Principles of FR analysis – part 2

Sample preparation – human samples

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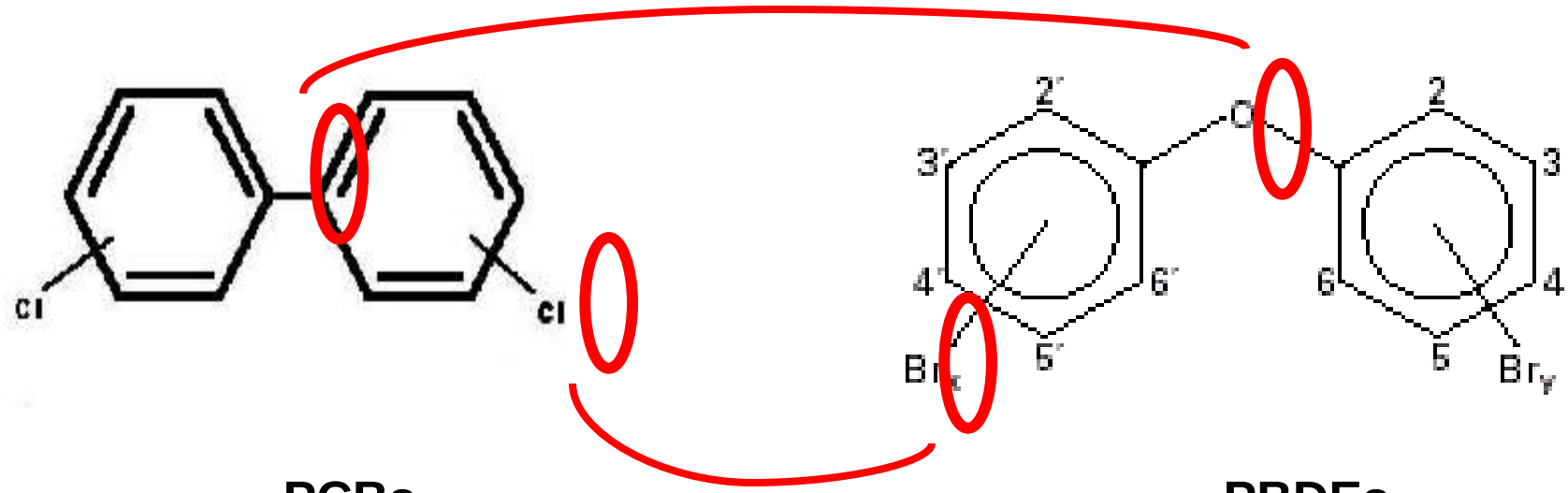
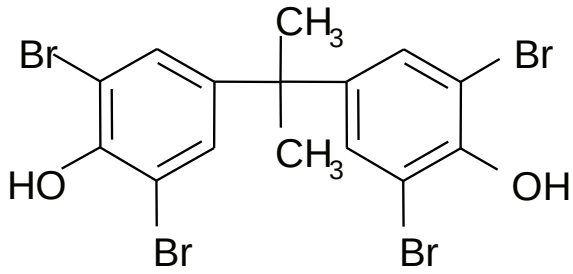
INFLAME – ATC1 – Mon 12-09-2011, Texel

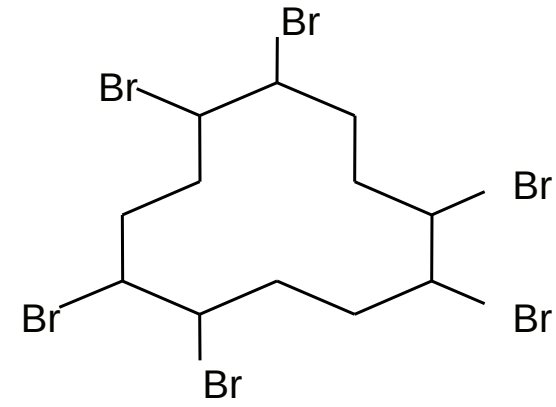
Structures of BFRs



PCBs **PBDEs**



TBBP-A

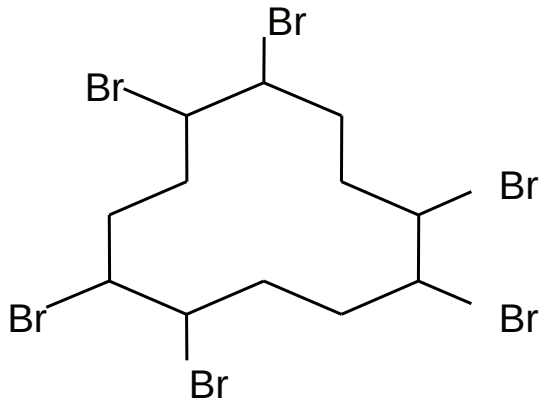


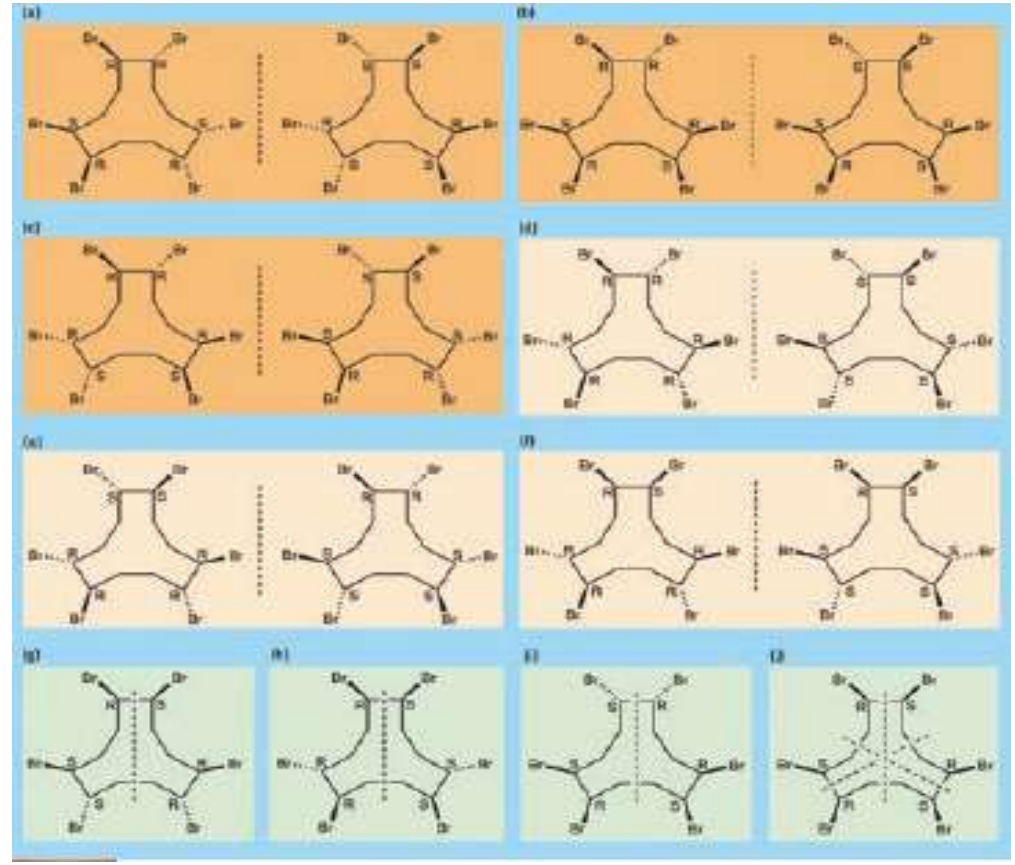
HBCD

Hexabromocyclododecanes (HBCDs)

