HIGH RESOLUTION MASS SPECTROMETRY PROVIDES NOVEL INSIGHTS INTO PRODUCTS OF HUMAN METABOLISM OF ORGANOPHOSPHATE AND BROMINATED FLAME RETARDANTS

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ADAPT

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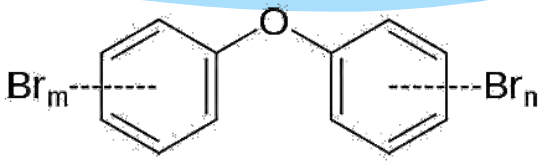
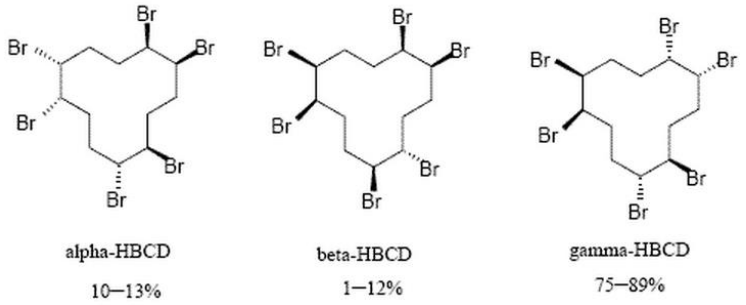
Organic Flame Retardants

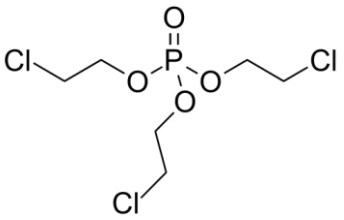
* Diverse group of halogenated chemicals.
* Widely applied in building materials and consumer products.
* Persistent, Bioaccumulative and Toxic (PBT) properties.
* Some are banned (e.g. Penta-BDEs) and others undergoing risk assessment.



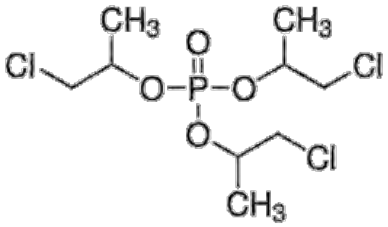
PBDEs



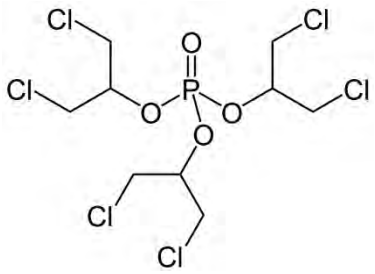
HBCDs



TCEP



TCiPP



TDCiPP

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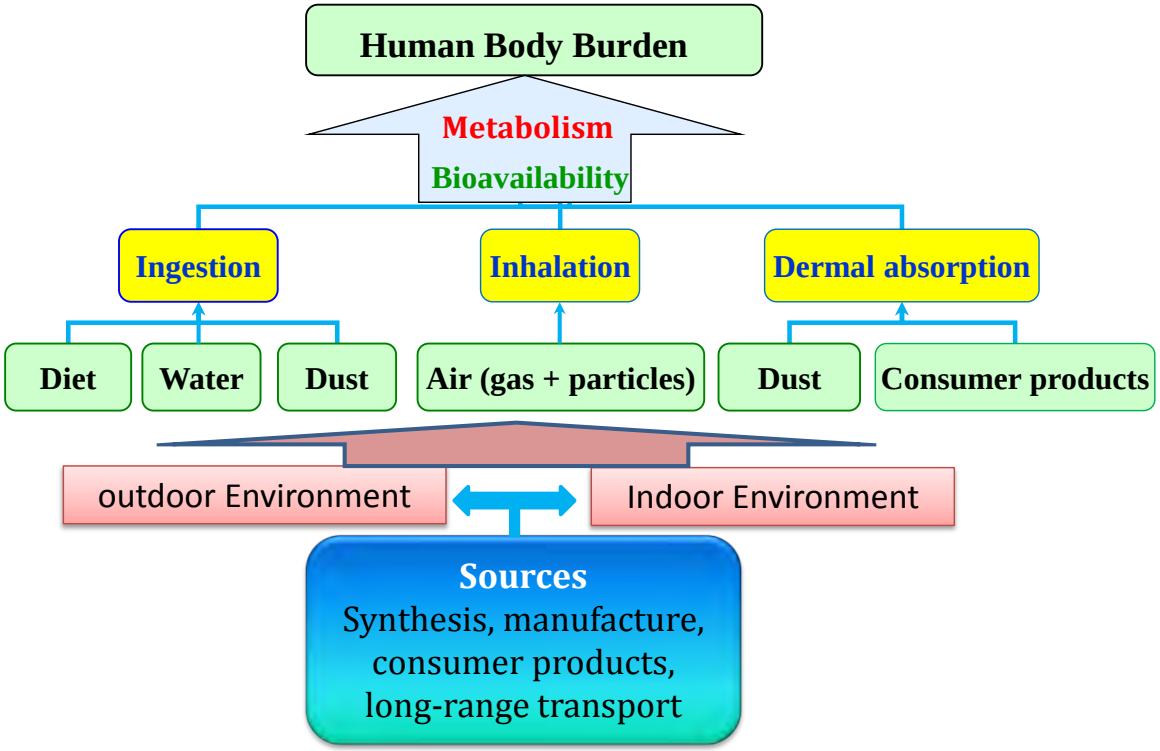
Organic Flame Retardants

* Most FRs are physically blended with rather than chemically bonded to polymers.
* Indoor dust has been consistently identified as a major pathway of human exposure to FRs.
* Higher BFRs can be biotransformed to more toxic and bioaccumulative metabolites.
* ***Further understanding of the metabolic process is essential for accurate risk assessment of these hazardous chemicals***



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Research Gap

* Few studies have investigated the metabolic pathways of different flame retardants present in indoor dust.
* Most of these studies have focused on PBDEs using animal or human LME, hepatic S9 fractions and rarely, human hepatocytes.

PBDEs are banned

➢ Very little is known about the metabolic pathways of alternative flame retardants in humans.

