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APPLICATION NOTE 35

Human Biomonitoring: Attogram Level Sensitivity and Consequences for Analytical Standards Purity

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Internal Versus External Dose in Human Exposure Assessment

The objectives of human exposure assessment to environmental chemicals are to quantify the magnitude, duration, frequency and routes of exposure, and to characterize and enumerate the exposed population. There are several ways to do human exposure assessment. The first is the external dose measurement process followed by modeling to predict the individual internal dose. This method usually involves the collection of questionnaire data and a measurement or estimation of concentrations of the chemical(s) in various environmental media such as air, water, soil, dust, food, consumer products, *etc*. This is followed by assumptions of media contact or intake routes that yield a level of applied dose. Predicting levels of toxicants in people using environmental media monitoring is very difficult and involves many assumptions such as: individual lung, intestine and skin absorption coefficients; genetic factors; personal habits; lifestyle factors; nutritional status; and many others.

A second approach to human exposure assessment is the biomonitoring approach which provides exposure estimates that are more directly related to concentrations of the active agent(s) at the target site or organ. Biomonitoring is an assessment of the internal dose by measuring a toxicant (or its metabolite or protein adduct) in human blood, urine, milk, saliva, adipose tissue, or other tissues. The biomonitoring approach provides a direct measure of exposure that integrates exposures from multiple pathways and sources. This approach decreases the uncertainty inherent in exposure assessment by the external dose method and provides a more biologically relevant measure of true exposure. Instead of predicting levels in people, this approach measures levels of toxicants in people and markedly decreases uncertainty in assessing human risk (Sexton *et al.* 2004). An example of the usefulness of the internal dose measurement versus the external dose process is shown in Figure 1.

The US Air Force conducted a 20-year prospective study examining the health, mortality and reproductive outcomes in US Air Force veterans of Operation Ranch Hand (RH), the unit responsible for the aerial spraying of herbicides, including 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)-contaminated Agent Orange, in Vietnam from 1962 to 1971 (Pavuk *et al.* 2007). Prior to beginning the study, the Air Force measured the levels of 2,3,7,8-TCDD in the serum (Patterson *et al.* 1987) of RH veterans and compared the levels to the external dose exposure index that had been developed for the Health Study. Figure 1 shows that the exposure index was poorly correlated with the internal dose TCDD measurements. Based on these results, the Air Force decided to use the internal dose TCDD serum measurements as the exposure index for the Health Study (Michalek 1989).

National Report on Human Exposure to Environmental Chemicals

Before what is "abnormal" may be determined, what is "normal" must be defined. The National Report on Human Exposure to Environmental Chemicals is an ongoing (every two years) biomonitoring assessment of the exposure of the US population to selected environmental chemicals, which are measured in urine, blood and its components. The goals of the National Report are to:

1) assess exposure to various chemicals;
2) establish national "reference ranges" of these chemicals;
3) track, over time, trends in these "reference ranges;"
4) help set priorities on linking exposure to health outcomes in the American population and subpopulations by age, sex and race/ethnicity.

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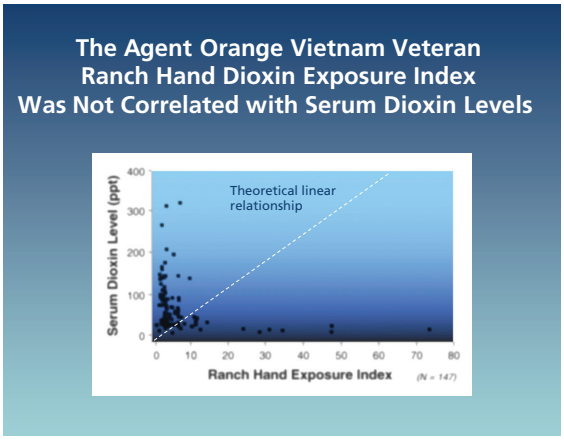

Figure 1.

