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REVERSED-PHASE LIQUID CHROMATOGRAPHY OF THE TWENTY-TWO TETRACHLORODIBENZO-*p*-DIOXIN ISOMERS ON PYRENYLETHYL- AND OCTADECYLSILYLATED SILICA GEL COLUMNS

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SUMMARY

To purify the tetrachlorobenzo-*p*-dioxins synthesized for chemical and biological reference standards, reversed-phase liquid chromatography on pyrenylethyl- and octadecylsilylated stationary phase (PYE and C_{18}) columns was employed with 100% methanol. The pyrenylethyl phase satisfactorily separated isomers resulting from mixtures of reaction products which had not been adequately separated with conventional C_{18} or silica gel. The use of a single chromatographic mode, liquid chromatography, and a single mobile phase with columns of distinctly different properties, C_{18} and PYE, separates 20 of the 22 isomers from each other and from the 1246/1249 pair. (The observed properties of the two columns afford insight into steric and polarizability differences among isomers.)

INTRODUCTION

The properties and epidemiological distribution of many chlorinated aromatic compounds have been of public health concern. Polychlorinated dibenzodioxins (PCDDs) and dibenzofurans rank high in this concern. The chemicals are apparently widespread in the environment[1–3]. Lack of sufficient quantities of pure compounds, however, has impeded investigations of their biological, chemical, and physical properties. The Centers for Disease Control/Toxicology Synthesis Laboratory is engaged mainly in the synthesis and purification of these and other polyhalogenated compounds of environmental and toxicological interest.

The 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (2378-TCDD) isomer is extremely toxic to many species of laboratory animals, far more so than any man-made compounds tested thus far[1,2]. To determine whether or not the human body burden of 2378-TCDD is associated with adverse health effects, the toxic isomer is being

quantified in serum and fat samples of humans[3]. Due to its high sensitivity and specificity, the gas chromatograph with mass spectrometer detection is the laboratory instrument system best suited for analyzing samples suspected of being contaminated with TCDDs. Detection and quantification of specific TCDD isomers are based on chromatographic indices and response factors that require calibration with authentic compounds of certified purity. Therefore, CDC projects included the synthesis[4], purification, and structural characterization of the 22 isomers. Synthesis procedures for TCDD involved union of potassium salts of two appropriately chlorinated phenols or of chlorinated catechol with chlorinated benzene, which resulted in appreciable quantities of more than one isomer because of alternate orientations of reactants and the Smiles rearrangement.

We have milligram quantities of chlorinated dioxin congeners to separate and chromatography seemed most amenable to this scale. Gas chromatography (GC) with packed or capillary columns did not afford the separation of all the TCDD isomers[5,6]. Moreover, the GC columns are not suitable for preparative separations. It would be convenient to develop a separation of all TCDD isomers in synthetic mixtures by liquid chromatography (LC) using a single mode. The combination of normal-phase and reversed-phase (RP) LC was examined at μg quantities[7–9] but difficulties were encountered in the separation of 1236/1239, 1237/1238, and 1247/1248.

It has been reported that the 2-(1-pyrenyl)ethylsilylated silica phase (PYE) showed a widely different selectivity from octadecylsilylated silica gel (C_{18}) in RPLC and proved to be useful for the separation of isomers of unsaturated compounds[10,11]. It has been difficult to separate isomers which were produced together during synthesis in large enough quantities (milligrams) for use as analytical reference standards and in quality control materials. In our previous work, TCDD synthesis products were fractionated by RPLC. Eluent volumes containing UV-absorbing species (detected at 313 nm) were fractionally collected from a C_{18} column with 85–100% acetonitrile[4]. Several of these fractions contained more than one isomer. After solvent evaporation, the residual compounds from the various fractions were characterized by gas chromatography–mass spectrometry (GC–MS)[12], proton nuclear magnetic resonance spectrometry (NMR)[12], and Fourier Transform infrared spectroscopy (FT-IR)[13]. Determinations of retention indices in various chromatographic modes also help to document authenticity. Here we report the isolation of TCDD isomers from all of the synthetic mixtures but one by RPLC using PYE. By combining the two systems, PYE and C_{18}, 20 of the 22 isomers could be isolated. This approach, LC mode alone with a single, simple mobile phase and two columns of distinctly different retention properties, greatly simplifies the separation of the TCDD isomers.

