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An Improved Analysis for Tetrachlorodibenzo-*p*-dioxins

ROBERT BAUGHMAN and MATTHEW MESELSON

Department of Chemistry and Department of Biochemistry and Molecular Biology, Harvard University, Cambridge, Mass. 02138

*A meaningful assessment of the environmental levels of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), an extraordinarily toxic compound present as an impurity in the herbicide 2,4,5-T and in some commercial chlorophenols, can be made only by evaluating representative samples with a sufficiently sensitive analytical method. The sensitivity required is well beyond that available with current methods. We report a procedure using time averaged high resolution mass spectroscopy with a sensitivity (10^{-12} gram) suitable for such an investigation. Interference from pentachlorobiphenyl in certain materials from the environment presently limits attaining full sensitivity of the method although we are working toward a resolution of this problem.*

Our interest in the chlorodioxin problem stems from our work with the Herbicide Assessment Commission of the American Association for the Advancement of Science which was organized in 1970 to initiate a study of the effects of herbicide use in Vietnam. As one part of that investigation we are analyzing various samples from Vietnam for TCDD, a known impurity in 2,4,5-T (*1, 2, 3, 4*). This herbicide in a one-to-one mixture with 2,4-D is a component of agent Orange, the herbicide that was used most widely in Vietnam. Our aim has been to determine whether TCDD has accumulated in food chains to any significant extent.

We were surprised to find that no method existed that was sensitive enough to detect TCDD in animal tissues even after administration in some species of lethal doses. An example is the guinea pig, the most susceptible species of the few that have been tested, and therefore a good choice for establishing desirable limits of detection. The lethal single oral dose (LD_{50}) in males of this species is 0.6 μg/kg body weight

In Chlorodioxins—Origin and Fate; Blair, E.;
Advances in Chemistry; American Chemical Society: Washington, DC, 1973.

(5). This means that if all of the TCDD were retained, the level of TCDD would be less than 1 part per billion (ppb) in the whole animal. The lowest reported limit of detection for TCDD in whole tissue is 50 ppb (6). Thus, a guinea pig could be killed with TCDD, and it would be impossible to establish this fact with the analytical procedures in current use.

Such analytical procedures are clearly of little value in monitoring food chains for the buildup of TCDD. This is even more apparent if one considers the possibility of sub-lethal toxic effects and allows a margin for a safety factor. If we provide a factor of about 100 for non-lethal toxicity (6) and a further factor of 10 for a safety margin and to allow for the possible existence of species even more sensitive than the guinea pig, we would require a level of detection of $(10^{-9})(10^{-2})(10^{-1}) = 10^{-12}$ or 1 ppt (1 part in 10^{12}) for environmental monitoring. For a 1 gram sample this would require a limit of detection of 1 pg (10^{-12} gram). The limit of detection of TCDD for the electron capture detector, the keystone of current analytical procedures, is not much less than 1 ng (10^{-9} gram).

In addition to high sensitivity, a requirement for any acceptable analytical method is high specificity because at very low levels few confirmatory procedures can be used to establish the identity of a particular compound. A method which uniquely combines high sensitivity with high specificity is high resolution mass spectrometry. We have used this method as the basis for an approach which we believe will make possible a meaningful assessment of TCDD levels in the environment.

Figure 1 shows that the mass spectrum of TCDD is relatively simple. (All the work reported here was done with an Associated Electrical In-

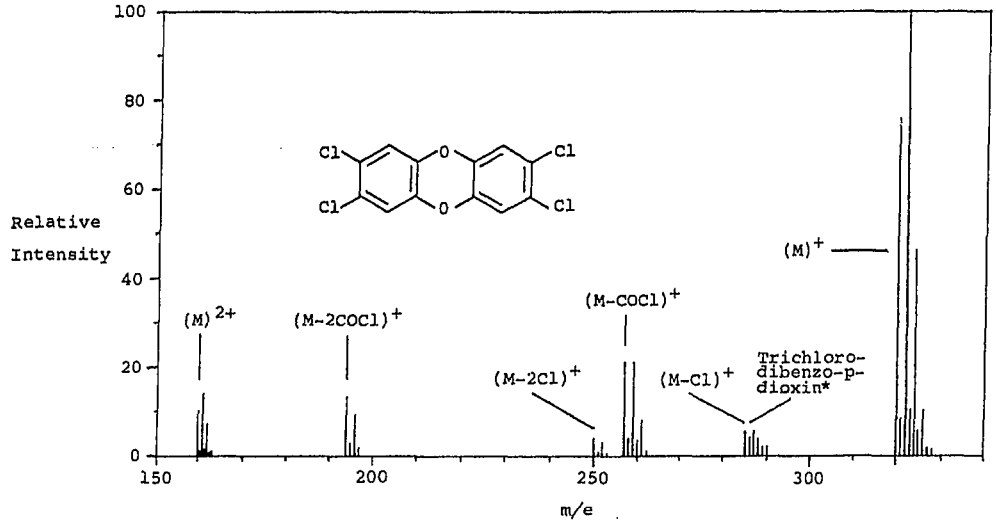

Figure 1. Mass spectrum of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). The molecular ion (M^+) is at m/e 320. Ionizing voltage 70 eV, source 150°C. Asterisk denotes impurity.

dustries MS-9 double focusing mass spectrometer.) The base peak is the molecular ion at m/e 320. As a result of the various possible combinations of the naturally occurring ^{35}Cl and ^{37}Cl isotopes in a tetrachloro compound, the signal for the molecular ion is a pentuplet with peaks at m/e 320, 322, 324, 326, and 328 with intensities in the ratio 77:100:49:10:1. In addition, the four chlorine atoms and the limited number of hydrogen atoms make the compound significantly mass deficient (the m/e 320 peak is actually 319.8956) and, therefore, relatively easily resolved from most other organic residues (for which m/e 320 would be 320.1–320.2).

