NON TARGET SCREENING OF HALOGENATED SUBSTANCES IN MARINE MAMMALS STRANDED ON FRENCH COASTS BASED ON LC-HRMS AND HALOSEEKER 1.0 SOFTWARE

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Introduction

Identifying environmental chemical contaminants in abiotic matrices, along the trophic chains and in human matrices, is a major scientific challenge undertaken to support risk assessment. Halogenated compounds, especially chlorinated and brominated ones, are a chemical class of particular concern. Indeed, some of anthropogenic origin like Persistent Organic Pollutants[1], together with emerging environmental contaminants exhibit harmful effects to the environment and human health. Others are naturally produced (HNPs), especially by marine organisms, and may pose a risk to human health as well. In the marine environment, top predators such as mammals are particularly exposed to bioamplified chemical hazards. Thus, within the exposomics concept, the comprehensive description of chemical hazards is a growing activity of the scientific community that will help guide future management policies.

In the present work, a Non Targeted Screening (NTS) strategy was applied to a set of blubber samples from marine mammals stranded or accidentally caught in net on the French coasts. The objective was to characterize polyhalogenated substances amenable by LC-ESI(-)-HRMS using a recently made publicly available open-source software, HaloSeeker 1.0, for assigning chemical formulae and select signals worth of further investigations.

Materials and methods

Samples

Twelve male individuals from five species were selected within the French sample bank of the national stranded marine mammals network (Table 1). Blubber was stored at -20 °C until analysis.

Table 1. Description of samples. Length in cm.

Acronym	Species	Common name	Year	Zone code	Length
Pp_1	*Phocoena phocoena*	Harbor porpoise	2012	Bay of Biscay	162
Pp_2	*Phocoena phocoena*	Harbor porpoise	2013	English Channel	154
Pp_3	*Phocoena phocoena*	Harbor porpoise	2015	English Channel	143
Pp_4	*Phocoena phocoena*	Harbor porpoise	2016	English Channel	155
Bp_1	*Balaenoptera physalus*	Fin whale	2010	Bay of Biscay	773
Bp_2	*Balaenoptera physalus*	Fin whale	2018	Bay of Biscay	1692
Tt_1	*Tursiops truncatus*	Bottlenose dolphin	2009	Bay of Biscay	185
Tt_2	*Tursiops truncatus*	Bottlenose dolphin	2018	Bay of Biscay	323
Pv_1	*Phoca vitulina*	Harbor seal	2017	English Channel	158
Pv_2	*Phoca vitulina*	Harbor seal	2016	Bay of Biscay	118
Pm_1	*Physeter macrocephalus*	Sperm whale	2001	Bay of Biscay	1045
Pm_2	*Physeter macrocephalus*	Sperm whale	2016	English Channel	1385

Sample preparation

Blubber sample was extracted with a mixture of toluene/acetone (7:3, v/v) using a Pressurized Liquid Extraction. Lipid fraction (500 mg) in hexane was subjected to partitioning with 4 × 1.5 mL concentrated sulfuric acid (1500 tr/min centrifugation steps) to remove lipids. The limpid organic layer was neutralized with 2 × 2 mL ultrapure water (up to pH=3) and dried with anhydrous sodium sulfate. Extract was reconstituted in a mixture of MeOH/H_2O 4:1 (v/v, 62.5 µL). Three procedural blanks were performed. $^{13}C_{12}$-γ-HBCDD (20 ng) and $^{2}H_{18}$-β-HBCDD (10 ng) were used as internal and recovery standards (IS, RS), respectively.