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Research Gap

* **Most *in vitro* biotransformation studies focus on exposing the metabolising system (LME, S9 or hepatocytes) to a single xenobiotic at a time which doesn't mimic the *in vivo* situation.**
* **No studies of HBCD metabolism in humans.**
* **No studies of TCEP, TCIPP and TDCPP in human hepatocytes which contain both Phase I and Phase II metabolic enzymes.**
* **The analytical capabilities and performance of the Orbitrap™-MS have not been fully evaluated in the field of POPs analysis.**

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The Orbitrap-MS (Exactive plus)

* High resolution (up to 140,000 FWHM).
* High mass accuracy (up to 1 ppm)-improved selectivity.
* High sensitivity- Adjustable AGC
* High scan rate.
* Rapid polarity switching of the ion source.
* Optional HCD cell- AIF spectra for structural confirmation.
* Optional quadropole for MS/MS analysis-*we didn't have that.*

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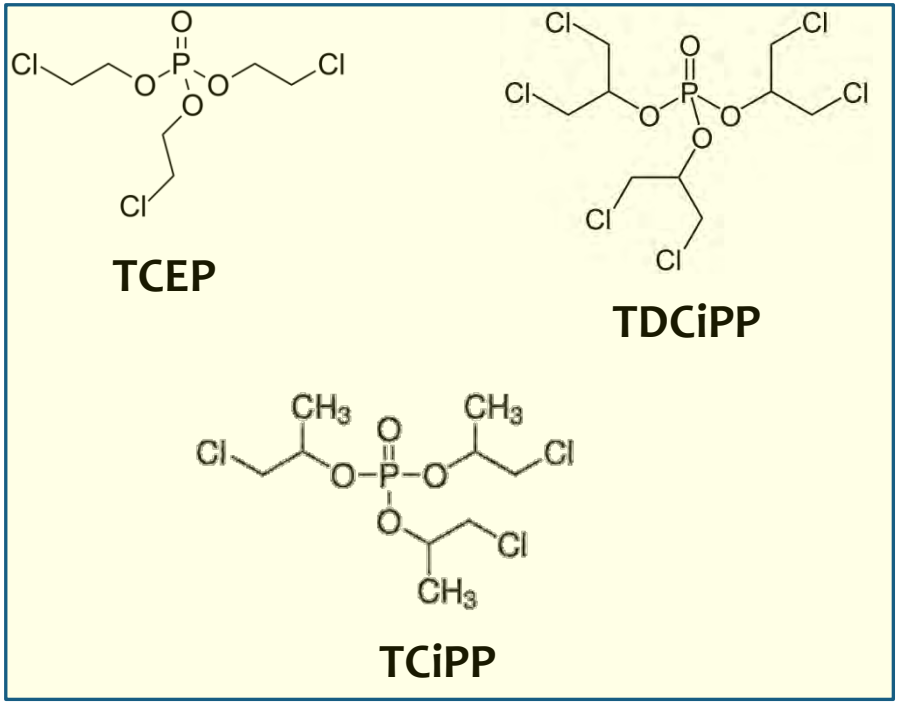
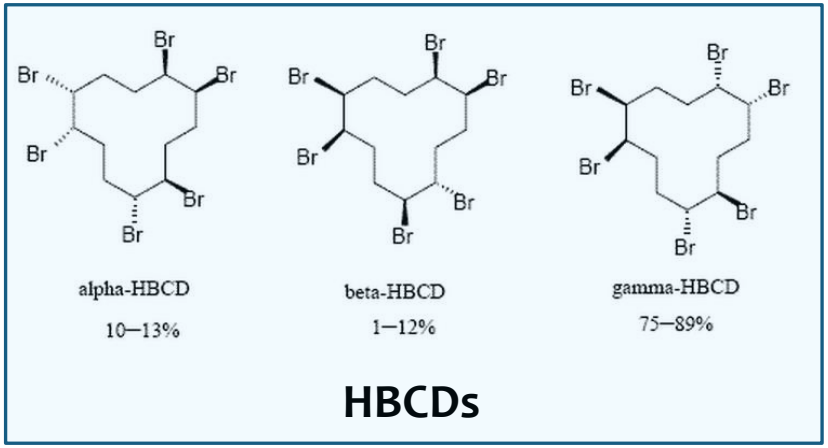
Aim

* **Study the metabolic profiles of HBCDs, TCEP, TCIPP and TDCPP in indoor dust (NIST SRM 2585), applied concomitantly to human hepatocyte cultures using UPLC-Orbitrap-MS.**





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Experimental

* **Cell cultures:** Human HEPG2/C3A cell lines were seeded and cultured in 6-well plates at 2 x 10^6 cells/well in modified William's E medium (containing 5% FBS).
* **Dosing Solutions:**
  * D1- SRM 2585 dust extract (using Dionex ASE 350)
  * D2-Synthetic mixture of the target compounds (HBCDs and 3 PFRs) with the same concentrations as in D1.
  * ***Exposure Scenario*:** 2 million cells exposed to the equivalent of 12 mg dust based on a 12.3 Kg toddler ingesting 200 mg dust/day.

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Experimental

Incubation at 37°C with humidified air containing 5% CO_2 for 24 hours

Extraction with methanol - *QUESHERS*

Instrumental Analysis- UPLC-ESI-Orbitrap MS

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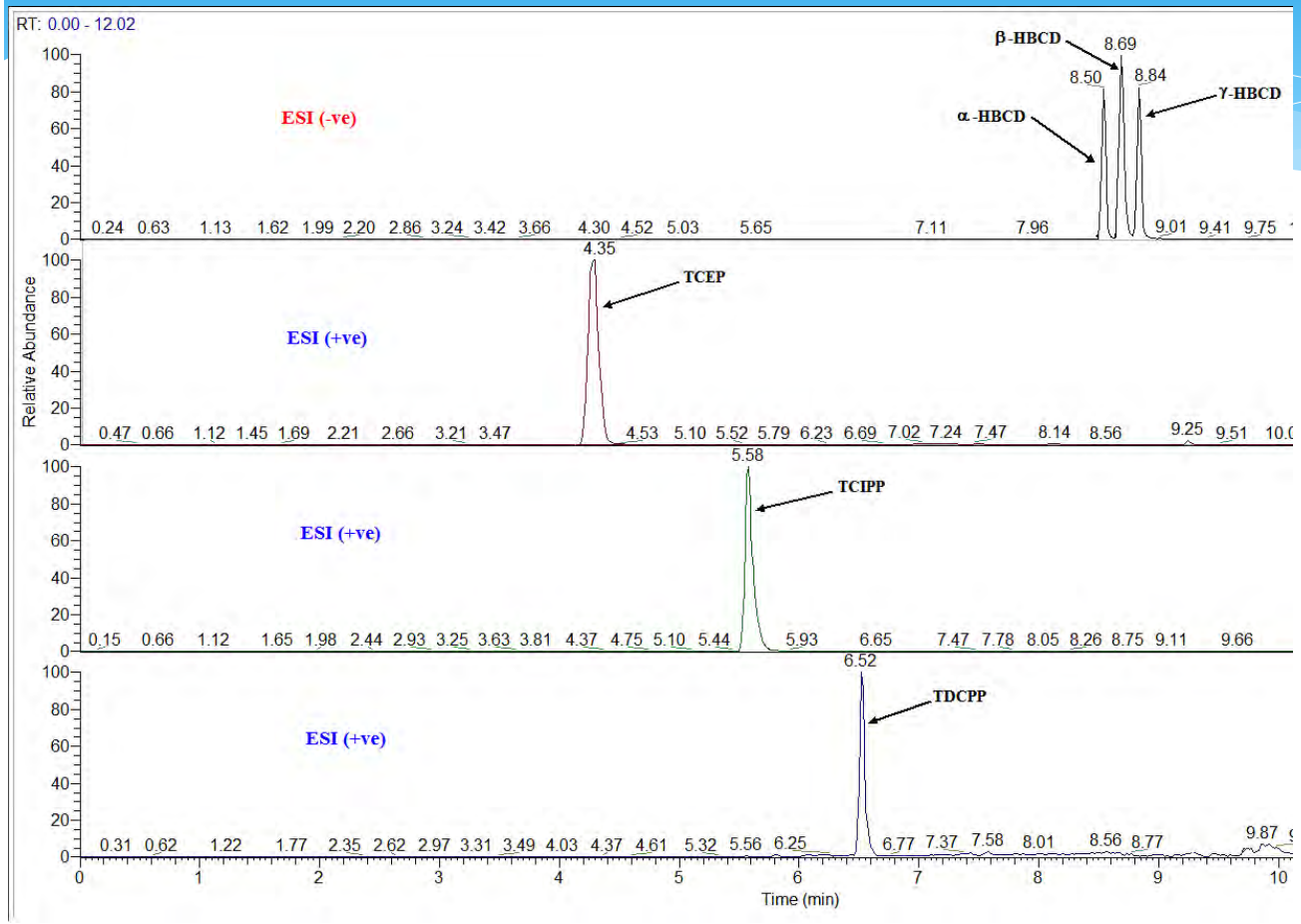
Results-Separation



