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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY IN THE ANALYSIS OF CHORINATED DIBENZODIOXINS AND DIBENZOFURANS IN CHICKEN LIVER AND WOOD SHAVING SAMPLES

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SUMMARY

High-performance liquid chromatography (HPLC) has been used to cleanup chicken liver and wood shaving samples for their subsequent analysis by mass spectrometry (MS). With a reversed-phase system and discrete fraction collection, hexa-, hepta-, octachlorodibenzodioxin and octachlorodibenzofuran have been detected in wood shavings and chicken livers to a lower level of 25 pg/g. With the use of HPLC as a cleanup tool, the MS determination both on the probe and by gas chromatography–MS resulted in an improved peak shape and a stronger more accurate signal for the dioxins and furans.

INTRODUCTION

For some time, we have been engaged in the analysis of the toxic polychlorinated dibenzodioxins (PCDD) and dibenzofurans (PCDF) in food and environmental samples. Our procedure, taken from several sources and modified to suit particular samples, used extraction with chloroform or chloroform–methanol mixtures in a neutral system similar to the extraction procedure as described by Albro and Corbett[1]. As we were interested in measuring the higher chlorinated congeners from pentachlorophenol contamination, as well as the tetrachlorodibenzodioxins, the use of an alkaline digestion[2–4] for defatting was specifically avoided. For cleanup, most of the lipid material was removed by partitioning with concentrated sulfuric acid. Further cleanup was affected by mini-columns of either Florisil or alumina which were sufficient to remove most of the polychlorinated biphenyls (PCB), DDE and remaining lipid material[3,5].

These techniques were suitable for analysing levels of PCDD and PCDF as low as 1 ng/g using gas chromatography with electron capture detection (GC–ECD). For the detection and determination of lower levels as would be found in most food and environmental samples, recourse had to be made to mass spectrometry (MS). This presented us with problems for the following reasons. The higher chlorinated dioxins, particularly octachlorodibenzodioxin (OCDD) did not pass efficiently through our GC–MS system using a separator based on diffusion through porous glass. When the

sample extract was placed directly on the probe at low levels, the MS peak shape was distorted due to background interference of co-extracted material. To overcome these problems, we investigated the use of high-performance liquid chromatography (HPLC) to purify the sample further.

This report shows the effect on the probe MS signal of the use of HPLC to purify food and environmental samples so that low levels (< 1 ng/g) can be determined. We find that HPLC with discrete fraction collection purifies the sample greatly as evidenced on the MS probe by the improved peak shape and a more accurate signal. In addition the technique is applicable to several types of dioxins and furans. At the same time, the introduction of a HPLC step increases the specificity of the measurement when used either alone with MS on the probe or in combination with capillary GC.

EXPERIMENTAL

Precautions

Chlorinated dibenzodioxins and -furans are extremely toxic substances. Personnel working with these compounds should be aware of the hazard. All work should be carried out in an isolated laboratory following a definite protocol designed to minimize possible exposure. The laboratory protocol followed at the Health Protection Branch, Ottawa, is available on request. Wastes should be treated separately from ordinary waste and be kept to a minimum. Disposal is best affected by high temperature ($>1000°C$) incineration.

Equipment

HPLC. A Waters Model 6000 chromatographic pump was used along with a Schoeffel variable-wavelength absorbance detector, Model SF-770, with an 8-μl flow cell, 10 mm pathlength and 0.3 mm I.D. tubing.

Sample injection was carried out using a Valco 6-port universal inlet injector for HPLC with a loop size of 100 μl.

Conditions. A LiChrosorb reversed-phase C_8 analytical column, 250 mm × 3.2 mm I.D., 10 μm particle size, was used with methanol–water (9:1; degassed) as the eluent at a flow-rate of 0.5 ml/min (pressure was usually less than 1000 p.s.i.). The wavelength maximum for measurement of the dioxins varied among congeners and was 233, 245, 225–245 (broad peak), 250, and 233 and 250 nm for 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), 1,2,3,6,7,8- and 1,2,3,6,7,9-hexachlorodibenzodioxin (HCDD), 1,2,3,4,6,7,8-heptachlorodibenzodioxin (HpCDD), OCDD and octachlorodibenzofuran (OCDF) (two peaks), respectively, in methanol. Routinely, a value of 235 nm was chosen at an absorbance of 0.01 a.u.f.s. for standards (5–15 ng) and 0.1 a.u.f.s. for samples.