LC-HRMS data acquisition

Extracts (7 µL) were analyzed with an UltiMate 3000 UHPLC pumping system coupled to an Orbitrap Q-Exactive mass spectrometer equipped with a heated ESI (HESI) source (Thermo Fischer Scientific, San Jose, CA, U.S.A.). The instrument was controlled with Chromeleon Xpress and Xcalibur softwares (Thermo Fischer Scientific). Chromatographic separation was performed on a Hypersil Gold analytical column (100 mm × 2.1 mm, 1.9 µm, Thermo Fischer Scientific) kept at 45 °C. A mobile phase consisting of acetonitrile (*v/v*, A) and water/acetonitrile

99:1 (B), both containing 10 mM ammonium acetate, was used. The gradient started with 20% A (0 to 2 min), was then increased linearly to 60% (10 min), 100% (40 to 46 min) and returner to the initial conditions (48 to 52 min). The flow rate was set at 0.4 mL/min. Data were recorded in negative mode with HESI parameters as follows: sheath gas flow, 50 arbitrary unit (AU); auxiliary gas flow, 5 AU; capillary temperature, 350 °C; source temperature, 150 °C; spray voltage, 2.5 kV; s-lens radio frequency, 50 AU. HRMS data were acquired in full-scan mode within the *m/z* range of 120−1000 at a resolving power of 140 000 at *m/z* 200, using *m/z* 305.02307 ($[CH_3COO•(NaCH_3CO_2)_3]^-$) as lock mass. The automatic gain control (AGC target) was set at 5×10^5, and the maximum injection time was set at 250 ms.

Post-acquisition data treatment

Data treatment was performed using HaloSeeker 1.0, an open source software aiming to seek halogenated signatures in full-scan HRMS fingerprints described in Léon et al., 2019[2]. The ergonomic web user interface in the R programing environment avoids any interactions with the coding component while allowing interactions with the data, including peak detection, deconvolution, and comprehensive manual review for chemical formula assignment.

Briefly, proprietary raw data were converted in an open format. Then, the application proceeded to the peak picking step using *xcms* 3.2.0 package[3] (mzTol=3, snthresh=10, prefilter step=3, peakwidth=10-60, prefilter level=20000, noise=0, mzdiff=0.001). Obtained features were paired according to precise mass differences between Cl and Br isotopes (tolerances: t_R =1 s, *m/z*=0.5 mDa). H/Cl-scale Mass Defect (MD) plots were drawn, using available data filters based on paired clusters as well as ion ratio rules. Finally, clusters of interest were investigated using the interactive pop-up window and chemical formulas were assigned. Ion formula decomposition was performed using ^1H, ^{12}C, ^{14}N, ^{16}O, ^{35}Cl and ^{79}Br, up to 50, 30, 10, 10, 15 and 10 elements, respectively. Ion ratio tolerance of scoring too was set at 20%.

Results and discussion

QA/QC

Retention times (t_R) were stable along the sequence (standards, procedural blanks and blubber). RS signal relative standard deviation (RSD) was ±25% along the sequence, sperm whale showing relatively low intensities. RSD decreased to ±12% when considering only the 4 other species. IS recoveries ranged from 50 to 93%, except for sperm whale at 44-46%. Considering slight losses and ion suppression phenomenon, results were not considered quantitative but qualitative only. Mass deviation for both IS and RS was in the range 0.40-0.81 ppm (0.23-0.51 mDa), allowing for sub-ppm formula searches.

Total ion chromatograms

Figure 1 shows obtained Total Ion Chromatograms for both blubber and procedural blank extracts. Unlike other species, which TICs exhibit a few signals between 7 and 37 min at relatively low intensities compared to baseline, sperm whale (Pm_1 and _2) TICs exhibit intense signals between 11 and 15 min with expected ions suppression (competition for charges), which is consistent with observed precipitate in the corresponding extract vials. These signals correspond to a series of $[C_nH_{2n+1}O_4S]^-$ ions, with $n \in [\![9\text{-}29]\!]$ (Figure 2), likely $[M - H]^-$ ions of alkyl sulfates with null Degree of Unsaturation (DoU). It could be related to the metabolism of spermaceti, a waxy substance found in the head cavities of the sperm whale.

