The Phantom Menace -- Determination of the True Method Detection Limit (MDL) for Background Levels of PCDDs, PCDFs, and cPCBs in Human Serum by High-resolution Mass Spectrometry

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Introduction

In 2000, the U.S. Environmental Protection Agency (EPA)[1] reported that the release of "dioxin-like" compounds into the environment decreased by almost 80% between 1987--1995. Comparable time-trend data for these toxicants in the environment were also observed in Germany[2] and Japan. [3] These declines in environmental levels are consistent with the assumption that regulatory efforts over the past several decades have been successful. Evidence that these regulatory efforts resulted in reducing human exposure was reported by Aylward and Hays. [4] In their review of studies from the United States, Canada, Germany, and France, they observed that mean 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) human serum levels have decreased by almost a factor of 10 over the past 30 years, and TCDD levels were about 2 parts per trillion (ppt or pg/g fat) in the general U.S. population in 2000. Based on the known pharmacokinetics in humans, Aylward and Hays[4] predicted that mean background TCDD levels will decrease to 0.5-1 ppt by 2015, even if current intake levels from diet do not change.

The recent worldwide decline in background serum levels of polychlorinated dibenzo-*p*-dioxins, furans, and coplanar biphenyls (PCDDs/PCDFs/cPCBs) is unquestionably an important finding. However, as serum levels continue to diminish, our analytical methods for measuring these toxicants will continue to be "pushed to their limit ." In a previous article,[5] we investigated some of the variables that influence the quantification of "ultra-trace" (fg/g) concentrations of PCDDs/PCDFs and cPCBs in human serum. In this report, we continue to explore parameters that can affect the determination of the

"true" detection limit of our method (MDL), using both analytical standards and matrix-based samples.

Methods and Materials

Sample Preparation

Multiple aliquots each of 5, 7.5, 10, 17.5, and 25 g of National Institute of Standards and Technology Standard Reference Material 1589a (NIST SRM 1589a; PCBs, Pesticides, and Dioxins/Furans in Human Serum, August 9, 2000) were prepared according to the procedure reported by Turner et al.[6] Samples were spiked with $^{13}C_{12}$-labled internal standards followed by C_{18} solid-phase extraction (SPE) and a multi-column automated cleanup and enrichment procedure using a Fluid Management Systems Power-Prep/6. An analytical run included one method blank, six NIST SRM 1589a aliquots, and two quality control samples. PCDDs/PCDFs/cPCBs were eluted from the AX-21 carbon columns in the reverse direction with 40 mL of toluene. We then added 1 µL of dodecane "keeper" to each eluant and the solvent evaporated to about 350 µL by using a Zymark TurboVap II. Residual toluene was transferred to silanized auto sampler vials, and was evaporated further to 1 µL at ambient temperature in a vented desiccator attached to a snorkel. Before analysis by high-resolution gas chromatography (HRGC) and high-resolution mass spectrometry (HRMS), vials were reconstituted with 5 µL of ^{13}C-labeled external standard in nonane.

Calibration Standards

Instrument calibration curves (slopes and intercepts) were calculated using replicate measurements of nine concentrations of PCDD/PCDF/cPCBs standards (21congeners) on five Thermo Electron MAT 95 XP high-resolution mass spectrometers over a period of 1 year. Due to space limitations, the only data presented in this report are standards for 0.04, 0.1, 0.2, 1, 2, 5, 20, 35, 50 pg/µL 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (2378D) and 0.04, 0.1, 0.2, 0.5, 1, 2, 5, 10, and 20 pg/µL 1,2,3,7,8-pentachlorodibenzo-*p*-dioxin (12378D) standards. Estimates of the limit of detection (LOD) for 2378D and 12378D were made using the five lowest standards. In addition, 0.01, 0.02, and 0.03 pg/µL 2378D and 12378D standards were prepared to evaluate measurements at or below the LOD.

