Problems Associated with the Determination of 2,3,7,8 Chlorine-Substituted Dioxins and Furans at the Part per Trillion Level in United States Beef Fat

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1. Introduction

As part of the USEPA Dioxin Reassessment Program[1)] the 2,3,7,8 chlorine (Cl)-substituted dibenzo-p-dioxins and furans were measured at part per trillion (ppt) levels in beef fat collected from slaughter facilities in the United States. This was a statistically designed national survey of these compounds in the US beef supply. Analyte concentrations were determined by gas chromatography/high resolution mass spectrometry (HRGC/HRMS), using isotope dilution methodology. This paper focuses on the (1) the basis for the establishment of the method's limits of detection and limits of quantitation (LODs/LOQs) , (2) the procedures used for background subtraction, and (3) the reporting of data.

The USEPA recently estimated that over 90% of human exposure to dioxins and dioxin-like compounds occurs through the diet, primarily, in the consumption of fatty foods of animal origin. An attempt to evaluate the literature to determine the concentration of dioxins/furans in beef from the United States revealed that none of the studies were based on a statistically derived sampling plan. In addition, limitations imposed by differences in analytical methodologies and a lack of adequately defined QA/QC procedures precluded an objective determination of dioxin/furan concentrations that were both statistically representative and technically defensible.[2-4)] The study described here is a statistically designed survey of the 2,3,7,8 Cl-substituted dibenzo-p-dioxins and furans in the back fat of beef slaughtered at federally inspected facilities. The analytical methods employed were validated prior to sample analysis and rigorous QA/QC procedures were maintained throughout the survey.

A sampling frame was constructed based on US 1993 slaughter information. The number of samples per animal class was as follows: two bulls, thirty-three steers, eighteen heifers, six dairy cows, and six beef cows. Due to practical considerations, a total of 65 samples were selected for the study. The number of samples taken per animal class was based on the proportion of each animal class slaughtered to the total. In order to ensure that each animal in the population had an approximately equal chance of being selected, establishments were randomly selected with

a probability in proportion to the total number of bulls, steers, heifers, cows, and dairy cows slaughtered.[5)]

Prior to the initiation of the study a detailed Quality Assurance Project Plan (QAPjP) was compiled and submitted to both the project QA Officer and a independent divisional QA Officer for review and approval.

2. **Methods**

Briefly, beef fat samples were homogenized and a 10 g subsample taken for analysis. The sample was fortified at 10 ppt with ^{13}C analogs of the various 2,3,7,8, Cl-substituted congeners prior to extraction. The crude extract was cleaned up using acid/base modified silica gel, alumina, and graphitized carbon column chromatography. The eluent was then concentrated, fortified with ^{13}C internal standards, and analyzed by HRGC/HRMS.

All analyses were performed on a Kratos Concept® high resolution mass spectrometer (HRMS) using isotope dilution. Chromatographic separations were achieved using a Hewlett Packard 5890 Series II high resolution gas chromatograph (HRGC), utilizing a 60 m x 0.32 mm (0.25 µm film thickness) DB5-MS® capillary column. The DB5-MS® column was found to have lower bleed than the standard DB-5® while providing complete separation of all 2,3,7,8 Cl-substituted congeners. Between four and six calibration standards with native analyte concentrations bracketing the expected analyte concentrations were analyzed prior to analyzing samples.

Prior to sample analysis the linearity of the calibration curve was verified by analyzing a calibration solution (200 fg TCDD) and calculating the RF as described previously. The mass chromatogram was also examined to ensure that all the 2,3,7,8 Cl substituted congeners were clearly separated. On the days that samples were analyzed, internal standards were added to each sample and the final volume adjusted to 20 µl. Once all QA/QC parameters had been verified to be within specified limits, sample analyses proceeded.

Samples were organized and analyzed in sets: method blank, matrix blank, laboratory control spike (LCS), and the nine samples.

3. **Results and Discussion**

The mean lipid adjusted concentrations of the analyses of the beef fat samples for the 2,3,7,8-Cl-substituted dioxins and furans are listed in Table 1. Mean concentrations were calculated in three ways: with non-detects = 0, with non-detect = 0.5 x LOD, and by ignoring non-detects and calculating a mean of the detected values. These values are arthimetric means and have not been weighed to represent the population statistics. The median of detected values together with the lowest and highest detected value of each congener and the frequency of detection are listed in Table 2. Reporting data in this manner provides the reader with all of the information required to objectively evaluate the results. Values reported without this supporting information is confusing and often leads to the misinterpretation of the data. For example, means are often listed without an explanation of how the "non-detects" were treated in the final calculation. Quite often no information is provided regarding the frequency of detection, the accuracy, the precision, or the method LOD/LOQ. As is evident from Table 1, the values for the means can be different and the magnitude of the differences increases as the frequency of detection decreases. In order to objectively evaluate the results or make meaningful comparisons to data in the literature, this type of information should always be provided.