EXPERIMENTAL*

Safety

Persons attempting laboratory manipulations of polychlorodibenzo-*p*-dioxins (PCDDs) should be aware of their high toxicity and be familiar with recommended procedures for safe handling and disposal.

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* Use of trade names is for identification only and does not constitute endorsement by the Public Health Service or the U.S. Department of Health and Human Services.

Samples

The 22 TCDDs were synthesized as previously reported[4]. Table I lists the isomer compositions of the aqueous acetonitrile/C_{18} fractions collected. Each sample was transferred to a vial in toluene, dried, and dissolved in 1,2-dimethoxyethane for RPLC injection.

GC–MS

For capillary GC the Hewlett-Packard 5840 was used with columns from Supelco (SP 2330) and J&W (DB-5). The columns, 60 m × 0.25 mm I.D., 0.20 μm d_f, were eluted with a helium flow at 23 cm/s and the oven temperature was programmed from 170°C to 220°C at 20°C/min and held. A VG mass spectrometer, ZAB-2F, was interfaced with the gas chromatograph. It was operated with the source at 200°C, 1500 resolution, 1 s/decade, electron impact mode, and mass range 50–700.

NMR

The Varian XL 300 NMR spectrometer, with a 7.0-T superconducting magnet, was used to obtain spectra of each TCDD sample in [$^2H_6$]acetone. Structural assignments were made by interpretations of the spectra[12].

RPLC

The system included a pump (Waters 6000A), injector (Rheodyne, 100 μl), a guard column (50 mm × 4.6 mm I.D.), and an analytical column (150 mm × 4.6 mm I.D.) of Cosmosil 5-PYE [2-(1-pyrenyl)ethylsilylated silica gel, particles sized at 5 μm,

TABLE I

RELATIVE RETENTION TIMES (RRT[§]) OF TCDD ISOMERS IN SYNTHETIC PREPARATIONS

TCDD isomer	*RRT (methanol elution)*			*RRT (hexane elution Silica gel***)*
	Pye	*C_{18} combination**	*C_{18} (DuPont ODS)***	
1234	1.152	1.065	1.15	1.248
1278	1.272	0.888	1.02	1.288
1378	1.067	0.964	1.01	1.000
2378	1.000	1.000	1.00	1.000
1239/1236	1.027/1.177	0.958	1.04/1.00	1.350/1.356
1247/1248	0.976/1.052	0.939	1.04	1.154/1.199
1237/1238	0.952/0.990	0.971	1.02	1.100/1.128
1246/1249	1.047	0.917	1.00	1.328/1.441
1268/1279	0.870/0.952	0.942	0.87	1.238/1.291
1368/1379	1.237/1.047	1.068/1.038	1.16/1.09	0.997/0.940
1267/1269/1289	0.818/0.928/0.693	0.803/0.780/0.803	0.88/0.87/0.88	1.627/1.702/1.795
1369/1268/1279	1.217/0.870/0.953	0.942	0.96	1.220/1.238/1.291
1478/1278	0.865/1.272	0.888	0.96/1.02	1.340/1.288
1469/1269	0.988/0.928	0.780	0.96	1.497/1.702

* Polymeric and monomeric C_{18} columns in series, see text.

** Calculated from ranges, ref. 7.

*** From ref. 7.

§ Relative to 2378-TCDD.