Mass Spectrometry

A Leap Technology GC Pal auto sampler was used to make 2 µL injections into an Agilent 6890 gas chromatograph (GC). The GC was operated in the splitless injection mode with a flow of 1 mL/min helium through a DB-5ms column (30 m

x 0.25 mm x 0.25 µm film). Seventeen 2,3,7,8-substituted PCDDs/PCDFs and four cPCBs congeners (using six MID groups) were quantified by isotope-dilution mass spectrometry, using selected ion monitoring (SIM) at 10,000 resolving power (10 % valley) on a Thermo Electron MAT 95 XP (5kV) magnetic sector field mass spectrometer (upgraded with a low-noise ion-detection system or "sensitivity" upgrade (part # 1150760), which operated in the electron impact (EI) mode at 40 eV.[7]

Results and Discussion

Previously we reported[5] a fourfold improvement in the signal-to-noise (S/N) ratio of the 319.8965 or M^+ ion (S/N ratio > 60:1 vs. > 15:1, one MID group) after injection of 20 fg TCDD (our daily instrument sensitivity check) after the installation of the new low-noise ion-detection systems. We concluded that the improvement in S/N was consistent with the claims made by Thermo Electron for the MAT 95 XP (S/N > 400:1 for 100 fg or > 40:1 for 10 fg TCDD mass 321.8939; one MID group) and was achieved based on the stated reduction in detector or electronic noise.

After the installation of the low-noise ion-detection systems, we also evaluated the LODs for 2387D and 12378D individually for each MAT 95XP and collectively by combining data from all five MAT 95 XPs using the extrapolation method of Taylor[8] to determine if additional enhancement in these parameters could also be observed. In Taylor's method, the standard deviation at any concentration (C) level represents the expected precision of measurement at that point. The value for S_0 obtained by extrapolation, is used to evaluate the LOD. Using this procedure, the LOD = $3S_0$ and limit of quanitation (LOQ) = $10S_0$. Extrapolated estimates of S_0 were made using data from replicate injections of 0.04, 0.1, 0.2, 1, and 2 pg/µL 2378D and 0.04, 0.1, 0.2, 0.5, 1, and 2 pg/µL 12378D standards. Plotting the standard deviation in pg/µL at each of the five levels (y-axis) vs. pg/µL (x-axis), the extrapolated estimates of S_0 were close to 0.0133 pg/2µL for each instrument individually and for all instruments collectively for 2378D and 12378D. These estimates resulted in an LOD ($3S_0$) = 0.04 pg and an LOQ ($10S_0$) = 0.133 pg or 40 and 133 fg injected onto the GC column. To check the dependability of our new LOD estimates, we prepared three additional 2378D and 12378D standards at concentrations below our lowest routine calibration standard (0.01, 0.02, and 0.03 pg/µL corresponding to 20, 40, and 60 fg/2µL injected on column). As predicted, we were not able to "detect" the 0.01 pg/µL standards for either 2378D or 12378D, but were able to "detect the 0.02 and 0.03 pg/µL standards. These new estimates

of LOD and LOQ are about half of the estimates observed prior to the installation of the sensitivity upgrades.