First, we tried scanning the region m/e 310–330 (Figure 2). As the sample was introduced into the mass spectrometer, signals appeared at m/e 320, 322, and 324 and then, as the sample became exhausted, disappeared. Under these conditions the limit of sensitivity was on the order of 100 pg. We next reduced the scanning interval to about one third of a mass unit. This allows the detector to spend more time in the region of interest, considerably increasing the signal. At a resolution of 10,000 a series of scans was made, alternately two at 322, two at 314 perfluorotributylamine (PFA) reference peak, two at 322, etc. (Figure 3). The PFA was bled in from an external reservoir at a constant rate, providing reference peaks that remain at the same height throughout the analysis while the sample peaks rise and then fall as the sample volatilizes. This procedure with a sensitivity of about 20 pg was still not adequate.

It is possible to obtain greater sensitivity from the repeated narrow scans shown in Figure 3 by combining them to produce a single time averaged scan. Procedures accomplishing this under low resolution conditions have been reported previously (*7, 8*). Under the present conditions a system was devised for doing this using a Varian 1024 averaging computer (CAT) in conjunction with the MS–9. The result is shown in Figure 4. The signal for a pair of peaks at the limit of detection for a single scan is shown in Figure 4A, and the averaged signal from sixty scans is shown in Figure 4B. The signal-to-noise ratio is expected to improve approximately as the square root of the number of scans (*9*). With 1 min of scanning at a rate of one scan per second, the observed improvement is approximately that expected. At very fast scan rates data is inefficiently transferred to the memory of the CAT, and resolution is decreased by damping caused by the time constant of the MS–9 circuitry. In the present system this limits the maximum scan rate to four scans per second. With very short volatilization times (< 10 sec) sensitivity is decreased, perhaps in part because of decreased ionization efficiency. With volatilization times longer than about 60 sec the drift in peak position from scan to scan is large enough to decrease significantly the resolution observed in the time averaged spectrum. The optimum volatilization time is from 30 to 60 sec.

The interfacing of the CAT with the MS–9 is illustrated in Figure 5. The ions in the *m/e* region of interest, after being focussed, pass by a small magnet coil which deflects the beam back and forth over the detector slit. After passing through the slit, the ions strike an electron