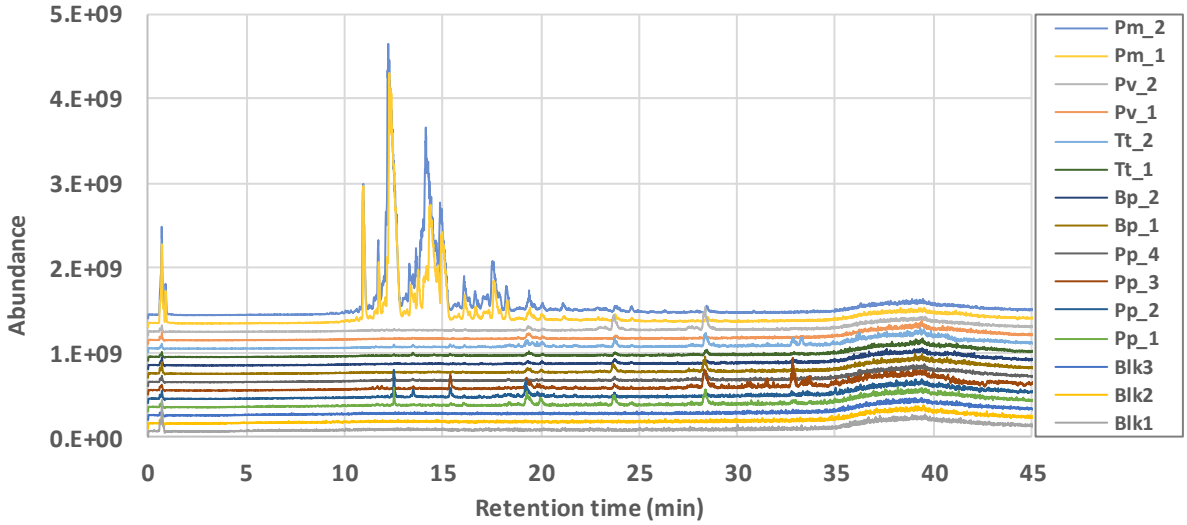

Figure 1. Total Ion Chromatograms obtained for blubber and procedural blank extracts.

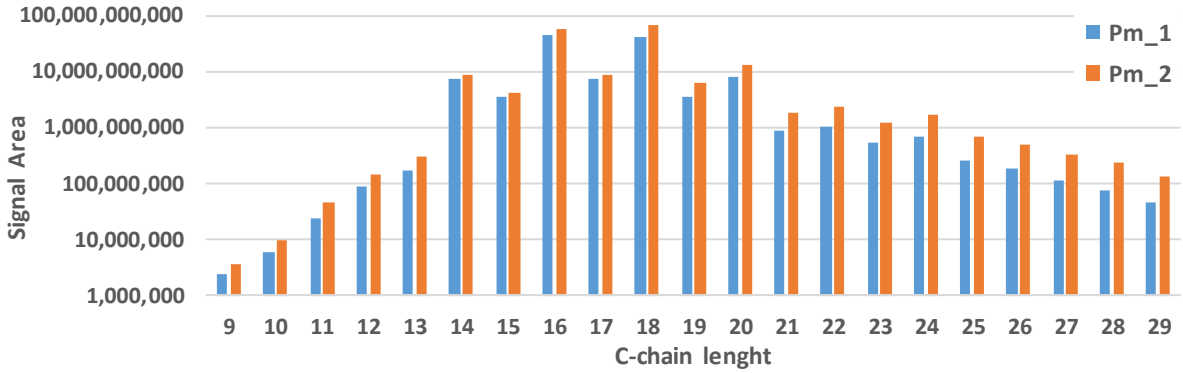

Figure 2. Log-scale signal intensities observed for alkyl sulfate series in sperm whale extracts, according to C-chain length.

Filters and enumeration of clusters of interest

For manual review of the data, 1-46 min was considered as t_R range, along with a cumulated intensity threshold of 10^6 for features/clusters. "F2+" filter of HaloSeeker allowed to focus on feature clusters paired according to precise mass differences between Cl and Br isotopes as well as ion ratio rules related to polyhalogenated ions. Table 2 shows that most features did not pass F2+ filter, illustrating efficiency of HaloSeeker algorithm. All F2+ clusters (n=888) were investigated through HaloSeeker interface and either discarded (RS, IS or obviously non halogenated) or assigned an ion formula (n=466). No cluster of interest was detected neither in standards nor in procedural blanks. In marine mammal blubber, 4 to 94 clusters of interest were assigned a polyhalogenated ion formula. In terms of number of clusters amenable by LC-ESI(-)-HRMS and cumulated intensities, harbor porpoise (Pp) and bottlenose dolphin (Tt) appeared as the most contaminated species.

