Electronic Supplementary Material (ESI) for Environmental Science: Advances.

SUPPORTING INFORMATION

New insight into the biodegradability of Complex Mixtures. UHPLC-qToF "all-ion MS/MS" acquisition technique to the Untargeted Metabolite Fingerprint and Targeted Analysis

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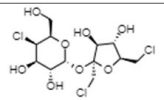
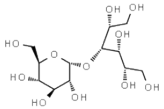
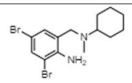
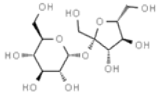
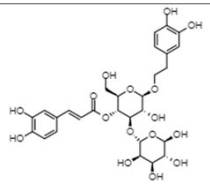
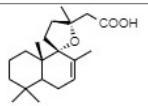
Table S1: Semi-quantitative evaluation of sucralose 1, maltitol 2 and Bromhexine 3 by Relative Ratio (RR) estimation and calculation of the reduction percentage [b]

Concentration	Sucralose (1)	Maltitol (2)	Bromhexine (3)
50 mg/L	RR at T0 0.049	RR at T0 2.801	RR at T0 0.064
	RR at T28 0.052	RR at T28 0.000	RR at T28 0.004
	No % reduction	*100% reduction*	*94 % reduction*
100 mg/L	RR at T0 0.100	RR at T0 4.932	RR at T0 0.102
	RR at T28 0.099	RR at T28 0.000	RR at T28 0.012
	0.56 % reduction	*100 % reduction*	*88 % reduction*
1000 mg/L	RR at T0 0.468	RR at T0 7.049	RR at T0 0.432
	RR at T28 0.483	RR at T28 4.344	RR at T28 0.054
	No % reduction	*38 % reduction*	*88 % reduction*
Bromhexine 0.7mg/L			RR at T0 17.826
			RR at T28 9.542
			46 % reduction

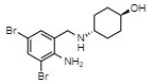
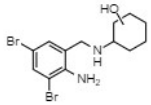
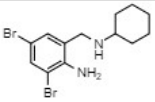
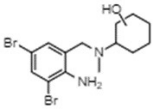
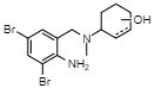
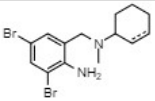
[b] The Relative Ratio (RR) is the ratio between the area counts of the compound and the area count of the internal standard (sulfadimethoxine-d6). The level of reduction at the end of the experiment is the ratio between the RR at T28 and the RR at T0 as a percentage.

Table S2. List of the main compounds studied in Mixture_A and Mixture_B

N°	Compounds	Structure/ elemental formula	Retention time (min)	Type of ionization	Calculated Mass $[M-X]^-$ or $[M+X]^+$ (m/z) (i)	Accurate Mass $[M-X]^-$ or $[M+X]^+$ (m/z) (ii)	Error (mDa) (a)	Score% (b)	Calculated mass-Fragment (m/z) (c)	Accurate mass-Fragment (m/z) (c)	Level of compound XXXdentification (d)	Products studied
1	Sucralose	 C12H19Cl3O8.	4,509	Pos	419,0038 $[M+Na]^+$	419,0045 $[M+Na]^+$	7	98.9	**238,9848***	**238,9846*** (CE30eV)	Level 1	Mixture_A
2	Maltitol	 C12H24O11	0,772	Pos	367,1211 $[M+Na]^+$	367,1218 $[M+Na]^+$	7	96.0	205,0683	**205,0685** (CE30eV)	Level 1	Mixture_A
3	Bromhexine	 C14H20Br2N2	9,655	Pos	377,0046 $[M+H+2]^+$	377,0048 $[M+H+2]^+$	2	98.2	263,8841* **114,1277**	263,8843* **114,1280** (CE30eV)	Level 1	Mixture _A
4	Sucrose	 C12H22O11	0,922	Neg	341,1089 $[M-H]^-$	341,1092 $[M-H]^-$	3	89.3	185.0415, **203.0518**	185,0415* **203,0518** (CE30eV)	Level 1	Mixture _B
5	Acteoside (syn. Verbascoside)	 C29H36O15	6,508	Neg	623,1981 $[M-H]^-$	623,1981 $[M-H]^-$	0	79.9	**161,0243** 461,1664*	**161,0243** 461,1658* (CE30eV)	Level 1	Mixture _B
6	Grindelic Acid	 C20H32O3	18,898	Neg	319,2279 $[M-H]^-$	319,2281 [M-H]-	2	83.3	205,1596*	**205,1596*** (CE30eV)	Level 1	Mixture _B

(a)Mass Difference between calculated mass or exact mass and the experimental value; (b) The reported value is the overall score to which contribute: the mass score, the isotope abundance score, the isotope spacing score and the retention time score; (c) Bold more abundant ions, asterisk qualifier ions. (d) Classification according to Sumner et al. [1]Level 1- validate identification with pure reference standard, Level 2- Putative identified compound MS/MS data match with the literature, Level 3- tentative structure identification match with the molecular formula; i) Mono isotopic mass of the most abundant ion of the isotopic cluster (ii) Accurate mass Value of the most abundant ion of the isotopic cluster

[1] Sumner LW, Amberg A, Barrett D, Beale MH, Beger R, Daykin CA, Fan TW, Fiehn O, Goodacre R, Griffin JL, Hankemeier T, Hardy N, Harnly J, Higashi R, Kopka J, Lane AN, Lindon JC, Marriott P, Nicholls AW, Reily MD, Thaden JJ, Viant MR. Proposed minimum reporting standards for chemical analysis Chemical Analysis Working Group (CAWG) Metabolomics Standards Initiative (MSI). Metabolomics 2007; 3: 211-221.

	Table S3. List of Bromhexine metabolites									
N°	**Compound Name**	**Retention time (min)**	**Putative Structure/ elemental formula**	**Type of ionization**	**Calculated Mass $[M-X]^-$ or $[M+X]^+$ m/z (ii)**	**Accurate Mass $[M-X]^-$ or $[M+X]^+$ (m/z) (iii)**	**Error (mDa) (a)**	**Calculated Mass m/z (c)**	**Accurate mass-Fragment m/z (c)**	**Level of compound identification (d)**
7	Ambroxol (E-4-HDMB)	6,146	 **C13H18Br2N2O**	Pos	378,9839 $[M+H+2]^+$	378,9836 $[M+H+2]^+$	-3	263,8841 116,1074	**263,8841*** 116,1069	Level 1
7°	[(2-amino-3,5 dibromobenzyl)-amino]-cyclohexanol (Other Ambroxol isomers with OH in different position on amino cyclohexane ring)	6,738; 7,094	 **C13H18Br2N2O**	Pos	378,9839 $[M+H+2]^+$	378,9836 378,9833 $[M+H+2]^+$	-3 -6	263,8841 116,1074	**263,8841*** 116,1069 **263,8842*** 116,1068 (CE30eV)	Level 2
8	(2-amino-3,5-dibromo-benzyl)-cyclohexyl-amine	9,633	 **C13H18Br2N2**	Pos	362,9889 $[M+H+2]^+$	362,9892 $[M+H+2]^+$	3	263,8841 100,1121	**263,8841*** 110,1119 (CE30eV)	Level 3
9	[(2-amino-3,5-dibromobenzyl)-(methyl)-amino]-cyclohexanol, (Different isomers with OH in different position on amino cyclohexane ring)	6,061; 6,924; 10,191	 **C14H20Br2N2O**	Pos	392,9995 $[M+H+2]^+$	392,9996 392,9998 393,0003 $[M+H+2]^+$	1 3 8	263,8841 130,1226	**263,88342*** 130,1226 **263,8839*** 130,1227 **263,8844*** 130,1228 (CE30eV)	Level 3
10	[(2-amino-3,5-dibromobenzyl)-(methyl)-amino]-cyclohexenol, (different isomers with OH in different position on the cyclohexane ring; a double bond in the cyclohexane ring in not defined position)	6,315; 6,738; 9,108	 **C14H18Br2N2O**	Pos	390,9839 $[M+H+2]^+$	390,9840 390,9843 390,9840 $[[M+H+2]^+$	1 4 1	263,8841 128,1070	**263,8847*** 128,1067 **263,8841*** 128,1068 **263,8839*** 128,1068 (CE30eV)	Level 3
11	2,4-dibromo-6-((cyclohex-2-en-1-yl-(methyl)-amino)-methyl)-aniline (double bond in the cyclohexane ring position not defined)	9,430	 **C14H18Br2N2**	Pos	374,9890 $[M+H+2]^+$	374,9890 $[M+H+2]^+$	0	263,8841 112,1121	**263,8839*** 112,1123 (CE30eV)	Level 3

iii) Normalized value obtained by the ratio of the area of the compound to the area of the internal standard.