Mass spectrometer. A Varian-Mat 311A high-resolution instrument with electron impact ionization was used. The source was maintained at 250°C with an ionization voltage of 70 eV. The resolution was at least 5,000 (10% valley) for HCDD, HpCDD, OCDD and OCDF and 10,000 for TCDD. The machine was equipped with a gold leaf probe which could be heated from 25 to 225°C in 20 sec. The single ion display response was monitored on a dual pen 10 mV recorder with range between

TABLE I

IONS MONITORED WITH MS

Peaks mentioned are base molecular ion peaks of full scan.

Dioxin	m/z monitored
TCDD	321.89
HCDD	389.82
HpCDD	423.78
OCDD	459.73
OCDF	443.74

0.01–1.0 V for full scale deflection. The total ion monitor between 0–100 V was made on the same recorder. The ions monitored are shown in Table I.

Materials

The solvents employed were chloroform, hexane, methanol, methylene chloride, and acetonitrile and were glass distilled grade. Water was doubly distilled. Anhydrous sodium sulfate (Baker analytical; J. T. Baker, Phillipsburgh, NJ, U.S.A.), Florisil (Floridin, Pittsburgh, PA, U.S.A.), alumina (A-540; Fisher Scientific, Pittsburgh, PA, U.S.A.) and glass wool were all extracted in a soxhlet with methylene chloride for 6 h to remove impurities. Sulfuric acid was Baker analytical grade. All glassware was routinely rinsed sequentially with toluene and methylene chloride before use.

Standards

Solids. TCDD was kindly supplied by Dow Chemical (Midland, MI, U.S.A.). OCDD and OCDF were purchased from New England Nuclear (Montreal, Canada); 1,2,3,6,7,8- and 1,2,3,6,7,9-HCDD were a generous gift of J. A. Moore (Research Triangle Park, NC, U.S.A.); HpCDD was obtained from R. Pike, (Canada Agriculture, Ottawa, Canada).

Solutions. GC: 0.1 ng/μl in hexane diluted from 20 ng/μl of either hexane or toluene stock solution. OCDD is slowly soluble in hexane to a maximum concentration of 20 ng/μl.

HPLC: 5 ng/μl in acetonitrile prepared by evaporation under pure nitrogen and redilution of 20 ng/μl hexane solution.

MS: 20 pg/μl in toluene diluted from 20 ng/μl hexane or toluene solution.

Procedure

Extraction. For liver samples, a homogenized ground sample (10 g) was blended with 30 ml chloroform and 60 ml methanol for 2 min. An additional 30 ml of chloroform were added and blended for 30 sec. A volume of 40–45 ml of water was added and a third blend carried out for 30 sec. The mixture was transferred to a 250-ml separatory funnel and the chloroform layer drawn off. The water and the insoluble material at the interface were returned to the blender jar and blended for 1 min with an additional 60 ml chloroform. The separated chloroform was then combined with the first fraction.

For wood shavings and litter, the ground material (10 g) was soaked with water (100 ml) for 1 h and blended with 50 ml chloroform. Shavings were filtered through a funnel containing a glass plug into a separatory funnel and litter was

centrifuged to effect phase separation. The non-chloroform phases were blended a second time with 50 ml additional chloroform and the chloroform fractions combined.

Acid partition. The chloroform phase from the neutral extraction was crudely dried by filtration under suction through a glass filter containing *ca.* 30 g disodium sulfate and the reagent rinsed with a few ml of chloroform. The organic solvent was evaporated to 7–10 ml in a rotary evaporator under vacuum at 30–40°C and 20 ml of hexane were added. After shaking and transferring to a separatory funnel, the non-hexane layers were re-extracted with a second 20 ml portion of hexane. The combined hexane phases were then shaken with 10 ml portions of conc. sulfuric acid until the acid was clear and pale yellow (2–6 times). The first extraction was shaken lightly to avoid strong emulsions. The hexane was washed with a little water, dried and concentrated to 5 ml in a rotary evaporator under vacuum at 30–40°C. The extract was finally reduced to 1 ml (not dryness) under nitrogen in a 15 ml centrifuge tube with washing of the sides of the tube during concentration.

