The Use of Sulfuric Acid as a Denaturing Agent in the Extraction of PCDDs/PCDFs from Milk and Blood Samples

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1. Introduction

Polychlorinated dibenzo-p-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) are two related classes of toxic compounds formed as by-products in the manufacture of industrial chemicals and in thermal processes such as incineration. They are widely distributed in the environment and have been found in the tissues of different species of biota including human population cohorts. Since they preferentially bioaccumulate in fatty tissues, lipid-containing body fluids such as breast milk and blood serum have been used extensively as media to determine body burdens in human and animal populations[1]. In the extraction of PCDDs/PCDFs from these fluids ethanol is frequently used to denature proteins and thereby release the compounds for extraction by a non-polar solvent such as hexane[2]. However there are several disadvantages to this process. In the first place emulsions can form which may not be readily broken by centrifugation. In the second place large volumes of solvents are required and under these circumstances we find that there is an increase in the background contribution from the laboratory of certain PCDDs/PCDFs such as octachlorodibenzo-p-dioxin (OCDD). In the present study we describe the use of sulfuric acid as an alternative to ethanol for denaturing proteins in cow's milk, human milk and human blood plasma prior to extraction of PCDDs/PCDFs. This reagent is used routinely in the dairy industry for fat determinations in milk (Babcock test)[3] and we have found that, when it is used instead of ethanol, stable emulsions are never encountered and solvent volumes are considerably reduced.

2. Methods

Cow's Milk. A 400 ml volume of milk was divided equally between six 150 ml Corex cenrtrifuge bottles. Each bottle was spiked with 83 pg/ isomer of a mixture of nine ^{13}C-labeled PCDDs/PCDFs in 160 μl of methanol. The milk in each centrifuge bottle was digested with 45 ml concentrated H_2SO_4 by swirling and then rapidly mixed with 20 ml iso-octane prior to centrifugation at 5,000 rpm for 5 min. The iso-octane layer was removed and combined with two additional rinses of the Corex bottle (no mixing). A fraction representing 2% of the extract was set aside for lipid analysis and the remaining portion of the extract was mixed with an equal volume of CH_2Cl_2 prior to cleanup as described below. The clean-up method was based on a procedure described in the literature[4] in which the extract was subjected to preliminary cleanup on a large column (5 x 60 cm) containing KOH-treated silica gel (25 ml) and silica gel (20 ml) followed by a smaller column (2.2 x 30 cm) containing the same adsorbents. Final cleanup was

accomplished on three columns connected in series: an AX- 21 carbon column, a H_2SO_4-treated silica gel/silica gel column and an acid alumina column. Solvent flow through the columns was controlled by series of valves.

The high-resolution system gas chromatography (GC) /mass spectrometry (MS) system used to analyze sample extracts for PCDDs and PCDFs was a Hewlett-Packard (HP) model 5890A GC (Hewlett-Packard Co., Palo Alto, CA) interfaced directly to the ion source of a FISONS/VG AUTOSPEC Q high resolution MS. The GC was equipped with a fused silica capillary column coated with cross-linked 5% phenyl methyl silicone (50 m x 0.2 mm id, 0.25 μm film thickness) for high resolution gas chromatography, (DB5, J & W Scientific, Inc., Rancho Cordova, CA). The GC/MS system was connected through SIOS and Ethernet to a DEC VAX Station 3100 running FISONS/VG OPUS data system software.

Human Milk and Blood Plasma. The sample size was reduced to 100 ml. Therefore, the volumes of the iso-octane extraction solvent and H_2SO_4 and the two precolumn dimensions were scaled down accordingly. Other steps in the clean-up/analysis method were as described above for cow's milk.

3. Results

Comparison of Lipid Levels in Extracts Obtained from Homogenized Cow's Milk using Ethanol and Sulfuric Acid as Denaturing Agents. Subsamples from a 500 ml carton of whole milk were extracted using hexane and ethanol or iso-octane and H_2SO_4 for extraction with the following results for % lipid by weight:

Hexane/Ethanol	Iso-octane/Sulfuric Acid
2.5 ± 0.1 (7)	2.9 ± 0.2 (6)

Theoretically whole milk in the USA should contain 4% lipid by weight and it is apparent from these results that the iso-octane/sulfuric acid method results in higher recovery of the lipid than the hexane/ethanol method.