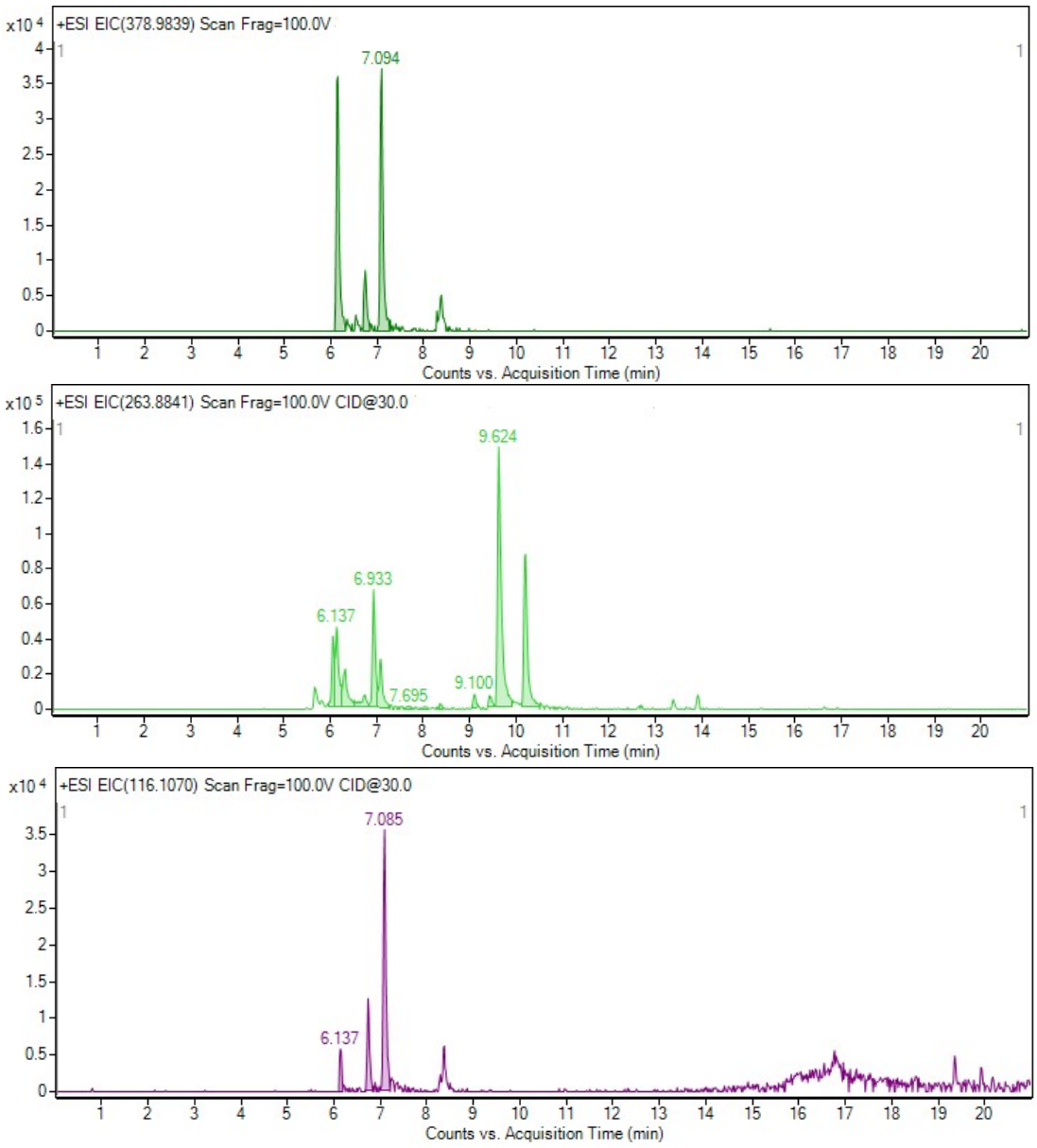


Figure S1 Mixture_A: Bromhexine metabolites at T28_ **2- and 3- and 4-[(2-Amino-3,5-dibromobenzyl)amino]cyclohexanol**_ EIC of the molecular ion at m/z 378.9839 (green line, ₍[M+H+2]⁺), and its fragments EICs at m/z 263.8841(30eV, light green line) and m/z 116.1070 (violet line). Different signals can be observed as consequence of microfauna hydroxylation on different position of amino-cyclohexane ring.
Ambroxol or **trans-4-[(2-Amino-3,5-dibromobenzyl)amino]cyclohexanol (7**) can be easily identified at **6.146** minutes by comparison with the retention time, accurate mass and accurate MS/MS fragments of the reference pure standard (see **Figure S1A**)