- C_{18}-RP columns (100 x 2.1 mm) :
  - Syncronis™ (1.7 µm)
  - Hypersil gold™ (1.9 µm)
  - Acclaim™ (2.2 µm)
  - Hypersil™ (2.4 µm)
  - ✓ Accucore™ (2.6 µm)
- Temp. 37°C
- Flow rate: 0.18 ml/min
- Inj. Vol. 10 µL

Mobile phase: 1 mM ammonium acetate (mobile phase A) and Methanol (mobile phase B), each modified with 0.1% formic acid. The elution programme commenced with 25% B ramped up to 50% B over 0.5 min, then increased linearly to 100% B over 6 minutes. This was held for 4 minutes, then decreased to 50% B over 0.5 min and kept at this composition (to equilibriate the column) for a further 1 minute.

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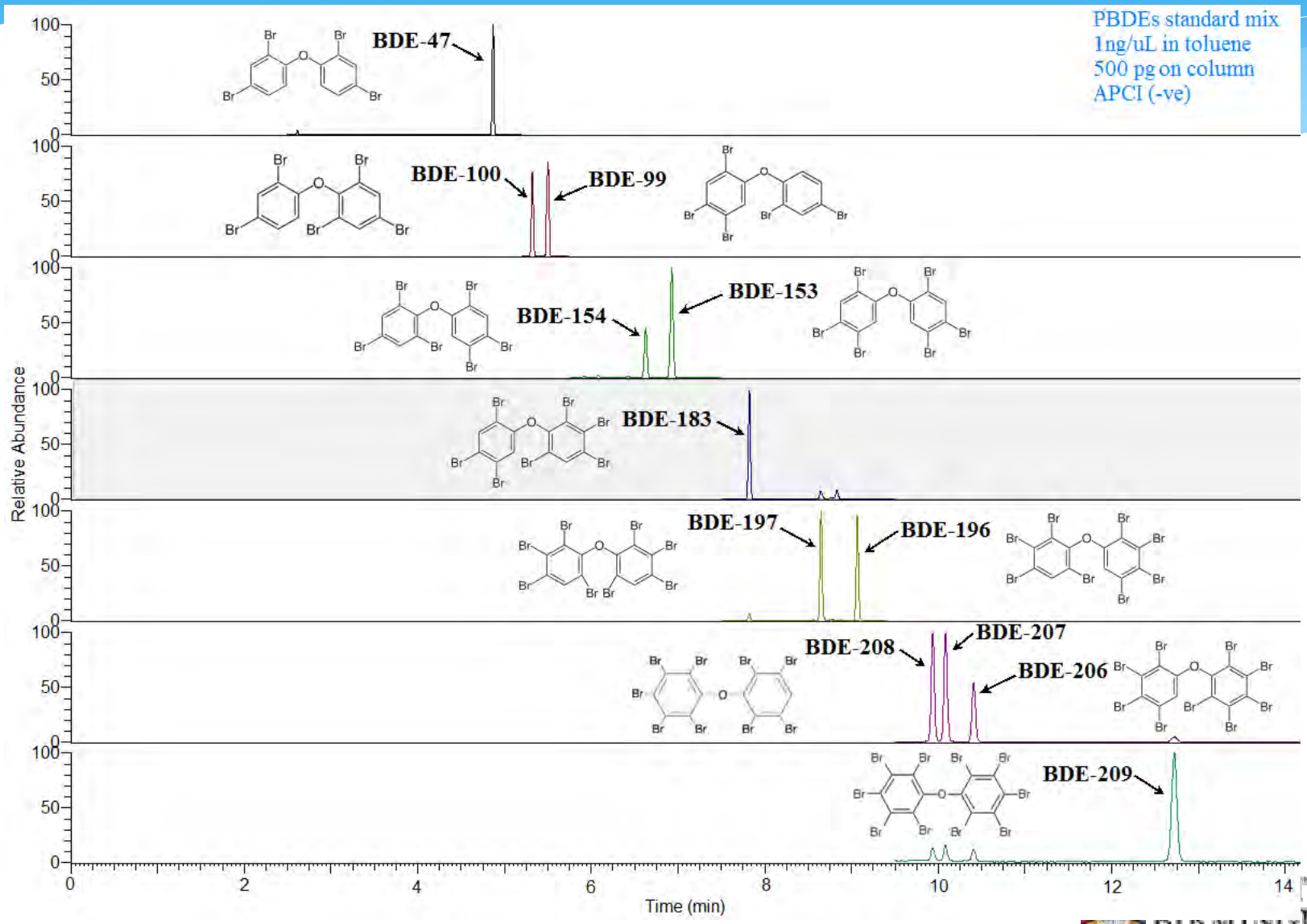
Results-Separation



Results-Optimisation

Capillary temperature (°C)	300
Source heater temperature (°C)	300
Electrospray voltage (V)	4500
Sheath gas flow (a.u.)*	15
Auxiliary gas flow (a.u.)*	10
S-lens frequence (Hz)	50
Maximum injection time (ms)	80
Automatic gain control (ions)	3×10^6
HCG energy (ev)	35
MS resolution (FWHM)	35000