Nakarai Chemicals, Kyoto, Japan]. Alternatively, we used a C_{18} guard column and either or both of two analytical columns connected in series (Cosmosil C_{18}, Nakarai Chemicals). One of the latter columns was prepared with a monofunctional octadecyl bonding reagent and the other with the trichloro, resulting in a monomeric and a polymeric C_{18}. The mobile phase was 100% methanol (Burdick & Jackson) at a flow-rate of 1.0 ml/min at room temperature. Other mobile phases tested consisted of organic solvents (Burdick and Jackson) or mixtures with deionized water, degassed by sonication. In some separations of 0.5-mg quantities of solutes, a Nakarai PYE column (250 mm × 10.0 mm I.D.) was employed with methanol at 3.0 ml/min. Compounds in the eluate were detected at 237 nm with either the Waters 990 photodiode array detector (courtesy John McKay, Waters Assoc.) or a spectrophotometer (Shimadzu UV-260).

Procedure

Aliquots of each of the fractions listed in Table I were dried under a stream of nitrogen and resuspended in 1,2-dimethoxyethane (glyme, Burdick and Jackson) containing a small amount of aqueous sodium nitrite for t_0 measurements. Injections of 10–20 μl containing 3–15 μg of the TCDDs were made in duplicate within a four-day period on PYE and C_{18} columns at room temperature. Retention times and UV spectra were recorded. Values for k' were calculated from chromatograms according to the equation: $k' = (t_R - t_0)/t_0$.

RESULTS AND DISCUSSION

TCDDs were synthesized from alkali metal salts of chlorinated catechol with either a chlorinated benzene or *o*-chloronitrobenzene. The reactions resulted in mixtures of TCDD isomers in most cases. In previous work, partial purification was effected with 85–100% acetonitrile in water on a C_{18} column[4]. However, many of these fractions contain more than one isomer. Conventional chromatographic approaches including GC[5,6], RPLC on C_{18}[7,8], and liquid–solid chromatography on silica[7,8], were reported to be unsuccessful for the separation of all the TCDD isomers.

Because of the need for pure compounds as standard reference material and the observed differences in PYE and C_{18} retention of aromatic compounds[10,11], we attempted RPLC separation of TCDD isomers by using both PYE and C_{18} columns. Twenty of the 22 TCDD isomers could be individually separated from synthesis product mixtures by RPLC with the 15-cm PYE column and methanol at 1.0 ml/min. Pure isomer fractions of consecutively eluting isomers with inadequate separation factors (relative retention indices of < 1.04) were obtained by collecting the early fraction of the first and the last half of the second peak (UV detector).

To evaluate the PYE column and retention mechanisms relative to the commercially available C_{18}, we measured isomer t_R values with methanol on C_{18} also. The elution orders of the TCDDs on C_{18} with methanol have been reported in the literature[7], and we tabulated these with the ones we obtained on the two C_{18} columns in series as well as those on PYE (Table I). Whether the two C_{18} columns in series were from DuPont (250 mm × 6.5 mm I.D.), with methanol at 2.0 ml/min or from Nakarai (150 mm × 4.6 mm I.D.), with methanol at 1.0 ml/min, elution of all 22 isomers occurred in 10–18 min with about 10 recognizable fractions. However, there were some

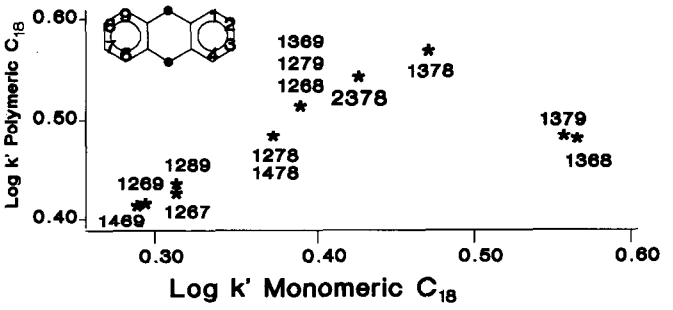

Fig. 1. Graph of log k' of 2:2 substituted TCDDs. Mobile phase, methanol; flow-rate, 1.0 ml/min; stationary phase, C_{18}.

differences in the two systems, presumably caused by the differences in the stationary phases. We used a monomeric and a polymeric C_{18} in series to get adequate retention of the TCDDs with methanol elution. Cosmosil C_{18} columns, especially the polymeric type, resulted in the late elution of 2,3,7,8-TCDD in comparison with the Zorbax ODS. The polymeric and monomeric C_{18} in the present study showed similar retention characteristics for the 2:2-substituted TCDDs except for two isomers (1368 and 1379) as can be seen in Fig. 1.