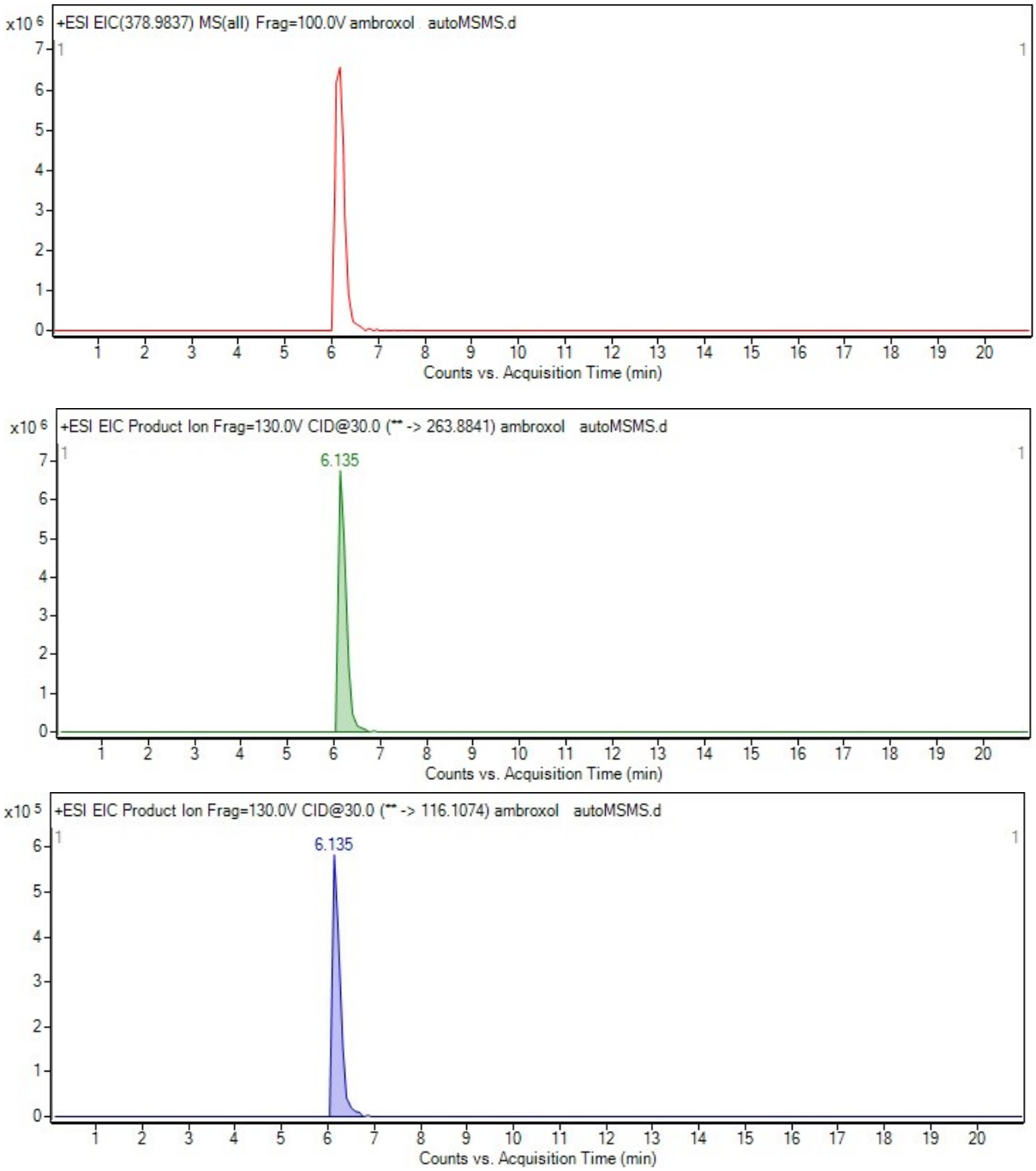


Figure S1A: trans-4-[(2-Amino-3,5-dibromobenzyl)amino]cyclohexanol, or **Ambroxol**, reference pure standard, EIC at m/z 378.9839 (red line), at m/z 263.8841(30eV, green line), and m/z 116.1070 (30eV, blue line).

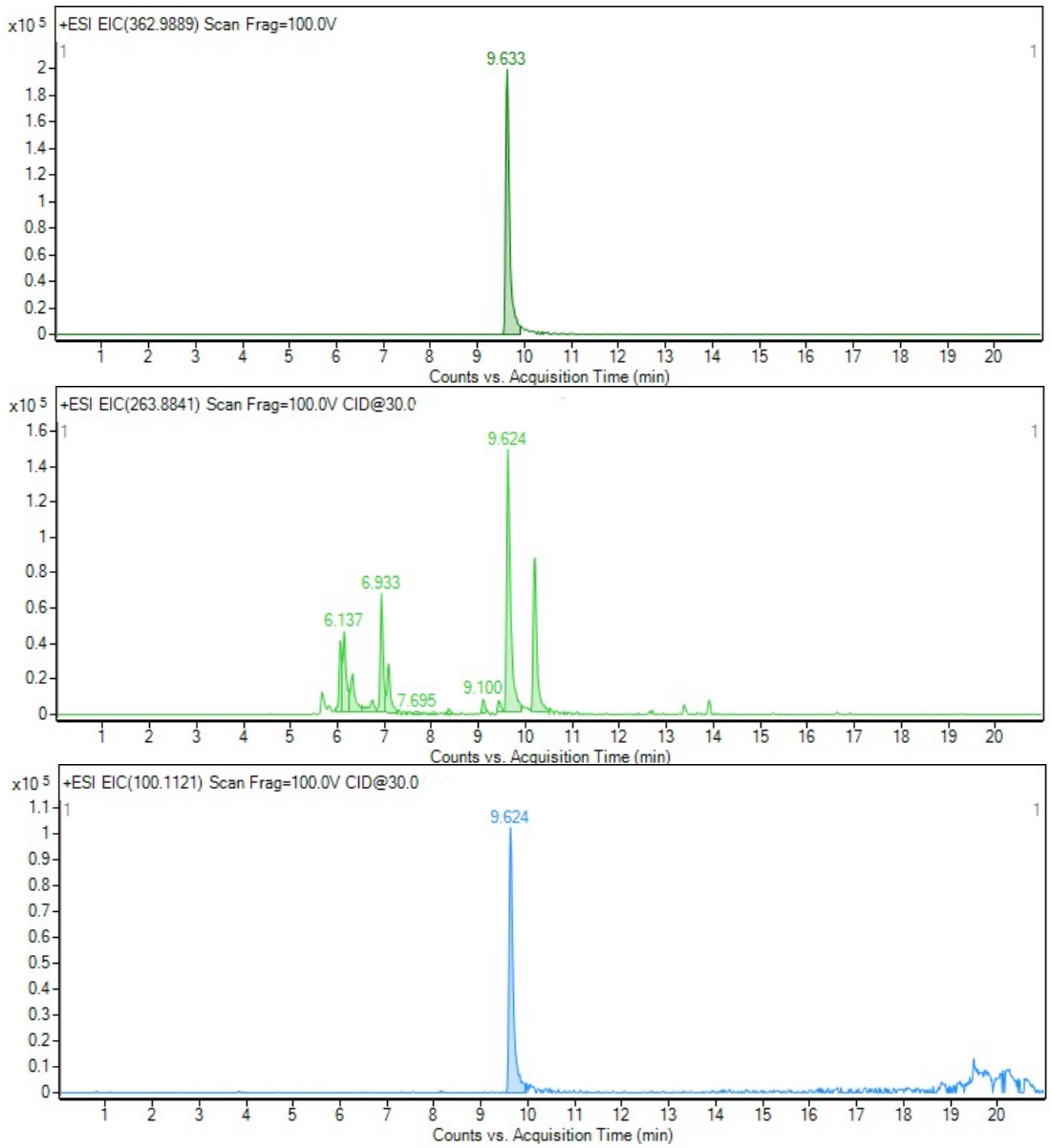


Figure S2 Mixture_A: Bromhexine metabolites at T28_**(2-amino-3,5-dibromo-benzyl)-cyclohexyl-amine, C13H18Br2N2** (**8**)_EIC at m/z 362,9889 (green line, $[M+H+2]^+$) and its fragments EICs at m/z 263.8841 (30eV, light green line) and 100.1121 (30eV, light blue line).

