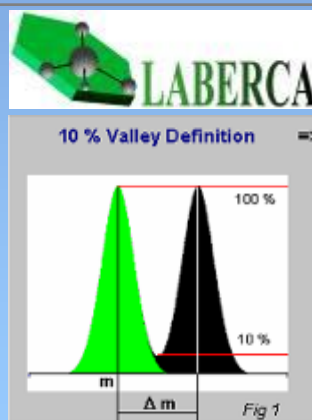




HIGHLIGHTING AND IDENTIFICATION TECHNIQUE OF CHLORINATION BY-PRODUCTS OF ETHINYLESTRADIOL IN DRINKING WATER TREATMENT BY UNTARGETED PROFILING METHOD BY LC-HR(MS)ⁿ

Gaël Gervais^a; Emmanuelle Bichon^a; Jean-Philippe Antignac^a; Fabrice Monteau^a; Valérie Ingrand^b; Gaëlle Leroy^b; Laurianne Barrिताud^b; Mathilde Chachignon^b; Pascal Roche^c; Luis Castillo^c & Bruno LeBizec^a

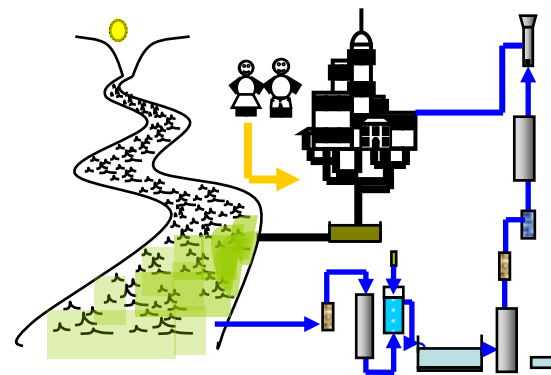


Introduction

Methodology

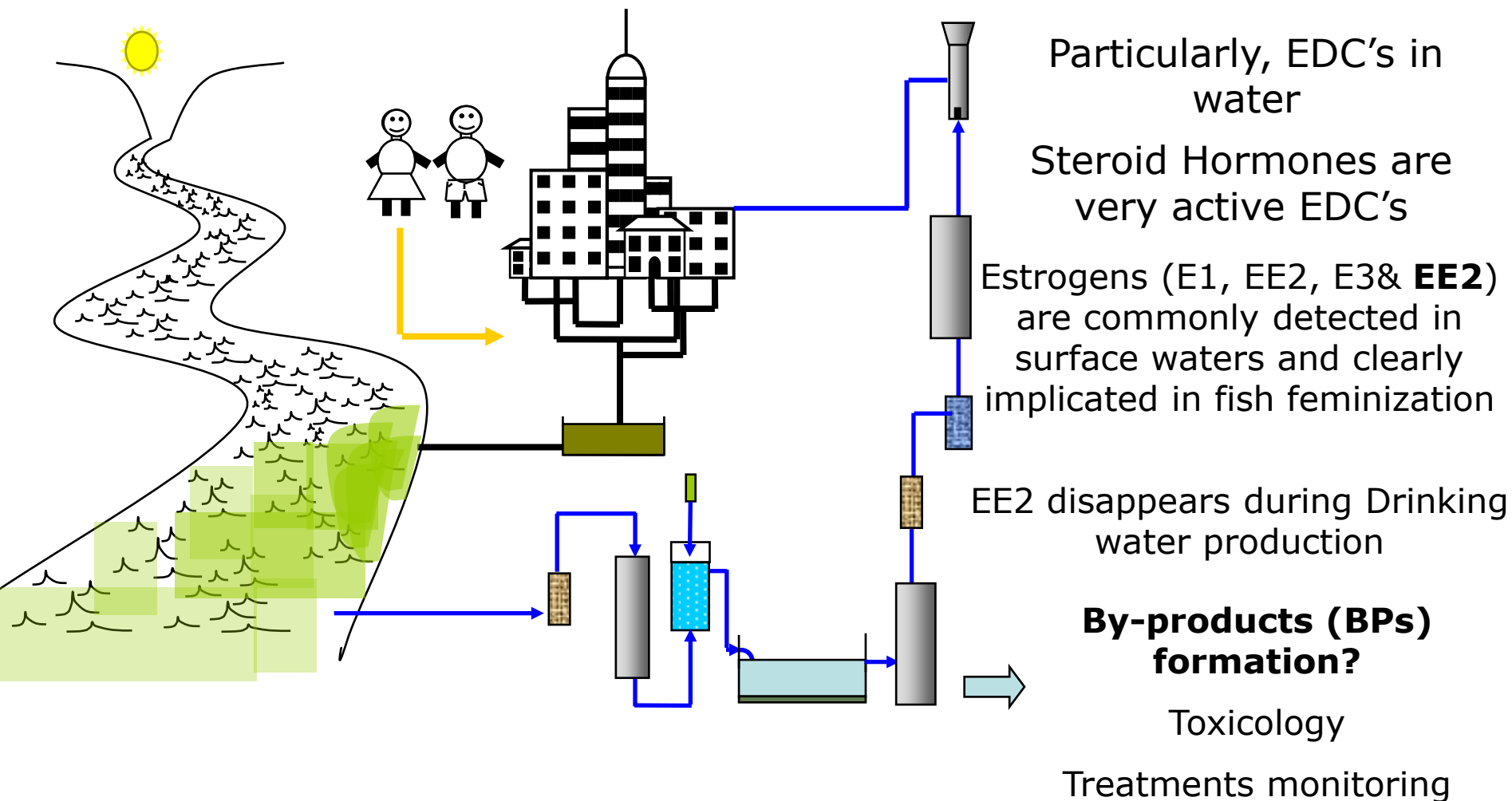
Results

Conclusion



Introduction

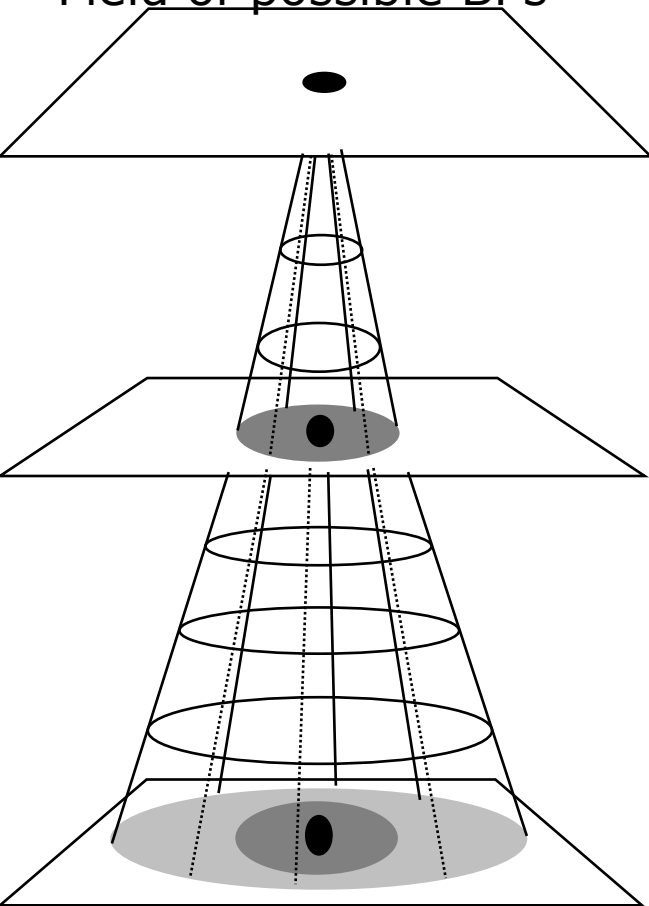
Endocrine Disruptors Compounds (EDCs) exposure is a major issue for Toxicology / Ecotoxicology / Food Safety Sciences



Introduction:

By-products Information

Field of possible BPs



Experiments

Water + EE2 + NaOCl

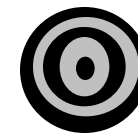


Water + EE2 + NaOCl +
Salts

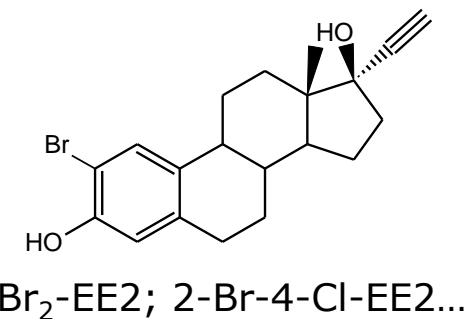
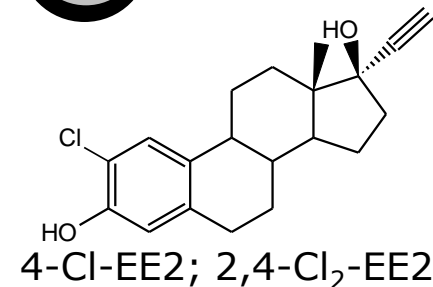


Real conditions:

Water + EE2 + NaOCl +
Salts + NOM + Humic acids
+ Fulvic aids+ Colloids...



Targets



?

**Exhaustiveness!
Of BPs**

Introduction:

Sampling

Classical treatment applied to EE2-spiked real water

Extraction:

Non selective extraction by SPE for polar & apolar compounds

Liquid chromatography:

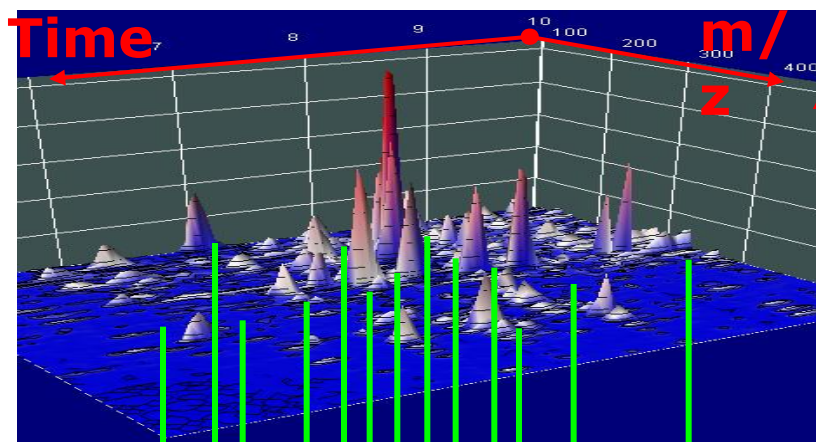
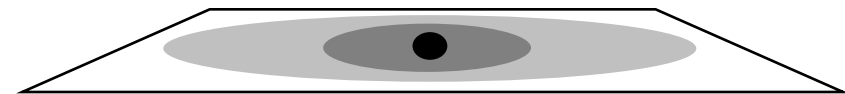
Non selective chromatographic gradient from 0% to 100% Organic

High Resolution Mass spectrometry:

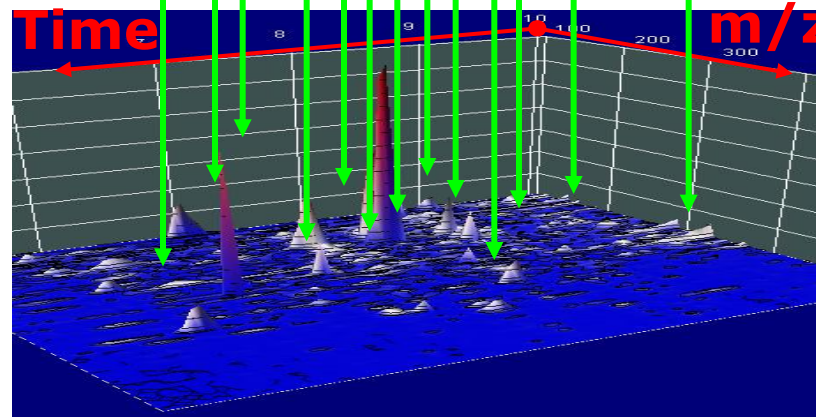
Use of Full Scan mode

Exhaustiveness of m/z

- **Comparison of groups of samples (Treated Vs. Untreated)**
- **Computational tool for data processing "fingerprints"**



Abundance



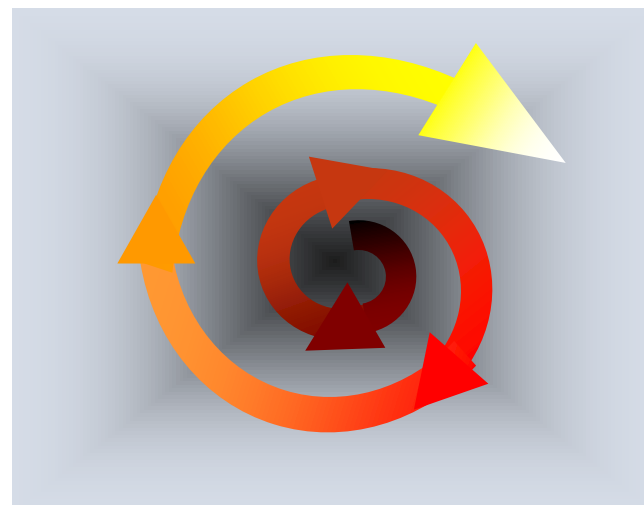
Abundance

Introduction

Methodology

Results

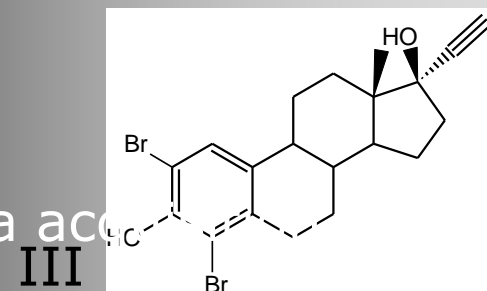
Conclusion



V – Structural hypothesis
(lit./frag./model.)

II – Data processing

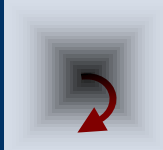
I – Data acquisition



measurement

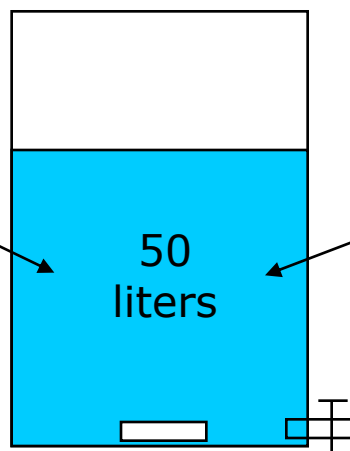
IV – raw formula
determination

"From shadow to light"



Real water from
DWTP

free from EE2



Spiking:
5 mg/L in Ultra
Pure Water

Chlorination:
NaOCl

Gr.1 "treated":

EE2-spiked (5 ppb) chlorinated (0.8 mg/L)
3 contact times:

- 10 min.
- 30 min.
- 120 min.