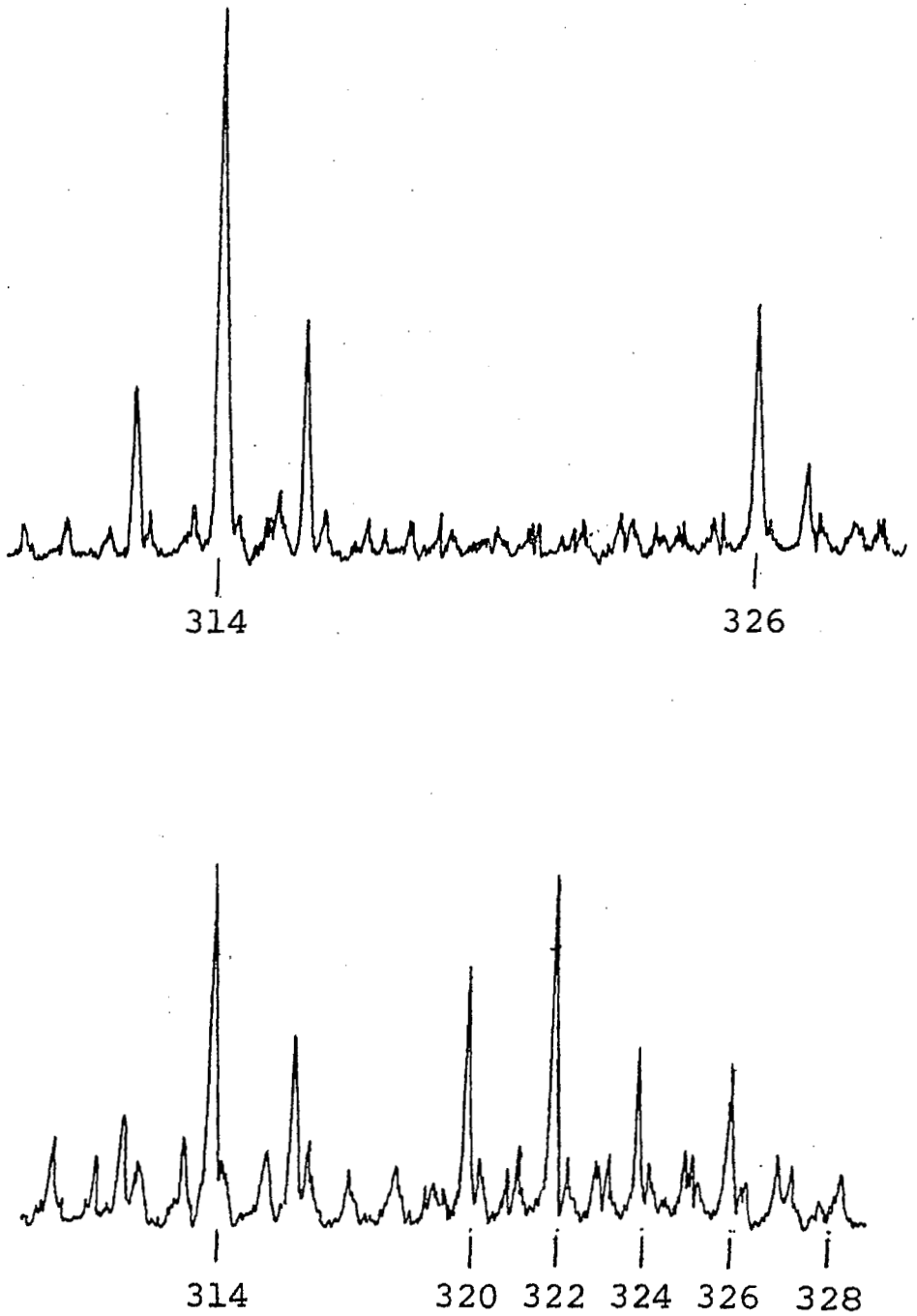

Figure 2. Repetitive scanning of m/e *310–330 (5 sec/scan). Standard conditions for this and all following figures: ionizing voltage 70 eV, accelerating voltage 8 kV, trap current 300 μA, multiplier 600, source 150°C.*

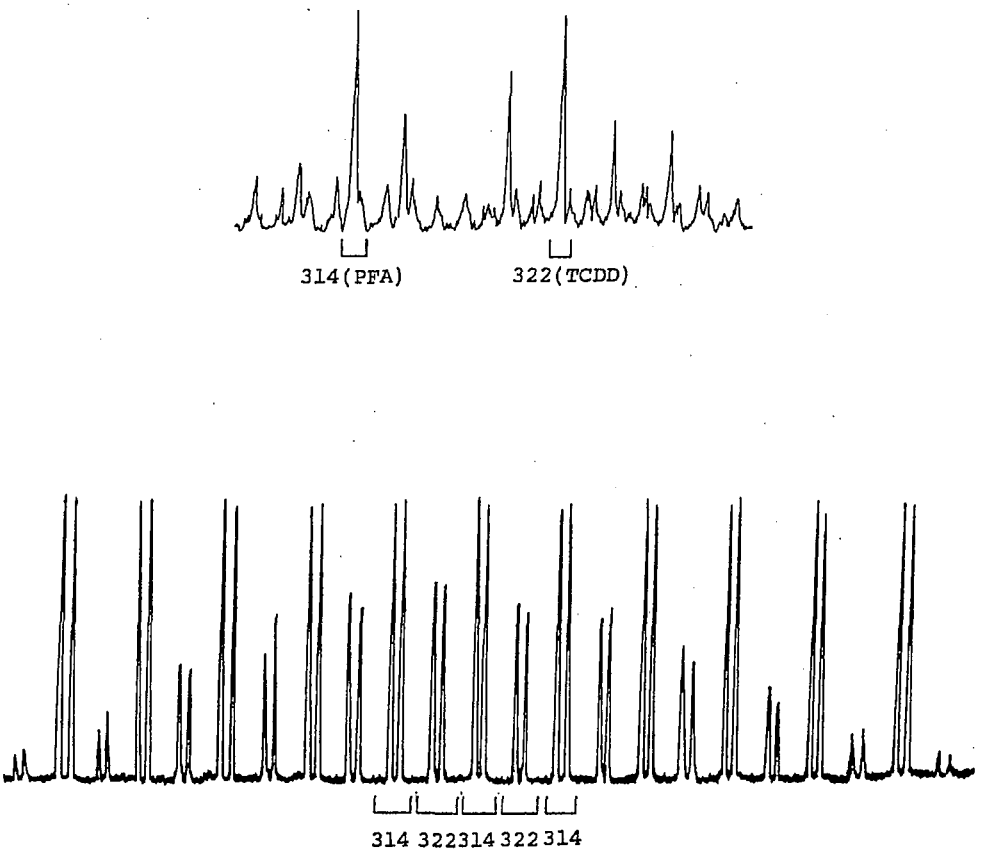

Figure 3. Alternating narrow scans

Top: regions scanned on each narrow scan. Bottom: alternating narrow scans (two half-second scans at 314, two at 322, etc.) (200 pg TCDD).

multiplier, producing a signal which is continuously displayed on an oscilloscope on the MS–9. This provides a means of monitoring each scan. Simultaneously, the signal is added to the 1024-channel memory of the CAT. An oscilloscope on the CAT continuously displays the total memory content which makes it possible to monitor the overall course of the analysis. A potential problem of phasing the beam deflection coil with the memory sweep circuit of the CAT is avoided by using the sweep voltage ramp of the CAT, *via* an amplifier and appropriate circuits of the MS–9, to drive the beam deflection coil. The coil is thus necessarily in synchrony with the CAT.