Stability of ^{13}C-OCDD in Cow's Milk Denatured by Sulfuric Acid. Considerable heat is generated when sulfuric acid is used to denature proteins in lipid-containing body fluids and we therefore investigated the possibility that higher molecular weight PCDDs/PCDFs could be dechlorinated to lower molecular weight PCDDs/PCDFs. However when the sulfuric acid extraction method was applied to a 400 ml volume of cow's milk spiked with 500 pg of ^{13}C-OCDD no ^{13}C-labeled tetra to hepta PCDDs were detected at a detection limit of approximately 1 pg/isomer. It has also been reported recently by Höckel et al[5] that PCDDs/PCDFs are not decomposed when heated to 80 °C for 20 min in the presence of concentrated sulfuric acid.

Application of the Method to Human Milk Samples. A study was carried out in our laboratory to determine PCDD/PCDF concentrations in selected groups of nursing mothers from rural areas of upstate New York and a summary of the results are shown in Table 1. There were no background contributions in the method blank for any of the 2,3,7,8-substituted compounds with the exception of OCDD and the contribution for this compound did not exceed 20% of the lowest OCDD concentration found in the samples. Recovery values for the nine ^{13}C-labeled internal standards varied from 83 ± 14% for 2,3,7,8-TCDD to 40 ± 27% for OCDD. The lower average recovery for OCDD reflects the low recovery of this internal standard in two samples.

Most of the not detected (ND) results were found in only one sample and other samples

had analyte concentrations which were generally not more than three times lower than the concentrations found in the sample containing the highest analyte concentrations. This range of PCDD/PCDF concentrations is within the range of values for breast milk samples obtained from human populations with no known accidental or occupational expoures[1].

Application of the Method to Cow's Milk Samples. Results from a study of the impact of municipal incinerator emissions on PCDD/PCDF concentrations in cow's milk are shown in Table 2. Since the analyte concentrations in cow's milk are close to an order of magnitude lower than those found in human milk it was necessary to use a 400 ml sample of cow's milk in order to obtain adequate detection limits. The detection limits for method blanks were found to be close to 0.01 pg/g fat and with these detection limits it was possible to detect most of the 2,3,7,8-substituted analytes in more than 90% of the control and study samples. Background contributions from the method blanks were minimal except in the case of OCDD and OCDF where the contributions could have been as high as 100% in some of the samples which contained low PCDD/PCDF concentrations. The precision of the results was determined by analyzing seven samples in duplicate. The average relative standard deviations from these duplicate analyses varied from 13 to 30% except in the case of OCDD and OCDF where the average relative standard deviations were 42% and 47% respectively. The higher relative standard deviations for OCDD and OCDF are probably a reflection of the high background contributions for each of these compounds.

4. Conclusions

Sulfuric acid was successfully used as a protein denaturing agent in the extraction of PCDD/PCDF compounds from cow's milk and human milk samples. Fat recoveries from homogenized cow's milk samples exceeded those found when ethanol was used as a denaturing agent It was also found, in comparison with ethanol, that solvent volumes were reduced by a factor of 7. The decrease in solvent volumes decreased the sample preparation time and also reduced the background contributions of PCDDs/PCDFs in method blanks. Experiments are currently underway to extend the iso-octane/sulfuric acid extraction method to the determination of PCDDs/PCDFs in human blood samples.

5. References

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Tabel 1. Summary of Results from a Study of PCDD/PCDF Levels (pg/g fat) in a Group of Human Milk Samples Collected in Upstate New York and Extracted using Sulfuric Acid as a Denaturing Agent.

