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DEVELOPMENT OF AN AUTOMATED SOLID PHASE EXTRACTION METHOD FOR THE MEASUREMENT OF HALOGENATED PHENOLIC COMPOUNDS FOR HUMAN BIOMONITORING USING HIGH-RESOLUTION MASS SPECTROMETRY DETECTION.

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Introduction

Halogenated phenolic compounds (HPCs) are a class of contaminants that have been associated with thyroid hormone status in both *in vitro* and laboratory animal studies. HPCs are primarily metabolites of persistent organic pollutants (POPs), especially PCBs, but include other compounds that may not be metabolites, such as pentachlorophenol (PCP) and other halogenated phenols. Hydroxylated metabolites of PCBs (HO-PCBs) are the most numerous of the HPCs in human and wildlife blood. The hydroxyl group on HPCs makes these compounds potential binding components to transthyretin (TTR), one of the main thyroid hormone transport proteins in humans. As shown in rat and mice dosing studies, HPCs can disrupt thyroid hormone transport by inhibiting the binding of thyroxine (T4) to TTR [1]. In addition, HPCs have been shown to affect brain deiodinase activity and thyroid and estrogen hormone metabolism in *in vitro* systems [2;3]. The toxicologic relevance of HPCs makes accurate determination in humans a priority for biomonitoring programs. This paper describes a new method developed for the extraction and measurement of HPCs in human serum samples.

Methods and Materials

Automated solid phase extraction (SPE) was completed on the Zymark Rapidtrace (Zymark Corporation, (Hopkinton, MA)). The SPE method optimization is a continuation of the semi-automated comprehensive extraction multiple fractionation (SACEMF) method described by Sandau *et al*.[4] The extract (10 mL, 10% methanol/dichloromethane) from the OASIS sorbent cartridge (540 mg, 3 mL cartridges, Waters Corporation, Milford, MA) was evaporated to ~ 500µL on a Labconco Rapidvap (Kansas City, MI) and applied to an activated silica cartridge (1 gram, 3 mL) using the automated SPE system. Two fractions were collected for each sample from each cartridge. The first fraction consisted of the load volume and dichloromethane:hexane (5%, 6 mL). The second fraction containing the polar compounds consisted of methanol:dichloromethane (10%, 8 mL). The first fraction is purified as described previously [4]. Both fractions were collected at a flow rate of 1 mL/min. Fraction 2 was reduced in volume, derivatized with diazomethane, and purified on a custom-made sulfuric acid:silica gel cartridge (22%, 0.3 g) topped with a potassium hydroxide:silica gel layer (1 M, 33%, 0.1 g). The eluant used to remove the analytes was dichloromethane (10 mL). The purified extract was then reduced to final volume and spiked with recovery standard for analysis.

All extracts for method development were measured using an HP5973 Mass Selective Detector (MSD) interfaced with an HP6890 gas chromatograph from Agilent Technologies

(Atlanta, GA). The injection of 50 µL was completed with an HP5723 auto sampler using programmable temperature vaporization (PTV) inlet. The GC column used was a DB-5MS (30 m, 0.25 mm i.d., 0.25 µm film thickness) from J&W Scientific (Folsom, CA). The GC ramp began at 80°C and ramped to 320°C over 30 minutes. The interface with the MSD was kept at a constant 280°C. The MSD was operated in negative chemical ionization mode with methane as the reagent gas. The source temperature was held at a constant 150°C. The MSD was operated in selected ion monitoring (SIM) mode with two ions selected per analyte.

An isotope dilution quantification method using labeled carbon-13 internal standards was developed on a gas chromatography high-resolution mass spectrometer (GC-HRMS) using an electron impact ionization method (VG Autospec – Micromass) using the same GC column and GC conditions described above. Two ions per compound were chosen, one for quantification and one as a qualifying ion for peak confirmation. Response ratios were calculated as the ratio of the molecular ion of the analyte to the molecular ion of the closest eluting carbon-13-labeled internal standard with the same number of chlorines. Instrumental quantification limits (IQL) were calculated as five times the signal to noise ratio for the lowest quantifiable standard from the calibration curve. All solvents were GC analysis-grade and reagents were highest purity available.

Results and Discussion

Recently, a method was developed at the Centers for Disease Control and Prevention (CDC) for the extraction of persistent pesticides and other POPs, including metabolites and HPCs[4]. This extraction technique was further adapted to include the isolation and purification of the HPCs for a separate analysis by GC-HRMS. This was accomplished by using a polar separation on custom packed activated silica gel cartridges as described above. Figure 1 shows the separation efficiency of the silica cartridge for most of the polar compounds, which included both HO-PCBs and methyl sulfonyl metabolites of PCB. The first fraction contained all the non-polar compounds, such as the chlorinated pesticides, PCBs, PCNs and PBDEs. The second fraction, after derivatization, containing the polar compounds was purified using a combination of acidic silica gel and basic silica gel to remove and purify the extract for final analysis. None of the compounds analyzed in this fraction were adversely affected by the aggressive cleanup column.

The newly developed SPE method was compared with a previously used liquid:liquid extraction method described by Hovander et al. [5] to compare extractability of HPCs between the two techniques. Both the liquid:liquid and SPE methods gave similar analyte concentrations (n=5) for the extraction of a real matrix (Figure 2).

The method has been used in a pilot project for the analysis of plasma from pigs exposed *in vivo* to PCBs and persistent pesticides. Recoveries of selected carbon-13-labeled internal recovery standards for the first 200 samples analyzed were acceptable (recovery ± standard deviation): hexachlorobenzene (99%±23), CB153 (73%±12), pentachlorophenol (95%±45), 4-HO-CB120 (64%±20), 4-HO-CB187 (65%±23). These recoveries demonstrate that the method has acceptable and reproducible recoveries for isotope dilution quantification. The large standard deviation for pentachlorophenol is a result of some co-elution problems while using the GC-MSD for analysis in some of the samples. The co-elution no longer exists when switching to high resolution mass spectrometry detection.