Cleanup. Florisil column chromatography. A Pasteur pipette containing a small plug of glass wool, 5 cm (*ca.* 1 g) of Florisil, and a second plug of wool, was activated at 140°C overnight. The column was prewashed with 5 ml methylene chloride and 2 ml hexane both of which were discarded. The sample in 1 ml hexane was adsorbed onto the column and the container washed twice with 2 ml portions of hexane which were added to the column. All the dioxins and furans were eluted with 8 ml of methylene chloride which was evaporated to a small volume under nitrogen in a 15 ml centrifuge tube with washing of the walls of the container. The sample was then transferred to a 0.5 ml conical tapered vial with two to three washings of 50–100 μl methylene chloride. The organic solvent was then evaporated just to dryness under nitrogen at room temperature and the sides of the vial washed once more with a little methylene chloride and the latter taken again to dryness.

. *HPLC procedure.* To ascertain the retention time of the dioxins for sample fraction collection, a mixed standard containing 5–15 ng each of TCDD, HCDD, HpCDD, OCDD, and OCDF in 50 μl acetonitrile and 50 μl mobile phase was injected onto the column via the 100-μl loop. These amounts of standards at 0.01 absorbance gave deflections of 15–40%. The sample loop was then thoroughly cleaned with three 100 μl injections of methylene chloride followed by three 100 μl injections of methanol.

The partially purified sample in a small tapered vial was completely dissolved in 40 μl acetonitrile. A 100-μl injection syringe was charged with 20 μl of methanol-water (9:1), then 40 μl of sample in acetonitrile. The sample vial was rinsed with a further 10 μl of acetonitrile and the syringe charged with this 10 μl wash and, finally, 10–15 μl methanol–water (9:1). The entire syringe load including the sample and flush volume was injected via the loop onto the column. At the retention time of each dioxin, a fraction of 1.5–2.0 ml (3–4 min elution time) was taken, usually 0.5 min before and 0.5 min after the standard peak. The fraction was collected in a 25-ml volumetric flask containing 1 ml of hexane. The flask was then made to volume with water, shaken several times, and the hexane transferred in steps with a pre-rinsed Pasteur pipette into a 0.5 ml conical vial. The aqueous phase was extracted with an additional 1 ml of hexane. The hexane washings were taken to dryness under nitrogen and the residue put into 25–40 μl of toluene for MS.

The HPLC analytical column was reconditioned after every second or third

sample by flushing the entire system with methylene chloride for 5–10 min followed by methanol–water (9:1) for 30 min.

Mass spectrometry procedure. A standard (20 pg/μl in toluene) of the dioxin of interest was injected onto the probe, a vacuum attained and heat applied. This standard of 100 pg typically gave 50–70% full scale on a 10-mV recorder at the 0.03-V range with a total ion current of less than 10^{-8} A corresponding to 1 V on the machine meter. The same procedure was repeated for a sample and the range adjusted if necessary. Sample concentration was estimated using peak heights taken from a standard curve. Each dioxin or furan from a single food or environmental sample required a separate probe analysis. Recovery values of the above HPLC procedure were obtained using glass capillary GC–MS. The column was a 15 m wall-coated open tubular column (0.25 mm I.D.) SP-2100 operated at 200, 220 and 250°C for TCDD, HCDD, OCDD, respectively with a flow of helium of 50 cm/sec. MS resolution in this case was 1000. The glass capillary GC system was interferfaced directly to the MS source via a platinum wire.

RESULTS AND DISCUSSION

Initial experiments in the analysis of PCDD and PCDF showed that the use of a neutral extraction combined with sulfuric acid and Florisil column chromatography (especially good for separation of dioxins from PCB[3,5] was sufficient to purify food and environmental samples so that they could be measured with GC–ECD down to 1 ng/g. For lower levels, it was necessary to use MS or GC–MS. With GC–MS with packed columns, we had problems of retention–adsorption on the porous-glass separator with the higher chlorinated congeners. With the MS alone on the probe, the samples were still so dirty that the total ion monitor was often above 50 V, the peak was broad and often multiple, and suppression of signal was evident, obviating quantitative measurements. The use of a second mini-column such as alumina for further purification did not appreciably reduce the total ion output of the sample extract on the mass spectrum. To circumvent these problems, we investigated the use of HPLC to purify the samples.