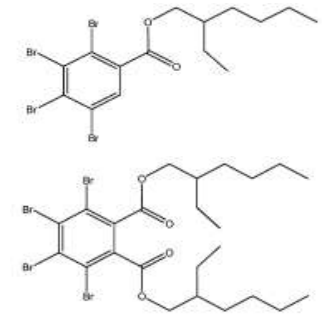
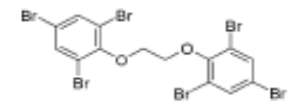
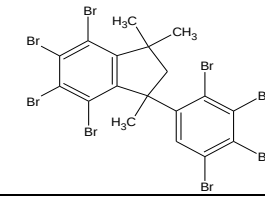
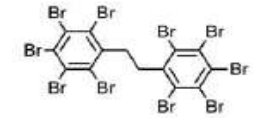
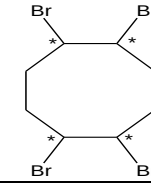
Congeners investigated in most studies

α-, β- and γ-HBCD present in the technical mixtures





Alternative BFRs

Short name	Chemical name	Technical name	Structure	Potential substitute for
NEW BFRs				
TBB + TBPH	TBB: 2-ethylhexyl 2,3,4,5-tetrabromobenzoate TBPH: 2-ethylhexyl) tetrabromophthalate	FR550		Penta-BDE
BTBPE	1,2-bis(2,4,6-tribromophenoxy) ethane	FR680		Octa-BDE
OBIND	Octabromotrimethyl-phenylindane			Octa-BDE
DBDPE	Decabrominated diphenyl ethane	SAYTEX 8010		Deca-BDE
TBCO	1,2,5,6-tetrabromocyclooctane			HBCD

Structure OPFRs

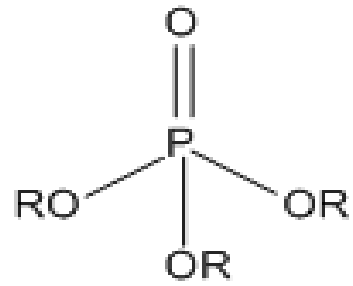
OPFRs:

R = alkyl: TEP,T*n*PP,T*i*BP,T*n*BP, TAP (IS1)

R = chloroalkyl: TCEP,TCPP,TDCPP

R = aryl: TPP, TTP, TPP-d15 (IS2)

R = ether: TBEP



E.g.: TDCPP – in PUF (replacement Penta-BDE)

Structure Chlorinated FRs

- **Polychlorinated parrafins (CPs)**

Chlorination degree of CPs can vary between 30 and 70%.

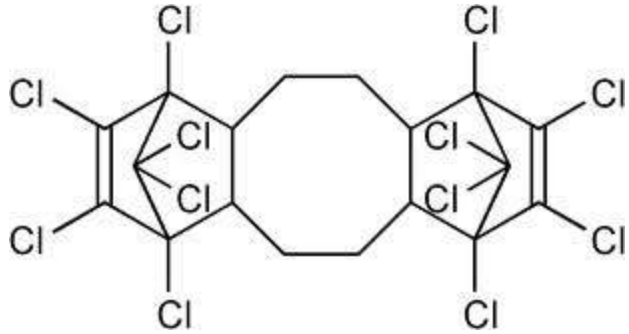
– CPs are subdivided according to their carbon chain length:

– short chain CPs (SCCPs, $C_{10\text{-}13}$)

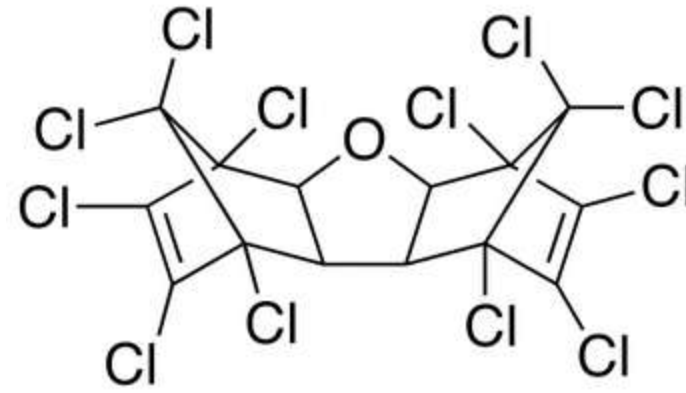
– medium chain CPs (MCCPs, $C_{14\text{-}17}$)

– long chain CPs (LCCPs, $C_{>17}$)

Dechlorane Plus



Dechlorane 602



Research questions

-Why do we want to analyse (B)FRs? (e.g. toxicological issues)

- Which (B)FRs can we analyse in human matrices? (most studies PBDEs and HBCDs), specific issues for TBBPA)

- At which concentration levels do we need to analyse these compounds (e.g. what are the currently reported/expected levels – pg/ml serum!!)?

- Which matrix is the most suitable? (re: sampling, relevance for exposure, accumulation of (B)FRs)

serum, milk for PBDEs and HBCDs; urine for OPFR metabolites

Samples

Biological samples (humans)

- serum

- milk

(these are the most used matrices for human biomonitoring) – WHY?

- fatty tissues (adipose tissue, liver, other organs)

- hair

- urine

- nails, skin, earwax

- saliva

Analysis

Chemical analysis

(1) Sample pre-treatment

(2) Extraction

(3) Clean-up

(4) Analysis: GC/MS or LC/MS

(5) Quality Control

Information:

-Concentrations of target analytes

Biological assays

(1) Sample pre-treatment

(2) Extraction

(3) Clean-up

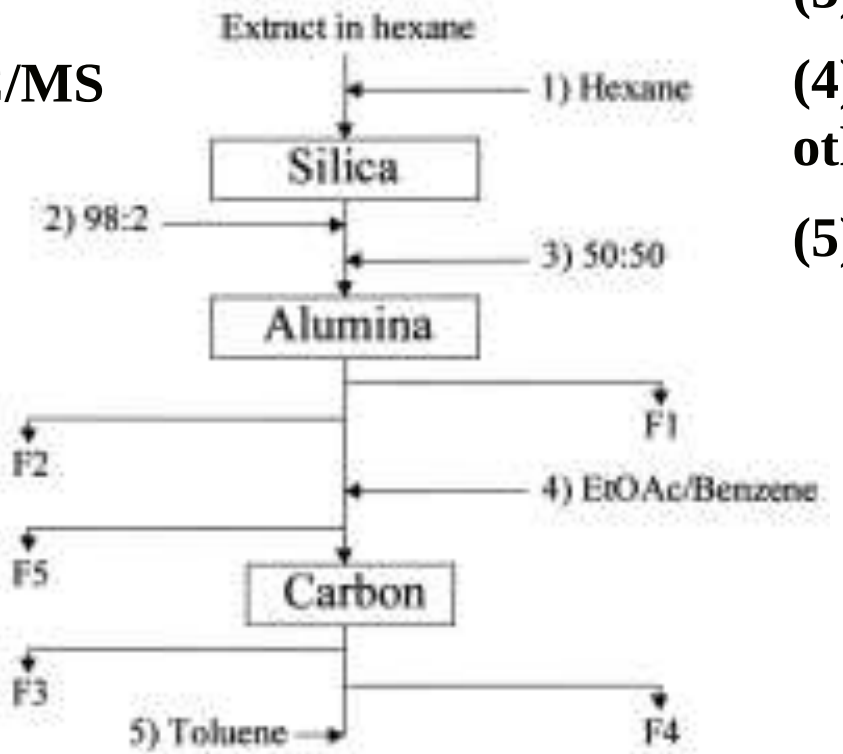
(4) Analysis: CALUX or other bioassays

(5) Quality Control

Information:

-Effects of extracts on specific cell lines

Clean-up



PART 1 - Sample preparation

- physical state: solid (organs, hair) or liquid (serum, milk)

Sample pre-treatment

- blood: centrifugation to obtain plasma or serum

- Serum is clearer than plasma because plasma has an additional protein (fibrinogen). Fibrinogen is not present in serum

- Serum/plasma:

- **protein precipitation (methanol or acetone- precipitation)**
- **deproteinization with formic acid (or other strong acids HCl), no precipitation**

PART 1 - Sample preparation

- physical state: solid (organs, hair) or liquid (serum, milk)

Sample pre-treatment

- fatty tissues: homogenization, sub-sampling, dehydration with anhydrous Na_2SO_4 or freeze-drying

- hair: washing, cutting

PART 1 - Sample preparation

2. Extraction

- Liquid samples: serum and milk

Liquid-liquid extraction (LLE)

Solid-phase extraction (SPE)

Molecular imprinted polymers (MIP)

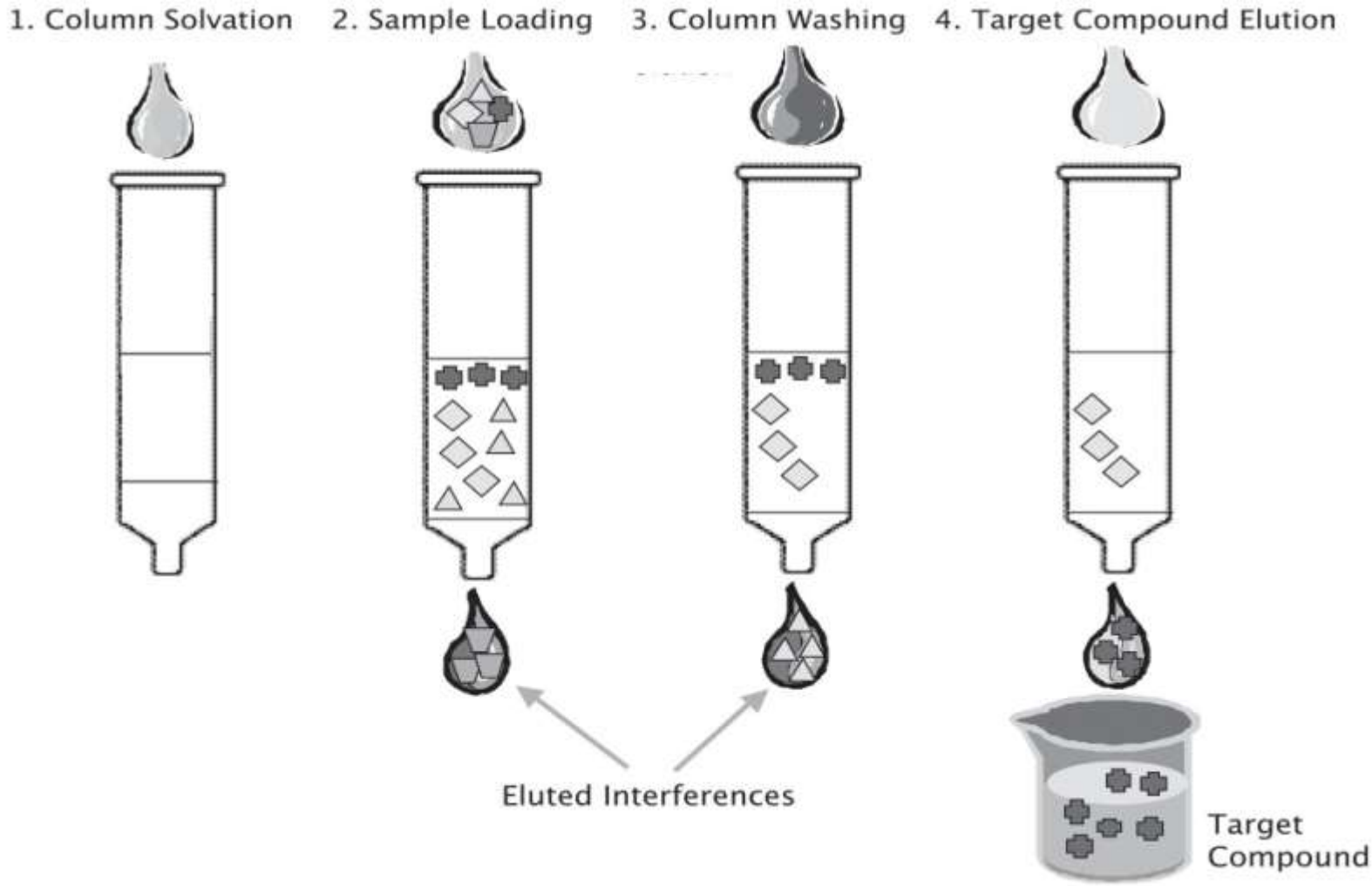
Solid-phase micro extraction (SPME)

Stir bar sorptive extraction (SBSE)

Liquid-liquid extraction (LLE)

- **Sample – liquid (serum, milk or urine)**
- **Bring to appropriate pH (if needed)**
- **Extraction with organic solvent (or mixtures) – choice depends on analytes and further clean-up**
- **Evaporate solvent**
- **Proceed to clean-up**

Solid phase extraction (SPE)



Solid phase extraction (SPE)

Vacuum manifold or positive pressure manifold

<u>TYPEs of cartridges</u> :

- <u>Silica-based C18, C8, C2</u> :

analytes are retained by:

» Non-polar interactions

» Van der Waals forces

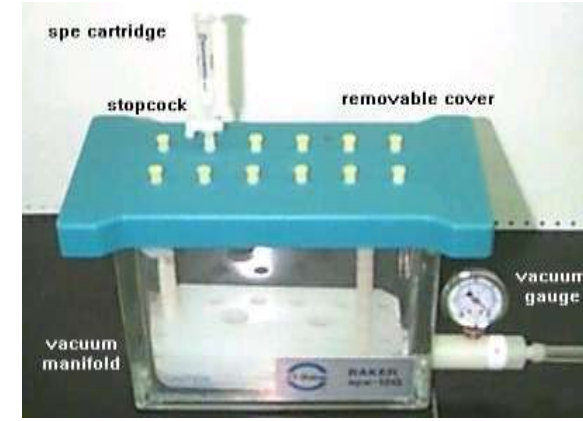
» Secundary interactions (hydrogen bridges)

– <u>Mixed-phase</u> cartridges :

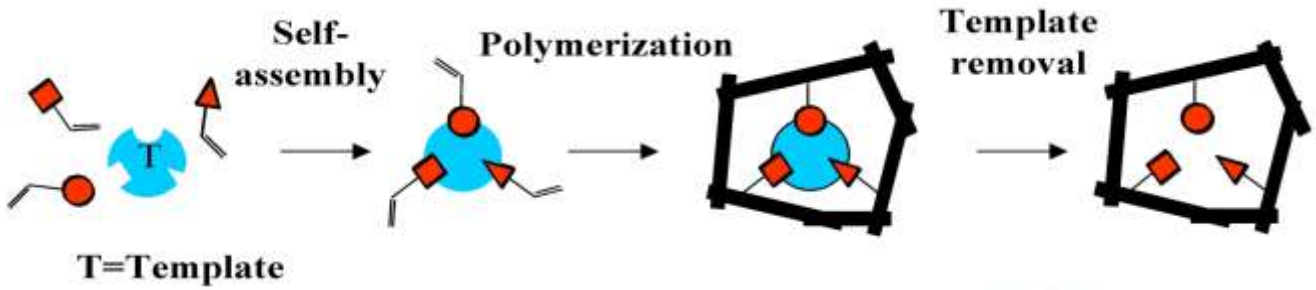
» Non-polar interactions

» Ion exchange interactions :

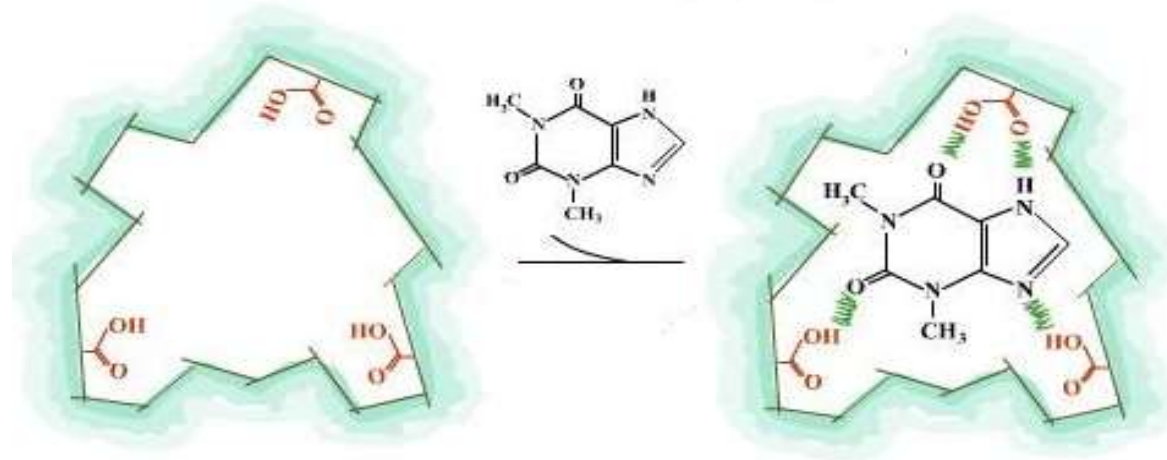
Cationic or anionic exchange sorbent



Molecular Imprinted Polymers (MIP)



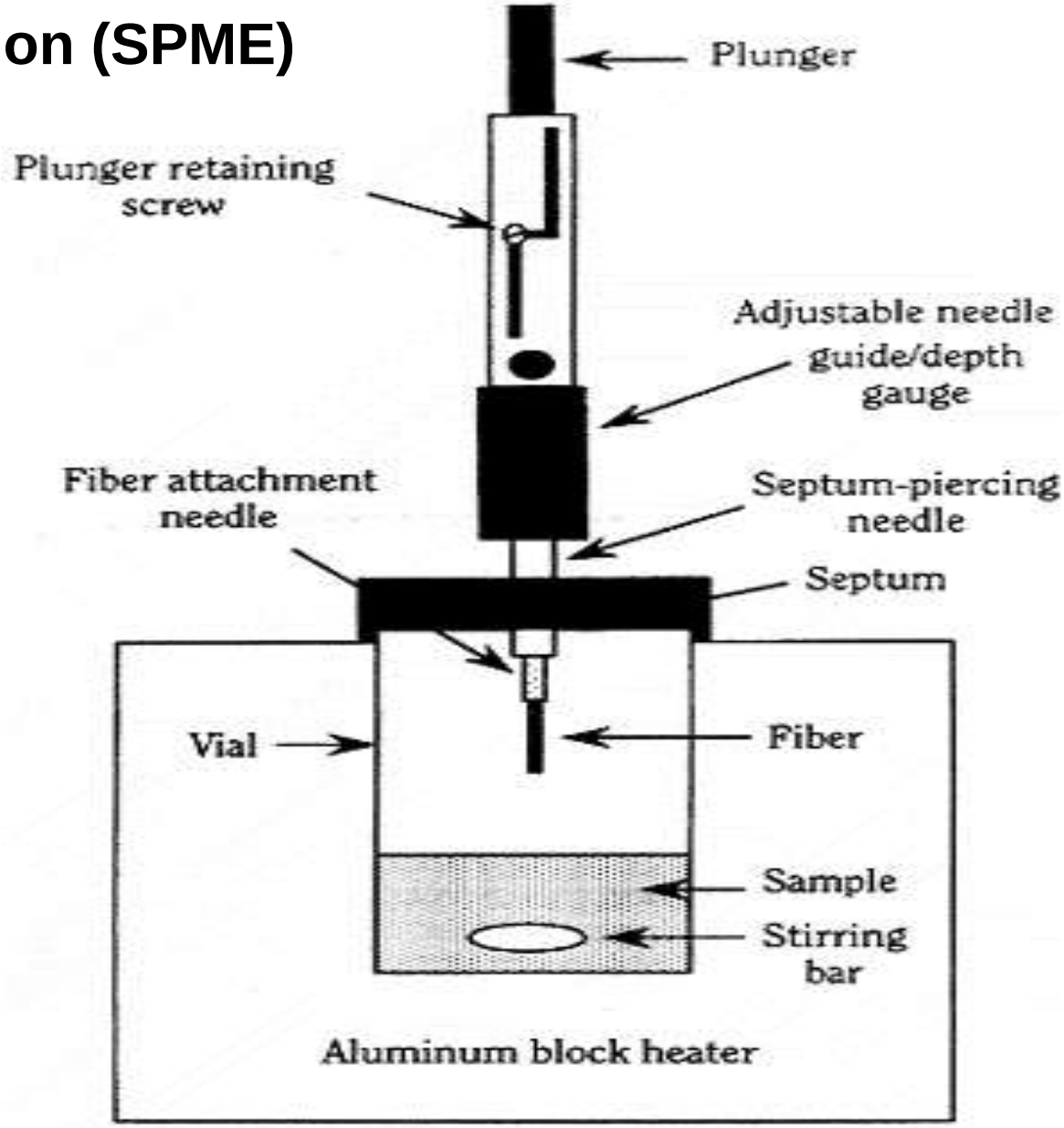
miofiline



Analyte-custom designed phases:

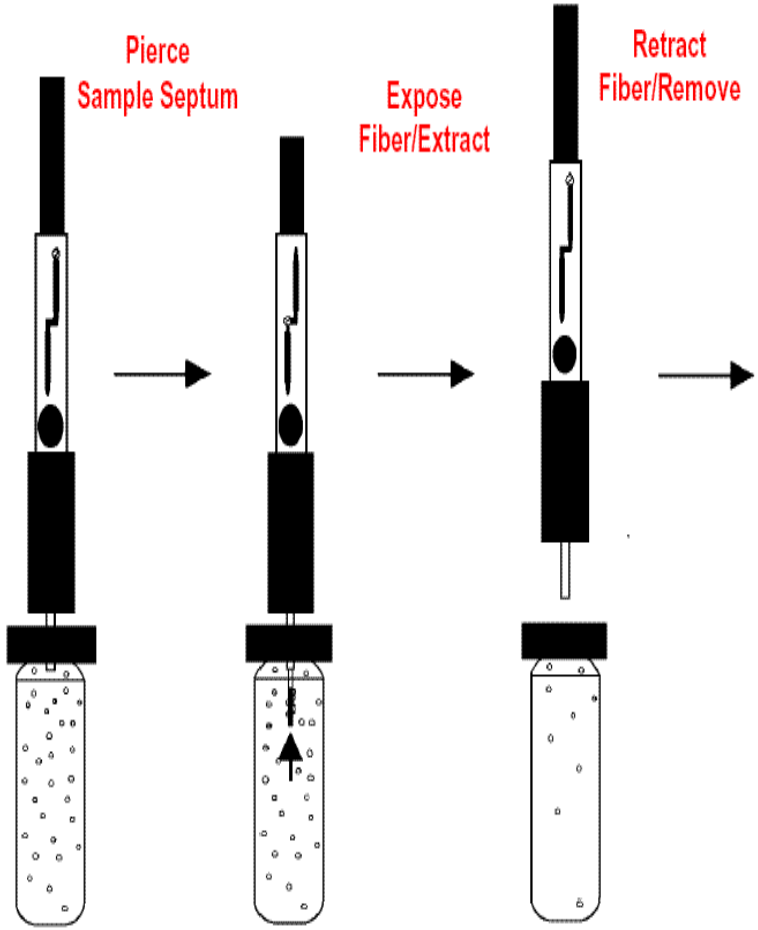
- Very high specificity!

Solid-phase microextraction (SPME)

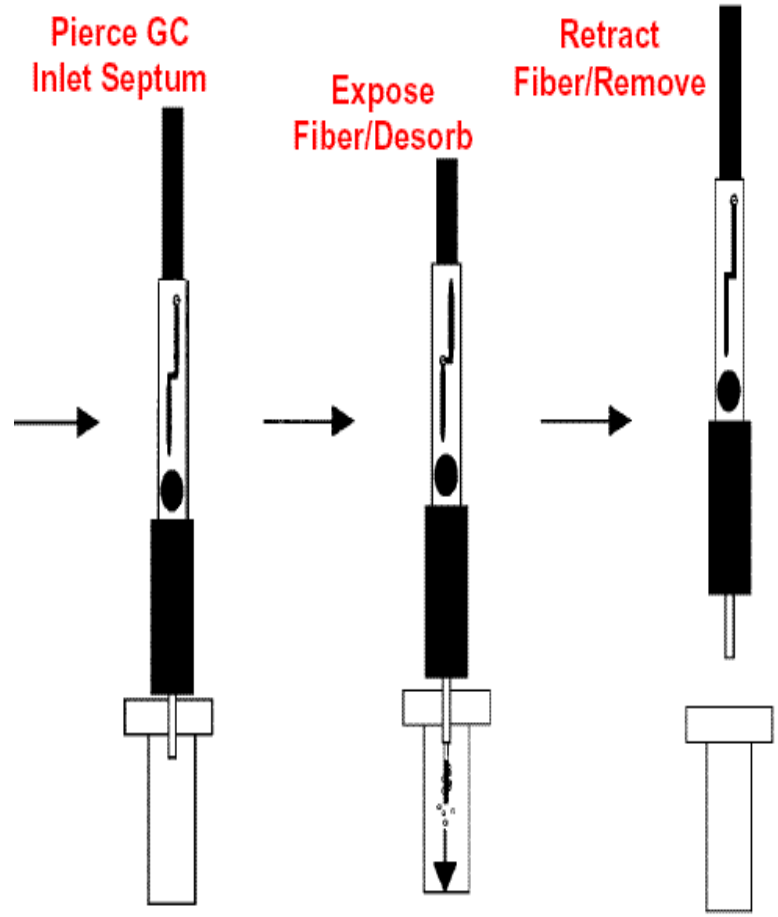


Solid-phase microextraction (SPME)

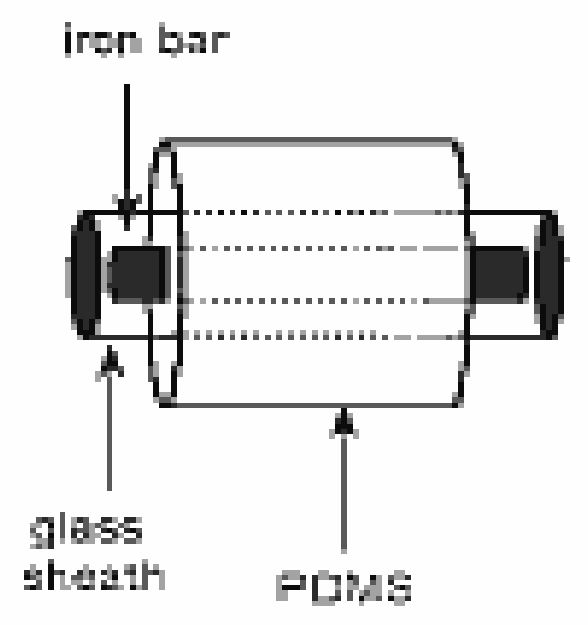
Extraction



Desorption / Injection



Stir bar sorptive extraction (SBSE)



- Solid magnetical stir bar coated with polymer - (polydimethylsiloxane (PDMS)

- After stirring, the stir bar is thermally desorbed in a GC.

- Due to the much larger volume of the PDMS-phase extraction efficiency is far better than for SPME.