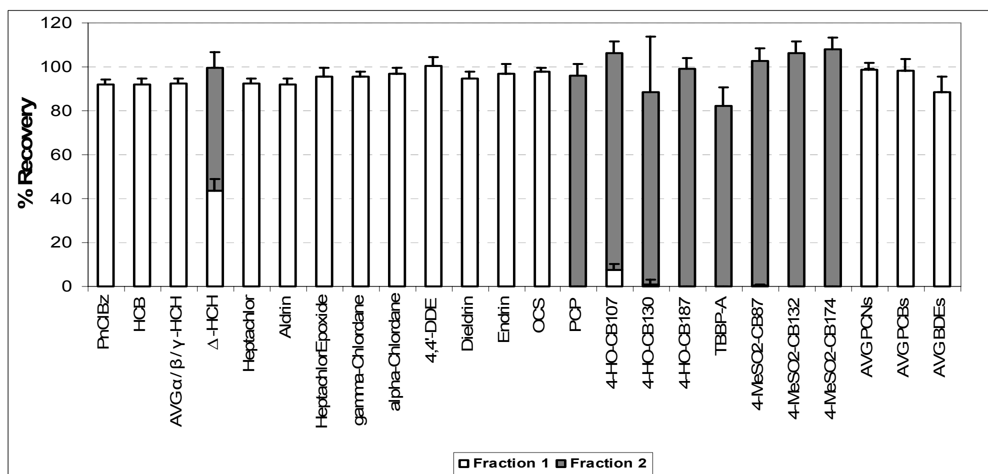

Figure 1 - Separation of polar and non-polar compounds using automated solid phase extraction with custom packed 3 mL silica gel (1 gram) cartridges.

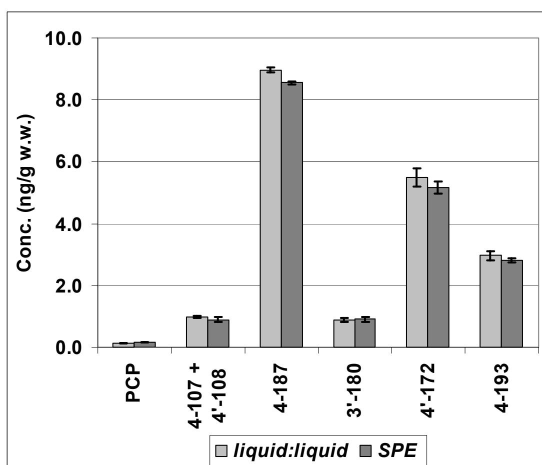

Figure 2 - Comparibility of quantification results using two different extraction

With these results, a complete SPE method using isotope dilution quantification was developed with detection on a GC-HRMS. Previous studies used electron-capture detection [6] or electron-capture negative chemical ionization (ECNI) mass spectrometry [7] for HPC measurement. Even though both offer adequate detection limits, ECD is limited by not offering confirmation ions for peak identification or overlapping peak determination, while ECNI has skewed responses for compounds containing different number of chlorines. The skewed responses of ECNI allow lower detection limits for higher chlorinated compounds while having impractical detection limits for lower chlorinated compounds, such as PCB metabolites with less than four chlorines. To address this issue, HRMS can be used to enhance specificity, maintain low detection limits and allow for the quantification of lower chlorinated compounds. Figure 3 shows the calibration curves for selected compounds representing tetra-, penta-, and hepta-chlorinated HO-PCBs. Response ratio with concentration for the higher chlorinated HO-PCB increased slightly (Figure 3) but slopes were not as different as previously used detection techniques (results not shown). The IQLs for the compounds shown in Figure 3 ranged from 17 to 30 fg/µL. This demonstrates that HRMS can be used for the detection of lower chlorinated compounds as easily

as higher chlorinated compounds and HRMS delivers detection limits adequate for human biomonitoring of HO-PCBs.

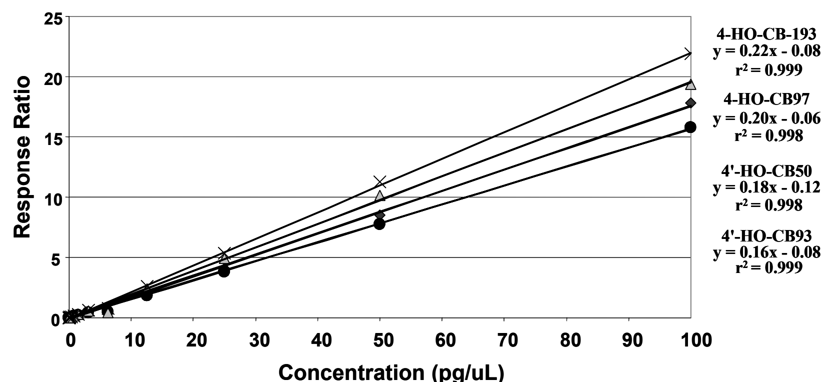

Figure 3 - GC-HRMS calibration curve for four selected HO-PCB analytes. Each point represents the average response ratio for curve which was run in triplicated triplicate.

With a new comprehensive list of recently synthesized HO-PCBs to cover the main HO-PCBs found at detectable concentrations in human serum [7;8], a sensitive, congener specific metabolite analysis method can be successfully applied to human samples. This will be another addition to a multi-fraction "universal-method", which will add an important class of compounds to CDC's human biomonitoring program.

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