As can be seen in Table I, a number of the TCDD pairs which were coproduced in synthesis were not separated by the C_{18} phases. Neither the monomeric nor the polymeric C_{18} phase was able to separate synthetic mixtures, namely 1246/1249, 1267/1289, 1237/1238, 1247/1248, and 1278/1478. The normal-phase LC (silica gel/hexane) retention values from the literature[7] are also included in the table to show the lack of adequate separation of isomers by any one of these systems.

Using one 150 mm × 4.6 mm I.D. PYE column, elution occurred between 27 and 55 min, and every isomer mixture but one fractionated into individual components. The 22 TCDDs were separated into at least eight distinct fractions by one PYE column. The chromatograms for two of the three isomer mixtures obtained with PYE are shown in Fig. 2. In this study PYE was superior to C_{18} in resolution and selectivity for the TCDDs.

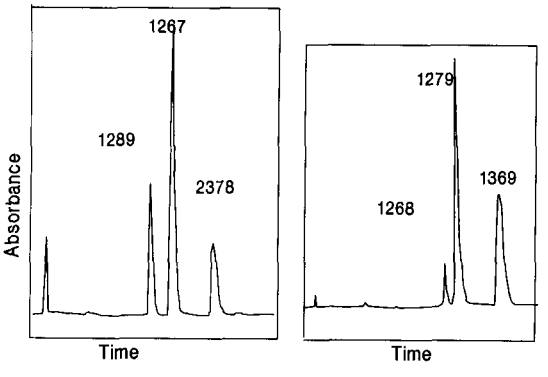

Fig. 2. Chromatograms of TCDD isomer mixtures on a PYE column with methanol as mobile phase. Flow-rate, 1.0 ml/min.

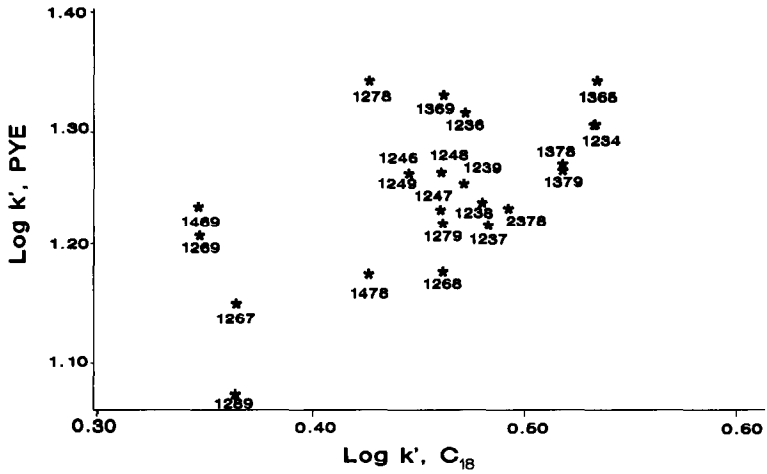

Fig. 3. Chromatographic index (log k') of TCDD isomers on C_{18} and PYE columns. Mobile phase, methanol.