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Results-Metabolites

* **Metabolite identification:**
  * **MS full scan, accurate mass (4 digits)-retention times.**
  * **Software (Analyst-Trace finder)**
  * **AIF spectra-useful for conjugates.**
  * **Confirmatory MS/MS analysis.**

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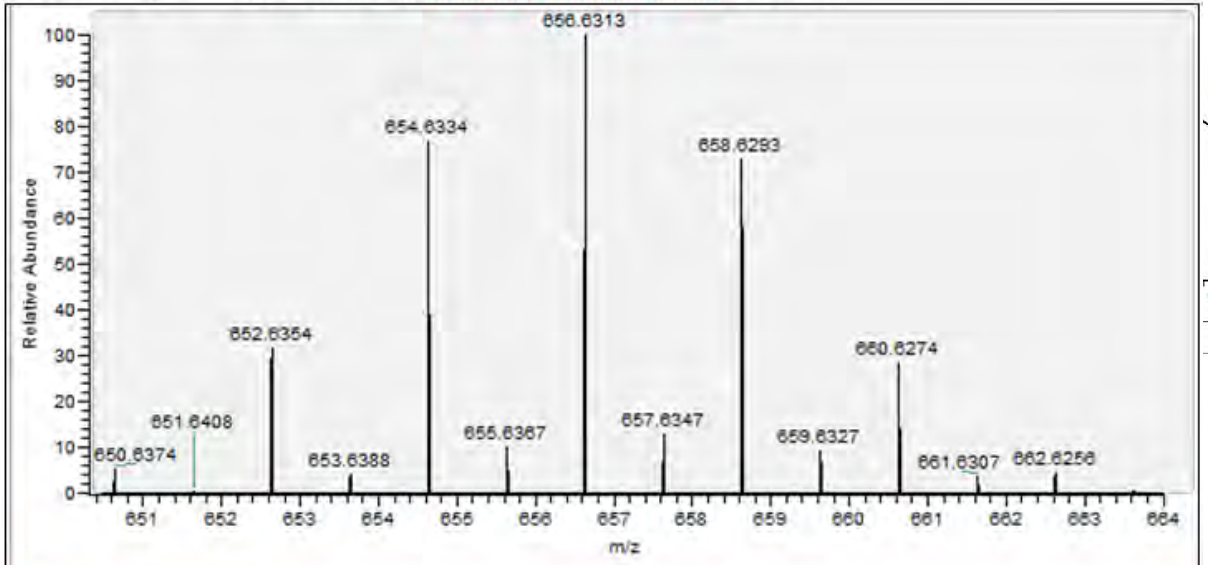
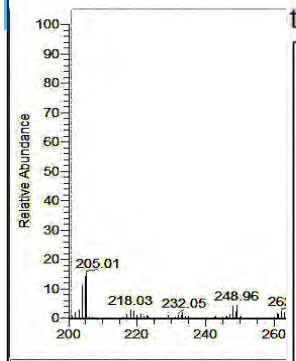
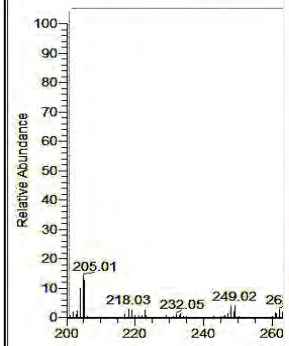
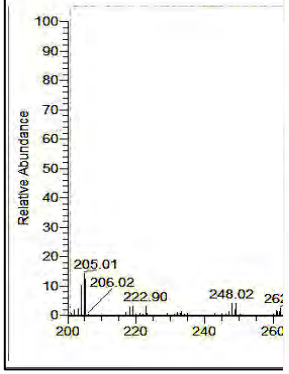
Results-Metabolites-Phase I

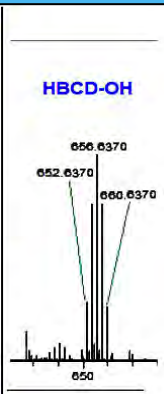
Isotope distribution of **HBCD-OH** obtained from full scan MS compared to theoretical pattern generated by **TraceFinder™** software.

