Comparison of GC-MS with an *in vitro* bioassay for PCDDs and related compounds in environmental samples

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Background

The determination of the TCDD Equivalent Concentration of the contaminants in an environmental matrix is time consuming and labour intensive, and hence expensive. Typically it involves the following steps: (1) spiking the sample with isotopically labelled dioxin surrogates such as [$^{13}C_{12}$]-TCDD (2) extraction of the PCDD/PCDF fraction into an organic solvent (3) multiple chromatographic procedures to isolate the PCDD/PCDF fraction from lipids, polycyclic aromatic compounds and PCBs (4) analysis of PCDDs, and PCDFs by capillary GC/high resolution MS (5) application of Toxic Equivalency Factors (TEFs)[1] for 17 PCDD and PCDF congeners which are chlorinated in the 2,3,7 and 8 positions in order to afford a TCDD Equivalent Concentration (TEQ) for the sample.

Both cost ($500-$2000 per sample) and time considerations make it impractical to mount extensive screening or monitoring programs for dioxin-like substances in the environment. In addition, the omission of other substances such as coplanar PCBs and coplanar polychlorinated azobenzenes from the I-TEF scheme could cause the toxicity of an environmental sample to be underestimated. For example, coplanar PCB congeners such as 3,3',4,4'-tetrachloro-, 3,3',4,4',5-pentachloro-, and 3,3',4,4',5,5'-hexachloro-biphenyl can be found in a wide variety of animal tissues at hundred- or thousand-fold higher concentrations than the 17 PCDD/PCDF compounds for which I-TEF values are available[2]. Although these congeners elicit toxic and biological responses by factors of 10 to 1,000 times less than TCDD, their higher environmental concentrations could make them equally or more important than the seventeen PCDD/PCDF congeners considered in the I-TEF scheme with respect to toxic risk.

The ideal assay of environmental samples for PCDDs, PCDFs and related compounds would be rapid and inexpensive, and would afford a TEQ value that is inclusive of all dioxin-like components of the sample. Previous assays suggested include methods which screen for an observable toxic endpoint in intact animals[3,4], cell culture assays involving morphological change[5], enzyme induction[6], and radioimmunoassays[7], but none have found widespead use. Bradfield and Poland[8] described an assay for TCDD and closely related halodibenzo-*p*-dioxins based upon the competitive binding of the analyte and a

reference radioligand to the intracellular Ah (aryl hydrocarbon) receptor protein. This method is attractive because the toxicity of dioxin-like compounds is mediated through the Ah receptor[9]: since toxicity is only observed following receptor-ligand binding[10], the extent of binding to the receptor might serve as a surrogate measure of toxicity. Correlations between the strength of binding to the Ah receptor *in vitro*, enzyme induction *in vitro*, and toxic potency *in vivo* have been established for numerous classes of halogenated aromatic compounds[11].

We previously extended the competition assay of Bradfield and Poland as follows[12]: (i) by studying dioxin-like compounds of widely varying structure, including PCDDs, PCDFs, PCBs, polychlorinated azo- and azoxy-benzenes, and *trans*-stilbenes; (ii) by demonstrating additive behaviour for a variety of mixtures of these dioxin-like compounds; (iii) by using commercially available [^{3}H]-TCDD as the radiolabel rather than [^{125}I]-2,3-dibromo-7-iododibenzo-*p*-dioxin as used previously.

Knowing that mixtures of dioxin-like compounds bind additively to the Ah receptor, we can assume that the assay results include all substances which interact with the receptor (and hence are potentially toxic), whether or not they have I-TEFs assigned, and without pre-separation of the mixture. Thus the total TEQ of the sample is obtained in a single step, although the chemical identities of the specific dioxin-like compounds present are not determined.

Specific objectives of the work

1. Extension of the assay from synthetic laboratory samples to environmental matrices
2. Comparison of the TEQs obtained by means of GC/MS analysis and the Ah receptor assay
3. Determination of the extent of chromatographic clean-up necessary prior to successful application of the Ah receptor assay to environmental samples.

Methodology

Environmental matrices studied were fish tissue (Lake Ontario lake-trout) and fly-ash.

Hepatic cytosol was obtained from immature male C57BL/6N mice. After sacrifice, the livers were immediately perfused *in situ* via the hepatic portal vein with fresh buffer (ice-cold 23 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid + 1 mM tetrasodium ethylenediaminetetraacetate + 10% v/v glycerol + 1 mM dithioerythritol, pH 7.6). The excised livers were rinsed once with 15 mL of fresh buffer, finely minced, rinsed with buffer and homogenized in buffer. The homogenates were centrifuged at 10,000 x g for 20 minutes at 4° C, and the supernatant was centrifuged at 100,000 x g for 60 minutes at 4° C. The cytosol fraction (supernatant) was collected by aspiration, and its protein concentration determined[13]. Aliquots were stored in small volumes at -70° C until used.