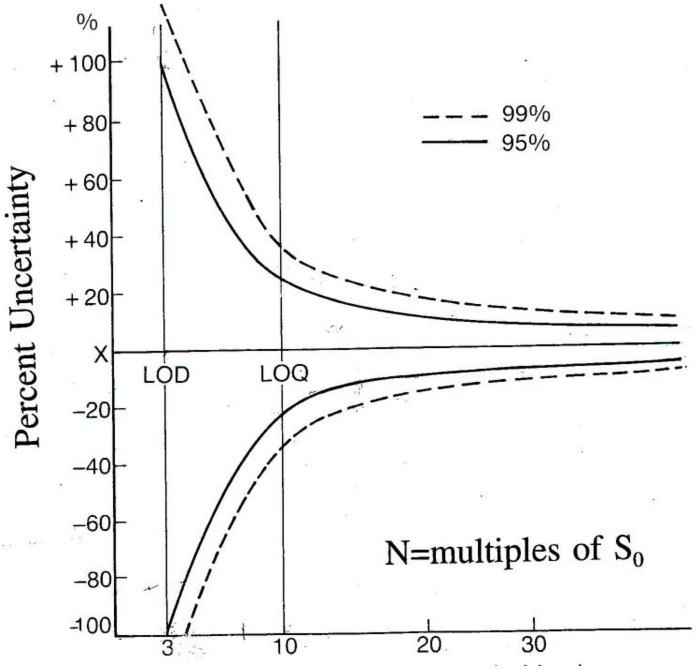

Figure 1. Uncertainty of measurements close to the limit of detection. Taken from Taylor J.K.[8] *Quality Assurance of Chemical Measurements;* 1987, p 82.

Figure 1 is a plot of the relative uncertainty (in percentage) of a measurement close to the LOD vs. the concentration of the analyte, expressed as multiples (N) of S_0. In Figure 1, the relative uncertainty is ± 100% at $3S_0$ or the LOD. Figure 2 is a plot of the 320/322 ion (M^+/M^{+2}) relative abundance ratios for 2378D vs. standard concentration, using 725 data points from mass-spectrometer calibration curves. Figure 2 provides another illustration of how rapidly measurement uncertainly increases near the LOD. In other experiments with standards, the 320/322 ion ratios for 2378D were unacceptable (greater than ± 20% compared with the theoretical abundance ratio) for amounts below 40 fg/2µL.

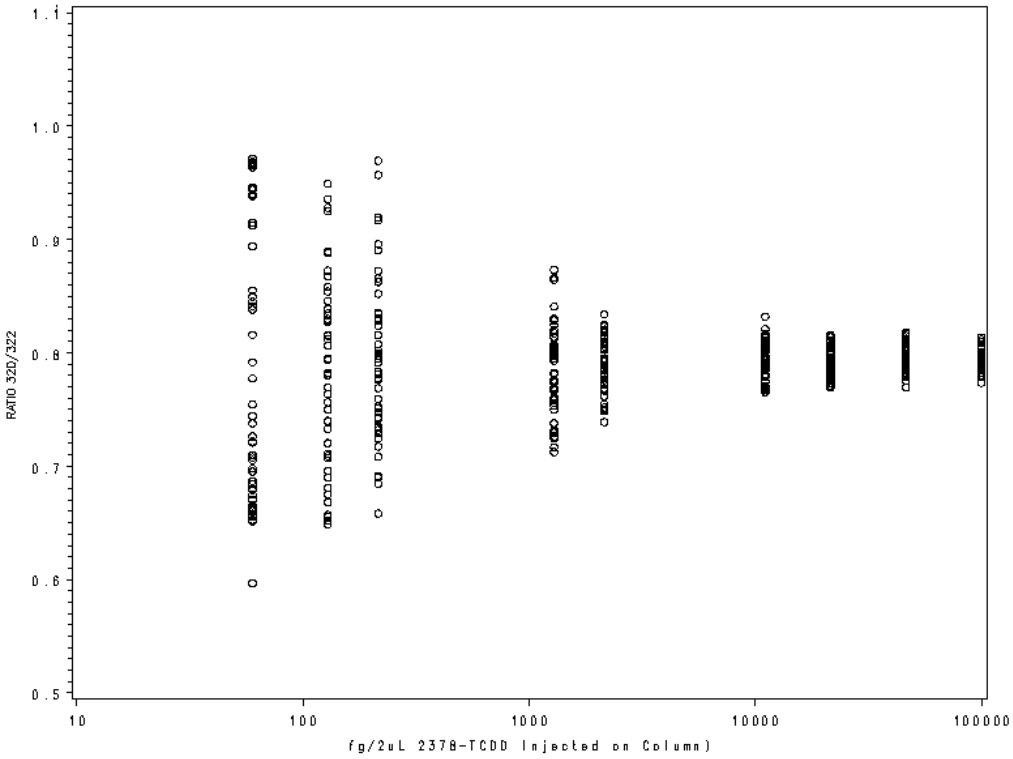

Figure 2. Plot of 320/322 (M^+/M^{+2}) ion-relative abundance ratios vs. fg/2µL 2378-TCDD injected on the DB-5ms GC Column.