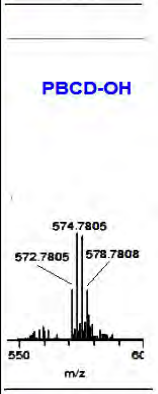


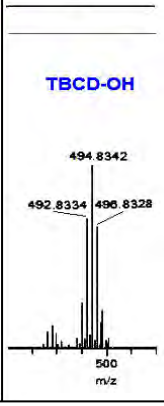






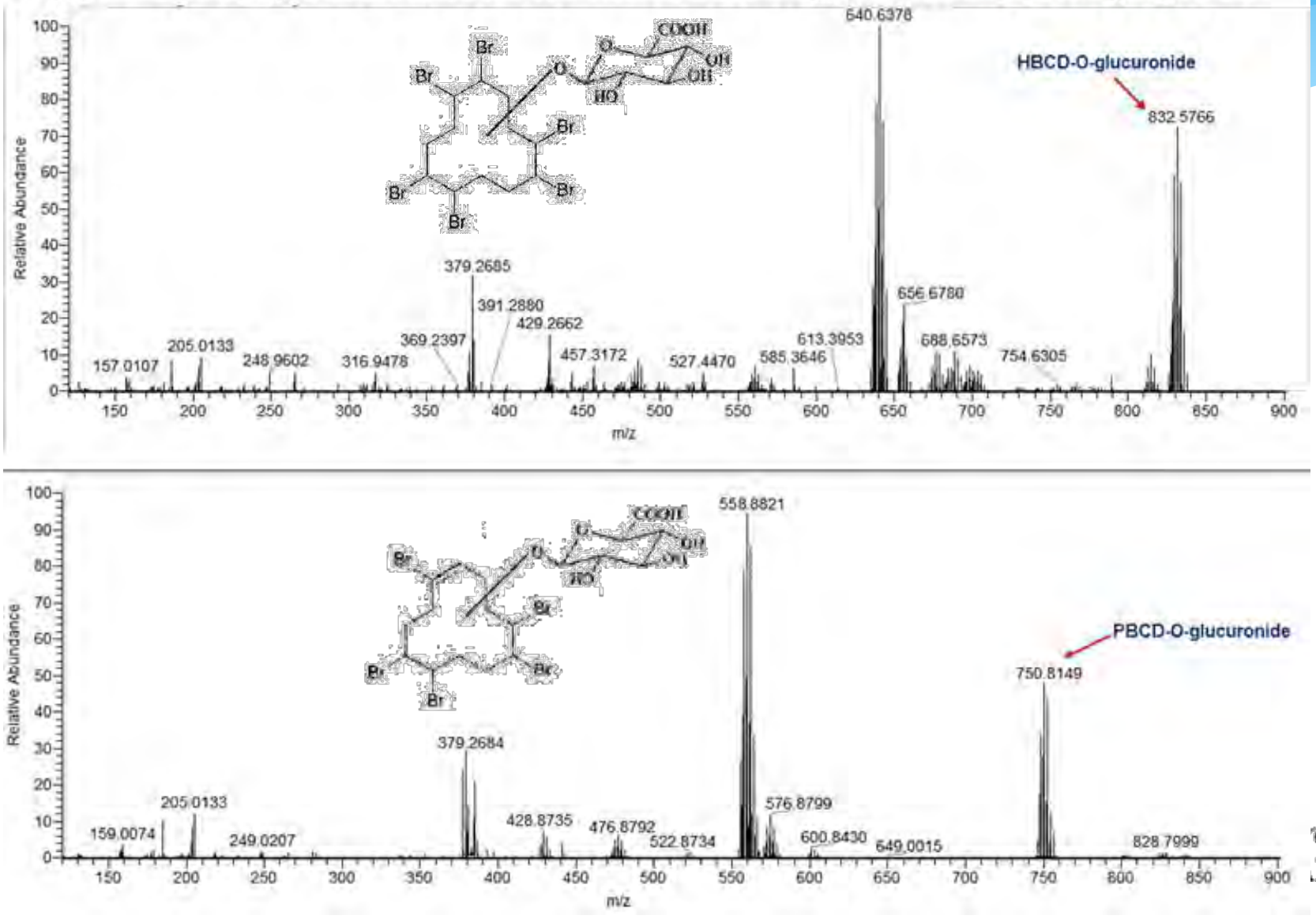






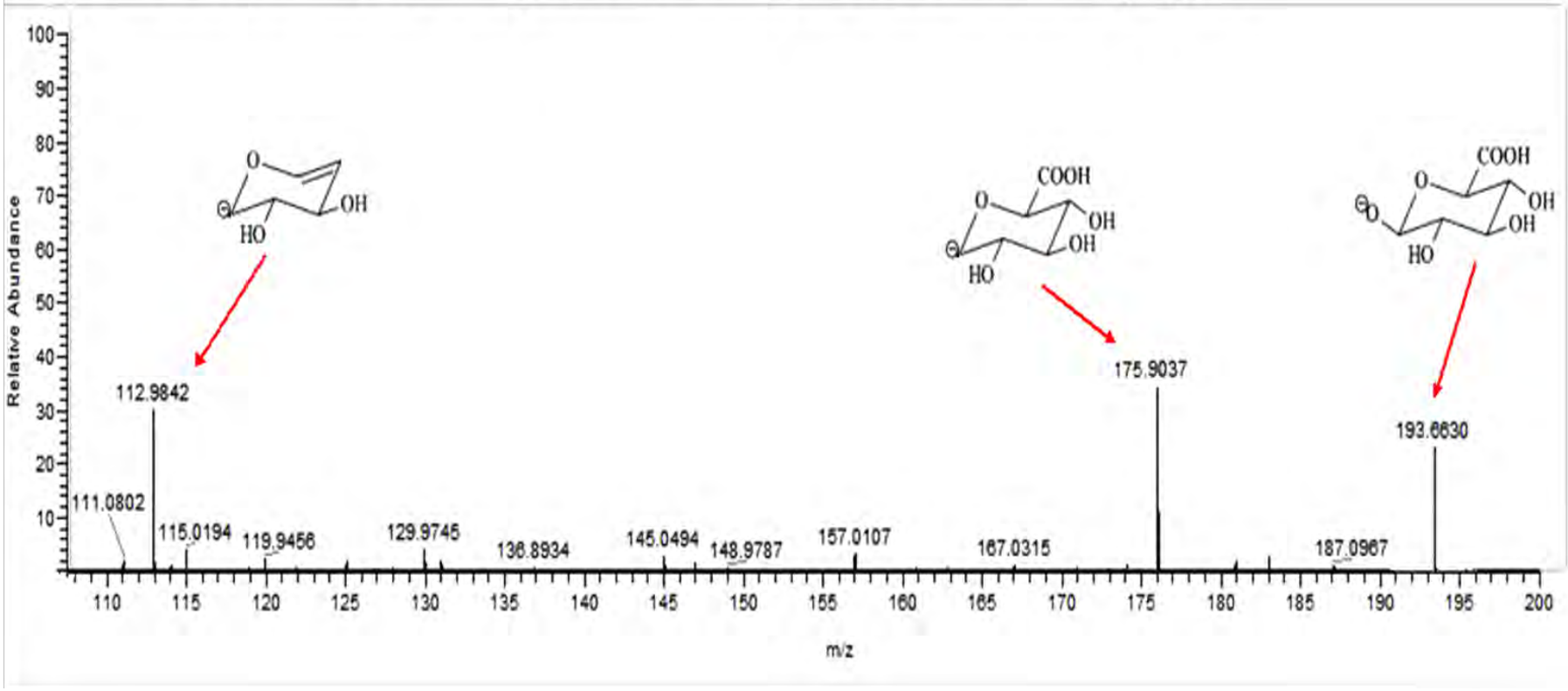
Results-Metabolites-Phase II

Fig. 3: Mass spectra of HBCD and PBCD glucuronide conjugates



Metabolites-Phase II-confirmation

Fig. 5: All ion fragmentation (AIF) spectrum showing the characteristic mass fragments of a glucuronide conjugate