The analysis of dioxins by HPLC with adsorption chromatography is restricted. These compounds elute in such a short time[6,7] that especially dry hexane as the elution solvent must be used. However, dioxins are more commonly separated and quantitated by HPLC using reversed-phase chromatography. Samples analysed have been pesticide formulations[8,9] for high levels ($> \mu$g/g), and, more recently, fish[10] where the single dioxin TCDD was measured using an elevated column temperature. When we used HPLC with reversed-phase systems, initially our standards of dioxins for retention time analyses were dissolved in methylene chloride since this solvent is one of the best for the higher chlorinated dioxins. However the use of as little as 20 μl of methylene chloride as standard solvent on our reversed-phase system caused premature elution and distortion of the dioxin peak. This effect on chromatography is illustrated in Fig. 1 for HCDD and OCDD (lower diagram for methylene chloride). When the standards were in either methanol, ethanol or acetonitrile (upper diagram), the peak shape was good and retention times consistent even when up to 50 μl of these injection solvents were used. Greater amounts of injection solvent resulted in unacceptable
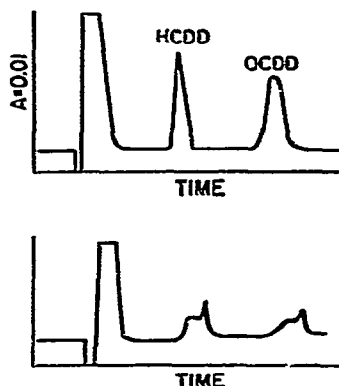
Fig. 1. Solvent effect on elution of dioxin standards (10–25 ng) on reversed-phase HPLC at 235 nm with methanol–water (9:1), 0.5 ml/min, on LiChrosorb C_3 column, 250 mm × 3.2 I.D., 10 μm. Upper diagram, with methanol or acetonitrile as standard solvent (50 μl injection); lower diagram, with methylene chloride as solvent (20 μl).

peak broadening. Acetonitrile was chosen as the solvent since it had better solvent properties than alcohol for partially purified samples.

To ensure high recovery of dioxins and accuracy of the procedure, there are several important points. Firstly, the injection valve must be thoroughly cleaned after the standards are run and before the sample is injected. Otherwise, a small amount of contamination in the valve (*e.g.* 0.1 ng corresponding to 10 pg/g) will give a positive result and preclude meaningful measurements at low levels. We have overcome this cross contamination problem by using two injection valves connected in parallel; one for the standards and one for the samples. Secondly, the retention time of the standards must be reproducible and stable otherwise recoveries will be low; constant flow, temperature, and pressure are necessary to achieve this. Thirdly, when analysing high levels of dioxins (*e.g.* in a formulation or grossly contaminated sample), the insolubility of the higher chlorinated congeners in the HPLC eluting solvent must be taken into account (*e.g.* OCDD has a solubility of 0.1 μg/ml in methanol–water (9:1)). In these cases, the analyst must make a judgement on how much sample to inject into the HPLC. Recoveries of 1 ng quantities of the three dioxins, TCDD, 1,2,3,6,7,8-HCDD and OCDD through the HPLC portion of the method alone as measured by glass capillary GC–MS at 1000 resolution are listed in Table II. The recoveries for spiked liver previously shown to be negative are good for TCDD and HCDD but lower and more variable for OCDD. The reason for the lower recovery for OCDD is not certain but the "lost" OCDD is not found in earlier or later eluting fractions of the HPLC as these were negative when monitored. This data shows there is little or no loss of dioxins through the HPLC step.

Fig. 2 illustrates the HPLC elution pattern of PCDD and PCDF in a standard solution (upper diagram) and of residues detected in a cleaned up extract of contaminated wood shavings used as poultry litter (lower diagram). With the wood shavings sample, due to the high levels present, the actual peaks for OCDD, OCDF, and HpCDD can be detected by their absorption at 235 nm. However, with liver and other samples containing low levels of contamination, this detection by UV is not possible. It is to be noted that the wood shavings sample has a UV peak on HPLC

