organic compounds from a capillary column into the mass spectrometer it was necessary to introduce a make-up gas on the high-pressure side of the separator. Capillary columns were prepared from soda glass etched with HCl gas[8] and deactivated with Carbowax 20M. The cyanosiloxane liquid phase, OV-275, was then coated onto the column walls by the dynamic mercury plug method[9]. The GC–MS system was operated in the ion-monitoring mode under the following conditions: column temperature, 80°C for 1 min, followed by programming to 180°C at 12°C/min; interface temperature, 250°C; mass resolution, 10,000; ionizing voltage, 70 eV; trap current, 0.5 mA; monitored ions (three per analysis), m/z 319.8965, 321.8935, 327.8845 ([2,3,7,8-^{37}Cl]TCDD), and 333.9338 ([U-^{13}C]TCDD). Ions were scanned over a 300 ppm mass window at a rate of 0.3 sec/scan. The total signals in individual scans were summed in real time for display as mass chromatograms. After completion of an analysis time-averaged mass profiles could be obtained for any selected number of scans[10].

RESULTS AND DISCUSSION

In testing the reversed-phase HPLC step of the published TCDD isomer separation method[4], we found that chromatographic efficiency could be maintained when the column temperature was reduced from 50 to 40°C. In our experience a lower temperature can prolong column life by minimizing degradation of the hydrocarbon chains bonded to the silica gel surface. To avoid photochemical decomposition of TCDD solutions on exposure to UV light, benzene was substituted for chloroform as a sample injection solvent for the reversed-phase columns. By placing plugs of a high-viscosity solvent, 2-propanol, in the injection syringe before and after the benzene solvent, extra column band broadening from the injector plumbing was reduced, as judged by a 40% reduction in the peak width at half height (Fig. 1).

For the second step, normal-phase HPLC, the original procedure required that the silica gel columns be highly activated. 2,2-Dimethoxypropane was used to remove water by a reverse ketal reaction, with formation of methanol and acetone[11]. Under these conditions we achieved separation between TCDD isomers, but the peaks displayed excessive tailing, and recoveries were reduced to $\leqslant$ 20% for $\leqslant$ 1 ng of 2,3,7,8-TCDD.

To improve separation we made use of the fact that the surface activity of silica gel plays a critical role in its chromatographic properties[12,13]. This is particularly true of the microporous silica gels used for HPLC, which contain many adsorptive sites of varying activity[13]. Nonlinear adsorption of solute molecules can be controlled by adding water molecules and to a certain extent by adding organic modifiers. Since the residual water content of the new columns was not specified by the manufacturer, we treated them chemically to remove water from the silica gel surface. The activated columns were then deactivated to the required level by adsorption of water from water-saturated hexane. At this stage the columns were ready for analysis of TCDD isomers with high-purity hexane (0.003 to 0.009% water) as an eluent. When additional uptake of water impaired the chromatographic resolution, the columns were reactivated with methylene chloride.