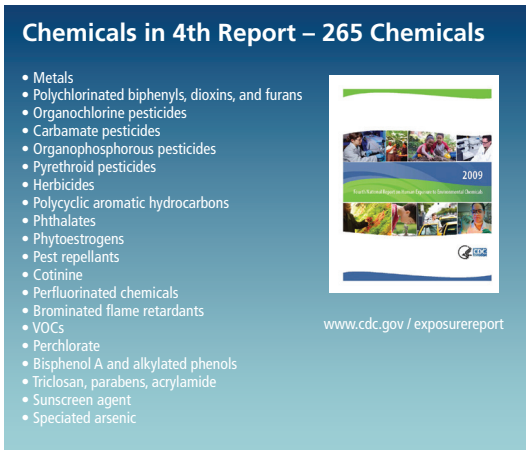

Figure 2. Aliphatic region of two-dimensional NCOCX chemical shift correlation experiment recorder at 600 MHz proton, 12.5 kHz spinning frequency, and with 30 ms of DARR carbon-carbon mixing. Acquisition lengths were 15 ms in t_1 and 22.3 ms in t_2 acquisition dimensions.

The samples for the National Report are obtained from the National Health and Nutrition Examination Survey (NHANES), which is conducted by the National Center for Health Statistics of the Centers for Disease Control and Prevention (CDC). The objective of this survey is to assess the health and nutritional status of adults and children in the United States. The NHANES sampling plan is a complex, stratified, multistage, probability cluster design that selects a representative sample of the civilian, non-institutionalized US population. The data collection includes information from questionnaires, physical examinations on individual participants, chemical measurements and clinical tests on samples collected from about 5,000 participants annually.

Since 1999, NHANES has incorporated a continuous annual survey of persistent organic pollutants (POPs), as well as other chemical measurements that are reported every two years from a random one-third subset of the collected samples. The reference range levels for a number of POPs, including various congeners of the polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), dioxin-like polychlorinated biphenyls (PCBs) and organochlorine pesticides have recently been published for the NHANES 2001-2002 study (Patterson *et al.* 2008) and the NHANES 2003-2004 study (Patterson *et al.* 2009). These results have been reported for the total US population (age 20+) and by age groups (ages 12-19, 20-39, 40-59 and 60+), sex, and race/ethnicity [Mexican American (MA), non-Hispanic blacks (NHB), and non-Hispanic whites (NHW)].

In addition to reporting the reference ranges for the individual congeners, Patterson *et al.* have also reported the total toxic equivalents (TEQ) reference ranges for the US population. Each of the individual PCDD, PCDF and PCB congeners has been assigned a toxic equivalency factor (TEF) relative to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (2,3,7,8-TCDD) by the World Health Organization (WHO). These TEF values (Van den Berg *et al.* 2006) are multiplied by the respective congener concentration to give the congener WHO toxic equivalency (TEQ), and these are summed together to give the total TEQ for each person. In addition, results from the NHANES 2003-2004 survey have been reported for polybrominated diphenyl ethers (Sjodin *et al.* 2008), polycyclic aromatic hydrocarbon metabolites (Li *et al.* 2008) and polyfluoroalkyl chemicals (Calafat *et al.* 2007). Additional classes of chemicals from the latest National Report on Human Exposure to Environmental Chemicals are listed in Figure 2.

Analytical Method Considerations and New Extremely Low Detection Limits

When we began our work on measuring dioxin in human tissues, we used adipose tissue because the levels were higher in this lipid-rich tissue (Patterson *et al.* 1986a).

Because of the invasive nature of the surgical procedure required to obtain the adipose tissue sample, we had a lower-than-expected participation rate for our first adipose tissue study in Times Beach, Missouri (Patterson *et al.* 1986b). We then turned our attention to developing a method using serum (Patterson *et al.* 1987) which was a less invasive matrix but the levels were much lower in serum due to the small amount of lipid (~0.6%) compared to adipose tissue (~95%). The methods required high-resolution mass spectrometry (HRMS) in order to have the sensitivity required to measure normal background dioxin levels in the picogram to femtogram range. For human studies, we needed the highest accuracy possible which required the use of isotopically labeled internal standards for our quantification scheme.