PART 1 - Sample preparation

2. Extraction

- Fatty tissues: liquid-solid extraction techniques

    - Classical: shaking with solvent, column percolation (tissues)

    - Ultrasonication

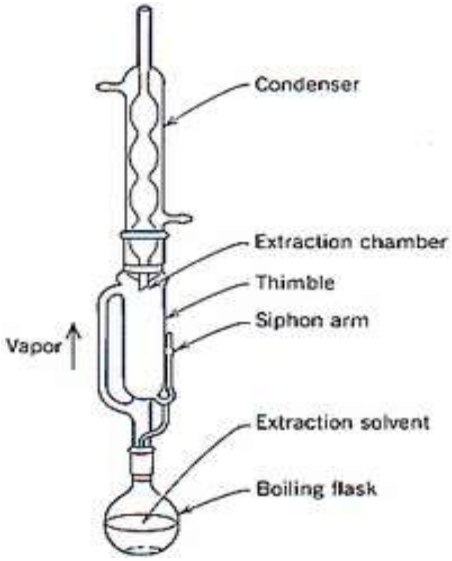
    - Soxhlet extraction : classical (cold) or hot : 2-24 h

    - Accelerated solvent extraction (ASE): temp, pres, time reduction (<1h)

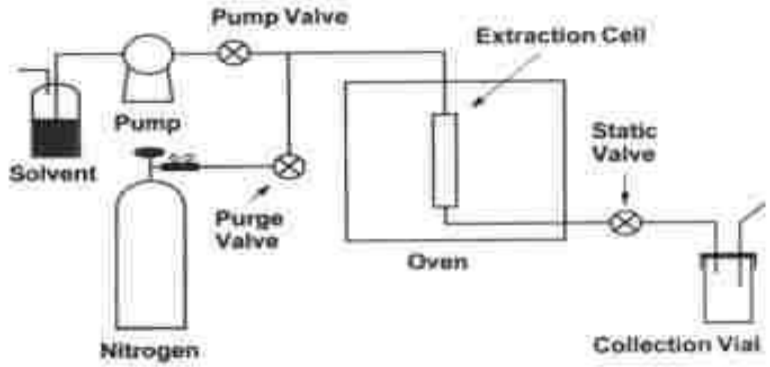
    - Microwave assisted extraction (MAE): time reduction (<1h)

    - Supercritical fluid extraction (SFE)

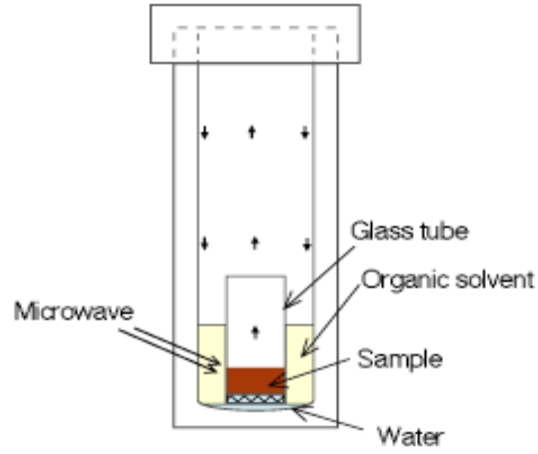
PART 1 - Sample preparation



Soxhlet extraction



Accelerated solvent extraction (ASE)



Microwave assisted extraction (MAE)

PART 1 - Sample preparation

3. Treatment of the raw extract

- Extract from fatty tissues: determination of lipid content
    - on an aliquot of extract, evaporation of solvent at 105°C, for 1h
    - on an aliquot of sample
        - Bligh & Dyer method (chloroform, methanol and water extraction)
        - Smedes method (cyclohexane, iso-propanol and water extraction)

(mixture of polar and non-polar solvent – to extract various lipids)

- Lipid determination for serum: enzymatically!! on a separate aliquot of sample

PART 1 - Sample preparation

4. Clean-up

- to remove interferences, especially lipids

- non-destructive techniques:

- adsorption chromatography: silica, Florisil, alumina, elution with non-polar solvents

- gel permeation chromatography

- dialysis

- destructive techniques:

- liquid partitioning with conc. sulfuric acid or KOH

- adsorption chromatography: acidified silica (with conc. sulfuric acid)

basic silica (with KOH)

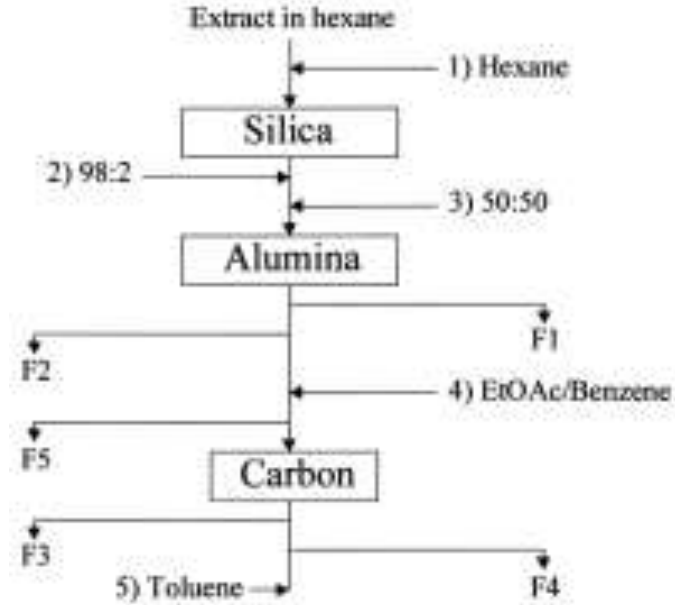
combination of both

some compounds may be completely destroyed:

TBPH - by conc. sulfuric acid

HBCD - by KOH

PART 1 - Sample preparation



5. Fractionation

- to separate the cleaned extract in specific classes

HPLC

- carbon columns: separation of bulk PCBs/PBDEs (hexane elution) from coplanar compounds (PBDDs and PBDFs) (toluene, reversed flow)