TABLE II

RECOVERY DATA OF 1.0 ng OF THREE CHLORINATED DIBENZODIOXINS FROM THE HPLC PROCEDURE

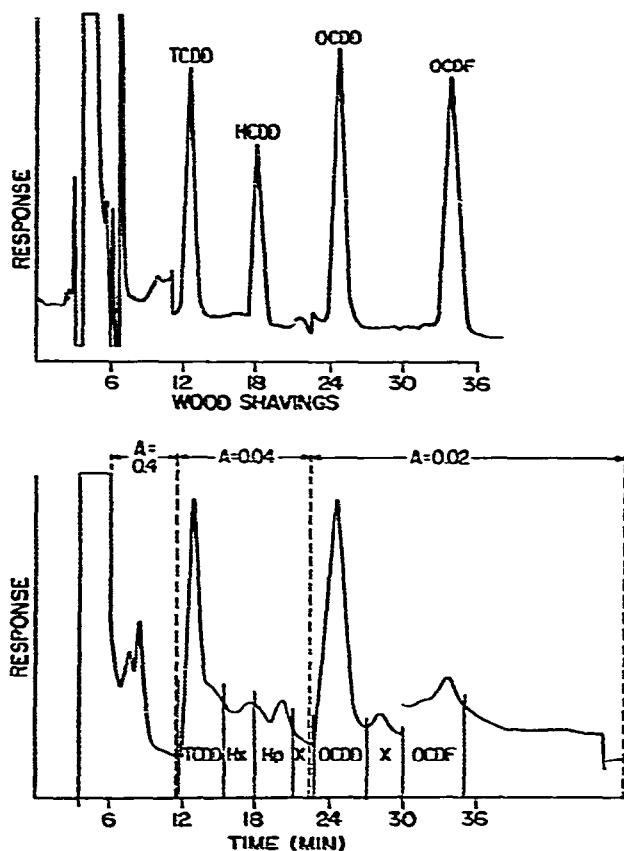| Sample		Recovery (%)		
		TCDD	1,2,3,6,7,8-HCDD	OCDD
Solvent	$\bar{x}$	100	95	92
	S.D.	7	20	16
	n	3	5	5
Chicken liver (10 g)	$\bar{x}$	102	101	94
	S.D.	13.8	14.5	26.9
	n	5	5	4

Fig. 2. Method used to collect dioxin fractions from HPLC prior to analysis by MS. Upper diagram, elution pattern of 10–25 ng standards at 235 nm and $A = 0.01$ to obtain retention times; lower diagram, actual tracing of extract of wood shavings at 235 nm and various absorbance values. Vertical lines are collection points of fractions for MS starting at about 11 min and ending at 35 min with five fractions collected. Hx = HCDD; Hp = HpCDD.

near the retention time of TCDD but subsequent analysis by MS gave a negative result.

The effect on the MS by probe analysis of the HPLC cleanup on liver samples is illustrated in Fig. 3. In this case the total ion monitor and peak shape of the MS both before (upper diagram) and after (lower diagram) HPLC is shown from three chicken liver samples taken from a flock reared on wood shavings contaminated with dioxins. The marked decrease in the total ion monitor and the improvement in peak shape is evident after HPLC. The measured levels of several dioxins and OCDF in two of the livers are listed in Table III both before and after HPLC. Before HPLC cleanup, the quenching of the signal at high resolution (attested by the high total ion current) gave either a lower value or no measureable value. This was rectified after HPLC to a more accurate value approaching more closely the standard since both the total ion decreased and the peak shape inproved. The nature of the interfering material is not certain except that it does give a strong total ion peak on MS and is removed by the above HPLC procedure. In this connection, Baughman and Meselson[11] found that the MS signal for TCDD was suppressed 50% by the presence of 5 μg of the
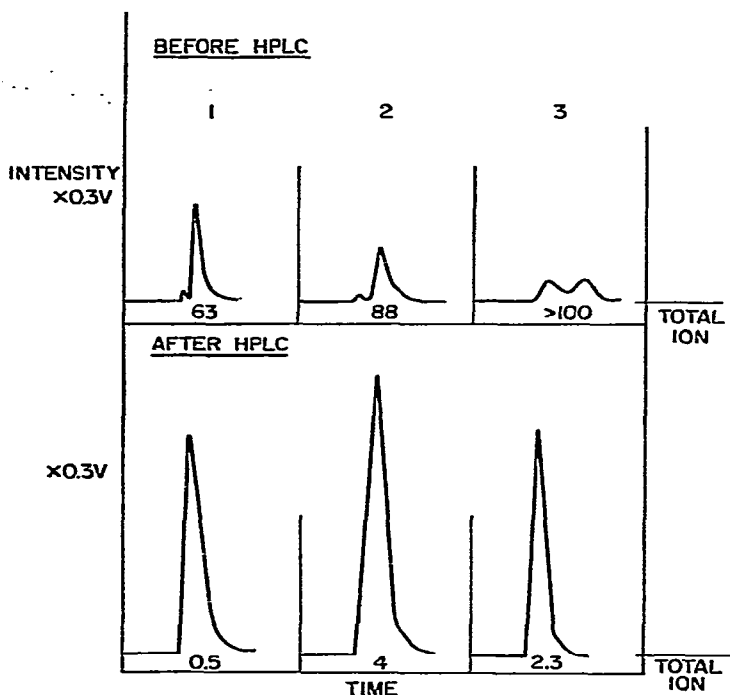