To evaluate whether the improvement in LODs obtained with standards after the installation of the sensitivity upgrades could also be achieved with serum extracts, we analyzed six aliquots each of 5, 10, 17.5, and 25 g and twelve 7.5 aliquots of NIST SRM 1589a. We selected the NIST SRM 1589a material for these experiments because the SRM was prepared by pooling serum from a large number of human donors (containing a combination of potential matrix effects); 2378D and 12378D were both present at background levels, and the concentration of 12378D was about three times that of 2378D. Table 1 shows mean concentrations were 16.8 fg/g (or 2.8 pg/g lipid) for 2378D and 50.4 fg/g (or 8.4 pg/g lipid) for 12378D, using 25 g NIST SRM 1859 samples. Table 1 also shows that 2378D could not be quantitated in any of the 5 g NIST samples because neither 320 nor 322 ion peaks were present in the chromatographic traces. Only three 12378D results for 5 g NIST samples could be quantitated because the 354

(M^+) masses were not present in the ion chromatograms. We also observed that several of the 7.5 g 2378D and 5 g 12387D samples had 320/322 or 354/356 (M^+/M^{+2}) ion ratios substantially greater than ± 20 %. The reasons for our inability to detect some of the peaks and the increased ion ratio variability in the 5 and 7.5 g samples were presumably due to some combination of chemical noise (matrix effects) and losses due to recovery in cleanup. These interferences and losses may also explain our frequent observation that S/N ratios do not always appear to be directly proportional to the sample weight. Factors, such as losses on the liner in the injection port, on the GC column, in the transfer line, and ionization efficiency of the ion source or of the detector, could also have contributed to the observed inconsistency of S/N ratios. We concluded that the MDL was not notably improved with real samples due to chemical noise.

Because of the observed differences in the chromatographic traces of samples compared with standards, we routinely multiply our LOD estimates by a factor of two to compensate for the matrix effects when calculating the method detection limit (MDL). We believe this procedure provides a more reliable estimation of the MDL. In reporting "non-detects" for samples, we also correct the MDL of each congener for both sample weight and recovery.

Table 1. Results of NIST SRM 1589a experiments using 5, 7.5, 10, 17.5, and 25 g aliquots

	Grams	Observed			Total		70% Recov	LOD	LOQ
Congener	NIST SRM	Mean(fg/g)	n	%CV	fg	fg/2µL	fg/2µL	fg/2µL	fg/2µL
2378D	25	16.8	6	15.9	420	168	117.6	40	133
2378D	17.5	16.8	6	17.9	294	117.6	82.3	40	133
2378D	10	17.4	6	14.1	174	69.6	48.7	40	133
2378D	7.5	17.4	12	23.1	131	52.2	36.5	40	133
2378D	5	0	6	NA	84	33.6	23.5	40	133
12378D	25	50.4	6	8.0	1260	504	352.8	40	133
12378D	17.5	52.2	6	7..9	914	365.4	255.8	40	133
12378D	10	49.8	6	18.7	498	199.2	139.4	40	133
12378D	7.5	63.6	12	14.1	477	190.8	133.6	40	133
12378D	5	63.6	12	25.5	318	127.2	89	40	133

Another case occurs when method blanks exceed the MDL for congeners, such as 1234678D, 1234678F, OCDD, OCDF, and 3,3',4,4'P (PCB 77). In this situation, MDL for congeners are estimated as described by Keith,[10] where MDL = $3S_{b,}$ and