- PYE columns : separation of PCBs function of their ortho-substitution

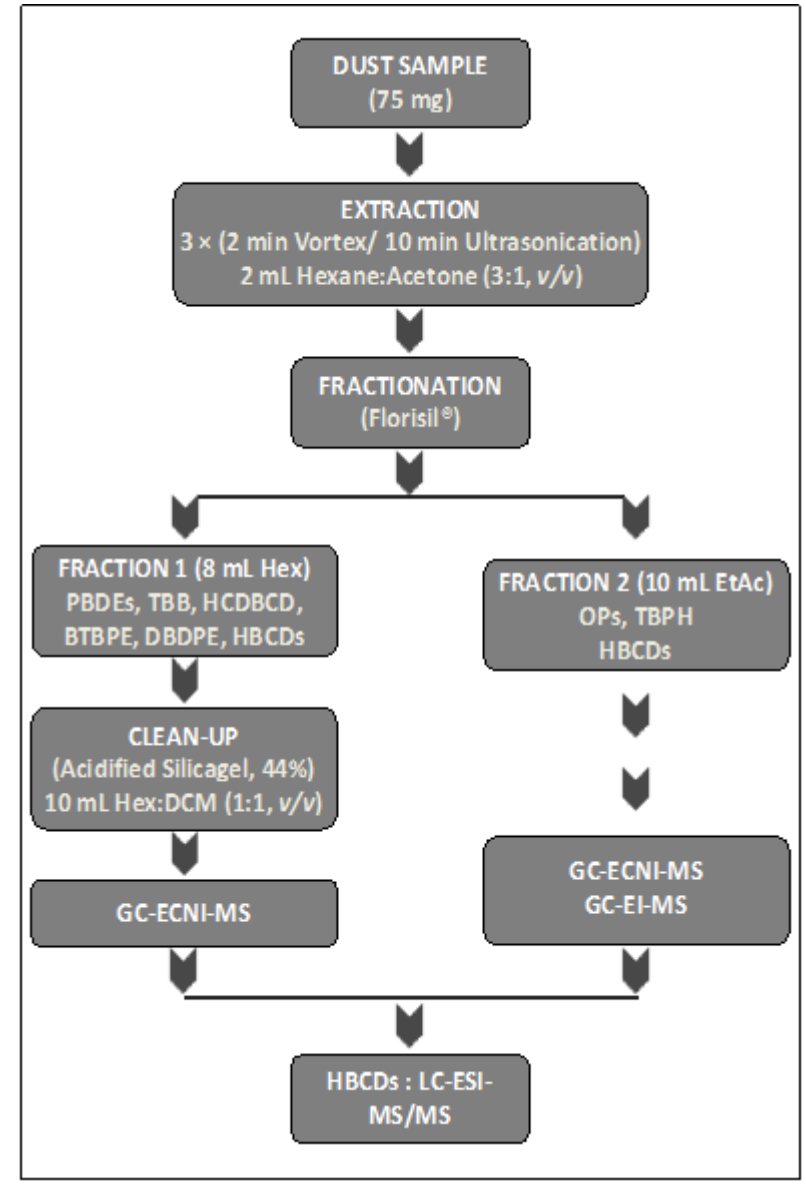
separation of chiral PCBs

PART 1 - Sample preparation

5. Fractionation

Florisil (or silica)

- separation of some groups of (B)FRs



Van den Eede et al., Talanta, submitted

Application of SPE for BFRs in serum (Tox Centrum)

- **4 mL serum + 4 ml water**
- **+ IS for PBDEs and HBCDs**
- **2 ml formic acid (for deproteinization)**
- **Sonication**

- **SPE cartridges (OASIS-HLB, 500mg, 6ml) conditionned with 3 ml DCM, 3 mL MeOH en 3 mL Milli-Q water**
- **Bring sample onto the SPE cartridge**
- **Wash columns with 3 mL water**
- **Dry columns under vacuum**

- **Eluate with 3x3 ml DCM and concentrate to 1ml**
- **Clean-up/fractionation on silica topped with 0.5 g acidified silica and 0.1 g anh. Na_2SO_4**
- **Elute with 6 ml hexane (contains PBDEs) – fraction 1**
- **Elute with 5 ml DCM (contains HBCDs) – fraction 2**

- **Concentrate each fraction to near dryness and reconstitute in**
- **100 µl ioctane/toluene (Fr1) (GC-MS analysis)**
- **100 µl methanol (Fr2) (LCMS analysis)**

Application of solid-liquid extraction for BFRs in fatty tissues (Tox Centrum)

- 3 g liver or 200 mg fat or 3-4 g organs)
- Mix with anh. Na_2SO_4, add IS for PBDEs and HBCDs
- Extract the mixture for 2h in a hot Soxhlet extractor

- Concentrate extract
- Lipid determination on 1/10 of the extract

- Rest of the extract cleaned-up with 8 g acid silica + 0.5 g anh. Na_2SO_4
- Elute analytes with 15 ml hexane and 10 ml DCM

- Concentrate eluate to 2 ml with rotavap
- Further fractionation on silica (see SPE for serum)
- Concentrate each fraction to dryness under stream of nitrogen and reconstitute in 100 µl ioctane (GC-MS analysis) or 100 µl MeOH (LC-MS analysis)

Specific issues

- **Metabolites (mostly hydroxylated) – may be present in blood and urine**
  - **Conjugated - conjugation with glucuronic acid or sulfate**
  - **increased water solubility**
  - **TBBPA can also be conjugated !!**

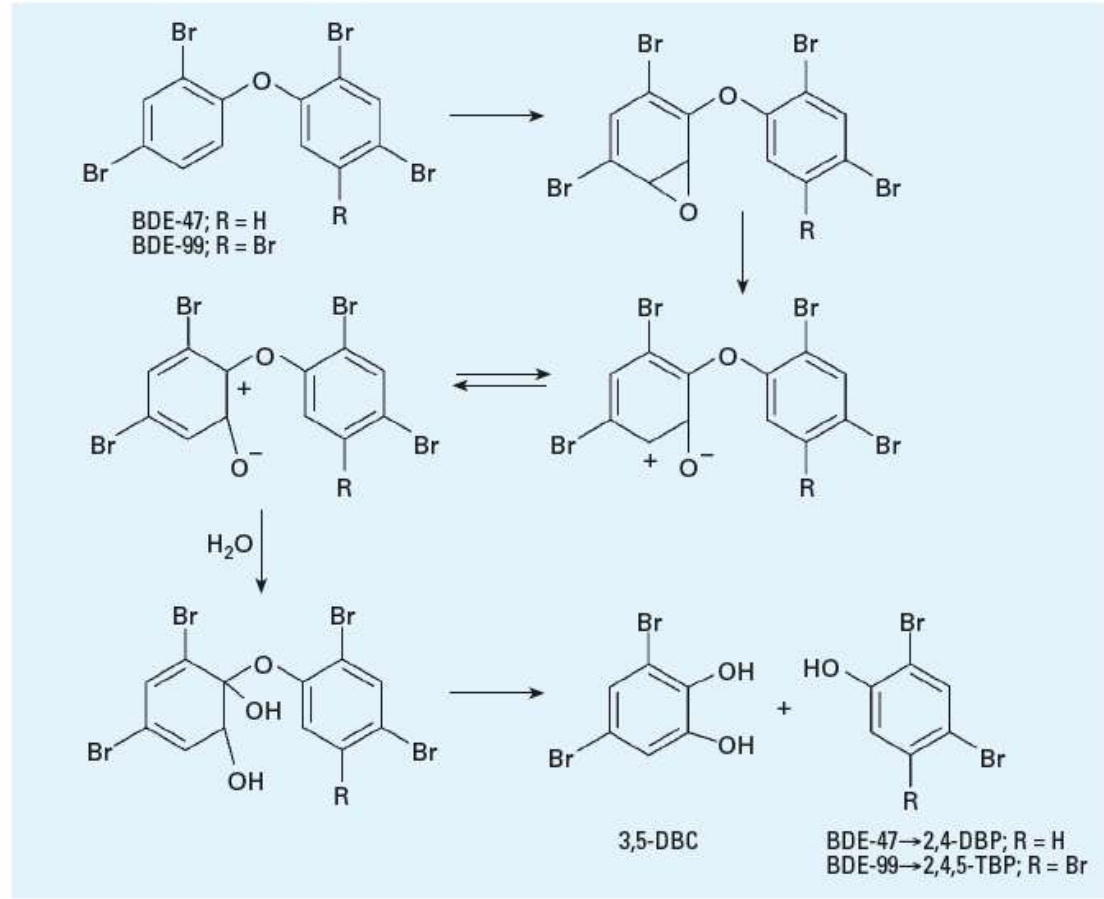


Figure 2. Proposed pathway of the cleavage of the diphenyl ether bond of BDE-47 and BDE-99 in mice.