Each sample extracted twice

Each extract injected twice

=12 fingerprints

Gr.2 "witnesses":

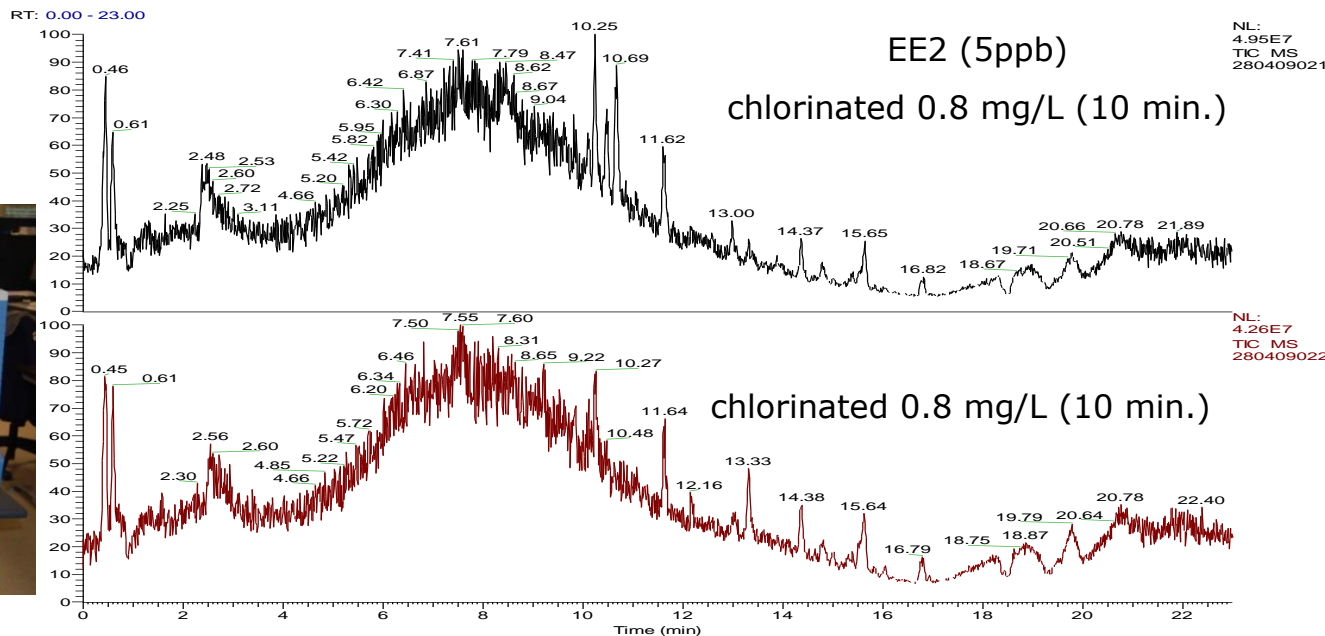
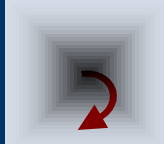
EE2-spiked & not-chlorinated

EE2-unspiked & chlorinated

EE2-unspiked & not-chlorinated

Ultra pure water

=16 fingerprints



Data acquisition of samples:

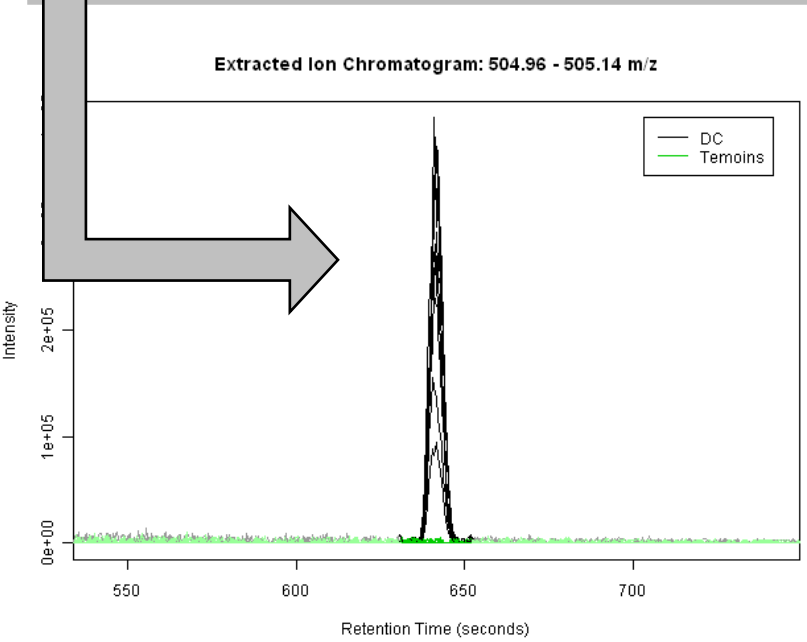
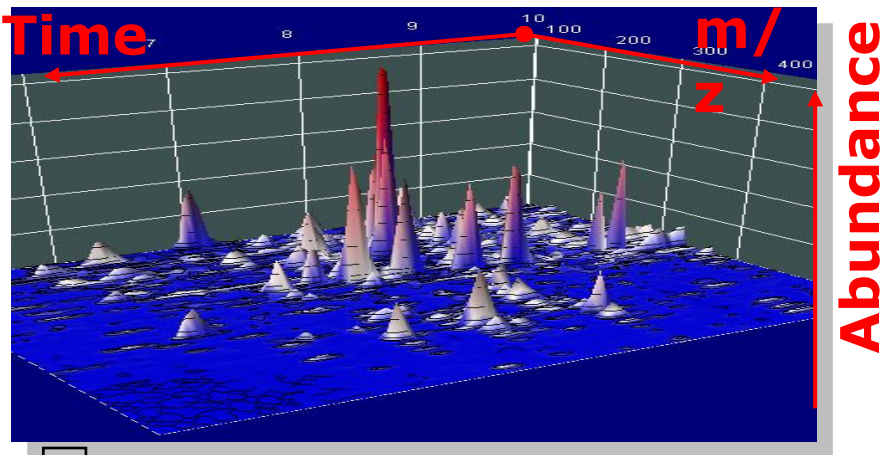
Extraction: SPE on Oasis HLB cartridge (500 mL -> 100 μ L)

HPLC: C18 50*2.1 mm (3 μ m)

UPW + 0.1% Acet. Acid / Acetonitrile

(HR)MS: LTQ-Orbitrap in ESI-

Full scan mode 70-800 Da



Abundance

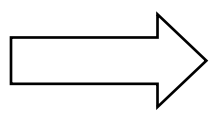
XCMS software:

Compares fingerprints of samples (m/z ; R_t & abundance) inside the group of samples and between groups.

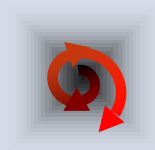
Ions statistically different between groups are listed by order of relevance (p-value)

The software creates Extracted Ion Chromatograms (EIC) for each relevant ion

Visual confirmation of ions relevance



List of ions that are different (over- or under-expressed) between "treated" and "untreated" samples



III-Exact mass measurement & isotopic pattern

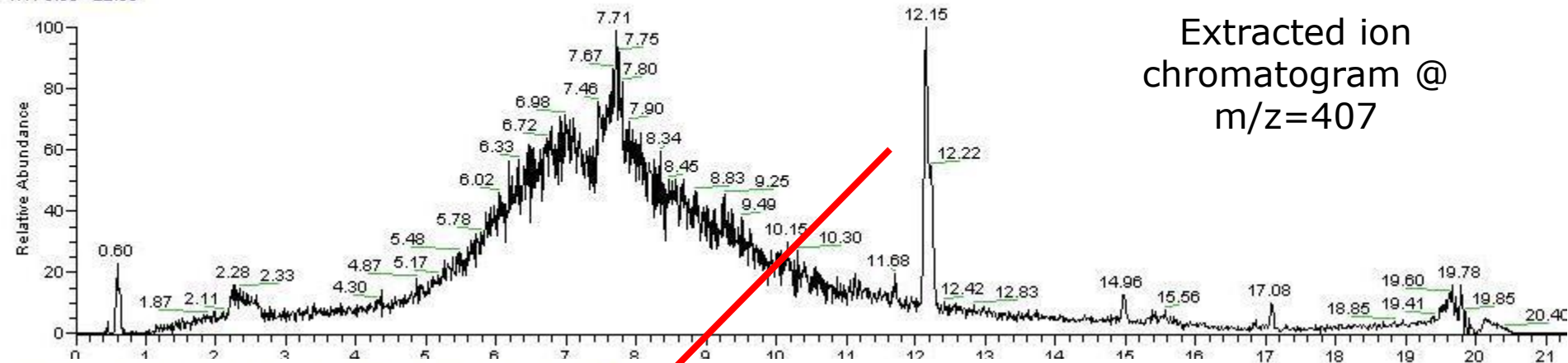
M407T730 : $m/z = 407$ & $R_t = 730$ sec (12.15min) in EE2-spiked chlorinated samples