Table 2. Enumeration of features, clusters and clusters with assigned formula according to Haloseeker Filter. SumInto: cumulated intensities ($\times 10^6$); Cumulated intensity threshold: 10^6; t_R range: 1-46 min; F0: all features; F2+: paired clusters complying polyhalogenated ion ratio rules.

	F0		F2+			F2+, assigned formula			
Sample	Features	SumInto	Features	Clusters	SumInto	Features	Clusters	SumInto	Into Ratio
Standards 1	4,015	63,156	117	38	1,005	0	-	-	
Standards 2	671	9,933	70	20	589	0	-	-	
Blank 1	3,355	54,463	76	28	1,138	0	-	-	
Blank 2	3,524	53,963	112	42	921	0	-	-	
Blank 3	697	10,308	41	8	98	0	-	-	
Pp_1	4,023	61,520	463	97	1,806	306	49	1,028	1.7%
Pp_2	1,187	12,411	633	108	3,236	518	83	2,931	24%
Pp_3	1,634	20,988	825	129	2,860	682	94	2,470	12%
Pp_4	3,719	51,906	412	84	1,522	302	46	823	1.6%
Bp_1	761	16,448	104	23	262	40	7	34	0.2%
Bp_2	785	15,457	97	20	261	31	4	32	0.2%
Tt_1	999	14,072	425	70	744	336	52	593	4.2%
Tt_2	2,612	24,899	692	113	2,388	551	77	1,966	7.9%
Pv_1	771	14,374	164	27	254	107	15	111	0.8%
Pv_2	801	20,475	173	29	273	102	14	110	0.5%
Pm_1	1,928	169,952	101	22	222	61	13	88	0.05%
Pm_2	2,030	244,503	91	20	164	58	12	96	0.04%
Standards 3	385	9,436	55	10	124	0	-	-	
				Minimum		31	4	32	
				Maximum		682	94	2,931	

Among the 466 clusters with an assigned formula, only 135 were different (including possible isomers), most compounds being observed in several species. *m/z* ranged from 203 to 809. t_R were either in the range 8.4-21.3 or 29.6-35.0 min, at 60-75% or 85-95% of organic mobile phase, respectively.

Remarkable cluster series

Then, exported data table was manually reviewed to group clusters according to remarkable patterns (e.g. homologues, isomers, adducts). More than 300 out of the 466 clusters, corresponding to >90% of cumulated intensities for each extract, were grouped into 16 cluster groups. These groups included the most intense clusters as well as most mixed halogenated ions. Here after are briefly described the most remarkable groups.

Group 1. The most intense cluster (SumInto ~1×10^9, t_R=12.53 min, *m/z*=370.78584), found in Pp_1 extract, was assigned the formula $[C_8Cl_7N_2]^-$. (ΔmDa=0.06, score=95%, DoU=6). Assuming a recovery and a response factor similar to IS, it could correspond to a concentration of 3.4 µg/g lw. This compound was also identified in other extracts, except sperm whale for which ion suppression could have occurred at this t_R. Mixed halogenated (Br_1Cl_6, Br_2Cl_5) homologues were also identified at slightly lower t_R, with relatively high correlations. We hypothesize that it could correspond to halogenobipyrroles. Tentative chemical structures of mono or dimethylated analogues, considered as HNPs in the marine environment, were previously suggested[4,5], but none of these analogues were detected in the present samples.

Group 2. The second most intense compound was identified as α-HBCDD in Pp_3 extract (SumInto ~0.9×10^9, t_R=15.40 min, ΔmDa=0.34), a well-known POP, detected in all blubber extract at concentrations in the 0.01 to 4 µg/g lw range.

