RECOVERY DETERMINATIONS FOR BIOASSAY ANALYSIS: CONSIDERATIONS AND RESULTS

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Introduction

Recovery determination is important for quantitative methods such as high resolution gas chromatography/high resolution mass spectrometry (HRGC/HRMS) so that it is possible to determine the losses associated with the sample clean up process. This is true for bioassay screening methods as well, however, it is not possible to use the same methods to determined recoveries as are used for chemical analysis of dioxins. In HRGC/HRMS, known amounts of isotopically labeled congeners are spiked into the sample prior to extraction and the recovery of these labeled compounds is determined based on the amount that is detected. The recoveries for the labeled congeners are then used to estimate the original concentration of the congeners from the sample. This is possible because the mass spectrometer detector can differentiate between the ^{13}C labeled spike and the unlabeled sample analytes. This method of determining recoveries is not possible in bioassays because they do not differentiate between isotopically labeled and unlabeled analytes. Adding a spike to the sample, whether labeled or not will only cause a corresponding increase in the response in the bioassays without providing information on the recovery. Therefore, we have previously employed a surrogate recovery sample; a duplicate aliquot of the sample that is spiked then treated in the same way as the unspiked unknown sample[1]. The recovery is determined for the spiked compound and assumed to relate to the recovery in the unspiked sample. This method has obvious difficulties including the increase in the number of samples that must be processed as well as the uncertainty in applying the recovery for one sample to a separate sample. Recently we have investigated the use of biologically inactive congeners as internal spikes. In this report the results of this research is presented as well as information collected from both HRGC/HRMS recoveries and radiolabeled spike experiments on the importance of appropriate extraction and clean up methods on recovery determination and sample quantification.

Materials and Methods

Extraction recoveries

Samples were spiked with ^{14}C labeled 2,3,7,8-tetrachlorodibenzo-p-dioxin (2,3,7,8-TCDD) and extracted with various solvents or solvent mixtures. Samples were incubated in an ultrasonic water bath with four solvent changes and the solvents were pooled. The resulting extracts were submitted to scintillation counting to determine extraction efficiencies.

Active congener separation and recovery

Samples were spiked with high-resolution gas chromatography/high-resolution mass spectrometry (HRGC/HRMS) recovery standards then submitted to extraction and clean up using

our patent pending sample clean up method[2]. Briefly the clean up method involves an acid silica column in series with an XCARB column. Following sample addition, the XCARB column is differentially eluted to yield a polyhalogenated biphenyl (PCB) and a dioxins/dibenzofuran (PCDD/F) fraction. Following clean up the two fractions were submitted to HRGC/HRMS for recovery determination.

Surrogate congener recovery

Four nanograms of 1,2,3,4-tetrachlorodibenzo-p-dioxin (1,2,3,4-TCDD) were added to each sample prior to extraction. The samples were extracted and cleaned up using the dual column (acid silica – XCARB) method and the dioxin/dibenzofuran fraction was concentrated under vacuum, then resuspended in toluene containing 15 ng/ml of each of four injection standards (2,3',6-PCB; 2,2',5,5'-PCB; 2,3',4,5'-PCB and 2,3,4,4',6-PCB). Samples were applied to a 60 m x 0.25 mm DB-5 column and separated with a 210 ^{o}C to 310 ^{o}C ramp in temperature at 4 ^{o}C/minutes. Detection was by ECD using a Hewlett Packard Electron Capture Detector. The areas under the peaks were determined by integration and standardized based on the injection standards. Recovery was calculated based on the comparison to the original 1,2,3,4-TCDD spiking solution.

Results and Discussion

Extraction method - considerations

Based on US EPA methods 1613 all samples except tissue samples should be extracted using toluene[3]. In an effort to minimize concentration times, various more volatile solvents were tested for extraction versus toluene. Comparison of recovery levels indicated that solvents such as hexane were not consistently efficient. Non-tissue samples may contain activated carbon, which will retain the analytes of interest unless extracted with an aromatic solvent. The wide differences in extraction efficiency seen when using non-aromatic solvents maybe associated with whether or not activated carbon is not present, on a sample to sample basis. Based on these results all solid samples with low moisture content are extracted first with a 20% methanol solution in toluene followed by three extractions with toluene. The first 20 % methanol solution extraction assists in the removal of traces of water from the sample. Use of other solvents for extraction such as hexane, acetone, or methylene chloride is reserved for tissue samples that should not have activated charcoal present, which could retain analytes of interest.

Surrogate congener recovery

At the concentrations of 1,2,3,4-TCDD used for spiking samples the peak for 1,2,3,4-TCDD recorded by GC/ECD was clear and easily measured (see figure 1). This concentration of 1,2,3,4-TCDD did not significantly increase the response of the bioassay to the sample extract. Based on these results the use of 1,2,3,4-TCDD was considered promising. Further studies comparing recoveries determined by 1,2,3,4-TCDD with paired samples spiked with ^{14}C- 2,3,7,8-TCDD indicated that the recoveries determined by the two methods were similar (table 1). Additional studies with various sample matrices are planned to further determine the applicability of using 1,2,3,4-TCDD as an internal recovery standard. If determined to be effective, 1,2,3,4-TCDD could be added to all samples prior to extraction with only those samples found to be near the TEQ regulation level further analyzed by GC/ECD for recovery determination. For example recoveries would only be determined for samples greater than 50 % of the regulated TEQ level. Samples measuring less than 50 % of the regulatory level would be unlikely to surpass the regulatory level following recovery correction as extraction using appropriate solvent systems generally results in recoveries of greater than 70 %.