S_b is the standard deviation of a well-characterized blank. In this case, the reported value for a congener is the observed value minus the average blank. For accuracy, Ferrario *et al.*[11] noted that "to define the level of background contamination and its variability over the course of a study, one must retrospectively examine the method blanks." We also recommend that the mean and S_b of blanks be updated using all the data collected over the course of a study before calculating results. This practice has worked well, given that various blanks are routinely present and are constantly changing for a variety of reasons. Whenever the blank correction becomes significant, it is necessary to measure with care, using the same amount of effort as with the sample itself, particularly in the case where the concentration in the sample approaches the concentration in the blank. In analytical chemistry, asymmetric, nongaussian blank distributions are common, especially where more than half of the blanks are negative (median = 0). After considering the later aspects, Linnet and Kondratovich[12] recently proposed a practical alternative approach for determining the MDL, using a partly nonparametric approach. In their procedure for sample size n, the nonparametrically determined 95th percentile of the blank measurements {obtained as the value of the [n(95/100) + 0.5]th ordered observation} defines the limit of the blank (LOB). The MDL is the lowest value that is likely to yield a result exceeding the LOD. Ferrario *et al.*[11] commented, "it is ironic that the advances in technology that have allowed the progressive lowering of detection limits have reached a limit imposed by the very contaminants the technology was designed to measure."

Unfortunately, we have also encountered a "worse-case scenario," in which the blank-derived MDL for OCDF and PCB77 exceeds the levels actually present in samples from studies with small volume, resulting in useless data that have to be acknowledged as "non-reportable."

The LOD is defined as the smallest concentration of some component of interest that can be measured by a single measurement with a stated level of confidence.[8] Thomsen, Schatzlein, and Mercuro[12] recently stated, "a common misconception is that the LOD is the smallest concentration that can be measured." If fact, the LOD is the point at which we decide whether a compound is present or not – that is, the point where we can just distinguish a signal from the background. As a rule of thumb, we have observed that MLD for PCDDs/PCDFs/cPCBs in real samples is typically two times higher than the mass spectrometer MDL for standards due to chemical noise and matrix effects in serum. To illustrate the "true" ability of our method to measure TCDD, it would require 9 mL of serum to detect 2 pg/g lipid,

18 mL for 1 pg/g lipid, and 36 mL for 0.5 pg/g lipid (assuming 70% recovery and a total lipid of 0.6%). Given the current "state-of-the-art", the only sensible way "to measure" the ever-decreasing concentrations of PCDDs/PCDFs/cPCBs in background level samples is to increase the sample size.

References

1. U.S. Environmental Protection Agency (2000). EPA-560/5-91-003.
2. Päpke O. (1998). *Environ. Health Perspect.* 106 (Suppl 2): 723-731.
3. Sakai, S., Hayakawa K., Okamoto K., and Takatsuki H. (2002). *Organohal. Comp.* 58: 189-192.
4. Aylward L.L. and Hayes S.M. (2002). *J. Expos. Anal. and Environ. Epidemiol.* 12: 319-328.
5. Turner W.E. Welch S.M., DiPietro E.S., Cash T.P., McClure P.C., Needham L.L., and Patterson D.D. Jr. (2003). *Organohal. Comp.* 60: 134-137.
6. Turner W.E., DiPietro, E.S., Cash, T.P., McClure P.C., Patterson D.G. Jr., and Shirkhan, H. (1994). *Organohal. Comp.* 19: 31-35.
7. Turner W.; DiPietro, E; Lapeza, C.; Green, V.; Gill, J.; Patterson, D.G. Jr. (1997). *Organohal. Comp.* 31: 26-31.
8. Taylor J.K. (1987). *Quality Assurance of Chemical Measurements*. Lewis Publisher, Inc. ISBN 087371-097-5
9. Keith L.H. (1991). *Environmental Sampling and Analysis: A Practical Guide*. Lewis Publishers, Inc. ISBN 0-87371-381-8.
10. Ferrario J., Byrne C, Dupuy A.E. Jr. (1997). *Chemosphere* 34: 2451-2465.
11. Linnet K. and Kondratovich (2004). *Clin. Chem.* 50/4: 732-740.
12. Thomsen V., Schatzlein D., and Mercuro D. (2003). *Spectrocopy* 18/12: 112-114.