Prior to analyzing real samples, a initial demonstration of the method's capabilities was performed by fortifying five subsamples of beef fat homogenate at five times the targeted limits of quantitation(LOQ). The results demonstrated that the accuracy and precision were excellent *with observed results within 20% of the actual values and a %RSD for all analytes less than 20%.* Recoveries were 48 to 139%. A laboratory control spike also fortified at five (5) times the LOQ was prepared and analyzed with each set of samples.

Target detection and quantitation limits (LODs, LOQs) were initially based on a level of performance we thought could be maintained for the duration of the project while satisfying the requirements of the stringent QA/QC program. These target LOQs, primarily based on instrument sensitivity, background, and the results of the demonstration phase, were tentatively set at 0.2 ppt for the tetras (TCDD & TCDF), 1.0 ppt for the pentas, hexas, heptas (PCDDs/PCDFs), HxCDDs/HxCDFs, HpCDDs/HpCDFs), and 6.0 ppt for the octas (OCDD/OCDF). The accuracy and precision at these target levels were verified by analyzing five replicates of the beef fat homogenate fortified at the specified limits. Considering the signal to noise ratios from the results of these analyses at the LOQs, the method limits of detection were estimated to be ½ of the target LOQs. These LODs/LOQs were to be reconsidered after completion of the project. We then verified the target LODs by fortifying replicate sub-samples at the appropriate level (i.e. 0.1 ppt for 2,3,7,8-TCDD). The samples were then processed and analyzed according to the procedure. For the majority of the analytes that were free from matrix or method blank background, the precision (± 20%) and accuracy (ranging from ± 8.0-44.0%) were quite good. For a few of the analytes (1,2,3,6,7,8-HxCDD, 1,2,3,4,6,7,8-HpCDD, and OCDD), the demonstrable accuracy declined to *between 50 and 100%. This apparent decline was due to the fact that these analytes were present* in the matrix at concentrations 2-5 times the fortification level and were routinely detected in the method blanks. It was extremely difficult to accurately measure such a relatively small "spike" when added to samples that contained background levels. Nonetheless, the results from the analytes that did not have background strongly suggested that the LODs could be achieved for all the analytes of interest, but it cannot be demonstrated when significant background exists.

We chose to determine the LODs/LOQs for each analyte in the method based on demonstrated performance criteria rather than calculate a variable LOD for each non-detected analyte in a particular sample. Even though this method of determining LODs is widespread and generally accepted, we have found in most cases that theoretical LODs based on signal to noise calculations from background noise result in LODs that are unrealistic.

Upon completion of the study we revised the method LODs/LOQs based on an evaluation of the methods overall performance for the duration of the study. Final LODs/LOQs were based on results from fortified samples done in replicate, the replicate analyses of real samples with analytes present at the specified limits, and from the calculation of background levels detected in method blanks.

Background contamination at part-per-trillion (ppt) detection limits is an important consideration when analyzing samples for compounds that are ubiquitously distributed in the environment. Background contamination did, in fact, define the lower limits of detection for several compounds in this study. In cases where background contamination is routinely present, the critical issue to be resolved is the level above background that can be reliably determined to be

"real" (i.e. contributed from the sample matrix). One must have some mechanism to define the level of background contamination and its variability over the course of the study. This can only done retrospectively by examing the method blanks.

Background levels for each of the native penta-hepta congeners were determined by calculating the mean concentration for each native analyte from the method blanks done with each sample set (n=14). Many of the analytes were often not detected in all of the method blanks. Therefore, we determined the average background of only those detected amounts. This resulted in a true arithmetic mean with a calculated value larger than had we substituted zeros or 0.5 x LOD for non detects (NDs) in the calculation of the mean. When subtracting background from an analyte in a particular sample, we subtracted either the average amount for that analyte or the amount found in the blank from that particular sample set, which ever was the greater value. No value for any analyte was reported unless it was ≥ 2 times the mean level of the blank, after background substraction. This procedure defined the method LOD in cases where background contamination was present for a particular analyte.

This method of background determination and subtraction is quite conservative and increases the possibility of reporting false negatives for values close to the detection limits. It also tends to increase method LODs/LOQs. However, it also increases the confidence associated with reported values near the LOD and minimizes the likelihood of false positives. As a result of these procedures, the method LOD/LOQ were 0.1/0.2 ppt for TCDF, 0.5/1 ppt for the pentas, hexas, heptas, and 3.0/6.0 ppt for the OCDD and OCDF.

