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DEVELOPMENT OF A COMPREHENSIVE NON-TARGETED SCREENING METHOD FOR ORGANIC CONTAMINANTS IN HUMAN PLASMA

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Introduction

When we begin to think of human exposure and chemical risk there is a list of compounds considered 'legacy' such as PCBs and dioxins and emerging contaminants of concern such as the phthalates and the parabens. But we live in a society that is immersed in chemicals. In 1979 the Toxic Substances Control Act, (TSCA) listed some 62'000 chemicals and today that inventory stands at 83'000 chemicals where more than 4500kg are imported or used in the United States1. In the EU, the Registration, Evaluation, Authorization and Restriction of Chemicals (REACH) listed some 143'000 compounds marketed within the European Union where >100 tons per year were imported or produced. Of these 143'000 compounds 168 were listed as substances of concern2. However more than 7 million chemicals have been identified so we can surmise that our potential exposure to a complex array of chemicals is high.

Traditional organic chemical environmental monitoring is limited to a target series of compounds and often chemists find that they are subject to the whim of popularity. For example, the identification of PFCs became a very popular topic and today phthalates are a class of compounds where there is much interest and thus potentially much funding. The problem however especially in the field of human exposure is that it is relatively expensive to collect a sample, and often the extraction method is tailored to a specific compound class. Archives of human samples may be the key to historical biomonitoring but are limited in the volume stored, difficult to access and expensive to maintain.

A major factor in contaminant monitoring is the wide range of physical and chemical properties. Over the last decade there has been a great deal of attention on non-target methods for the determination of compounds. High-resolution mass spectrometry is a powerful tool for non-target detection enabling the qualification and quantification of a wide range of organic compounds and their metabolic transformation products3. However most often the samples screened tend to be those extracted previously for a targeted analysis, thus they may be subject to cleanup processes and unknown recovery. To make the full use of high resolution non-target screening, sample preparation must capture the widest range of compounds while eliminating matrix interference4.

By using GC-Orbitrap-MS and LC-Time of Flight-MS (LC-QTOF-MS) along with the analysis of differing sources this project aims to develop a robust operating procedure that will minimize the number of instrument runs while maximizing the information for a given sample. A complex mixture of >200 compounds including POPs, flame retardants, pesticides, plasticizers and pharmaceuticals was analyzed by 6 source conditions on LC-TOF and 3 on GC-Orbitrap operating in full scan. This same mixture was spiked in triplicate to human serum and extracted through 5 traditional extraction methods used in human exposure monitoring. Both target/suspect screening and non-target screening techniques were then applied to these samples for the determination of recovery of compounds and the detection of unknown compounds.

Method

Extraction

A mixture of more than 200 compounds including PCBs (n=10), PAHs and their metabolites (n=48), Pesticides, herbicides and insecticides (n=>50), BFRs (n=22), phthalates (n=21), siloxanes (n=7), musks (n=6) steroids and pharmaceuticals (n=10) was prepared, and further used as calibration curve with mass labeled compounds.

Two types of methods were considered, liquid/liquid (LLE) and solid phase extraction (SPE), with the process described in figure 1. For LLE two solvents, Dichloromethane (DCM) and Hexane, were chosen and for SPE 3 cartridges with similar hydrophilic, lipophilic properties; HLB (Waters Oasis, Millford MA), Hypersep Retain (Thermo-Fisher) and Bond Elut Plexa (PCX; Agilent). All cartridges contained 60mg of sorbent within 3mL cartridges. In triplicate serum, (Male AB serum from plasma; Sigma Aldrich) were fortified with the spike mix and extracted either by LLE or SPE along with triplicate plasma blanks and triplicate solvent blanks. After extraction and concentration, a mix of 10 Mass labeled BFRs, pesticides and hormones were introduced as instrument standards.