At the time we began our work, very few unlabeled and isotopically labeled dioxins, furans and PCBs were commercially available. We therefore constructed at the Division of Laboratory Sciences at CDC a special Chemical Toxicant Laboratory (CTL) (Myers and Patterson 1987) and synthesized unlabeled and ^{13}C-labeled PCDD, PCDF and PCB congeners (Figure 3).

Figure 3.

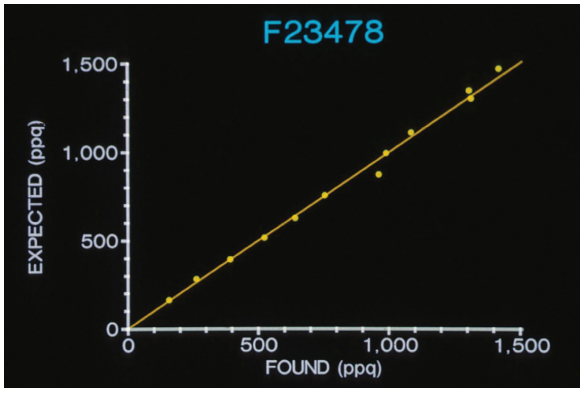

Figure 4a.

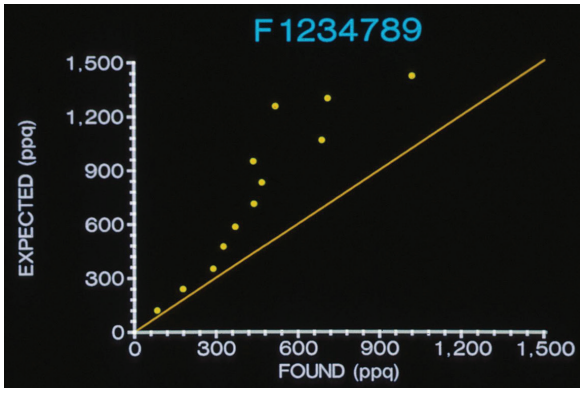

Figure 4b.

The utility of using isotope-dilution quantification is apparent in Figure 4. The 2,3,4,7,8-PeCDF congener (Figure 4a) had a $^{13}C_{12}$-2,3,4,7,8-PeCDF congener as an internal standard and the accuracy of the measured concentration versus the expected concentration is apparent.

Figure 4b shows the quantitative results for the 1,2,3,4,7,8,9-HpCDF congener which did not have a ^{13}C-labeled internal standard. The inaccuracy for this congener is apparent in Figure 4b. Over the years, unlabeled and isotopically labeled standards became available from Cambridge Isotope Laboratories, Inc. (CIL) Many of the analytes measured by CDC in the NHANES surveys described above use CIL unlabeled and ^{13}C-labeled standards. The CIL unlabeled standards provide the accuracy base for all these analytes in the NHANES studies which provide background national reference ranges for these chemicals in people from the United States.

For a number of reasons, it is important to continue to try to develop more sensitive analytical methods for environmental chemicals:

1) to determine the normal human background levels of chemicals shown to be toxic to certain animals that we cannot detect with current methods;
2) to continue monitoring chemical levels that are decreasing in the US population (dioxins, furans, PCBs, pesticides);
3) to provide better analytical CVs of chemicals that we can measure which will translate into lower measurement uncertainties; and
4) a lower analytical CV translates directly into higher statistical power in epidemiological studies.

A lower analytical CV allows a higher statistical power for a given number of samples in an epidemiological study. Also, a lower analytical CV can provide the same statistical power using a smaller numbers of samples in a study (generating a cost savings).

Newer, more sensitive analytical techniques are currently being developed (Patterson *et al.* 2011) using cryogenic zone compression and loop modulation coupled with high resolution mass spectrometry to measure persistent organic pollutants. A chromatogram showing the signal from a standard of 2,3,7,8-TCDD (313 attogram) using this newer technique is depicted in Figure 5.

A modification of this technique, called time-controlled cryogenic zone compression, being developed by Thermo Scientific, is shown in Figure 6.