The LOD for 2,3,7,8, TCDD was not determined in the manner just described. No background levels were subtracted from TCDD values, since we did not routinely detect TCDD in the blanks. We had no detectable TCDD background at the onset of the project and therefore did not feel we had to initiate the procedure described above. However, we did sporadically detect TCDD at approximately the detection limit in some subsequent blanks. Since the frequency of detection of TCDD in both the blanks and the samples was low (17%), we decided to reprocess and reanalyze all samples that were positive for TCDD. As a result of these replicate analyses, the LOD/LOQ for 2,3,7,8, TCDD was found to be 0.05/0.1 ppt. A positive response for TCDD below the detection limit could not be verified within the guidelines established in the QA/QC plan. Therefore, no value was assigned.

This method of reporting data results in values falling into one of two categories: values at or above the LOQs which have a defined and demonstrated precision and accuracy of within 20% or values that lie between the LOQ and LOD which are flagged denoting their status. It should be understood that the uncertainty associated with reported values below the LOQ increases as they approach the method detection limit at which point no value should be reported. The methods defined herein for background subtraction and defining LODs/LOQs were our best attempt to report technically defensible values. We invite and encourage any constructive criticism or dialogue regarding these topics that might help to further define and standardize these procedures.

Table 1. Lipid Adjusted/Unweighted Mean Concentrations of PCDDs and PCDFs in Beef Fat (picogram/gram, ppt)

Compound	N	Detects	Mean[1]	Mean [2]	Mean [3]
2,3,7,8-TCDD	63	11	0.030	0.056	0.173
1,2,3,7,8-PeCDD	63	2	0.059	0.364	1.862
1,2,3,4,7,8-HxCDD	63	8	0.219	0.495	1.728
1,2,3,6,7,8-HxCDD	63	21	1.310	1.521	3.929
1,2,3,7,8,9-HxCDD	63	9	0.283	0.553	1.981
1,2,3,4,6,7,8-HpCDD	63	45	4.676	4.855	6.673
1,2,3,4,6,7,8-OCDD	63	13	3.778	5.279	18.309
2,3,7,8-TCDF	63	0	0.000	0.031	ND
1,2,3,7,8-PeCDF	63	0	0.000	0.315	ND
2,3,4,7,8-PeCDF	63	4	0.061	0.357	0.968
1,2,3,4,7,8-HxCDF	63	8	0.283	0.558	2.226
1,2,3,6,7,8-HxCDF	63	7	0.121	0.402	1.086
1,2,3,7,8,9-HxCDF	63	0	0.000	0.315	ND
2,3,4,6,7,8-HxCDF	63	5	0.097	0.388	1.224
1,2,3,4,6,7,8-HpCDF	63	14	0.836	1.082	3.763
1,2,3,4,7,8,9-HpCDF	63	0	0.000	0.315	ND
1,2,3,4,6,7,8,9-OCDF	63	0	0.000	1.887	ND

N - Number of Samples Analyzed during Study
Mean[1] - Using 0 for non-detects
Mean[2] - Using 0.5 LOD for non-detects
Mean[3] - Mean of detected values only

Table 2. Lipid Adjusted/Unweighted Median Concentrations of PCDDs and PCDFs in Beef Fat (picogram/gram, ppt) with Minimum and Maximum Detected Values

Compound	N	Detects	Minimum	Median	Maximum
2,3,7,8-TCDD	63	11	0.069	0.104	0.736
1,2,3,7,8-PeCDD	63	2	0.689	1.862	3.035
1,2,3,4,7,8-HxCDD	63	8	0.728	1.144	4.691
1,2,3,6,7,8-HxCDD	63	21	0.701	3.935	12.459
1,2,3,7,8,9-HxCDD	63	9	0.784	1.787	3.679
1,2,3,4,6,7,8-HpCDD	63	45	0.775	2.576	47.557
1,2,3,4,6,7,8-OCDD	63	13	4.221	11.733	71.839
2,3,7,8-TCDF	63	0	ND	ND	ND
1,2,3,7,8-PeCDF	63	0	ND	ND	ND
2,3,4,7,8-PeCDF	63	4	0.860	0.960	1.094
1,2,3,4,7,8-HxCDF	63	8	0.894	1.812	4.291
1,2,3,6,7,8-HxCDF	63	7	0.710	1.039	1.964
1,2,3,7,8,9-HxCDF	63	0	ND	ND	ND
2,3,4,6,7,8-HxCDF	63	5	0.710	1.158	1.753
1,2,3,4,6,7,8-HpCDF	63	14	0.770	2.990	10.115
1,2,3,4,7,8,9-HpCDF	63	0	ND	ND	ND
1,2,3,4,6,7,8,9-OCDF	63	0	ND	ND	ND

4. References

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