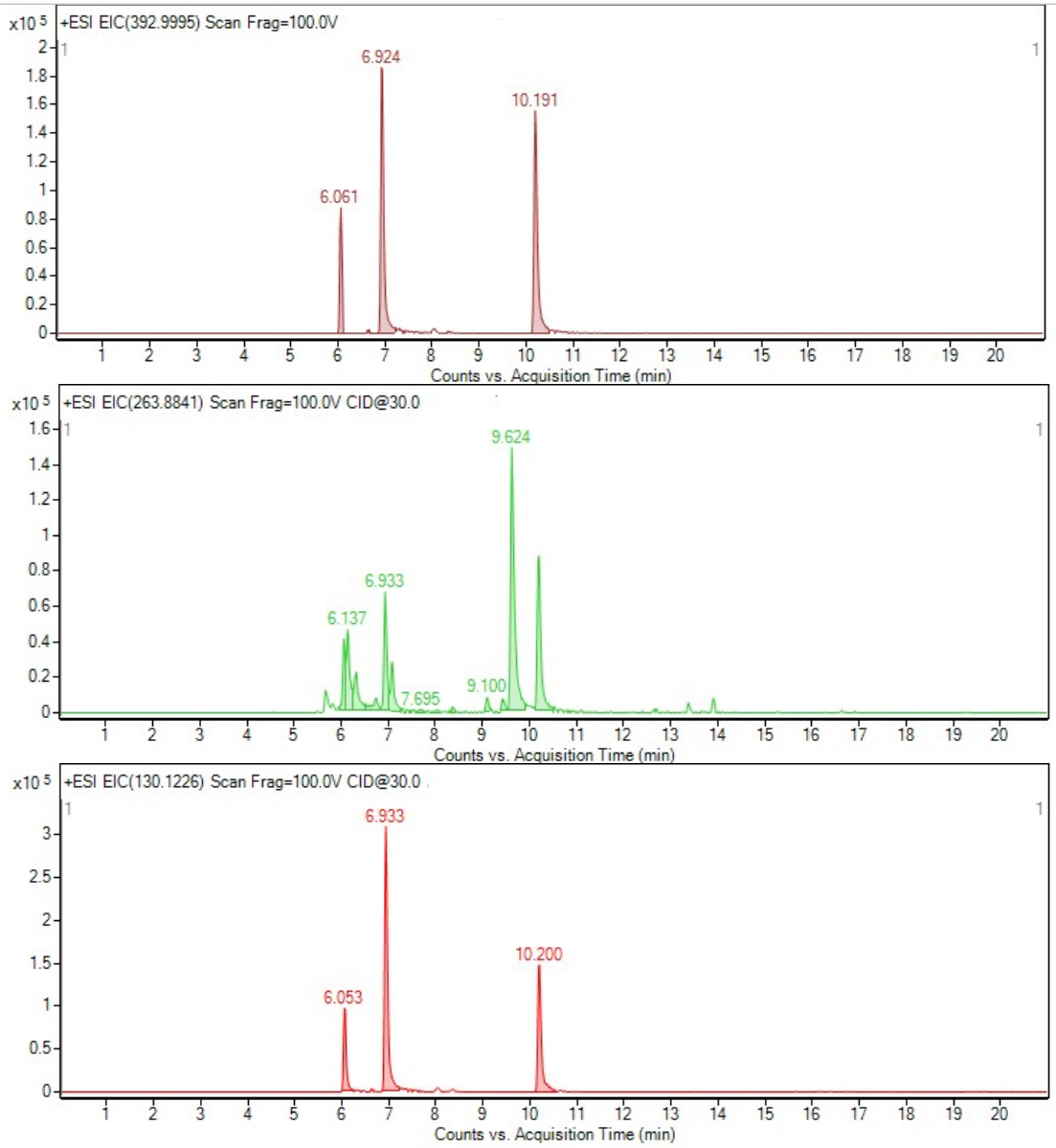


Figure S3 Mixture_A: Bromhexine metabolite at T28_ **2- and 3- and 4-[(2-amino-3,5-dibromobenzyl)-(methyl)-amino]-cyclohexanol** (**9**) EIC of the molecular ion at m/z 392.9995 (brown line, $[M+H+2]^+$), and its fragments EICs at m/z 263.8841(30eV, light green line) and m/z 130.1226 (30eV, red line). Different signals can be observed as consequence of microfauna hydroxylation on different position of amino-cyclohexane ring.

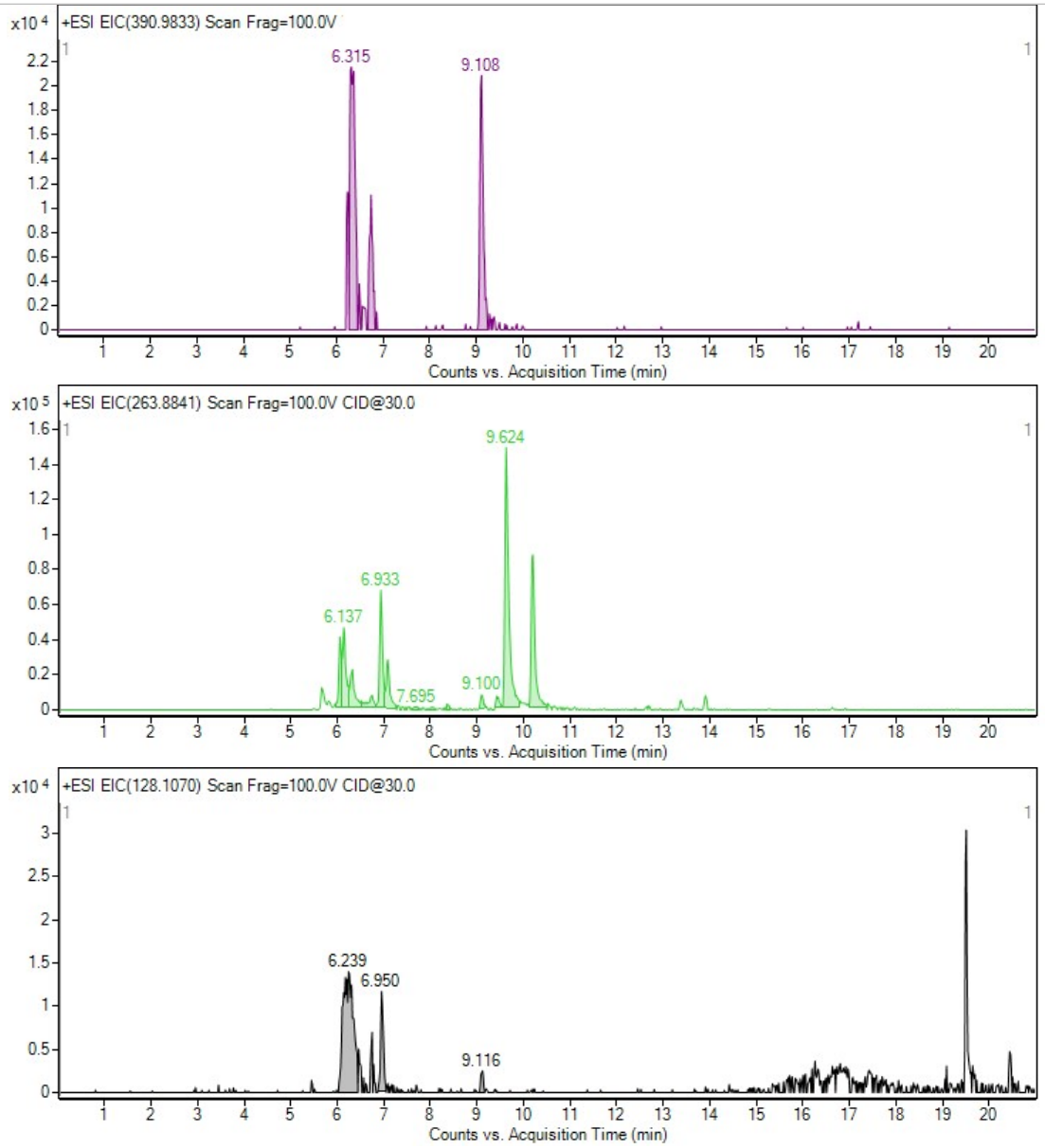


Figure S4 Mixture_A: Bromhexine metabolite at T28_ **2- and 3- and 4-[(2-amino-3,5-dibromobenzyl)-(methyl)-amino]-cyclohexenol (10)**_EIC of the molecular ion at m/z 390.9839 (violet line, $[M+H+2]^+$), and its fragments EICs at m/z 263.8841(30eV, light green line) and m/z 128.1070 (30eV, grey line). Different signals can be observed as consequence of microfauna hydroxylation and double bond formation on different position of amino-cyclohexane ring.