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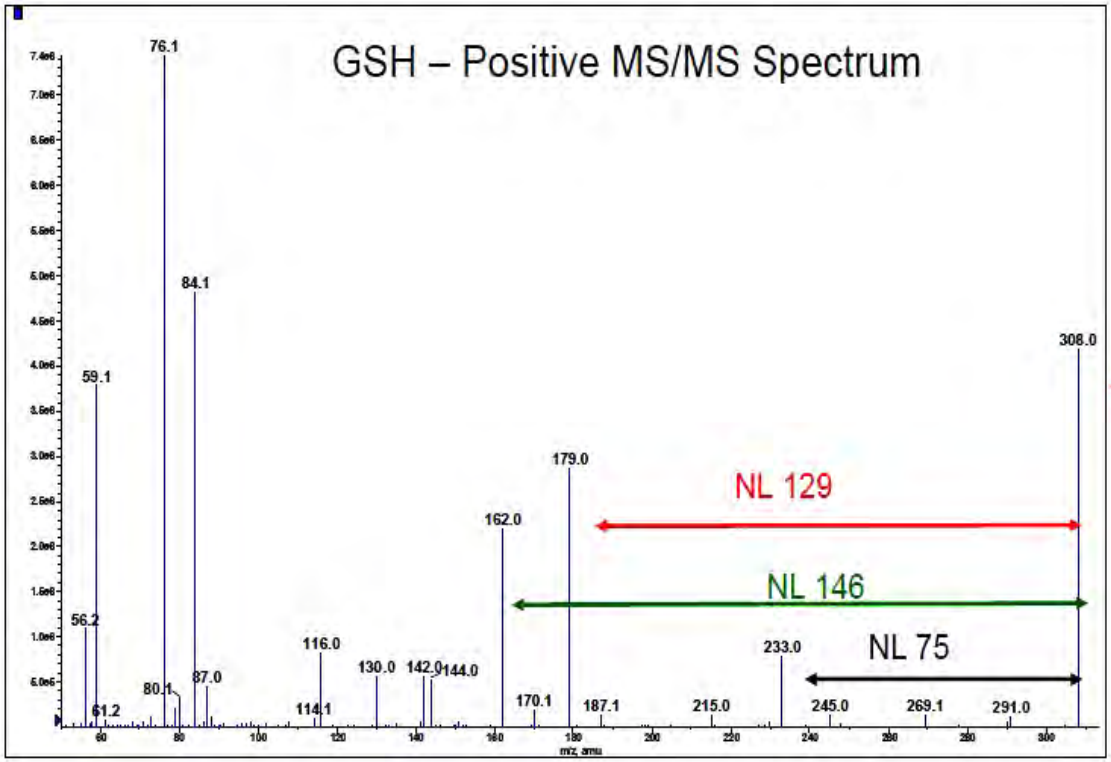
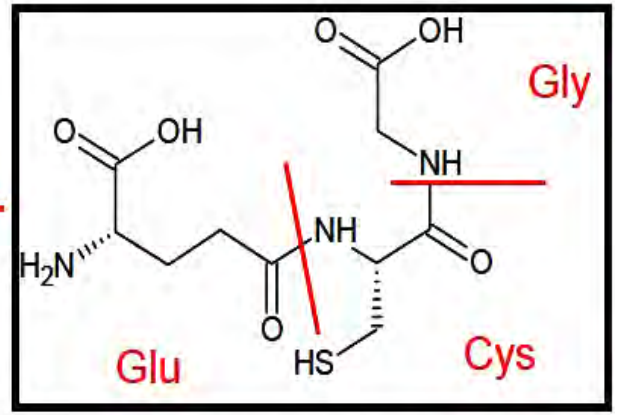
Metabolites-Phase II-confirmation





GSH, γ-glutamylcysteinylglycine

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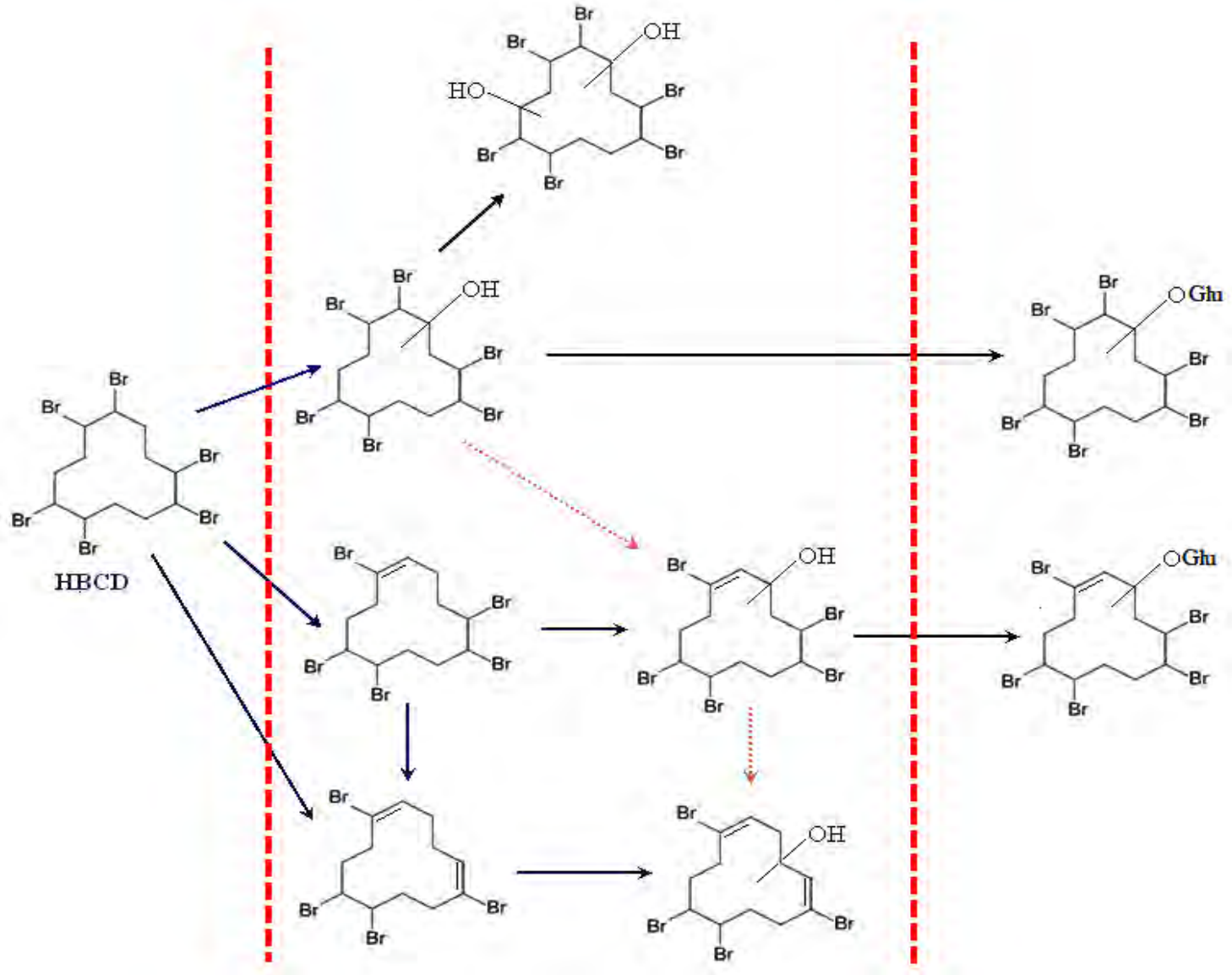
Metabolites-HBCDs