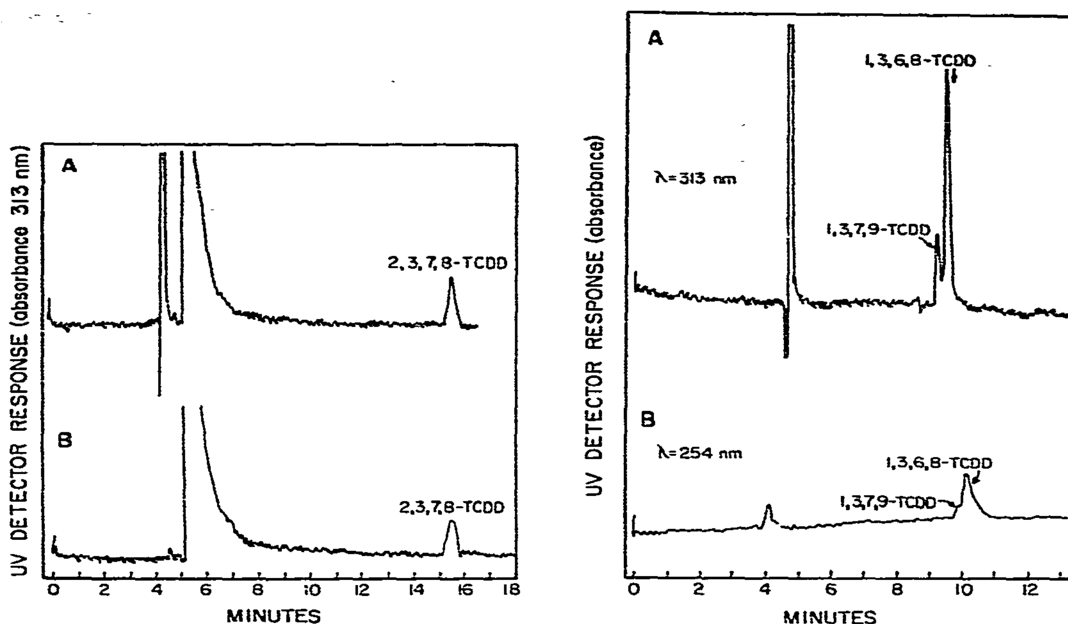

Fig. 1. Effects of injection solvents on peak shapes of TCDD isomers analyzed by reversed-phase HPLC. A 2,3,7,8-TCDD standard, dissolved in benzene (0.6 ng/μl), was drawn into the syringe between plugs in the following sequences: (A) 10 μl of 2-propanol, 30 μl of standard solution, 10 μl of 2-propanol; (B) 10 μl of benzene, 30 μl of standard solution, 10 μl of benzene.

Fig. 2. Effects of silica gel activity and eluent polarity on separation of TCDD isomers by normal-phase HPLC. (A) Water-deactivated silica gel, 0.4% toluene in hexane at 2.0 ml/min; (B) silica gel activated by chemical removal of water, hexane at 2.5 ml/min.

Organic modifiers were also necessary for optimal separation of TCDD isomers. For this purpose a mixture of 0.3% benzene and 0.4% methylene chloride (water-saturated) was added to the hexane eluent. We later found that the same effect could be produced by 0.4% toluene (water-saturated), which is simpler to prepare and less toxic.

The improvement in resolution made possible by these combined changes is illustrated in Fig. 2. Recovery of subnanogram quantities of TCDD was also increased from 20 to 80%.

In the original procedure final separation of TCDD isomers was carried out on a packed GC column, prepared by using a two-component liquid phase and a support material to which a deactivating agent had been chemically bonded[4]. These high-efficiency columns had sufficient resolution to separate the few isomers which coeluted through the two HPLC steps. However, we believe that complete separation of all 22 TCDD isomers will ultimately be achieved by high-resolution capillary GC alone. For this reason we decided to incorporate capillary columns into the modified procedure.

Testing of a number of liquid phases (including OV-17, OV-101, OV-225, Poly-MPE, and Poly-S-179) showed that separation of TCDD isomers was optimal with columns coated with the dicyanoallyl siloxane polymer, OV-275. Narrow-bore (0.25 mm I.D.) capillary columns prepared with a related cyano liquid phase, Silar 10C,