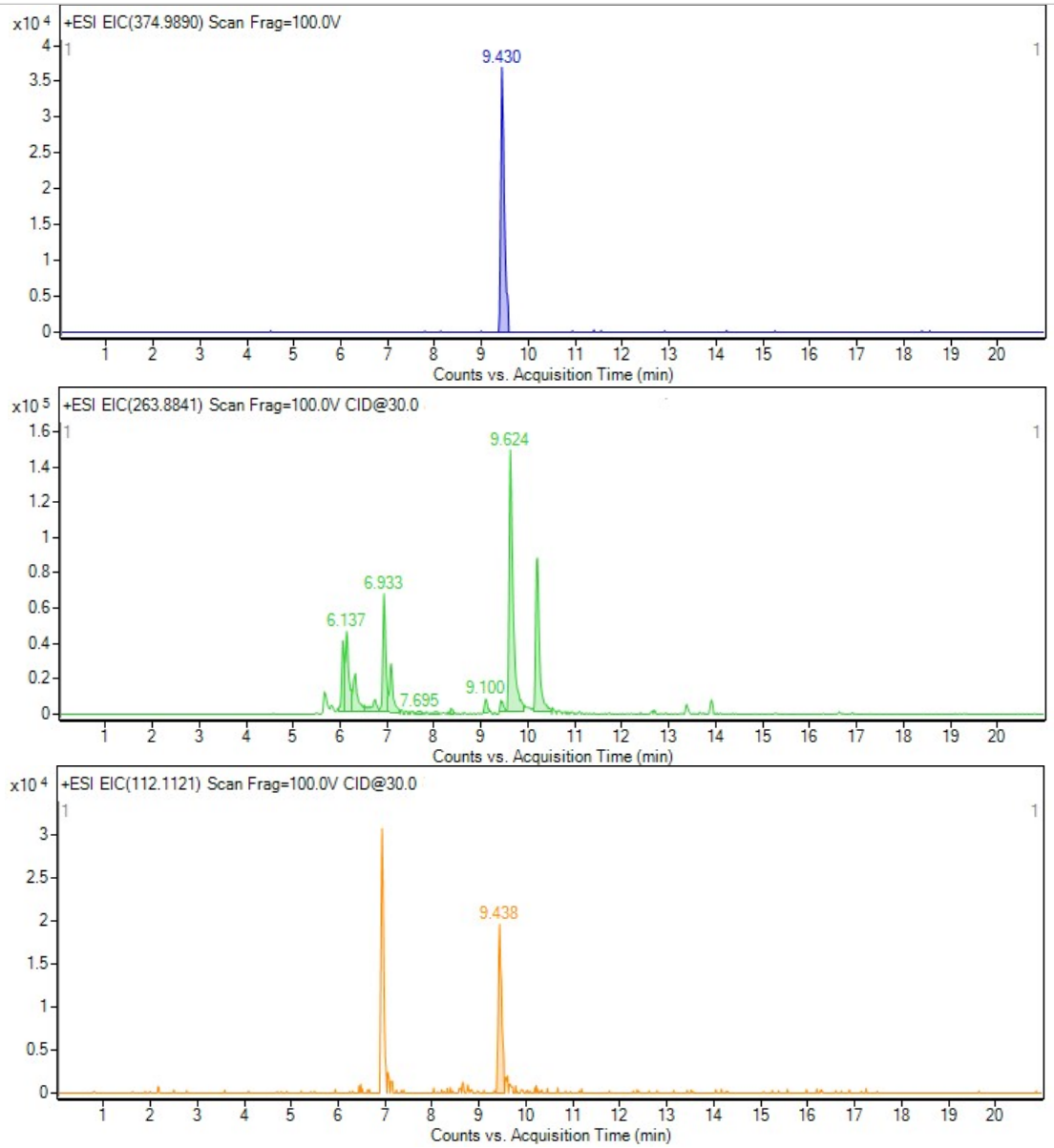


Figure S5 Mixture_A: Bromhexine metabolite at T28_**2,4-dibromo-6-((cyclohex-2-en-1-yl-(methyl)-amino)-methyl)-aniline (11)**_EIC of the molecular ion at m/z 374.9890 (blue line, $[M+H+2]^+$), and its fragments EICs at m/z 263.8841(30eV, light green line) and m/z 112.1121 (30eV, orange line). Double bond in the cyclohexane ring position was not defined. Different signals in the EIC chromatogram of the fragment ion at m/z 263.8841 can be observed as the fragment is common to other bromhexine metabolites. Potentially, several metabolites with a double bond in the amino-cyclohexane ring not well separated under the same chromatographic peak can be presents.

Table S4. Experimental parameters for the respirometric test

Sample concentration	Volume of mineral media	Volume of the vessel
50 mg/L	400 ml	1L
100 mg/L	400 ml	1L
1 g/L	100 ml	1L

Table S5. *UHPLC ESI-qToF Methods:* Elution gradient

Time (min)	A%	B%
0,0	99	1
1,0	99	1
2,0	75	25
10,0	50	50
11,0	50	50
15,0	25	75
17,0	15	85
19,0	1	99
19,5	1	99
21,0	99	1
24,0 (post run)	99	1

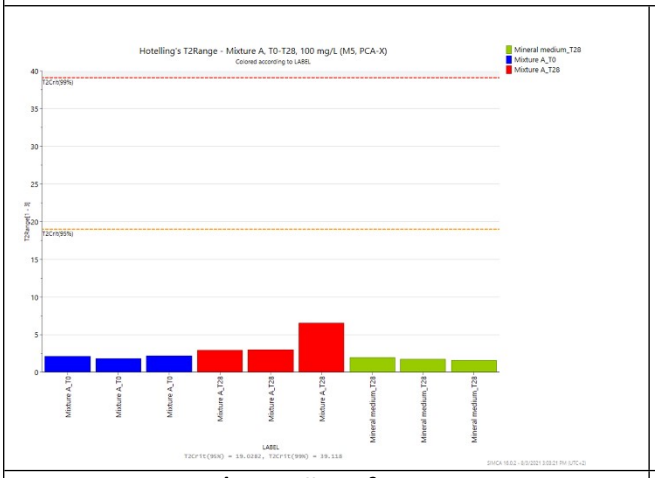
Table S6. *UHPLC ESI- qToF Methods:* MS parameters

Instrumental parameters	ESI-	ESI+
Gas temperature (°C)	325	325
Gas flow (L min-1)	11	11
Nebulizer (psig)	35	35
Sheat gas temperature (°C)	350	350
Sheat gas flow (L min-1)	12	12
Vcap	3500	3500
Nozzle voltage (V)	0	1000
Fragmentor	100	100
Skimmer	65	65
Octopole RF Peak	750	750

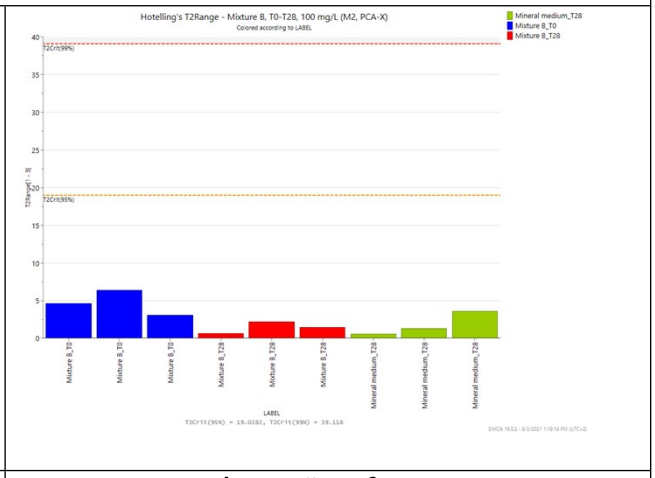
Table S7. Statistical Model performance evaluation by means of Q^2 and R^2X,