Certain isomers seem to be preferentially retained by PYE, as can be seen when log k' on PYE is plotted against log k' on C_{18} (Fig. 3). PYE is less hydrophobic than C_{18} and can participate in more specific interactions, such as charge transfer[10,11,14]. Both C_{18} and PYE columns selectively retard planar compounds but have certain differences in steric requirements[10,11]. Among the TCDDs, C_{18} shows the least affinity for those congeners having chlorine substituents in the *para* positions (necessarily alpha to the C–O bond); affinity increases with *ortho* chloro substitutions alpha to C–O and becomes greater for *ortho* beta to C–O and the greatest for *meta* chloro patterns. TCDDs with these substituent patterns are diverse in affinities for PYE. However, the C_{18} correlation is to be expected in reversed phase because steric congestion between *ortho*-chlorine substituents lowers the hydrophobicity relative to

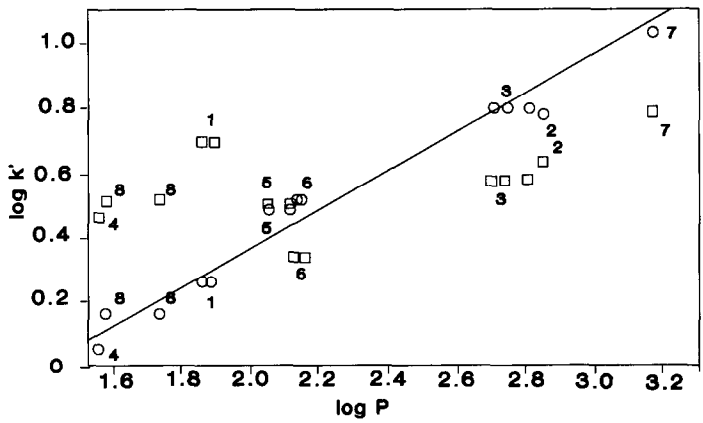

Fig. 4. Graph of column retention (log k') *versus* log P (from ref. 14, includes all available values per compound) of monosubstituted benzenes. Substituents: 1 = NO_2; 2 = Cl; 3 = CH_3; 4 = CN; 5 = OCH_3; 6 = H; 7 = C_2H_5; 8 = $COCH_3$. (○) C_{18}; (□) PYE.

the *meta* positions. Because the effectiveness of a hydrophobic group (chlorine on benzene ring) vicinal to hydrophilic functional groups has been shown to decrease[14], we assume that the hydrophobic character of TCDDs is minimized in isomers substituted 1,4 because of proximities of chlorine to oxygen.

The effect of the hydrophobic character of solutes is greater on C_{18} retention, as is expected, considering Fig. 4, the plot of k' *versus* log P of substituted benzene. The *n*-octanol–water partition coefficient, P, has been routinely used to describe the hydrophobicity of molecules. Fig. 4 includes values (and for some compounds more than one was available and, therefore, plotted) for the monosubstituted benzenes (data from the two different phases are indicated by different symbols). With C_{18} there is a high correlation between the logarithms of the capacity factors in RPLC and partition coefficients in liquid–liquid two-phase systems, signifying an increasing affinity with hydrophobicity. There is a poor correlation of these parameters with PYE, with all other conditions held constant. This indicates the contribution of other specific interactions of the solute molecules with PYE.

The characteristic attributes of the two columns are further exposed when log k' on PYE *versus* log k' on C_{18} is plotted for monosubstituted and disubstituted benzenes. We find increasing retention on PYE with electron-withdrawing substituents, especially in the *para* and *meta* positions in the disubstituted isomers (Fig. 5). Distortions of the aromatic sextet electronic configuration, especially by *para* or *meta* substituents, apparently enhance charge-transfer complexing with the pyrene moieties, which are fused-ring systems with partial bond fixations[15]. Molecules with regions of relative abundance and paucity of electron density afford donor and acceptor pockets for charge transfer. Furthermore, we see that the retention of disubstituted benzenes on C_{18} could be predicted from the results with monosubstituted benzenes. Fig. 5

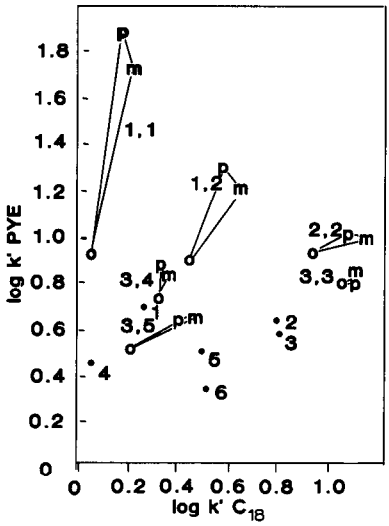

Fig. 5. Retention (log k') of substituted benzenes on PYE and C_{18}. Mobile phase; methanol–water (60:40). Substituents: 1 = NO_2; 2 = Cl; 3 = CH_3; 4 = CN; 5 = OCH_3; 6 = H. o = *ortho*; m = *meta*; p = *para*; ● = mono.