C:\Xcalibur\20-PEVEO\Data\280409002

28/04/2009 20:52:57

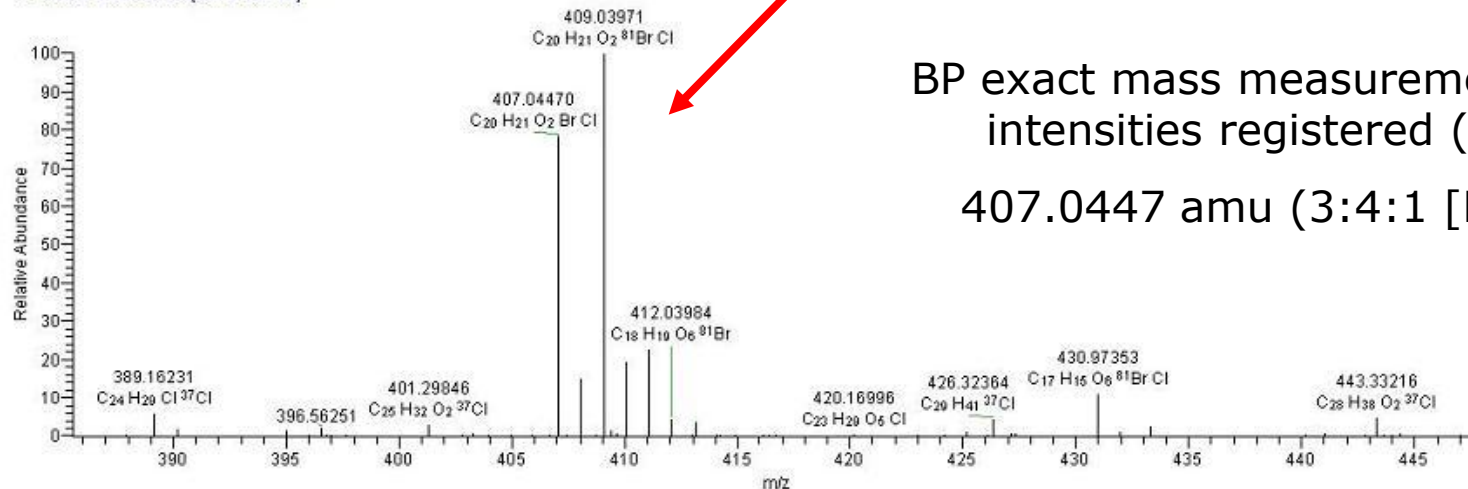
DCT10-1

RT: 0.00 - 22.99



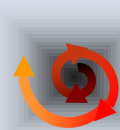
Extracted ion chromatogram @ $m/z=407$

280409002 #1176-1187 RT: 12.13-12.25 AV: 12 SB: 246 12.80-14.37 , 10.53-11.82 NL: 1.10E5
T: FTMS - c ESI Full ms [50.00-800.00]



BP exact mass measurement & isotopic peaks intensities registered (isotopic pattern)

407.0447 amu (3:4:1 [M]/[M+2]/[M+4])



407.0447 amu (3:4:1 [M]/[M+2]/[M+4])

Rt (min.)	observed m/z	raw formula	theoretical m/z	delta(mmu)	R	RDB eq.
12.15	407.0447	C20H22O2ClBr	407.0419	2.8	0.9994	9
		C21H26OBrCl	407.0784	-33.7	0.9993	8
		C18H24O4Cl3	407.0892	-44.5	0.9788	6
		C20H19BrCl2	406.9973	47.4	0.9449	10
		C19H22O3Cl3	407.0954	-50.7	0.9792	5
		C18H14O4ClBr	406.9689	75.8	0.9992	11
		C18H18OClBr2	406.9650	79.7	0.8951	9

Criteria of selection:

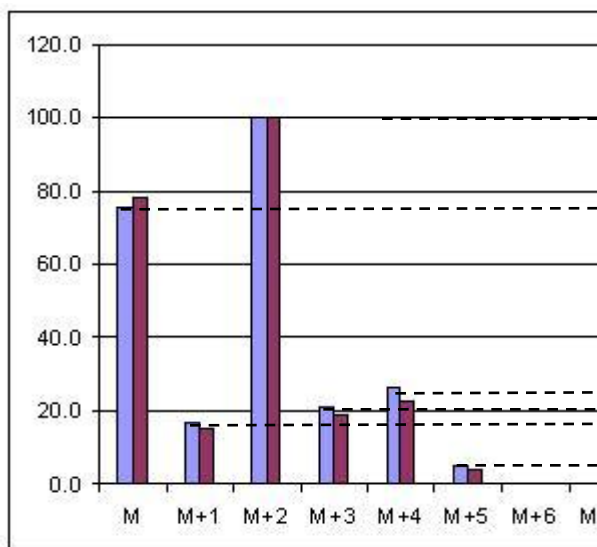
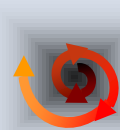
Limited number of elements: C, N, O, H, Br, Cl, P, S,

mass precision: $\Delta\text{mmu} < 5$ mmu

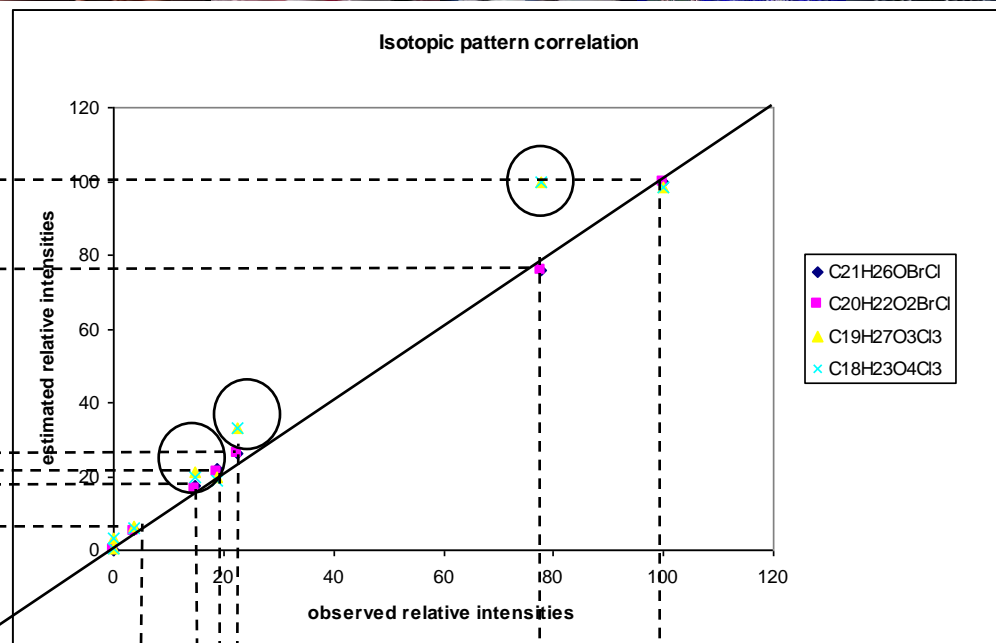
Isotopic pattern: R² highest possible

RDBeq. Close to EE2 (9)

Nitrogen rules



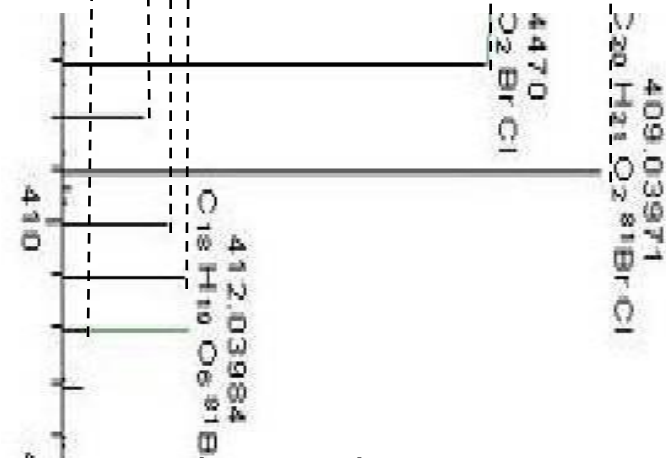
Theoretical pattern



R=1

Correlation coefficient as high as possible

Especially with halogenated compounds



Observed isotopic pattern



Propose a structure

Reduce the number of hypothetical structures

Fragmentation MS²:

Any specific fragments?