The procedure we have adopted for introducing samples into the MS–9 is shown in Figure 6. It provides reproducible analyses at a high level of sensitivity. The sample tubes are made from 1 mm id melting point capillaries. A Hamilton 10-μl syringe is used to introduce a 3–4 μl portion of the residue into the sample tube. With a small flame the sample tube is drawn out just above the level of the liquid to produce a capillary constriction about 20 mm long. The solvent is then

removed at reduced pressure. Bumping is prevented by the capillary constriction. The sample tube is then sealed with a flame. At the time of analysis the capillary is broken off 2–3 mm above the constriction to give the tube configuration shown in Figure 6. The tubes are introduced into the MS–9 with a wire holder on the tip of a standard MS–9 direct insertion probe. To aid reproducibility all analyses are started at the same time after insertion of the sample tube into the MS–9 source. The temperature of the source heating block is adjusted to give a sample volatilization time of 30 to 60 seconds.

The result of combining these various components in the analysis of a 2-pg sample of TCDD is illustrated in Figure 7. An internal standard is given by a PFA fragmentation peak which is a known distance, 85 mmu (1 millimass unit or mmu = 10^{-3} atomic mass unit), from the TCDD peak. In its present form the MS–9–CAT system has a limit of detection for TCDD of about 1 pg.

The procedure we have described retains the generality of normal mass spectral analysis. It is particularly suited, however, to compounds

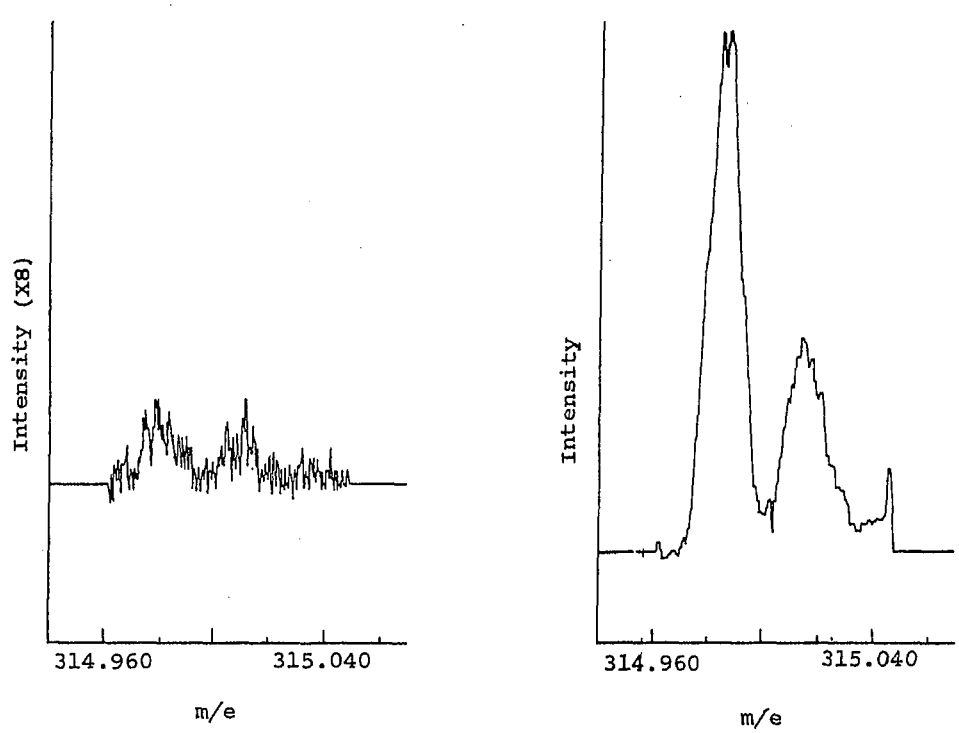

Figure 4. Improvement in sensitivity with the CAT. PFA and a reference peak at m/e *315. The observed improvement in signal-to-noise ratio results from the longer total scanning time and also the fact that many sweeps are made during this time. The overall improvement in signal-to-noise ratio depends on the detailed power spectrum of the noise* (9). *Resolution 12,000 here and for all following time averaged spectra*

Left: one scan. Right: 60 scans (one scan/sec).

containing atoms with significant mass defects, such as heavy metal or organochlorine compounds, which are easily resolved from other residues.

Before the procedure is applied to tissue or other samples from the environment, some potential complications must be taken into account. One is the possibility that other chlorinated organic compounds present in the environment might interfere with or obscure the TCDD peaks. To test this, we obtained mass spectra on the MS–9 for most of the common organochlorine pesticides including lindane, aldrin, dieldrin, mirex, heptachlor, DDD, DDE, and DDT, as well as various polychlorinated biphenyl (PCB) mixtures. In the TCDD mass range DDE from its molecular ion has isotopic isomer peaks at *m/e* 320, 322, and weakly 324. The molecular ion of pentachlorobiphenyl, a component of some PCB mixtures, has a peak at *m/e* 324, and this compound has a weak fragmentation peak at *m/e* 322. DDT has weak fragmentation peaks at *m/e* 320, 322, and 324. As shown for *m/e* 322 in Figure 8, all of these compounds can be resolved from TCDD at our normal resolution of 12,000 (27 mmu at *m/e* 322). The relative input amounts of each compound producing the peaks shown are: DDT, 250; DDE, 25; TCDD, 1; PCB (Arochlor 1254), 250. Even though moderately large excesses of these interferences can be tolerated, it is necessary to use highly