This technique allows targeted cryofocusing of certain peaks that might need enhanced sensitivity while allowing the remainder of the chromatographic separation to proceed unaltered. Tables 1 and 2 summarize the current state of the art in sensitivity for measurements of dioxin and dioxin-like chemicals.

Table 1

Sensitivity for 2,3,7,8-TCDD using various GC–MS techniques

Technique	Sample amount on column	S/N (4σ)
GC (MAT95XP)–HRMS	Standard 20 fg	43
GC (DFS)–HRMS	Standard 20 fg	604
CZC-GC (MAT95XP)–HRMS	Standard 313 ag	400
CZC-GC (MAT95XP)–HRMS	Serum 325 ag	161
GCxGC-LRTOFMS	Standard 500 fg	6

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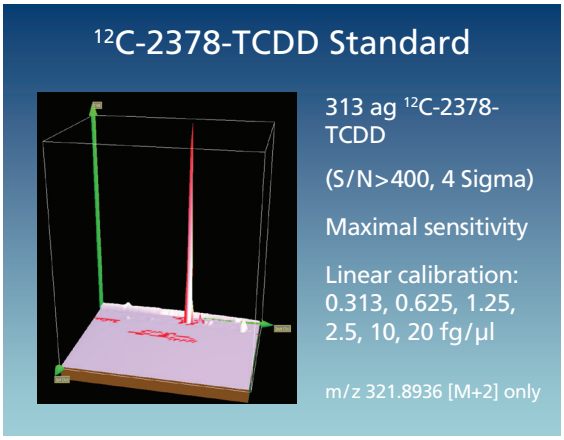

Figure 5.

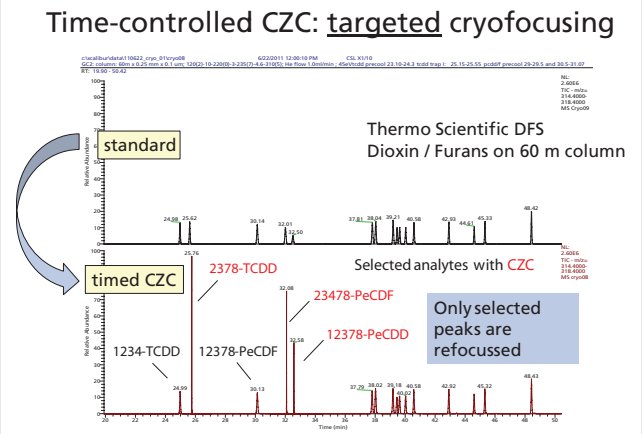

Figure 6.

Table 2

Current state of the art for the measurement of 2,3,7,8-TCDD and the potential detection limits and numbers of molecules (calculations based on M+2 321.8936 m/z ion).

Quantity	Notation	Number of moles	Number of molecules
1 nanogram (ng) 10^{-9} g	ppb	3.1×10^{-12} (3.1 picomoles)	1,870,000,000,000 (1.87×10^{12})
1 picogram (pg) 10^{-12} g	ppt	3.1×10^{-15} (3.1 femtomoles)	1,870,000,000 (1.87×10^{9})
1 femtogram (fg) 10^{-15} g	ppq	3.1×10^{-18} (3.1 attomoles)	1,870,000 (1.87×10^{6})
313 attogram (ag) 10^{-18} g	**ppquint**	**9.7×10^{-19} (972 zeptomoles)**	**586,000 (5.86×10^{5})**

The consequences of the use of these newer analytical techniques for CIL and other laboratories producing and supplying analytical standards is that the purity of the standards will most likely have to be improved. Even very small amounts of the unlabeled compound or partially labeled compound in isotopically labeled standards will be detectable and interfere with accurate quantification. For example, in 1 ng of a standard, 0.00001% impurity is 100 attograms! Impurities at these levels will be detectable and will have to be eliminated. This could be a time-consuming and costly process for standard producers which could require extensive laboratory facility cleanup and extensive quality assurance/quality control procedures.

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