Sample	Q^2	R^2X
Mixture_A 100 mg/L	0,849	0,937
Mixture_B 100 mg/L	0,818	0,953
Mixture_A 1000 mg/L	0,669	0,937
Mixture_B 1000 mg/L	0,864	0,989

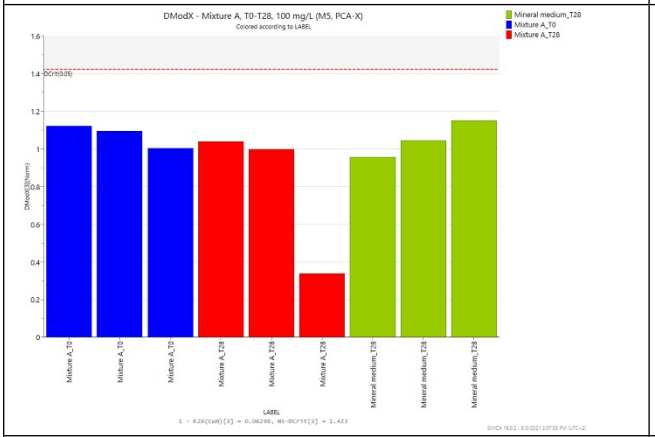
Table S8: Hotelling T^2 and DModX



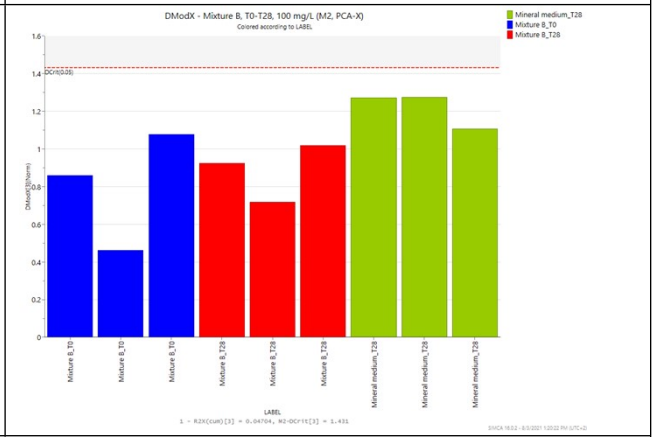
Mixture_A 100 mg/L_Hotelling T^2



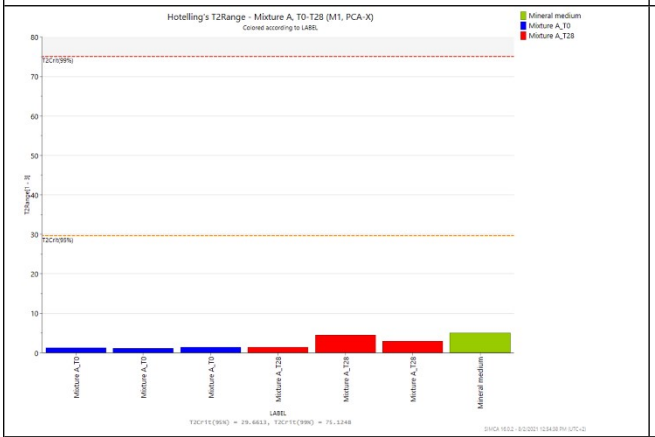
Mixture_B 100 mg/L_Hotelling T^2



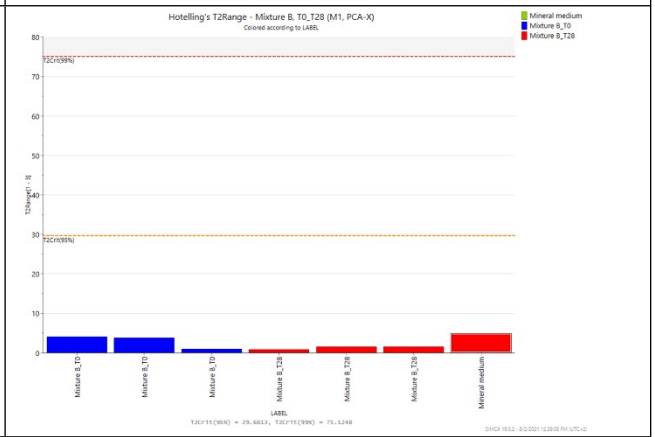
Mixture_A 100 mg/L _DMod X



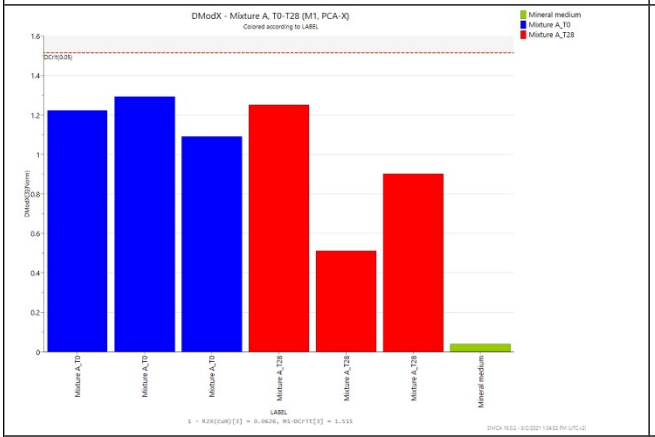
Mixture_B 100 mg/L_ DMod X



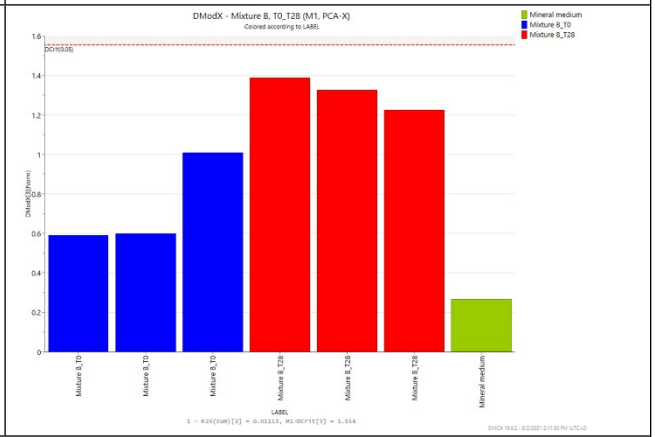
Mixture_A 1000 mg/L_Hotelling T^2



Mixture_B 1000 mg/L_Hotelling T^2



Mixture_A 1000 mg/L _DMod X



Mixture_B 1000 mg/L_ DMod X

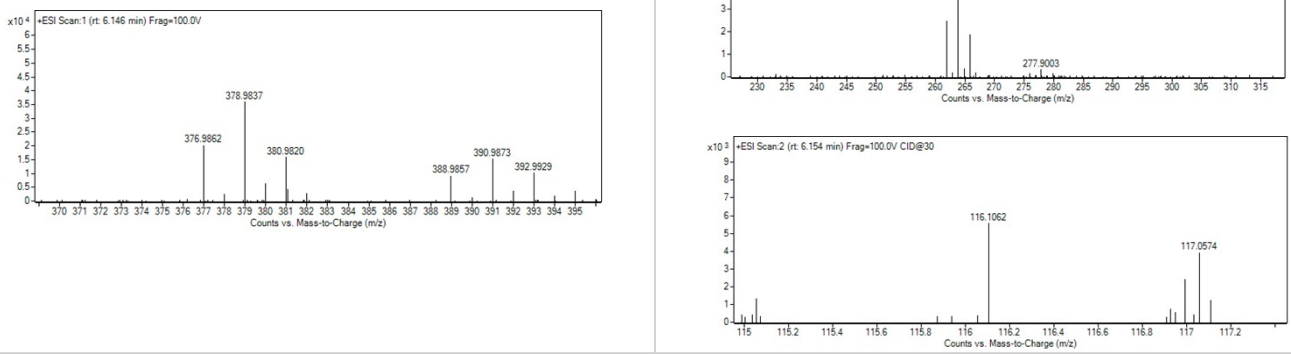
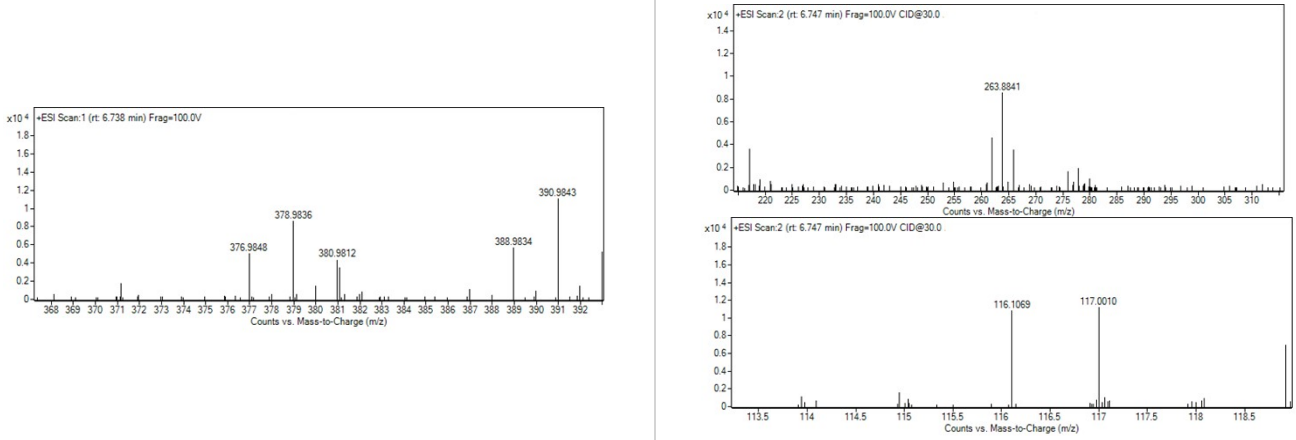
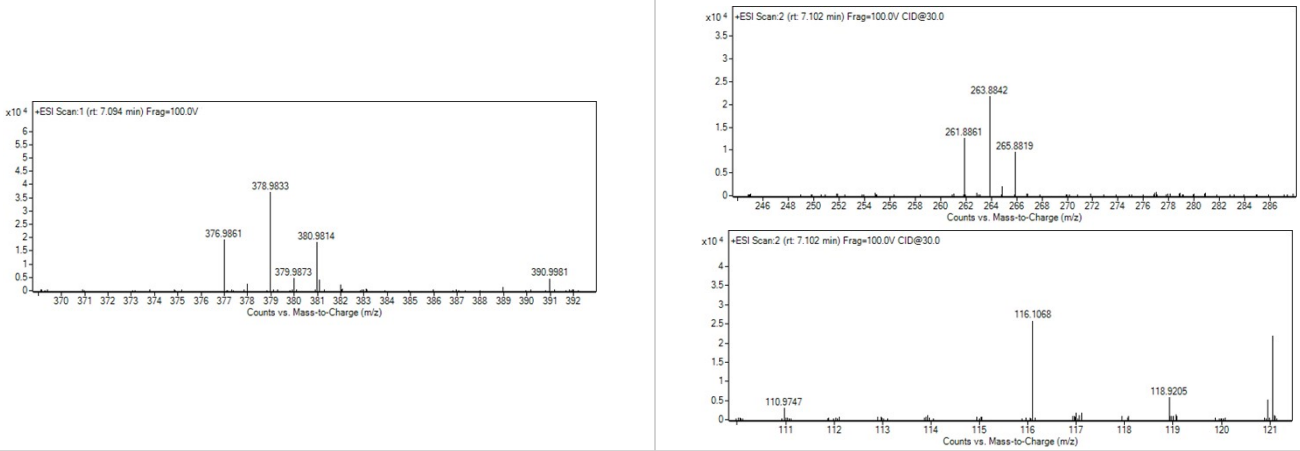