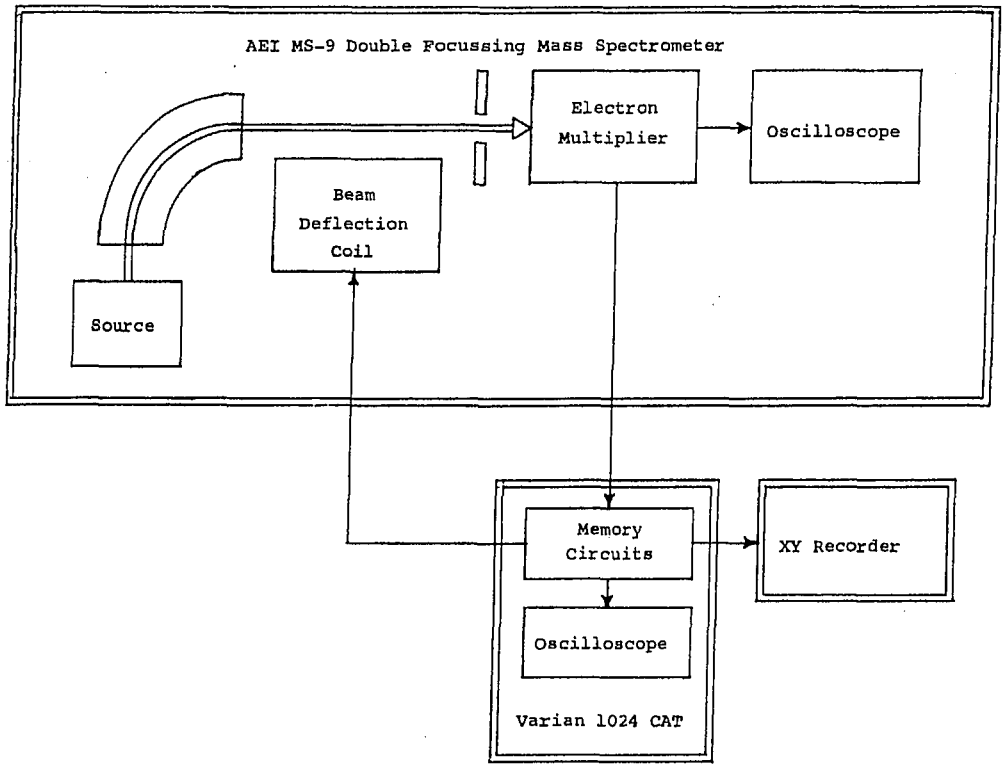

Figure 5. CAT–MS-9 interfacing

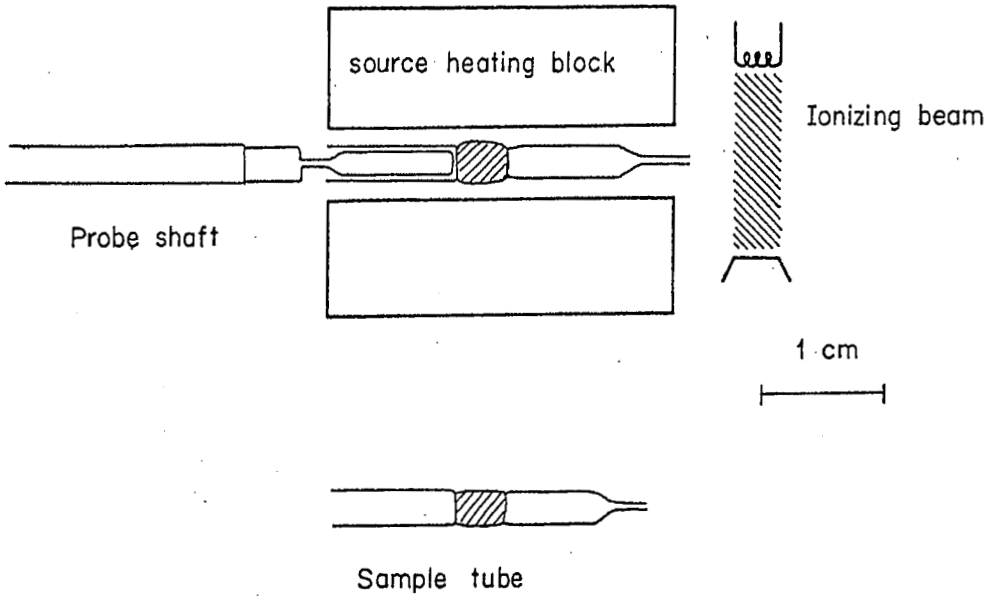

Figure 6. Sample introduction

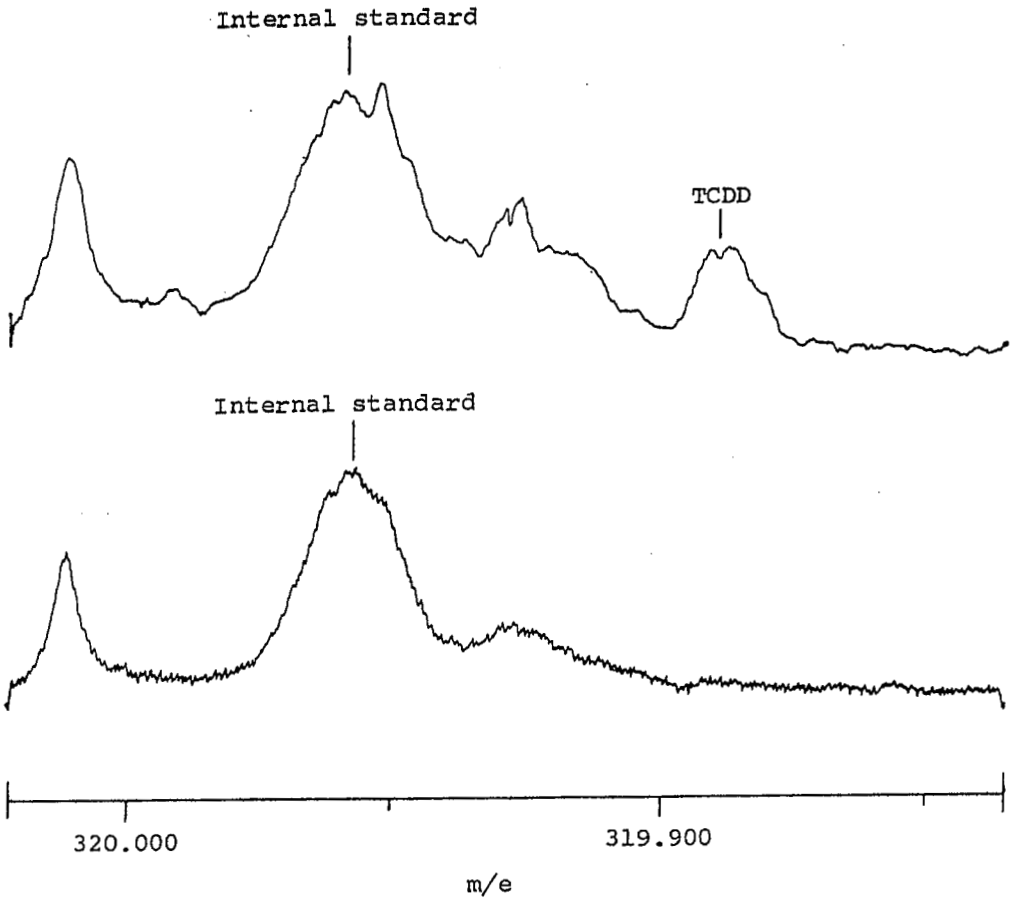

Figure 7. Limit of detection

Top: 2 pg (2×10^{-12} gram) TCDD. Bottom: background.

efficient cleanup procedures to carry out analyses for TCDD at very low levels.

Another complicating characteristic of materials from the environment is that the size and nature of the residue to be analyzed in the mass spectrometer will change from sample to sample. To determine if this might have an effect on the observed TCDD signal, we analyzed identical samples of TCDD with differing amounts of squalane, a saturated hydrocarbon selected as a model for residues obtained from standard extraction and cleanup procedures. As is indicated in Table I (Part A), there was