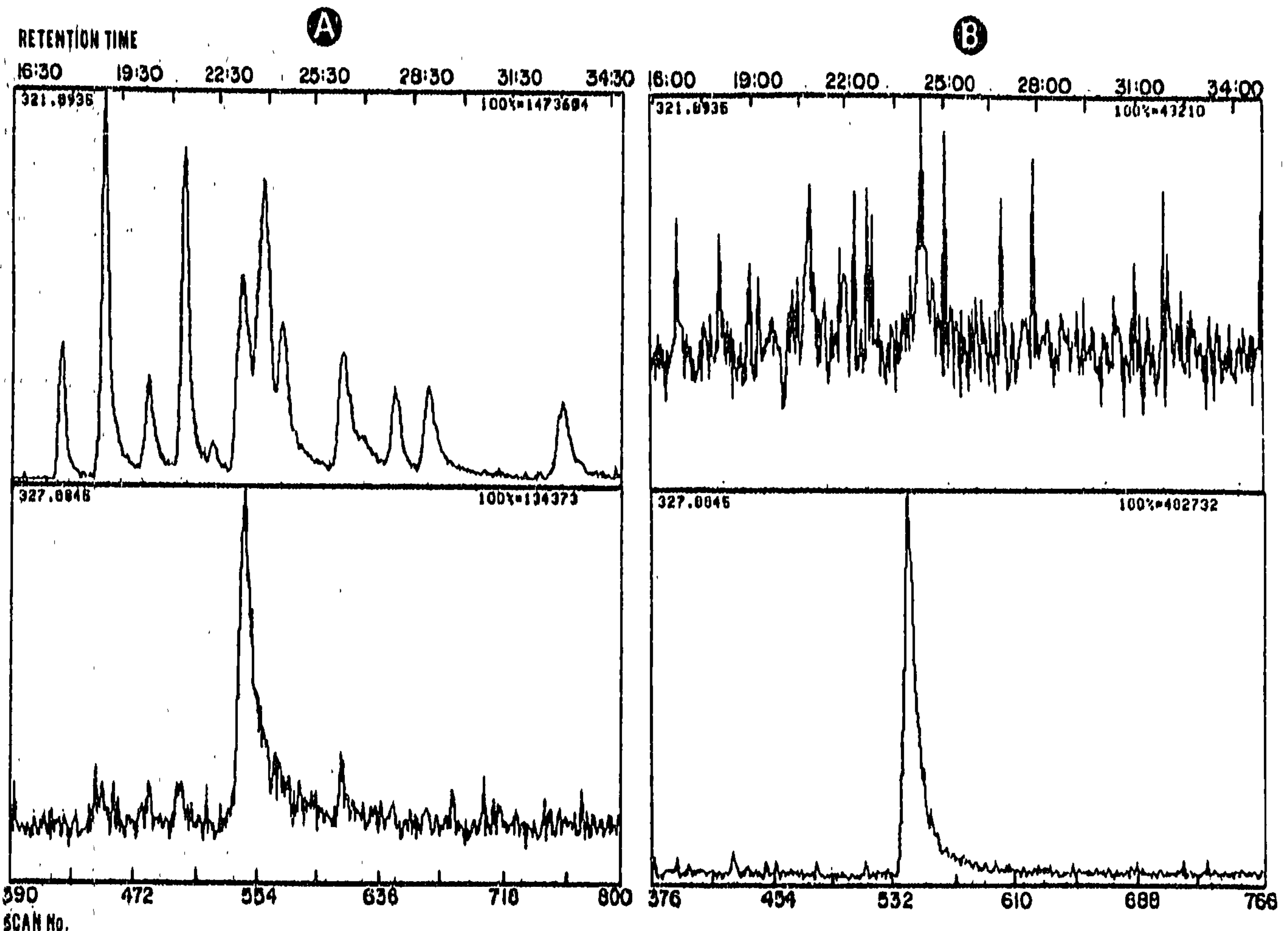

Fig. 3. Ion-monitoring mass chromatograms from analysis of TCDD isomers by capillary GC–high-resolution MS. (A) Analysis of a mixture of the 22 TCDD isomers and [2,3,7,8-^{37}Cl]TCDD; (B) analysis of the 2,3,7,8-TCDD fractions collected sequentially from reversed-phase and normal-phase HPLC of a mixture of 21 TCDD isomers (all except 2,3,7,8-TCDD) and [2,3,7,8-^{37}Cl]TCDD. Unlabeled isomers (0.5 to 5 ng/isomer) were monitored at m/z 321.8935 (upper) and the ^{37}Cl-labeled internal standard (480 pg per mixture) at m/z 327.8845 (lower). Injection aliquots (30 μl) contained 10% and 50% of sample concentrates for analyses A and B, respectively.