illustrates that the k' of *ortho*-substituted benzene is smaller than that of *meta*- or *para*-substituted benzene on C_{18}, as expected. The graph of TCDDs (Fig. 3) shows the same trends for molecules with two dichloro-rings. It is readily seen that 1469, the TCDD of least planarity, is eluted first from C_{18} but interacts rather strongly with PYE. The latter interaction may be due to easy accommodation of charge transfer as a result of the flexibility and spatial arrangement of donor and acceptor regions relative to those available on the molecular principals on PYE. The earliest eluting TCDD on PYE is 1289, and yet 1278 is one of the latest. The substitution pattern differs in only one position, which greatly affects retention of the species on PYE and only minimally affects retention on C_{18}. Since both steric and electronic interactions have been attributed to PYE[10] and the less polar of the two, 1278-TCDD, is the more planar and probably the more amenable to charge transfer, PYE retention is greater.

The pair of isomers, 1246/1249, that ordinarily co-elute on both C_{18} and PYE analytical scale systems, exhibited two distinguishable maxima when only a few micrograms were chromatographed with 5% dichloromethane in methanol on the 250-mm semipreparative column. By NMR quantification, the purity of each isomer is about 80% in the fractions collected (front and rear of peaks).

Because of the difficulty in separating 1247 and 1248 from synthesis mixtures, IR spectral features of the pair had been determined by spectral subtraction before the separation on the semiprep scale PYE column[13]. After the individual isomers were characterized by FT-IR and NMR spectroscopists, isomer assignments were contradictory[16,17] for the early (and late) eluter on the chromatogram of Fig. 6. If the same retention mechanisms that separate 1237 from 1238 operate on PYE with 1247/1248, then these data support assignment of 1247 to the earlier eluter in agreement with FT-IR calculations[16]. Further support for this prediction comes from a consideration of the more stable intermediate in synthesis, based on degrees of dichlorophenol proton dissociation, which predicts a relatively greater yield of 1248 and has been used to accurately predict which products would be most numerous in two other TCDD synthetic reactions. The second eluting compound (Fig. 6) displays a higher absorbance peak at 237 nm, as expected for the isomer in greater abundance in the

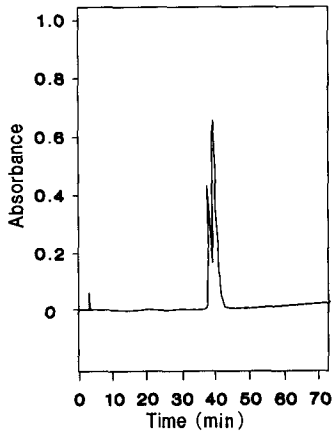

Fig. 6. Chromatogram of the 1247/1248 TCDD isomer mixture on PYE.

mixture. Crystal growth experiments are under way for X-ray crystallography analysis to assess the correct assignments.

Several mobile phase compositions were attempted in the 1247/1248 separation on PYE. Methanol seems to be the most effective mobile phase for separation of this isomer pair, although slightly improved resolution was obtained with the 25-cm column by adding 5% dichloromethane. The separation needs further optimization.