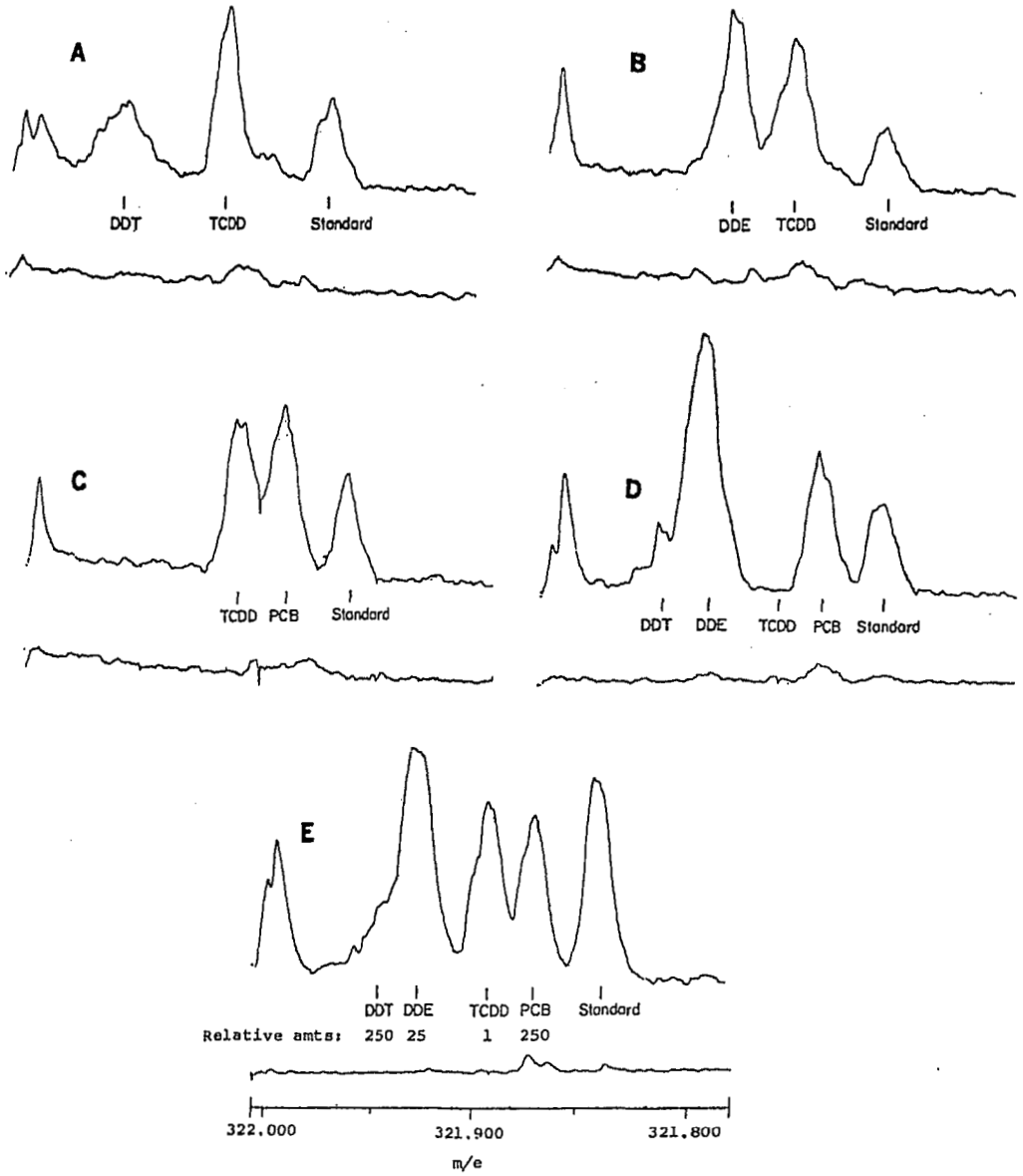

Figure 8. Resolution of TCDD from interferences

A: DDT + TCDD. B: DDE + TCDD. C: PCB (Arochlor 1254) + TCDD. D: DDT + DDE + PCB. E: DDT + DDE + PCB + TCDD.

Table I. Effect of Size of Total Residue

A. Response for TCDD

Squalane added (micrograms)	*Relative response for 20 pg TCDD*
0	100
1	75
5	50
25	15

B. Ratio of two components [a]

Squalane added (micrograms)	*TCDD/TBB*
1	11
3	5
9	1

[a] Ratio of response for 20 pg TCDD to response for 200 pg of 2,3,5,6-tetrachloro-4-bromoethylbenzene (TBB).

a significant effect on the response to TCDD. This eliminates any hope of determining the amount of TCDD present directly from the area of the TCDD peak. In an attempt to resolve this difficulty, we added an internal standard which does not coincide with TCDD or with peaks from any of the potential chlorinated hydrocarbon impurities and measured the ratio between TCDD and the standard in the presence of various amounts of squalane. A compound with a suitable mass, 2,3,5,6-tetrachloro-4-bromoethylbenzene (TBB), was synthesized for this purpose. However, as is apparent from Table I (Part B), the ratio of its signal to that of TCDD was far from constant, and this procedure was ruled out. Table I also suggests that in order not to cause a significant loss of sensitivity, the total sample size should be kept under 5 μg.

Some alternative method had to be devised to quantify the TCDD measurements. The problem was solved with the observation, illustrated in Figure 9, that the response to TCDD is linear over a wide concentration range as long as the size and nature of the sample matrix remain the same. Thus, it is possible to divide a sample into two equal portions, run one, then add an appropriate known amount of TCDD to the other, run it, and by simply noting the increase in area caused by the added TCDD to calculate the amount of TCDD present in the first portion. Figure 9 illustrates the reproducibility of the system. Each point was obtained from four or five independent analyses with an error (root mean square) of 5–10%, as indicated by the error flags, which is acceptable for the present purposes.

To satisfy the requirement of having a total residue of only a few micrograms, the sample cleanup must be very thorough (*10*). The procedure which we have used to accomplish this is the following. A 10-