Figure S6 Spectrum of Structure 7_ Ambroxol (E-4-HDMB). Accurate mass spectrum at m/z 378,9837 and 6.146 min (right) then the corresponding MS/MS fragmentation pattern at 30eV (left) with ions at m/z 263,8842 and 116,1062

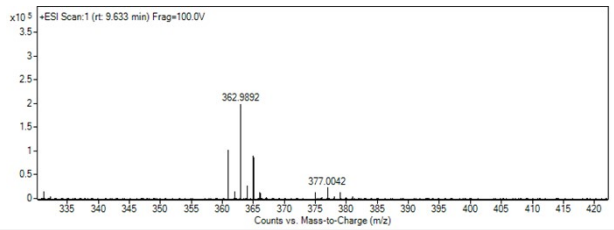


i) Metabolite at 6.738 min, Accurate mass spectrum at m/z 378,9836 (right) then the corresponding MS/MS fragmentation pattern at 30eV (left) with ions at m/z 263,8841 and 116,1069



ii) Metabolite at 7.094 min, Accurate mass spectrum at m/z 378,9833 (right) then the corresponding MS/MS fragmentation pattern at 30eV (left) with ions at m/z 263,8842 and 116,1068

Figure S7 Spectrum of Structures 7a_ [(2-amino-3,5 dibromobenzyl)-amino]- cyclohexanol
(Other Ambroxol isomers with OH in different position on amino cyclohexane ring).



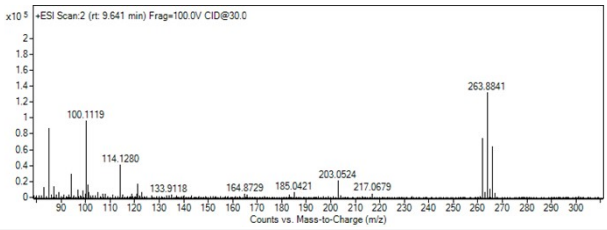
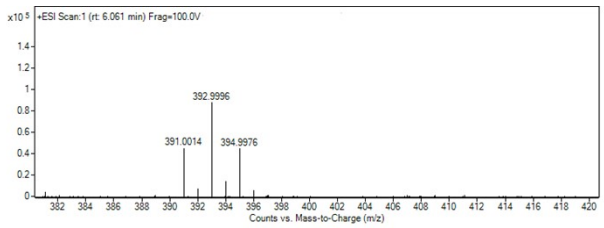
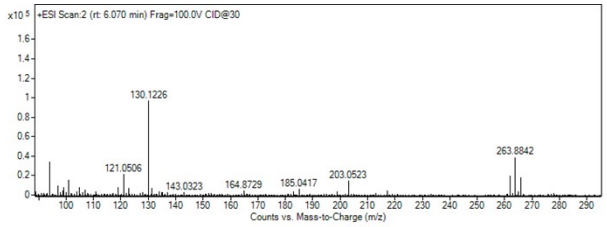
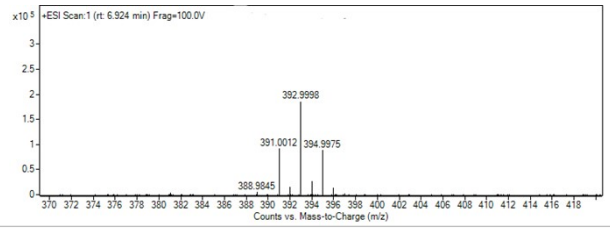