Fig. 3. Mass chromatograms of three liver samples for OCDD at m/z 459.7; Resolution 5000 both before (upper) and after (lower) HPLC. 1 g total sample extract on probe heated from 25 to 225°C in 20 sec; measurements on 10-mV recorder and total ion monitor in volts shown below peak.

TABLE III

PCDD AND PCDF LEVELS OF TWO LIVER SAMPLES ANALYSED BY MS BOTH BEFORE AND AFTER HPLC

Dashes indicate no analysis was carried out.

Compound	Before HPLC		After HPLC	
	Concentration (pg/g)	Total ion (V)	Concentration (pg/g)	Total ion (V)
Liver 1				
HCDD	0	90	43	5
HpCDD	26	40	243	3
OCDD	169	63	433	3.5
OCDF	—	—	87	3.5
Liver 2				
HCDD	—	—	35	2
HpCDD	—	—	82	3
OCDD	92	88	673	4
OCDF	—	—	130	2.3

lipid squalane. The cleanliness of the final extract was further attested to by its response on GC with flame ionization detection where no positive peaks were observed after the solvent peak. The limit of detection in 10 g liver samples varied depending both on the dioxin and the specific sample. Using a criterium of signal-to-noise ratio of 3:1, it was at least 25 pg/g and in most cases lower.

This HPLC procedure was also used in analysis of TCDD in fish. Application to over thirty samples showed that it was possible to inject large amounts (1–2 g of extracts) onto a glass capillary column directly interfaced with a MS system without affecting either GC or MS. Injection of such large amounts of concentrated extract into the MS without prior HPLC cleanup was not possible either due to poor GC resolution and rapid column deterioration or contamination of the MS source. Although the procedure of manually collecting fractions from an HPLC column and evaporating the solvent before analysis by MS or GC–MS is tedious, the technique not only adds specificity to the measurement, but also improves the purification of the sample so that low levels can be detected, and is applicable to many types of PCDD and PCDF. Until the commercial models for achieving direct interfacing of HPLC with MS are more reliable and versatile[12], we believe this described procedure has definite advantages. A possible improvement would be automatic collection of sample fractions.

REFERENCES

1 P. W. Albro and B. J. Corbett, *Chemosphere*, 6 (1977) 381.
2 R. A. Hummel, *J. Agr. Food Chem.*, 25 (1977) 1049.

3 D. A. Firestone, *J. Agr. Food Chem.*, 25 (1977) 1274.
4 L. L. Lamparski, N. H. Mahle and L. A. Shadoff, *J. Agr. Food Chem.*, 26 (1978) 1113.
5 P. W. O'Keefe, M. S. Meselson and R. W. Baughman, *J. Ass. Offic. Anal. Chem.*, 61 (1978) 621.
6 R. J. Dolphin and F. W. Willmott, *J. Chromatogr.*, 149 (1978) 161.
7 T. J. Nestrick, L. L. Lamparski and R. H. Stehl, *Anal. Chem.*, 51 (1979) 2273.
8 C. D. Pfeiffer, *J. Chromatogr. Sci.*, 14 (1976) 386.
9 C. D. Pfeiffer, T. J. Nestrick and C. W. Kocher, *Anal. Chem.*, 50 (1978) 800.
10 L. L. Lamparski, T. J. Nestrick and R. H. Stehl, *Anal. Chem.*, 51 (1979) 1453.
11 R. Baughman and M. Meselson, *Advan. Chem. Ser.*, 120 (1973) 92.
12 P. J. Arpino and G. Guiochon, *Anal. Chem.*, 51 (1979) 683A.