TABLE I

TCDD ISOMER RETENTION INDEXES

TCDD isomer	*Relative retention index*		
	*Reversed-phase HPLC**	*Normal-phase HPLC**	*Capillary GC (OV-275)***
1,2,6,7/1,2,8,9	0.853	1.471	1.208
	0.853	1.689	1.365
1,2,6,8/1,2,7,9	0.953	1.191	1.037
	0.997	1.240	0.963
1,2,6,9	0.833	1.670	1.170
1,2,7,8	0.956	1.240	1.118
1,3,6,8	1.100	0.963	0.795
1,3,6,9/1,4,7,8	0.950	1.294	0.992
	0.963	1.183	0.923
1,3,7,8	1.068	1.001	0.896
1,3,7,9	1.100	0.933	0.838
1,4,6,9	0.833	1.582	1.212
2,3,7,8	1.000	1.000	1.004
1,2,3,6/1,2,3,9	1.027	1.331	1.036
	1.016	1.331	1.130
1,2,3,7/1,2,3,8	1.007	1.086	1.013
	1.007	1.100	1.021
1,2,4,6/1,2,4,9	0.983	1.289	1.009
	0.983	1.367	1.014
1,2,4,7/1,2,4,8	1.013	1.114	0.928
	1.013	1.159	0.930
1,2,3,4	1.132	1.238	1.002

* Retention time relative to absolute retention time of 2,3,7,8-TCDD.

** Retention time relative to absolute retention time of [^{13}C]2,3,7,8-TCDD and [2,3,7,8-^{37}Cl]TCDD.

were recently reported[5] to separate 2,3,7,8-TCDD from the 21 other TCDD isomers by GC alone. Except for 1,4,6,9-TCDD the elution order with the Silar 10C column[5] is identical to that with our OV-275 columns (Table I). Therefore it is conceivable that 2,3,7,8-TCDD could also be separated from the other isomers on a narrow-bore OV-275 column alone. However, the on-column injection system of our GC instrument requires wide-bore capillary columns ($\geqslant$ 0.33 mm I.D.), which have lower resolution than narrow-bore columns. With the present instrument we cannot realize the ultimate potential of OV-275 for TCDD isomer separation.

As with the original procedure the retention indexes from the modified HPLC cystems and GC column provide a unique fingerprint for each TCDD isomer (Table I). However, for seven pairs of isomers produced in the same reaction, retention indexes could not be assigned to individual compounds without additional structural information. The order of elution of TCDD isomers from the HPLC columns is in general agreement with that reported previously. Any variations are probably a result of modifications in the chromatographic procedure.

Since our major objective was distinct identification of 2,3,7,8-TCDD, we prepared two mixtures of TCDD isomers, one complete and one without 2,3,7,8-TCDD. An internal reference, [2,3,7,8-^{37}Cl]TCDD, was included in each mixture. As shown

by the signals at *m/z* 321.8935, the 22 isomers were separated into 12 peaks by capillary GC–high-resolution MS (Fig. 3A). Five isomers (1,2,3,7/1,2,3,8; 1,2,4,6/1,2,4,9 and 1,2,3,4) elute close to 2,3,7,8-TCDD (Table I) and may not be separated from the 2,3,7,8-TCDD peak. However, the retention time for 2,3,7,8-TCDD can be determined from the response at *m/z* 327.8845 for the internal standard.

The mixture which contained all isomers except 2,3,7,8-TCDD was passed through the reversed-phase HPLC system, and the fraction corresponding to the retention time for 2,3,7,8-TCDD was collected. This fraction was further analyzed by normal-phase HPLC, and the 2,3,7,8-TCDD fraction was collected for final analysis by capillary GC–high-resolution MS. There was a peak at *m/z* 327.8845 for the internal standard, as expected, but at *m/z* 321.8935 there was only one trace signal from the 21 TCDD isomers (Fig. 3B). The relative retention index for this *m/z* 321.8935 signal (1.019) was close to that obtained for one isomer from the pair 1,2,3,7/1,2,3,8-TCDD. Based on the retention indexes for the two HPLC columns this isomer would appear to have the greatest potential for interference with 2,3,7,8-TCDD. However, after correction for recovery of [2,3,7,8-^{37}Cl]TCDD (45%) the trace signal accounted for only 0.5% of the isomer added to the mixture. In the absence of any other signals at *m/z* 321.8935 even greater removal efficiency must have been achieved for the remaining 20 isomers.

With additional extraction and preliminary clean-up steps the modified TCDD isomer separation procedure is being used in our laboratory for determination of 2,3,7,8-TCDD in a variety of environmental samples.

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