Instrument Analysis
Both LC-QTOF (Agilent 6550 iFunnel Q-TOF LC/MS) and GC-Orbitrap (Q Exactive™ GC Orbitrap™ GC-MS/MS) were used for non-targeted screening of plasma with instruments operating in EI+, NCI and PCI on the GC-Orbitrap and in both + and – modes for ESI, APPI and APCI on the LC-QTOF.
The GC-Orbitrap was ran at 60'000 resolutions with a run time of 6-60 min and a range of 66-1000 AMU. A 60mTrace Gold column was used (film 0.25 µm, id 25mm) for separation of analytes, with an initial temperature of 80°C holding for 1 minute followed by a ramp of 7°C to 310°C. In CI, methane was used at a flow of 1.25 mL min-1.
For the LC-QTOF solvents consisted of 10% MeOH, 90% H2O, 0.1% FA (A) and 100% MeOH, 0.1% FA (B) with an initial gradient of 0% B raising to 100% B over 15 minutes holding for 7.5 minutes before returning to 100% A at a constant flow of 0.3 mL min-1. A C18 column (Waters Acquity UPLC, BEH C18, 1.7 µm, 2.1x100mm) was used for the separation of analytes. The MS ran in auto ms/ms with a range of 50-1000 AMU.
Determination of compounds
Determination of spiked compounds was performed initially using MassHunter Qual software for LC-QTOF and on the GC-Orbitrap by an in-house library with Excalibur software using additional validation via NIST and peer reviewed literature. Further screening on the LC-QTOF was required for positive identification of some compounds not determined through software processing.
Non-targeted screening for ions was performed on both GC and LC separated samples using Sieve for the GC and Agilent MassHunter Profinder on the LC, with 3 times the baseline as a minimum criterion for inclusion. Ions not present in all 3 replicates of any sample were subsequently excluded from the data as were peaks <2.5 times greater than the solvent blanks. Further exclusion of samples with >30% standard deviation between replicates was performed.

Results and discussion
Of the 244 compounds, 156 compounds were positively identified by GC-EI/MS +ve using an in-house library in EI mode and 45 compounds by LC-ESI +ve. The low determination of compounds in LC is not unexpected as a wide range of compounds are more readily ionized by GC. Plasma samples were further tested for non-target peak identification of ions using the differing extraction methods. Criteria for positive detection was limited to only ions found in all triplicates at intensities greater than 2.5 times the solvent blank with a standard deviation of <40%. Adjustment to the ratio of internal standard may further improve the standard deviation.
A clear distinction is determined between LLE extraction and SPE methodology (figure 2) indicating that use of either method for non-target determination of compounds will produce potentially different results. Identification of the 'best' ion source will depend largely upon if specific compounds are of interest however the three SPE cartridges showed a large degree of cross over in GC-NCI and LC- ESI, but less so for GC-EI +Ve (figure 3). Further analysis however of GC-EI indicates that much variability may be due to software generating different peaks for compounds with 0.01s drift.
By assessment of both the GC-Orbitrap and LC-TOF in tandem and differing modes it is clear that though current non-target screening identifies a range of compounds, combining these powerful tools creates a comprehensive data set that not only will determine the current list of compounds of concern but also the chromatograms will provide an opportunity for qualitative or semi-quantitative identification of emerging compounds without the need for a whole new biomonitoring program. Current work is to further develop the identification process and identify the ionization and instruments that will produce he most comprehensive data set with the minimum amount of processing time.
A further 300 compounds of concern have since been purchased including a greater number of LC amenable compounds and these will be tested against the extraction methods for greater understanding of the recovery for each method. Also use of an additional solvent in SPE elution will be introduced for better recovery of compounds such as PCBs. This work has two valuable contributions in that for existing non-target publications that use similar methods to those tested here the limitations can be seen and for future biomonitoring studies it is possible to create a more comprehensive non-target screening process that will identify the greatest number of organic compounds.