PYE retention of the more highly chlorinated dioxins has been preliminary tested. The hexachlorinated and octachlorinated congeners are not retained by PYE, and certain of the pentachorinated compounds are retained to a lesser degree than some of the TCDDs. In general, isomeric PCDDs with 5–8 chlorine atoms on the nucleus were discriminated by PYE even more than the TCDDs were. There is also a dramatic difference in elution orders on C_{18} and Pye. With PYE, the bonded planar moieties in the stationary phase apparently exclude the bulkier molecules from sequestration between the planes of the pyrene moieties. Study of the separation of these PCDDs as well as other isomeric compounds is currently under way[18]. Models of PCDD structures suggested by molecular mechanical calculations[19], might facilitate studies on structural effects and hydrophobicity among substituted dioxins such as has been accomplished with benzenes[20] so that hydrophobic effects might be dissected from other interactions with PYE.

We conclude that the availability of RPLC on PYE greatly facilitates the separation of PCDD synthesis products. Development of both the theory and practice of PCDD differentiation, although far from complete, is significantly enhanced by RPLC using PYE.

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REFERENCES

1 J. A. Moore, E. E. McConnell, D. W. Dalgard and M. W. Harris, *Ann. N.Y. Acad. Sci.*, 320 (1979) 151.
2 E. E. McConnell, J. A. Moore, J. K. Haseman and M. W. Harris, *Toxcol. Appl. Pharmacol.*, 44 (1978) 355.
3 D. G. Patterson, Jr., L. Hampton, C. R. Lapeza, Jr., W. T. Belser, V. E. Green, L. R. Alexnder and L. L. Needham, *Anal. Chem.*, 59 (1987) 2000.
4 L. T. Gelbaum, D. G. Patterson and D. F. Groce, in C. Rappe, G. Choudhary and L. H. Keith (Editors), *Chlorinated Dioxins and Dibenzofurans in Perspective*, Lewis Publishers, Chelsea, MI, 1986, pp. 479–483.
5 P. W. O'Keefe, R. Smith, C. Meyer, D. Hilker, K. Aldous and B. Jelus-Tyror, *J. Chromatogr.*, 242 (1982) 305.
6 M. Oehme and P. Kirshmer, *Anal. Chem.*, 56 (1984) 2754.

7 T. J. Nestrick, L. L. Lamparski and R. H. Stehl, *Anal. Chem.*, 51 (1979) 2273.
8 D. Wagel, T. O. Tiernan, B. Ramalingam, J. H. Garrett and J. G. Solch, in C. Rappe, G. Choudhary and L. H. Keith (Editors), *Chlorinated Dioxins and Dibenzofurans in Perspective*, Lewis Publishers, Chelsea, MI, 1986, pp. 305–327.
9 M. Swerev and K. Ballschmiter, *Chemosphere*, 15 (1986) 1123.
10 N. Tanaka, Y. Tokuda, Y. Iwakuchi and M. Araki, *J. Chromatogr.*, 239 (1982) 761.
11 N. Tanaka, Y. Tachibana and M. Araki, *Nippon Kagaku Kaishi*, (1986) 993.
12 L. T. Gelbaum, D. G. Patterson, D. L. Ashley and D. F. Groce, *Chemosphere*, 17 (1988) 551.
13 J. Grainger and L. T. Gelbaum, *Appl. Spectrosc.*, 41 (1987) 809.
14 N. Tanaka and E. R. Thornton, *J. Am. Chem. Soc.*, 99 (1977) 7300.
15 F. A. Carey and R. J. Sundberg, *Advanced Organic Chemistry*, Part A, Plenum Press, New York, 2nd ed., 1984, Ch. 9.
16 J. Gainger, E. R. Barnhart, D. G. Patterson, Jr. and D. Presser, *Appl. Spectrosc.*, 42 (1988) 321.
17 D. L. Ashley, V. V. Reddy and D. G. Patterson, Jr., *Magn. Res. Chem.*, submitted for publication.
18 E. R. Barnhart, D. O. Ashley, V. V. Reddy and D. G. Patterson, Jr., *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 9 (1986) 528.
19 D. G. Patterson, Jr., J. Grainger and J. P. Bowen, *Chemosphere*, (1988) in press.
20 A. Tsantili-Kakoulidou, N. El. Tayar, H. Van de Waterbeemd and B. Testa, J. Chromatogr., 389 (1987) 33.