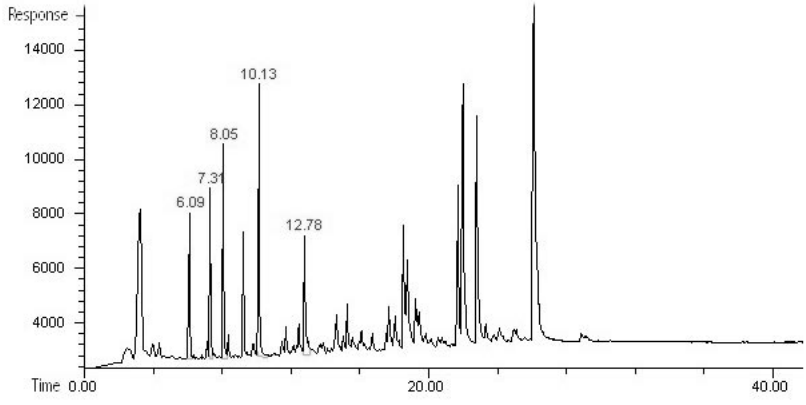

Figure 1. GC/ECD trace for a sample spiked with 1,2,3,4-TCDD. Peaks for the injections standards (2,3',6-PCB = 6.09 min.; 2,2',5,5'-PCB = 7.31 min.; 2,3',4,5'-PCB = 8.05 min. and 2,3,4,4',6-PCB= 10.13 min.) and for 1,2,3,4-TCDD = 12.78 min. are identified.

Table 1. Comparison of 1,2,3,4-TCDD recoveries determined by GC/ECD and ^{14}C labeled 2,3,7,8-TCDD recoveries determined by scintillations counting.

Sample	1234-TCDD by GC/ECD			^{14}C 2,3,7,8-TCDD by scintillation counting		
1	76.3 %	+/-	1.1 %	91.2 %	+/-	0.9 %
2	95.4 %	+/-	1.7 %	84.1 %	+/-	2.9 %
3	91.6 %	+/-	1.8 %	89.9 %	+/-	2.4 %
4	90.6 %	+/-	0.3 %	83.5 %	+/-	3.4 %

Active congener separation and recovery

^{13}C labeled congeners were subjected to our sample clean up and separation method and the resulting fractions were submitted to HRGC/HRMS for recovery determination. The PCDD/F congeners were found only in the PCDD/F fraction (see table 2), however, two of the PCB congeners (PCB #126 and 169) were not completely eluted in the PCB fraction. These two PCB congeners are retained by the XCARB column and tail into the PCDD/F fraction. Attempts were made to increase the elution of these compounds by increasing the toluene concentration used to elute the PCB fraction. This resulted in nearly complete elution (greater than 90 % recovery in the PCB fraction) of these two PCB congeners, however, it also resulted in the tetrachlorinated dioxin and dibenzofuran being partially eluted in the PCB fraction (24 % and 27 % of 2,3,7,8-TCDD and 2,3,7,8-TCDF were recovered in the PCB fraction respectively). Further studies indicated that it was not possible to consistently and completely separate the two classes of compounds, therefore minor contamination of the PCDD/F fraction by PCB 126 and 169 were accepted in order to obtain consistent recovery of the dioxin and dibenzofuran congeners in the PCDD/F fraction.

Table 2. Congener specific recoveries for sample clean up and separation.

	^{13}C		Recovery (%)		WHO-TEF
			PCB Fraction	PCDD/Fs Fraction	
PCDDs	2378-TCDD		0	92	1
	12378-PeCDD		0	99	1
	123678HxCDD		0	96	0.1
	1234678HpCDD		0	102	0.01
	OCDD		0	97	0.0001
PCDF	2378TCDF		0	98	0.1
	12378PeCDF		0	99	0.05
	123478HxCDF		1	106	0.1
	1234678HpCDF		0	98	0.01
	OCDF		0	95	0.0001
non-ortho	33’44’-TCB	#77	101	5	0.0001
	344’5-TCB	#81	96	3	0.0001
mono-ortho	33’44’5-PeCB	#126	70	12	0.1
	33’44’55’-HxCB	#169	72	34	0.01
	2344’5-PeCB	#114	100	1	0.0005
	233’44’5-HxCB	#157	106	2	0.0005
	233’44’55’HpCB	#189	118	2	0.0001

Conclusions

Because of differences in the way that bioassays and chemical analysis methods detect compounds it is not possible to directly use extraction and clean up recovery methods from chemical analysis in sample preparation for bioassay analysis. However, it is necessary to include recovery determination in order to avoid under reporting the TEQ contamination of samples. Ignoring recovery will tend to increase the rate of false negatives, especially for samples that are more difficult to extract. Here we report promising preliminary data on the use of a surrogate congener internal spike that is appropriate for bioassays. The use of an inactive congener as a recovery spike allows for estimation of the recovery efficiency for samples that are close to the regulated level, while not interfering with the detection of the bioassay.

Dioxin bioassays do not normally allow for differentiation between difference types of compounds, as they respond to all compounds that can bind to and activate the receptor. However, by coupling the bioassay with a sample clean up method that allows for the separation of PCB and PCDD/F fractions it is possible to obtain additional information about a sample.

Acknowledgements

This study was funded in part by a Small Business Innovation Research Grant from the National Institutes of Environmental Health Sciences (ES 08372-03).

References

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3. EPA method 1613, US Environmental Protection Agency, 40 CFR Part 136.