Acknowledgements
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Reference

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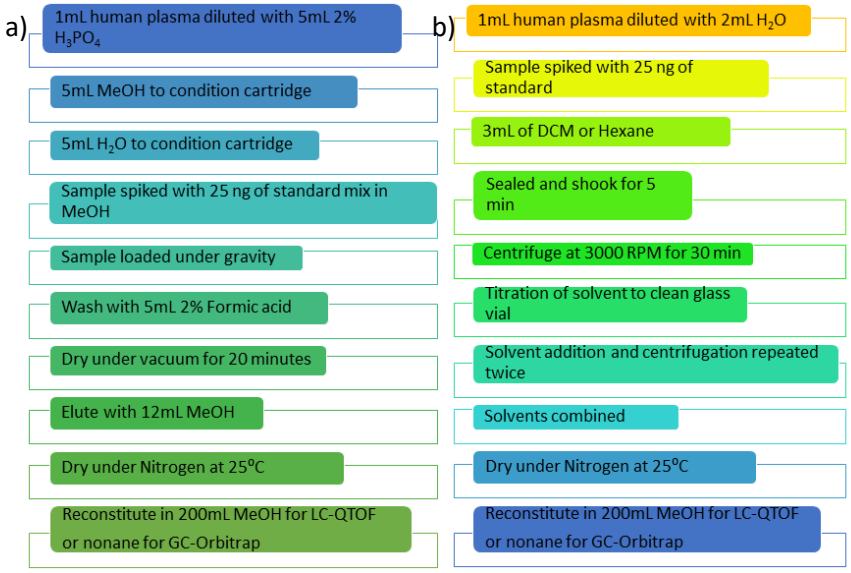

Extraction Method	LC-QTOF ESI Positive			GC-Orbitrap EI Positive		
	average recovery	Std dev	compounds	average recovery	Std dev	compounds
Solid Phase Extraction						
Plexa	69%	33%	31	42%	8%	84
HyperSep	87%	25%	36	57%	8%	91
HLB	91%	16%	36	82%	14%	89
Solvent Extraction						
DCM	90%	27%	34	78%	13%	86
Hexane	59%	22%	26	32%	25%	72

Table 1: Recovery of spike compounds from solid phase extraction (SPE) and liquid-liquid extraction (LLE)

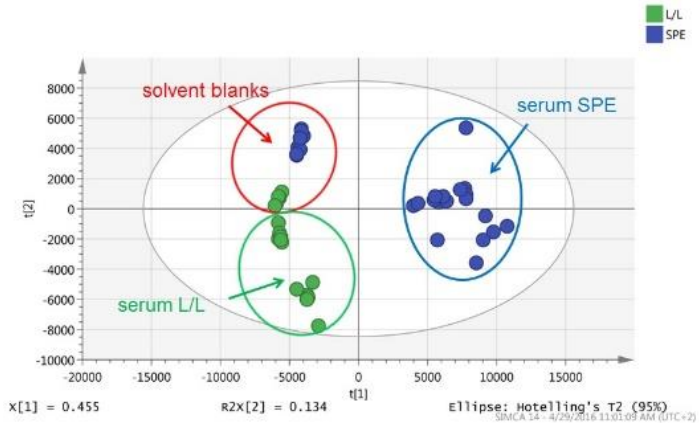

Figure 2: Variation in LLE and SPE determination for LC-ESI

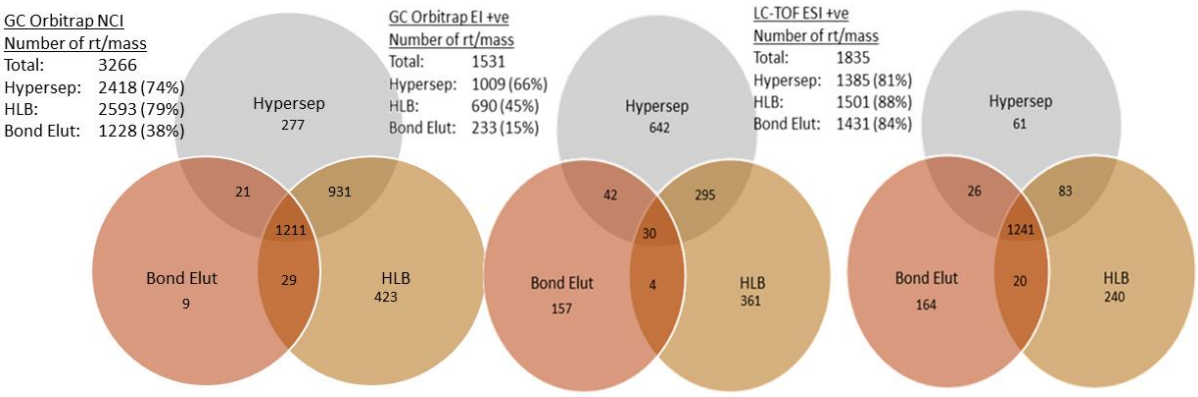

Figure 3: Venn diagrams of Ion peaks detected on LC-TOF ESI +ve and GC-Orbitrap EI in positive and NCI.