Reaction	Abbreviation	Chemical formula	Mol. ion $[M-H]^-$	Main fragment	Ret. time (min)
Phase I Reductive debromination	PBCD (2 isomers)	$C_6H_{17}Br_5$	560.6388	80.9153	7.19, 7.58
Reductive debromination	TBCD	$C_6H_{16}Br_4$	480.6618	80.9152	6.63
Hydroxylation	HBCD-OH (5 isomers)	$C_6H_{17}Br_6O$	656.6376	80.9152	5.89, 6.09, 6.38, 6.72, 7.11
Hydroxylation	Di-hydroxyl HBCD	$C_6H_{17}Br_6O_2$	672.6412	80.9152	5.08
Hydroxylation	PBCD-OH (2 isomers)	$C_6H_{16}Br_5O$	576.6780	80.9154	5.48, 5.71
Hydroxylationn	TBCD-OH	$C_6H_{15}Br_4O$	496.6778	80.9153	5.29
Phase II Glucuronidation	HBCD-O-Glu	$C_{12}H_{26}Br_6O_6$	832.5766	80.9153	4.68
Glucuronidation	PBCD-O-Glu	$C_{12}H_{25}Br_5O_6$	750.8149	80.9153	3.22

Metabolic Profile-HBCD



Metabolites-chlorinated PFRs

* **Phase I:**
  * **Dealkylation: Formation of the Di-phosphate ester.**
  * **Oxidative dehalogenation: replacement of Cl with OH**
* **Phase II:**
  * **Glucuronide conjugates**
  * **Glutathione conjugates.**

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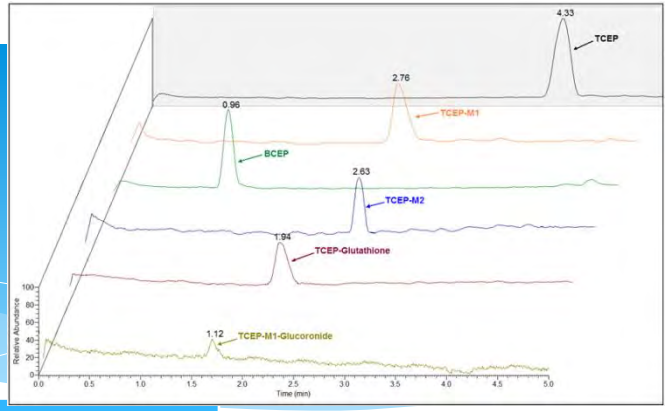
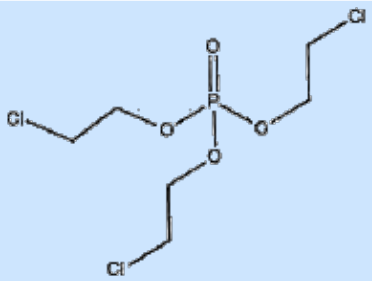
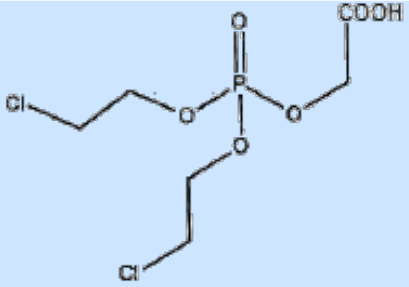
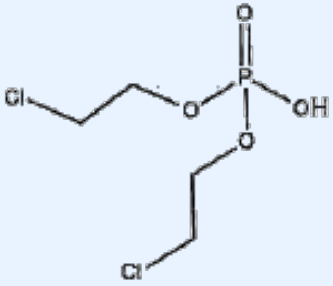
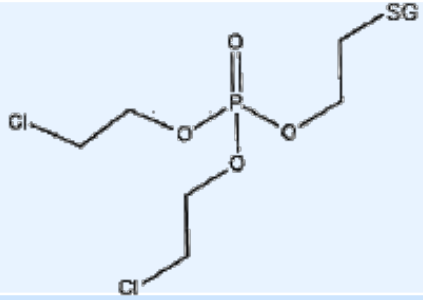
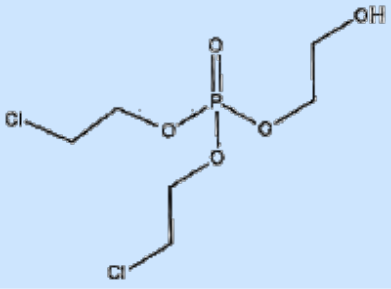
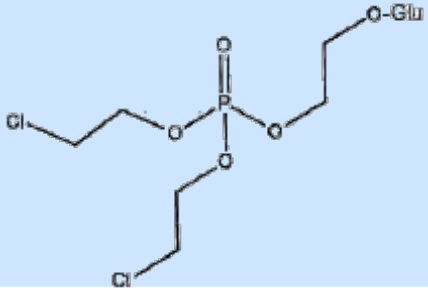
Metabolic Profile-TCEP



Name	Chemical structure	Name	Chemical structure
TCEP		TCEP-M2	
BCEP		TCEP-Glutathione	
TCEP-M1		TCEP-M1-Glucoronide	

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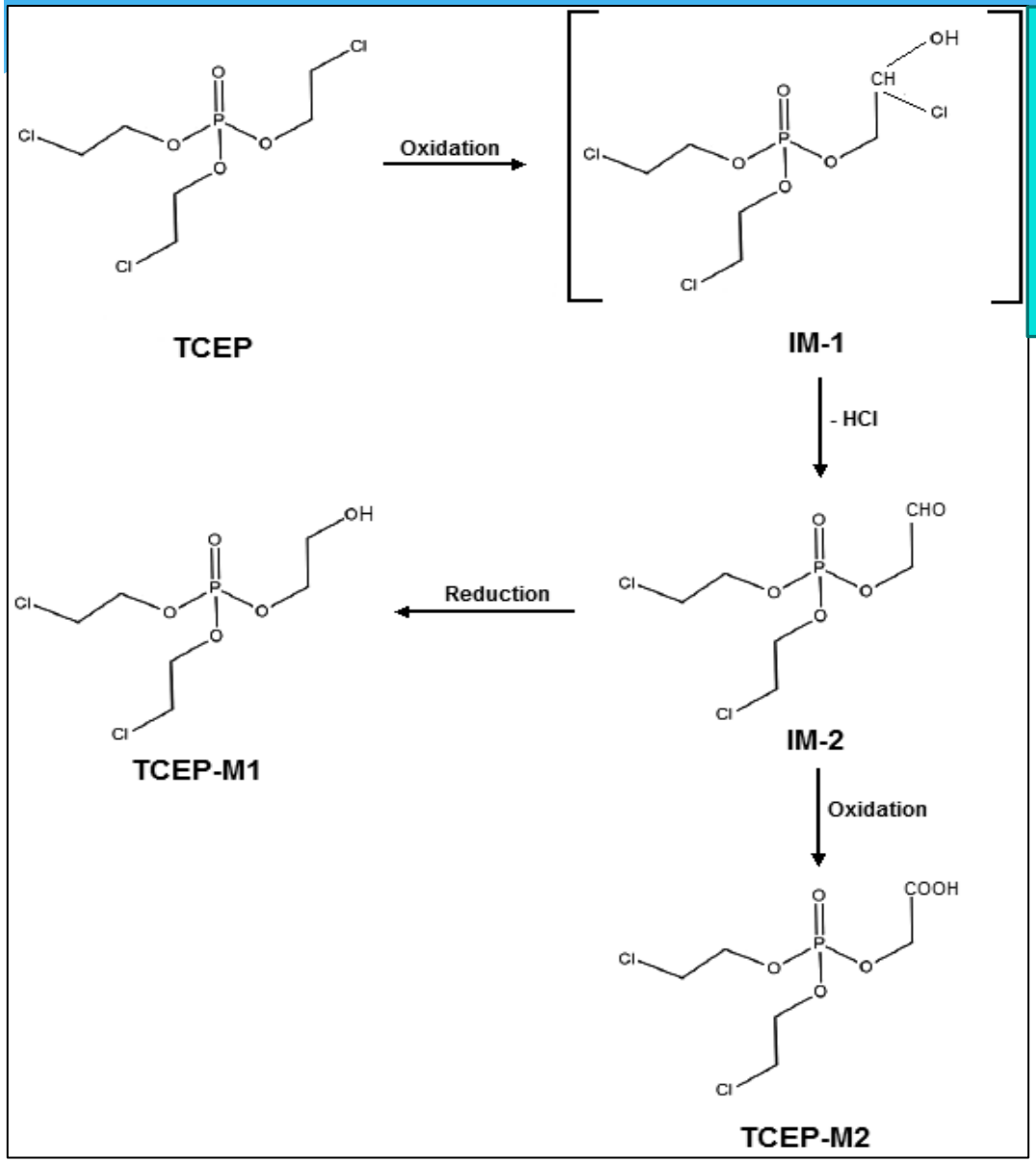
Metabolic Profile-TCEP