Literature study:

Described compound?

Chemical reaction
already observed?

Molecular modeling:

Initial state (e-density)

Transitional state

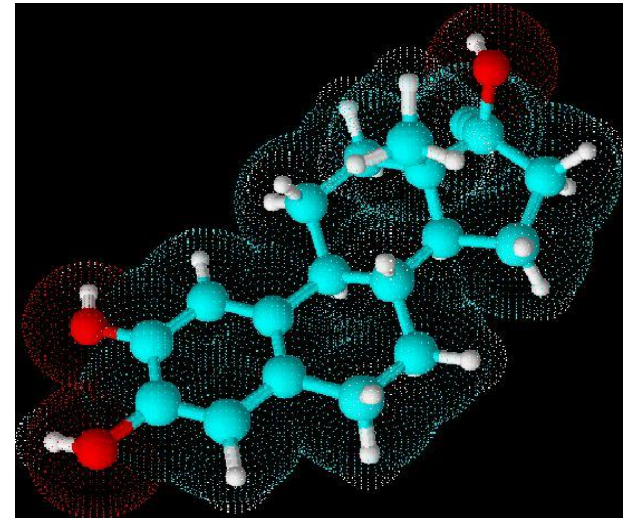
Final state
(thermodynamic)

Introduction

Methodology

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Conclusion



Highlighted ions & raw formulae selected

Code	Rt (min)	m/z	adduct	Raw formula	Δ	R ²	RDB eq.	Intensity (Arb.unit)	Confidence (b. unit)	evolution
X10 & X9										
X1	8,58	418/420	yes	C ₂₀ H ₂₂ O ₄ NBr	0.900	0.99952	10	*	*	+
X2	9,8	402/404	yes	C ₂₀ H ₂₂ O ₃ NBr	1.005	0.99926	10	*	*	+
X3	10,32	379/381/383	yes	C ₂₀ H ₂₂ O ₃ Cl ₂	26.569	0.99900	9	**	**	=
X4	10,5	423/425/427	yes	C ₂₀ H ₂₂ O ₃ BrCl	0.262	0.99795	9	***	**	=
X5	10,7	467/469/471	yes	C ₂₀ H ₂₂ O ₃ Br ₂	0.221	0.99915	9	***	**	=
X6	10,85	543/545/547	no	C ₂₂ H ₂₆ O ₆ Br ₂	0.466	0.99706	9	***	**	+
X7	11,62	501/503/505/507	yes	C ₂₀ H ₂₂ O ₆ N ₂ Br ₂	0.566	0.99970	9	***	**	-
X8	11,74	545/547/549/551	yes	C ₂₀ H ₂₂ O ₆ N ₂ Br ₂	0.600	0.99970	9	***	**	---
X9	12,15	407/409/411	yes	C ₂₀ H ₂₂ O ₂ BrCl	0.307	0.99909	9	*	***	--
X10	12,42	451/453/455	yes	C ₂₀ H ₂₂ O ₂ Br ₂	0.774	0.99937	9	***	***	--



• X10 decreased along the treatment. This is in accordance with disappearance kinetic (*Lee, 2009*)

• Bromination of EE2 already observed (*Nakamura, 2006; Lee, 2009; Flores, 2008*)

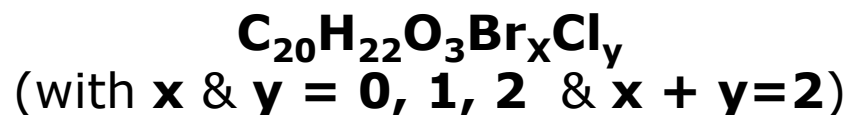
• ortho positions favored for halogenation (mol. Model.)

Validation of the Identification process

Highlighted ions & raw formulae selected

Code	Rt (min)	m/z	adduct	Raw formula	$\Delta m m u$	R ²	RDB eq.	Intensity (Arb.unit)	Confidence (Arb. unit)	evolution
X1	8,58	418/420	yes	C20H22O4NBr	0.900	0.99952	10	*	**	+
X2	9,8	402/404	yes	C20H22O3NBr	1.005	0.99926	10	*	*	+
X3	10,32	379/381/383	yes	C20H22O3Cl2	26.569	0.99900	9	**	**	=
X4	10,5	423/425/427	yes	C20H22O3BrCl	0.262	0.99795	9	***	**	=
X5	10,7	467/469/471	yes	C20H22O3Br2	0.221	0.99915	9	****	***	=
X6	10,85	543/545/547	no	C22H26O6Br2 (adduct) C20H22O6N2Br2	0.166 25.086	0.99706 0.99713	9 10	**	*	+
X7	11,62	501/503/505/507	yes	C20H21O3ClBr2	0.324	0.99717	9	*	*	-
X8	11,74	545/547/549/551	yes	C20H21O3Br3	0.607	0.99886	9	***	**	---
X9	12,15	407/409/411	yes	C20H22O2BrCl	0.307	0.99909	9	*	***	--
X10	12,42	451/453/455	yes	C20H22O2Br2	0.774	0.99937	9	***	***	--

Discussion of X3, X4 and X5 compounds



One more Oxygen, where?

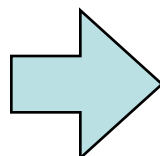
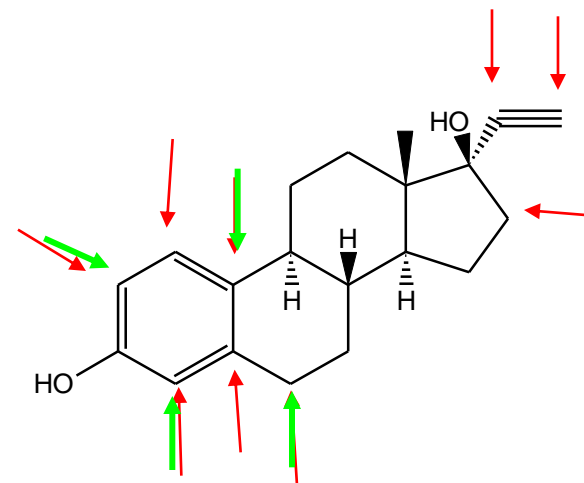
2 halogens, Where?