Congener	^{13}C - Labeled Internal Standard Recoveries (n = 10)	Iso-octane/H_2SO_4 Extraction Method Blank[a, b]	Data Range for Study Samples (n = 10)[c]
2,3,7,8-TCDF	72 ± 11	ND (0.3)	ND - 0.8
1,2,3,7,8-PeCDF	78 ± 15	ND (0.3)	ND - 0.2
2,3,4,7,8-PeCDF		ND (0.4)	ND - 4.2
1,2,3,4,7,8-HxCDF	61 ± 17	ND (0.4)	ND - 3.6
1,2,3,6,7,8-HxCDF		ND (0.4)	ND - 2.3
2,3,4,6,7,8-HxCDF		ND (0.4)	ND - 0.9
1,2,3,7,8,9-HxCDF		ND (0.4)	ND (0.4)
1,2,3,4,6,7,8-HpCDF	53 ± 24	ND (0.6)	1.8 - 5.3
OCDF		ND (2.8)	ND - 4.1
2,3,7,8-TCDD	83 ± 14	ND (0.4)	ND - 1.8
1,2,3,7,8-PeCDD	76 ± 16	ND (0.9)	ND - 2.8
1,2,3,4,7,8-HxCDD		ND (0.4)	ND - 1.4
1,2,3,6,7,8-HxCDD	61 ± 20	ND (0.4)	4.2 - 19
1,2,3,7,8,9-HxCDD		ND (0.4)	ND - 3.3
1,2,3,4,6,7,8-HpCDD	51 ± 27	ND (0.6)	6.9 - 27
OCDD	40 ± 27	7.8	44 - 129

[a] Cleanup carried out in the absence of sample matrix. Data are normlized to 2 g lipid, the average lipid content of a 100 g milk sample.
[b] ND = not detected with dection limit in parenthesis for method blanks
[c] Detection limits are similar to those found for the iso-octane/H_2SO_4 method blank.

Table 2. Summary of Results from a Study of the Impact of Municipal Incinerators on PCDD/PCDF Levels in Cow's Milk Samples in New York State. Sulfuric Acid was used as a Denaturing Agent during Extraction.

	Concentration Ranges (pg/g fat)[a]		Relative (%)
	Method Blanks[b]	Control and Study Samples	Std. Deviation of Duplicates
Congener	(n = 10)	(n = 30)	(n = 7)
2,3,7,8-TCDF	ND (0.02) - 0.05	ND(0.02) - 0.16	30 ± 9.3
1,2,3,7,8-PeCDF	ND (0.01) - ND(0.1)	ND (0.01) - 0.05	
2,3,4,7,8-PeCDF	ND (0.01) - ND (0.1)	0.1 - 1.3	16 ± 12
1,2,3,4,7,8-HxCDF	ND (0.01) - 0.03	ND(0.1) - 2.0	17 ± 13
1,2,3,6,7,8-HxCDF	ND (0.01) - 0.03	ND (0.12) - 1.8	19 ± 15
2,3,4,6,7,8-HxCDF	ND (0.01) - 0.02	ND (0.08) - 0.9	13 ± 10
1,2,3,7,8,9-HxCDF	ND (0.01) - 0.02	ND (0.01) - 0.02	
1,2,3,4,6,7,8-HpCDF	ND (0.01) - 0.11	ND (0.07) - 5.1	27 ± 17
OCDF	0.24 - 1.17	0.4 - 6.1	47 ± 36
2,3,7,8-TCDD	ND (0.01) - 0.06	ND (0.02) - 0.2	20 ± 9.0
1,2,3,7,8-PeCDD	ND (0.01) - 0.02	ND (0.05) - 2.1	25 ± 15
1,2,3,4,7,8-HxCDD	ND(0.01) - ND(0.06)	ND (0.07) - 3.6	21 ± 17
1,2,3,6,7,8-HxCDD	ND (0.01) - ND (0.05)	0.1 - 13	20 ± 14
1,2,3,7,8,9-HxCDD	ND (0.01) - ND (0.05)	ND (0.15) - 6.8	14 ± 7.6
1,2,3,4,6,7,8-HpCDD	ND (0.16) - 0.22	0.3 - 41	21 ± 15
OCDD	1.4 - 6.8	2.4 - 58	42 ± 35

[a] ND = not detected with detection limit in parenthesis.

[b] Cleanup carried out in the absence of sample matrix. Data are normalized to 12 g lipid, the average lipid content of a 400 g milk sample.