Group 3. The third group of interest was assigned $[M + C_2H_3O_2]^-$ and less intense $[M - H]^-$ ions of mixed pentahalogenated compounds, mainly $C_{10}H_{13}Br_2Cl_3$ (SumInto ~0.45×10^9 in Pp_2 extract, t_R=13.51 min) but also $C_{10}H_{13}Br_3Cl_2$. According to the literature[6], it could correspond to a naturally occurring monoterpene.

These 3 groups accounted for 11 to 81% of cumulated intensities of clusters of interest and other groups belong to various other polyhalogenated families. As an example, Figure 3 shows the MD-plot obtained for Pp_2 extract.

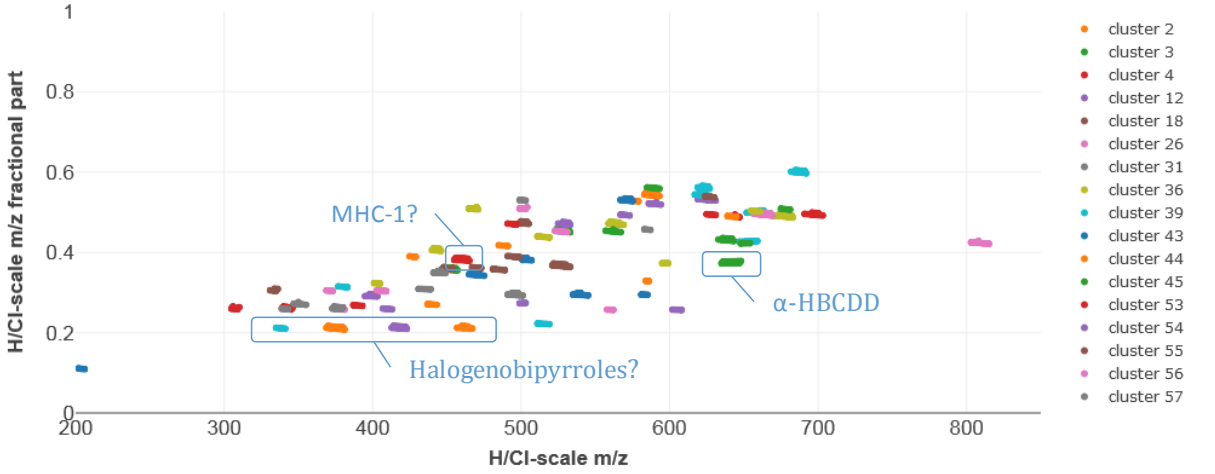

Figure 3. H/Cl-scale MD-plot obtained with HaloSeeker 1.0 for Pp_2 extract. Clusters with assigned formula; F2+ filter.

Conclusion and perspectives

The applied NTS analytical strategy proved to be efficient in highlighting polyhalogenated substances in marine mammal blubber samples using LC-ESI(-)-HRMS and an open-source post-acquisition data treatment software. Clusters of interest were grouped in consistent series according to their assigned formulae and other criterion. Further investigations are required for structural elucidation and determination of natural or anthropogenic origin. Ultimately, most relevant series, e.g. in terms of concentrations or active functions, could raise toxicological concern.

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References

[1]. UNEP. Convention on Persistent Organic Pollutants, Stockholm, Sweden, 2001.
[2]. Léon A, Cariou R, Hutinet S, et al. (2019) *Anal. Chem.* 91(5): 3500-3507.
[3]. Tautenhahn R, Böttcher C, Neumann S (2008) *BMC Bioinf.* 9: 504.
[4]. Hauler C, Martin R, Knöler HJ, Gaus C, Mueller JF, Vetter W (2013) *Environ. Pollut.*, 178: 329-335.
[5]. Vetter W, Stoll E (2002) *Eur. Food Res. Technol.*, 215: 523-528.
[6]. Vetter W, Hiebl J, Oldham NJ (2001) *Environ. Sci. Technol.*, 35: 4157-4162.