Literature study:

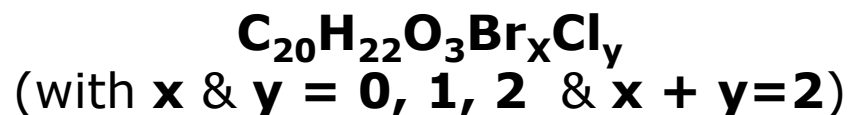
Oxidation in position 2, 4, 6 & 10

Halogenation is an electrophilic substitution on the A-ring

No reactions on the ethynyl moiety



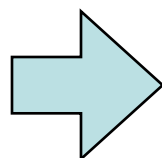
Discussion of X3,X4 and X5 compounds



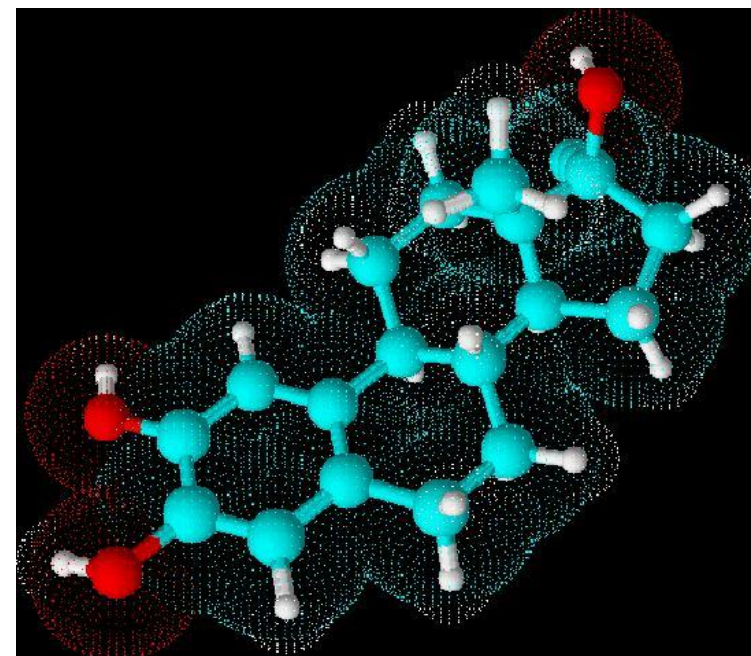
One more Oxygen, where?

2 halogens, Where?

Molecular modeling:

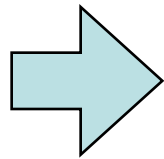
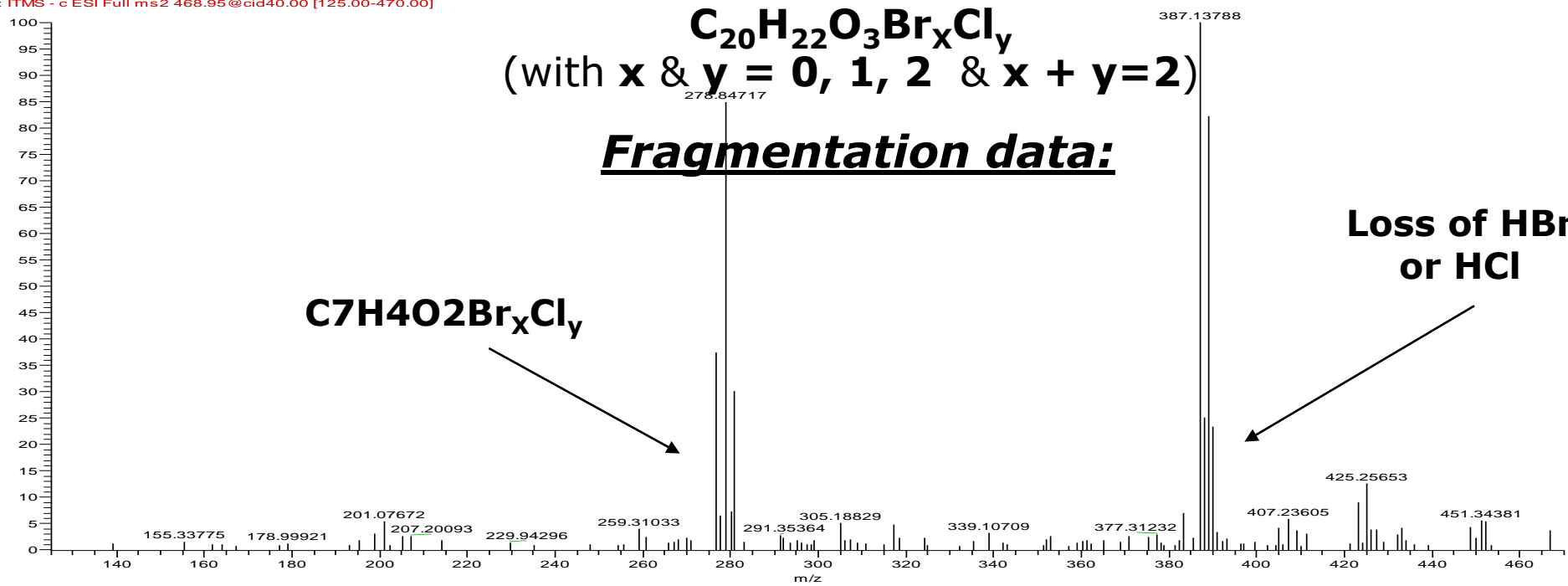


- Transitional state favored halogenations on the A-ring
- Final state favored halogenations on ethynyl group
- Oxidation favorable in C2 position

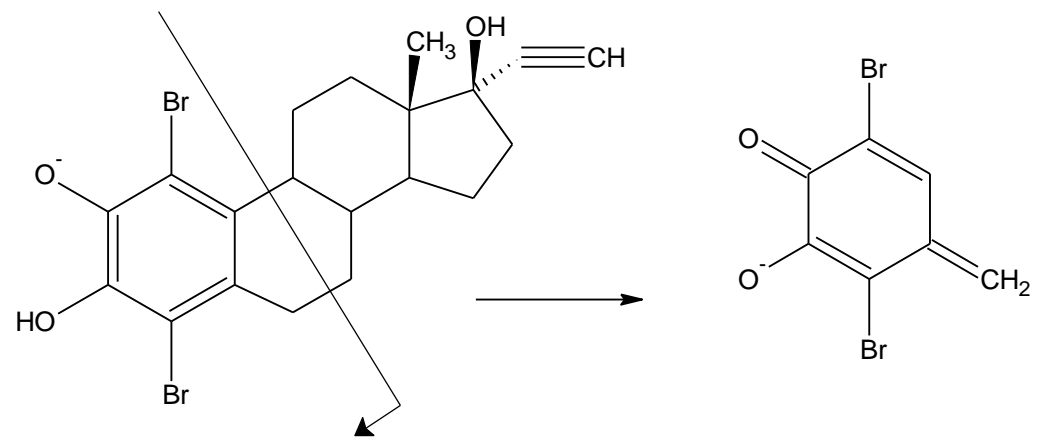


Additional Hydroxyl-group position on EE2	1	2	4	6	10
Heat of formation, ΔH_f° (kcal)	-82,94	-85,67	-83,06	-82,56	-60,23

090609004 #621 RT: 10.78 AV: 1 NL: 6.18E2
 F: ITMS - c ESI Full ms2 468.95 @cid40.00 [125.00-470.00]



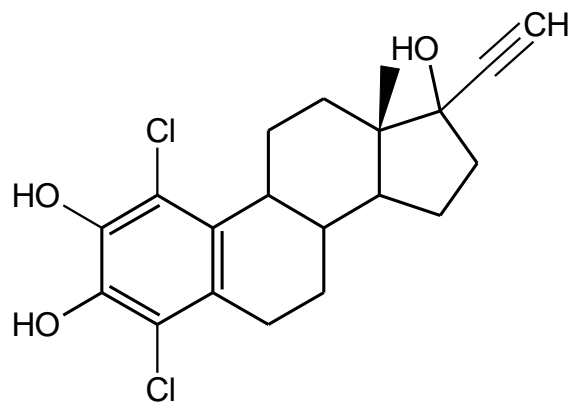
With previous information, only one possibility