Schematic representation of α-oxidation proposed as a mechanism for biotransformation of TCEP by human hepatocytes.

* *Recently identified in urine of Australian adults (Van den Eede et al., **Env Int** , (2015) 74, 1-8.*

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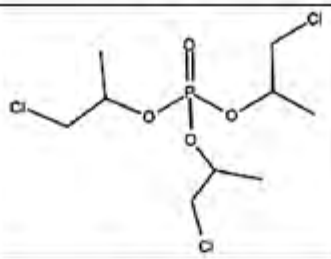
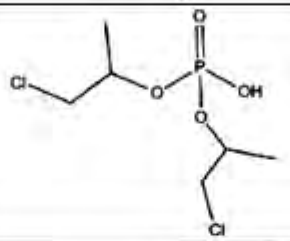
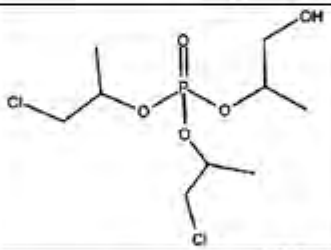
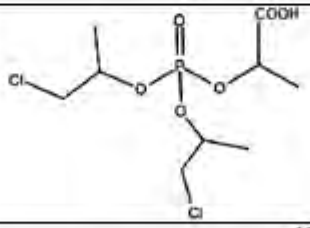
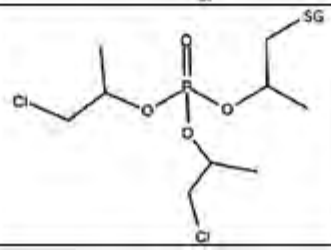
Metabolic Profile of TCiPP

Name	Molecular formula	Chemical structure	Molecular ion $[M+H]^+$	Theoretical mass
TCIPP	$C_9H_{18}Cl_3O_4P$		327.0081	327.0009
BCIPP	$C_6H_{13}Cl_2O_4P$		250.9929	251.0002
TCIPP-M1	$C_9H_{19}Cl_2O_5P$		309.0402	309.0348
TCIPP-M2	$C_6H_{12}Cl_2O_6P$		322.9892	323.0140
TCIPP-Glutathione	$C_{19}H_{34}Cl_2N_3O_{10}PS$		598.1295	598.1080

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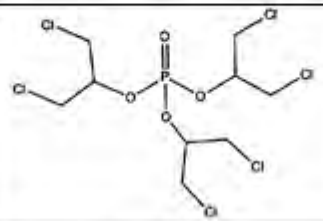
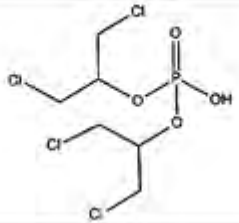
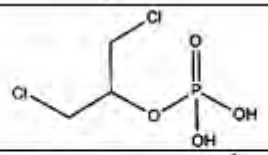
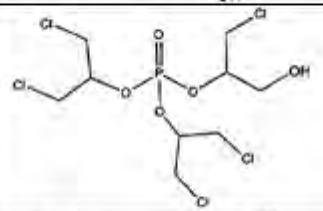
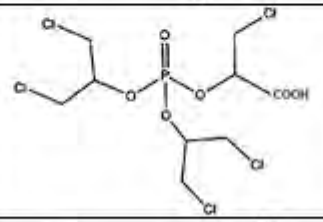
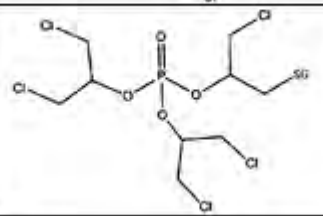
Metabolic profile of TDCiPP

Name	Molecular formula	Chemical structure	Molecular ion $[M+H]^+$	Theoretical mass
TDCIPP	$C_9H_{15}Cl_6O_4P$		430.8882	430.8809
BDCIPP	$C_6H_{11}Cl_4O_4P$		320.9192	320.9120
DCIPP	$C_3H_7Cl_2O_4P$		208.9533	208.9459
TDCIPP-M1	$C_9H_{16}Cl_5O_5P$		412.9062	412.9149
TDCIPP-M2	$C_9H_{14}Cl_5O_6P$		426.8787	426.8942
TDCIPP-Glutathione	$C_{19}H_{31}Cl_5N_3O_{10}PS$		702.0208	701.9982

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Conclusions

* **Human HepG2 cell lines can metabolise HBCDs, TCEP, TCIPP and TDCPP present in indoor dust.**

* **HBCDs undergoes oxidative hydroxylation and reductive debromination during phase I metabolism. Penta- and Tetra-brominated derivatives were detected together with their hydroxylated metabolites. Phase II glucuronidation was observed for both HBCDs and PBCDs.**

* **The biphosphate ester was the major metabolite observed for TCEP, TCIPP and TDCPP followed by the oxidative dechlorinated metabolite. Both glucuronide and glutathione conjugates were detected as a result of Phase II metabolism.**

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Conclusions

* **α-oxidation was proposed as a mechanism for biotransformation of PFRs by human hepatocytes.**

* **In total, 6 different brominated and chlorinated FRs and their 37 metabolites were *simultaneously* separated and identified in one run.**

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