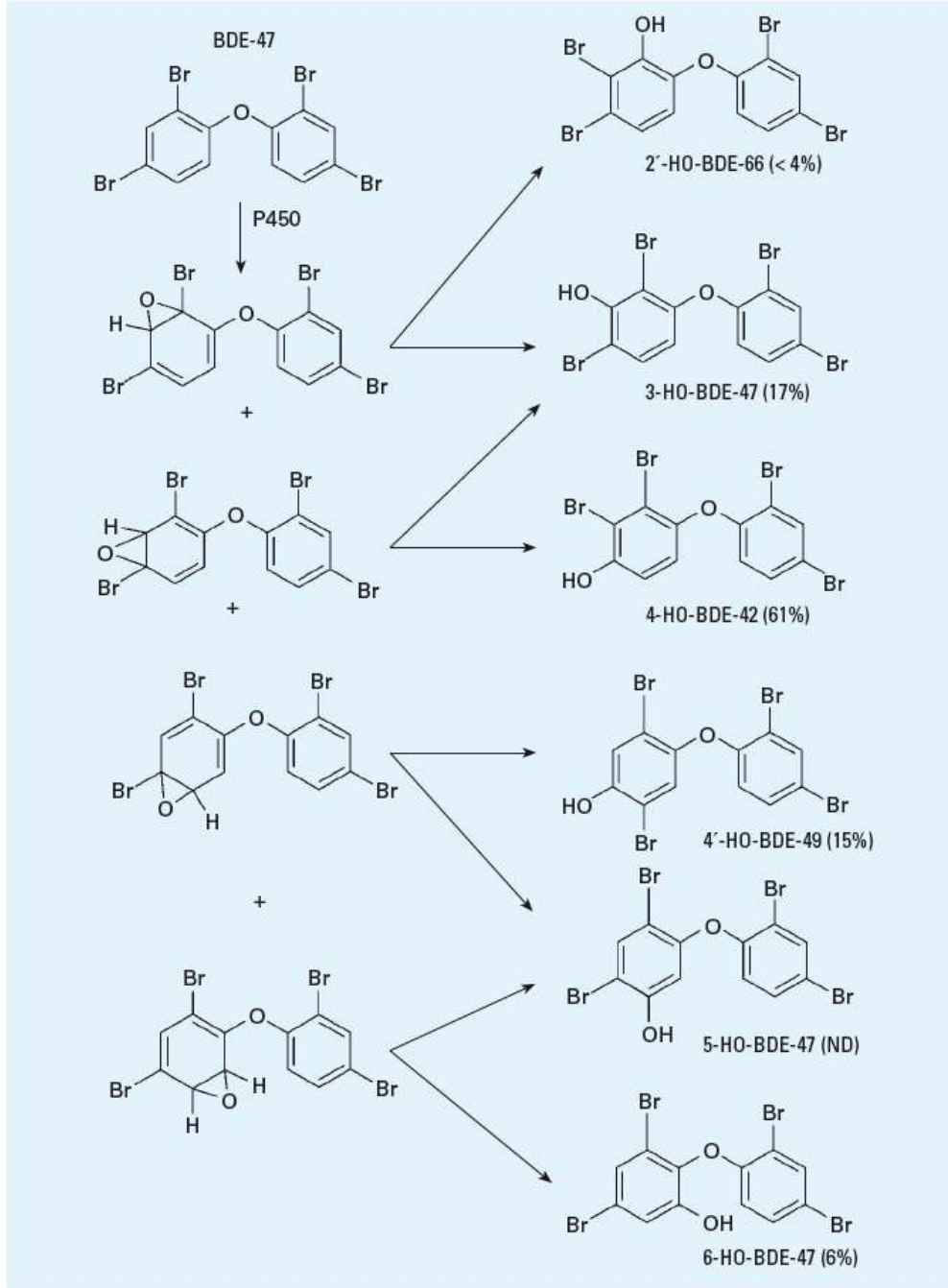


Figure 3. Proposed hydroxylation pathway of BDE-47 in mice and the percentage of metabolites based on the measurements in this study. ND, not detected.

Exposure of mice to Penta-BDE mixture

Table 1. Concentrations (ng/g wet weight) of neutral and phenolic compounds in mouse plasma.

Compound/congener	Oral gavage samples ($n = 15$) Mean ± SD	Oral gavage samples ($n = 15$) Percent of total	sc injection samples ($n = 14$) Mean ± SD	sc injection samples ($n = 14$) Percent of total	Blank and control samples ($n = 19$) Mean ± SD
Neutral compounds[a]					
BDE-28 (0.3)	4.4 ± 1.1	0.2	5.3 ± 1.6	0.5	0.6 ± 0.8
BDE-47 (36)	390 ± 100	18	360 ± 77	31	3.0 ± 6.3
BDE-85 (2.6)	57 ± 19	2.6	32 ± 5	2.8	ND
BDE-99 (44)	410 ± 120	19	330 ± 70	29	2.3 ± 3.9
BDE-100 (9.1)	140 ± 40	6.4	110 ± 20	9.2	0.4 ± 1.3
BDE-153 (4.3)	1,100 ± 380	52	290 ± 80	25	0.6 ± 0.8
BDE-154 (3.3)	22 ± 7	1.0	20 ± 4	1.7	0.1 ± 0.2
Total	2,150 ± 410	100	1,150 ± 130	100	
Phenolic compounds					
2,4-DBP	72 ± 23	15	62 ± 25	17	1.4 ± 3.8
2,4,5-TBP	79 ± 29	16	86 ± 40	24	0.3 ± 0.6
2,4,6-TBP	5.3 ± 3.4	1.1	6.0 ± 6.0	1.6	3.3 ± 3.2
4′-HO-BDE-17	17 ± 10	3.5	11 ± 7	3.0	ND
2′-HO-BDE-28	11 ± 6	2.3	5.2 ± 2.5	1.4	0.1 ± 0.2
4-HO-BDE-42	180 ± 120	38	120 ± 88	32	1.1 ± 2.7
3-HO-BDE-47	53 ± 25	11	33 ± 17	9.1	ND
6-HO-BDE-47	22 ± 12	4.6	8.5 ± 4.0	2.3	ND
4′-HO-BDE-49	42 ± 22	8.7	34 ± 19	9.3	0.3 ± 0.8
Total	480 ± 130	100	360 ± 104	100	

ND, not detected.
[a]Congeners (percentages) found in DE-71.

Background contamination

- Lab contamination

- Air/dust in the lab may contain FRs

- dust-free

Please read further:

Covaci et al. J Chromatogr A, 1153 (1-2), 145-171 (2007).

Hair analysis

- We will discuss this topic more in details during the ATC 2 in Antwerp