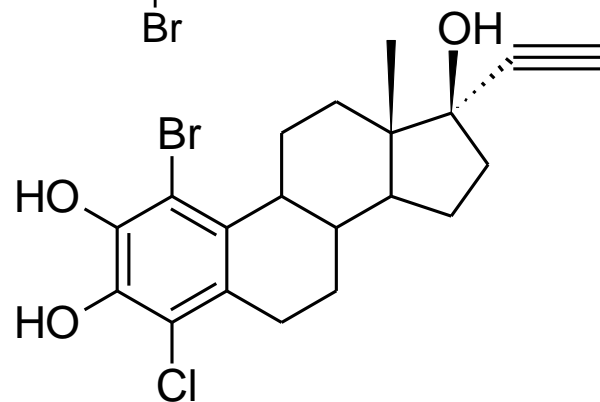
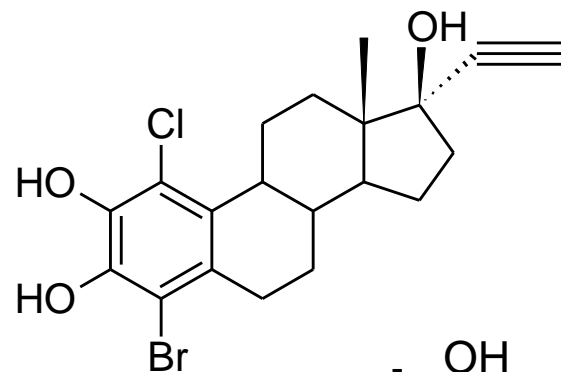
Discussion of X3, X4 and X5 compounds

X3, X4 & X5

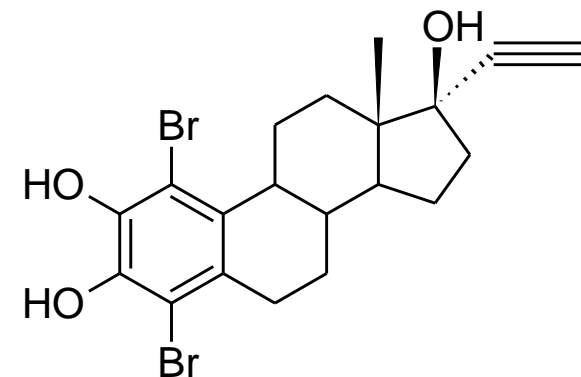
$C_{20}H_{22}O_3Br_xCl_y$
(with x & $y = 0, 1, 2$ & $x + y = 2$)



$m/z = 379/381/383$



$m/z = 423/425/427$



$m/z = 467/469/471$



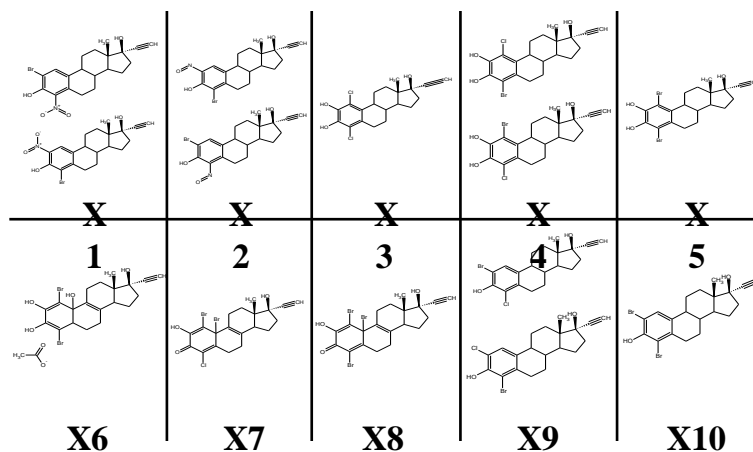
**Main By-products of
EE2**

Introduction

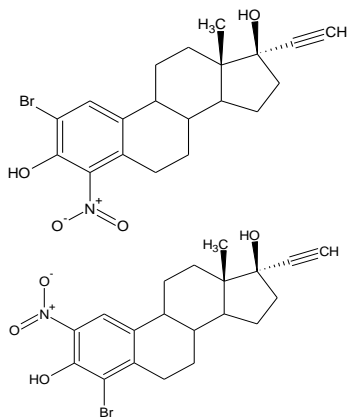
Methodology

Results

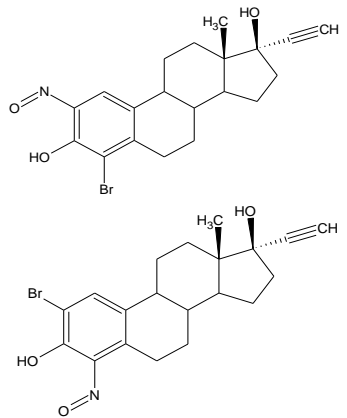
Conclusion



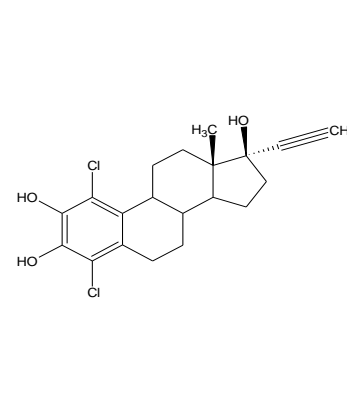
Conclusion



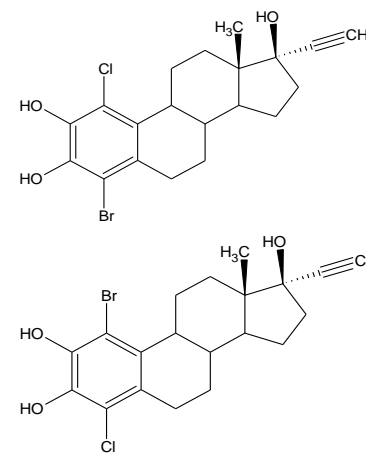
X1



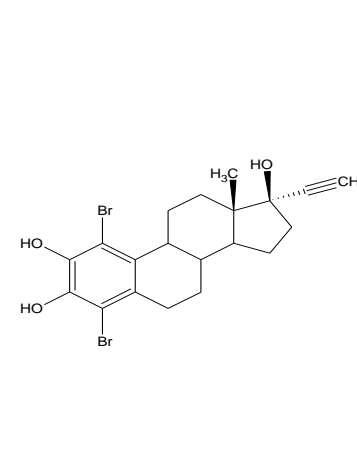
X2



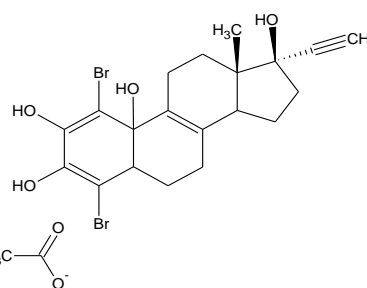
X3



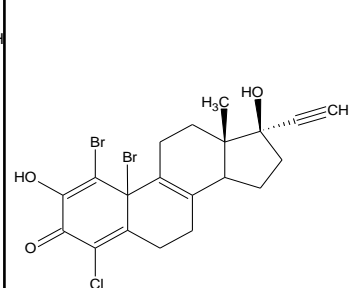
X4



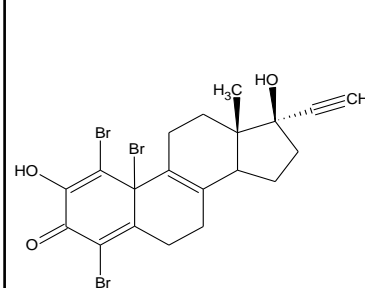
X5



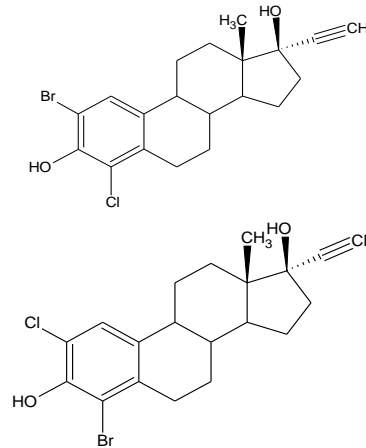
X6



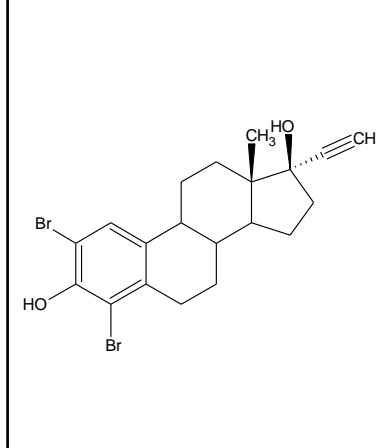
X7



X8



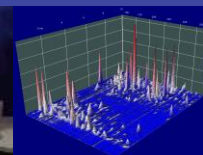
X9



X10

Perspectives:

- Develop targeted analysis of identified BPs to reach observable amounts (<1 ng/L)
- Confirm these BPs in DWTP by (LC-MS/MS)
- Apply this identification process to other kind of treatment (ozonation, etc.)
- Apply this identification process to other EDCs (Estrone-sulfate, etc.)
- Evaluate BPs Toxicity (probably lower than EE2)



LABERCA:

Antignac Jean-Philippe
Bichon Emmanuelle
Monteau Fabrice
Le Bizec Bruno

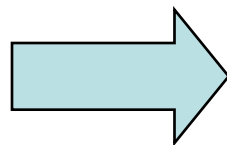
CAE:

Ingrand Valérie
Leroy Gaëla
Barritaud Lauriane
Chachignon Mathilde

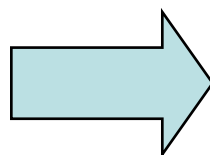
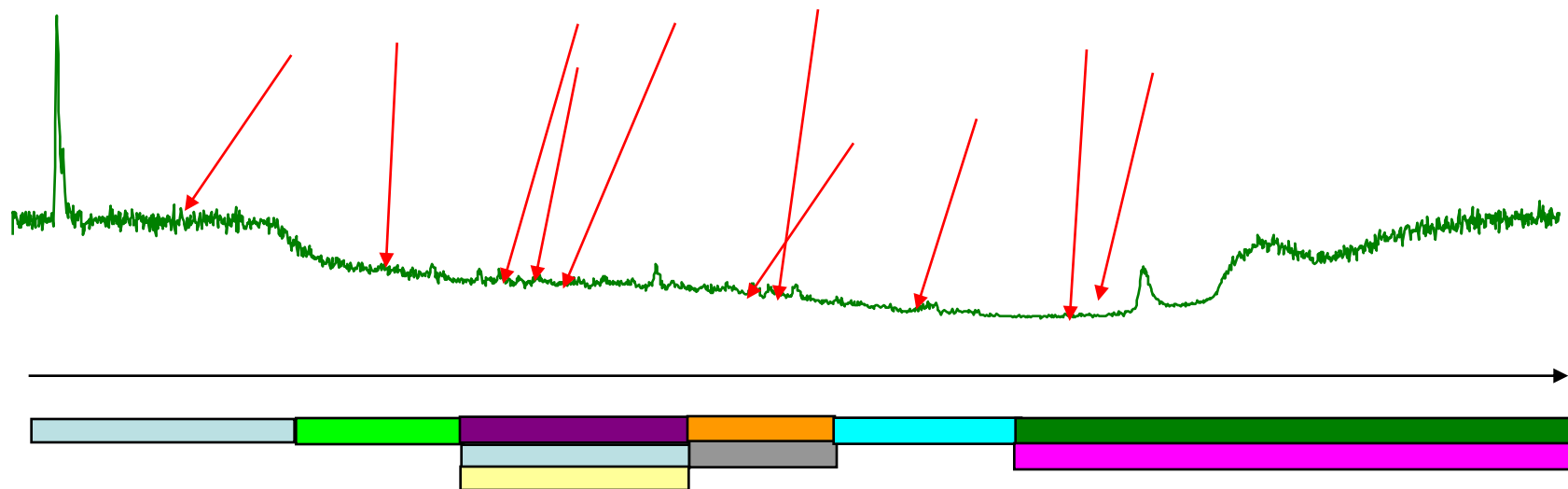
Anjou-Recherche:

Roche Pascal
Castillo Luis
Bourdin Delphine

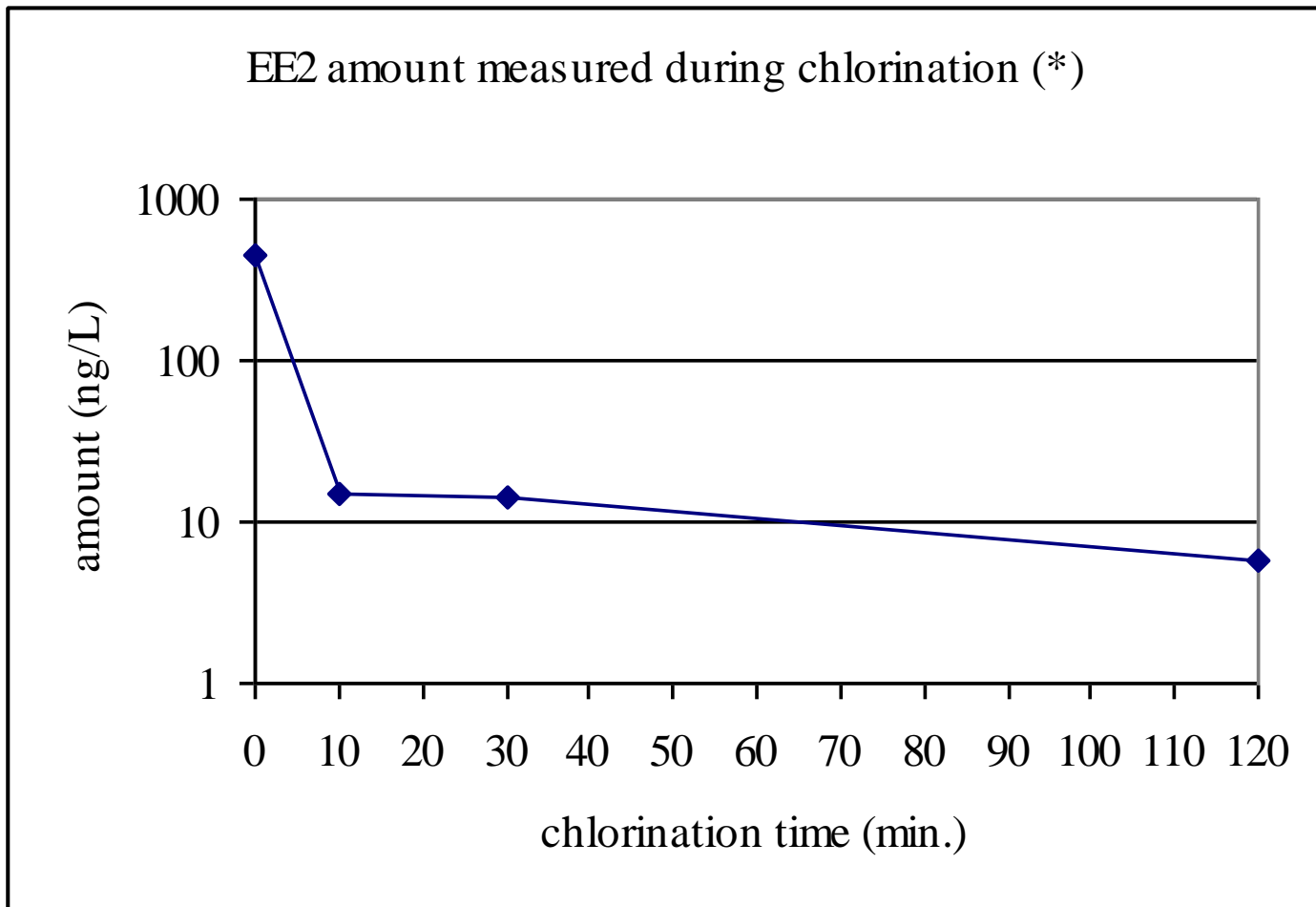
Thank you!



If XCMS highlights 10 compounds



Create 10 MS/MS functions centered on the isotopi pattern of the parent ion with a 6 Da « Iso-width »



*data from CAE, VEOLIA Environment