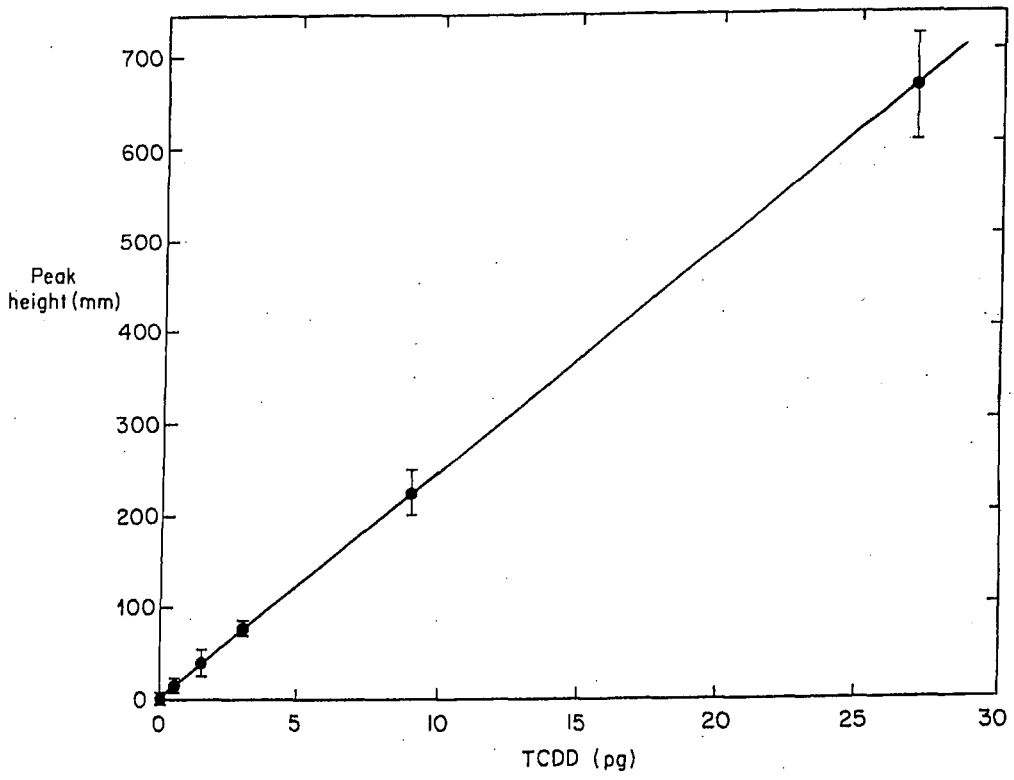

Figure 9. Linearity of response and reproducibility. The error flags indicate the root mean square error for five measurements at each value. The average relative error is about 10%.

gram sample of human milk was combined with 10 ml of ethanol (all solvents were of pesticide grade) and 20 ml of aqueous 40% KOH and refluxed for 6 hours. This solution was then extracted with four 6-ml portions of 5% benzene in hexane. The organic phase was extracted with four 8-ml portions of 85% H_2SO_4, filtered through a 10 mm id column of 10 grams of powdered Na_2CO_3, concentrated carefully to about 10 μl, and preparatively chromatographed by gas–liquid chromatography on a 1 m $\times$ 1/4" column of 3% OV–101 methyl silicone polymer liquid phase on 50/60 mesh Anakrom AB solid support (200°C column, 30 ml/min He). The GLC trap consisted of a 150 mm $\times$ 1.5 mm id borosilicate glass tube packed with 30 mm of 100/120 mesh glass beads retained with glass wool plugs (*11, 12*). The trap was wetted with hexane and cooled in dry ice. The GLC cleanup is carried out through a thermal conductivity detector. A small amount of an internal standard, *m*-terphenyl, with a known retention time relative to TCDD was added to make certain that the TCDD collection was carried out at the right retention time. The total residue from the GLC cleanup when divided into twelve fractions provided a suitable sample size.

Figure 10 illustrates the results of a typical analysis of a 10-gram sample of human milk to which 0.1 ppb TCDD has been added. Blank

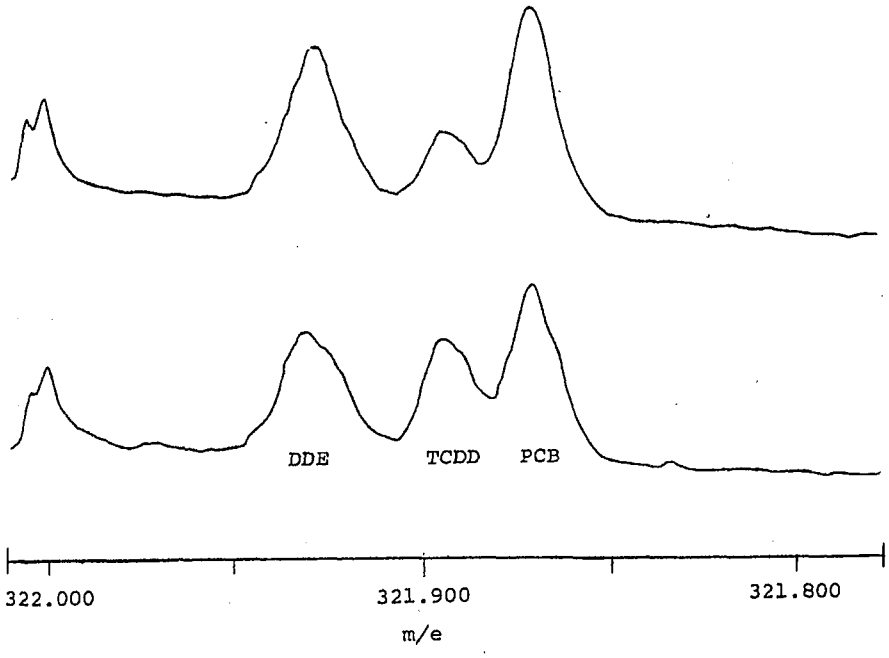

Figure 10. TCDD recovery in 10 gram human milk to which 10^{-9} gram (0.1 ppb) TCDD was added. Each trace represents 8% of the total residue. Top: residue (25% recovery). Bottom: residue + 20 pg TCDD.

samples showed no indication of TCDD at this level. Each trace represents 8% of the total sample and since the area under the unknown TCDD peak, calculated from the run with TCDD added, corresponds to approximately 20 pg, the recovery is about 25%. The other signals observed in the TCDD mass region are probably from DDE and pentachlorobiphenyl. At the present time the major impediment to extending the procedure to the desired level of 1 ppt is interference from pentachlorobiphenyl. We are investigating changes in the cleanup procedure which show excellent promise of reducing the level of this and other interferences in the final residue. We are also developing a procedure for directly measuring the recovery of TCDD in each cleanup by adding chlorine–[^{37}Cl] labelled TCDD to the sample before cleanup.

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