Extraction: The samples to be analyzed by GC/MS (but not those for the screening assay) were spiked with ^{13}C-labelled PCDD and PCDF surrogates prior to extraction.

The fish tissue was homogenized; a subsample mixed with preextracted and dried sodium sulphate; Soxhlet-extracted with dichloromethane (DCM) for 16 hours;

and the DCM extract concentrated with solvent exchange into DCM:cyclohexane (1:1). Lipids were removed using gel permeation chromatography (GPC). Chromatography involved the successive use of (1) multilayer silica, acid-treated silica and base-treated silica for removal of polar, acidic and basic organics; (2) alumina for removal of nonpolar, nonplanar compounds such as aliphatic hydrocarbons, chlorobenzenes and PCBs; (3) Carbopack C to isolate the PCDDs and PCDFs from 'nonplanar' compounds.

The fly ash was treated with dilute acid, filtered, dried, and Soxhlet extracted with toluene prior to the same chromatographic methods as those just described. GC/MS was based on US EPA Method 1613. The eluate from the carbon column was concentrated with solvent exchange into nonane, and an injection/performance standard was added. The HRGC/HRMS system was a Hewlett Packard 5890 Series II capillary GC (60 m DB-5 column) coupled to a VG 70SE magnetic sector MS, with DEC 3100 model 38 workstation/OPUS 2000 operating system.

Receptor assay: The extracts or eluates were evaporated to dryness, and taken up in 100 μL of DMSO. Four 10 μL samples were withdrawn; two were used in duplicate assays at their original concentrations; the other two were serially diluted x 10 with DMSO to provide a dilution series ranging in concentration from 10^{-1} to 10^{-4} x the original concentration. Each of these samples was analysed by the hydroxylapatite assay[14] using a 10 μL aliquot of Ah receptor preparation and a reference radioligand concentration of 1 nM. This series of dilutions was used to estimate (to an order of magnitude) the dilution needed to afford a solution having a TCDD equivalent concentration corresponding to the EC_{50}. The TEQ of each sample was compared with that obtained by GC/MS. A second dilution series was then carried out over a narrower concentration range to locate the EC_{50} more precisely. Assays were carried out after Soxhet extraction (fly ash) or GPC (fish), and also after each of the 3 stages of chromatography.

Results and Discussion

Analyses of two fish and two fly ash samples are reported below.

Sample		TEQ(bioassay)		TEQ(GC/MS)	
Fly ash		**#1**	**#2**	**#1**	**#2**
#1	initial extract	394 ppb	355 ppb		
	stage 1	301	265		
	stage 2	283	291		
	stage 3	224	260	115 ppb	109 ppb
Fish					
#1	gpc	23,000 ppt	8,600 ppt		
	stage 1	4,900	4,900		
	stage 2	910	3,100		
	stage 3	490	950	55 ppt	89 ppt

Fly ash samples

1. There is little change (< x 2) in the TEQ obtained by bioassay as the clean-up procedure progresses. Little chromatographic clean-up is necessary with these

samples in order to carry out the bioassay successfully.
2. The bioassay and GC/MS results agree within a factor of 2 for these samples.
3. The bioassay always gives a higher TEQ than GC/MS, suggesting that there are other dioxin-like substances present in addition to the 17 2,3,7,8-substituted PCDDs and PCDFs.

Fish samples

1. There is a large diminution in the bioassay result as the clean-up proceeds. Extensive chromatographic clean-up of these samples is essential prior to bioassay.
2. The TEQ obtained from the bioassay was a factor of 10 greater than that obtained by GC/MS. This suggested extensive contamination with dioxin-like substances other than PCDDs and PCDFs.

Further analysis showed that the fish samples were contaminated with coplanar PCBs. Using the provisional TEFs suggested by Safe[11], we obtained the following results.

Sample	TEQ(GC/MS)	PCB:77	126	169	ΣPCB	ΣTEQ	Bioassay
1	55	54	690	29	773	828	490
2	89	57	496	35	588	677	950

Analysis of fish samples only for the 17 priority PCDDs and PCDFs thus substantially underestimates their load of dioxin-like compounds. After inclusion of the coplanar PCBs the GC/MS and bioassay methods are seen to be in much closer agreement.

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