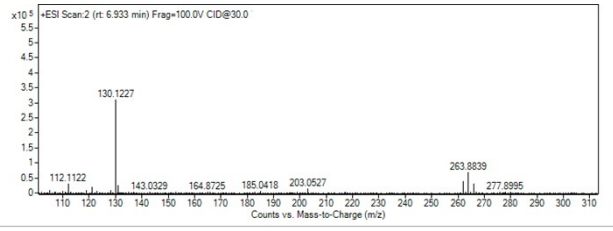
Figure S8 Spectrum of Structure 8_(2-amino-3,5-dibromo-benzyl)-cyclohexyl-amine. Accurate mass spectrum at m/z 362,9992 and 9.633 min (right) then the corresponding MS/MS fragmentation pattern at 30eV (left) with ions at m/z 263,8841 and 110,1119



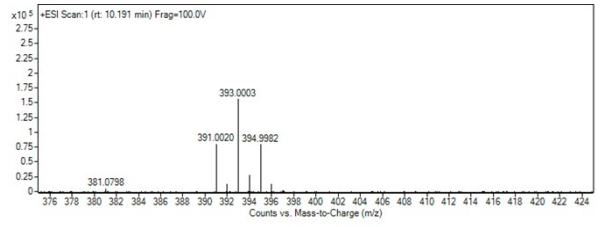


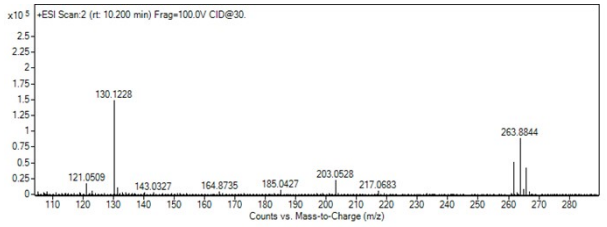
i) Metabolite at 6.061 min. Accurate mass spectrum at m/z 392,9996 (right), then the corresponding MS/MS fragmentation pattern at 30eV (left) with ions at m/z 263,8842 and 130,1226





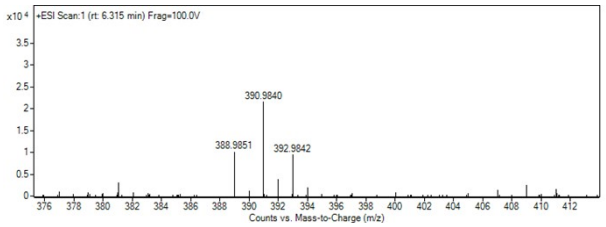
ii) Metabolite at 6.924 min. Accurate mass spectrum at m/z 392,9998 (right) then the corresponding MS/MS fragmentation pattern at 30eV (left) with ions at m/z 263,8839 and 130,1227

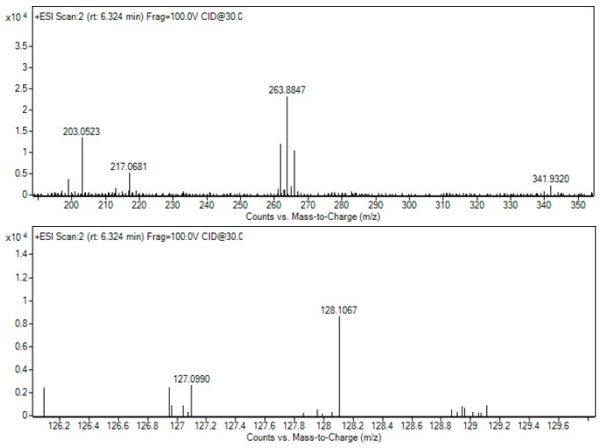




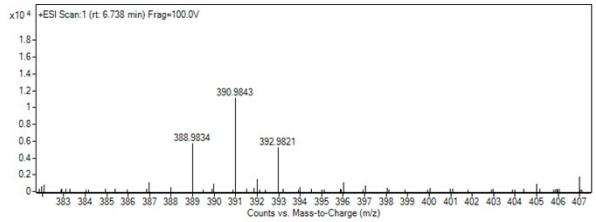
iii) Metabolite at 10.191 min. Accurate mass spectrum at m/z 393,0003 (right) then the corresponding MS/MS fragmentation pattern at 30eV (left) with ions at m/z 263,8844 and 130,1228

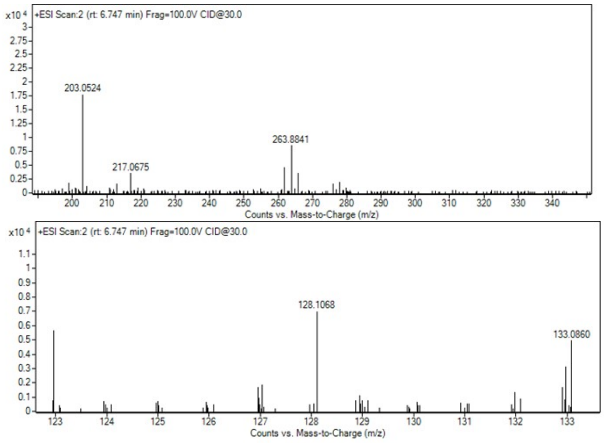
Figure S9 Spectrum of Structures 9_[(2-amino-3,5-dibromobenzyl)-(methyl)-amino]-cyclohexanol, (Different isomers with OH in different position on amino cyclohexane ring).



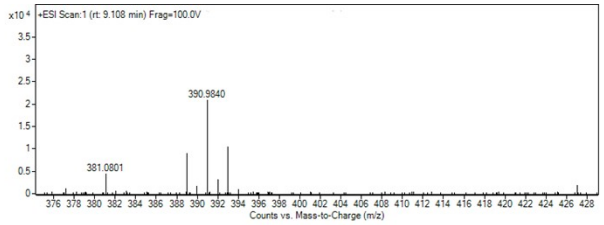


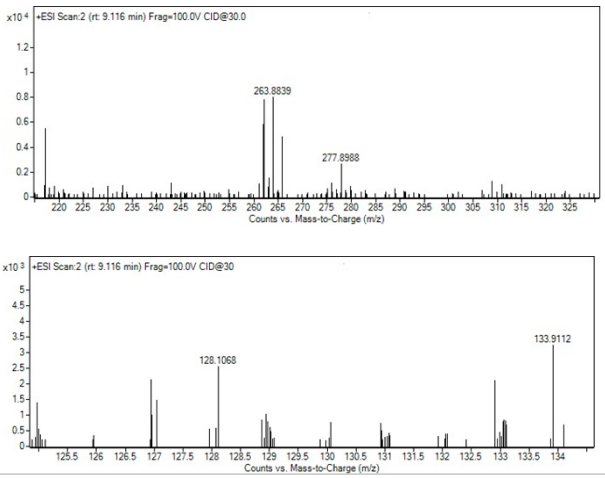
i) Metabolite at 6.315 min. Accurate mass spectrum at m/z 390,9840 (right) then the corresponding MS/MS fragmentation pattern at 30eV (left) with ions at m/z 263,8847 and 128,1067





ii) Metabolite at 6.738 min. Accurate mass spectrum at m/z 390,9843 (right) then the corresponding MS/MS fragmentation pattern at 30eV (left) with ions at m/z 263,8841 and 128,1068





iii) Metabolite at 9.108 min. Accurate mass spectrum at m/z 390,9840 (right) then the corresponding MS/MS fragmentation pattern at 30eV (left) with ions at m/z 263,8839 and 128,1068

Figure S10 Spectrum of Structure 10_[(2-amino-3,5-dibromobenzyl)-(methyl)-amino]-cyclohexenol, (different isomers with OH in ortho, meta or para position and a double bond in the cyclohexane ring in position not defined).

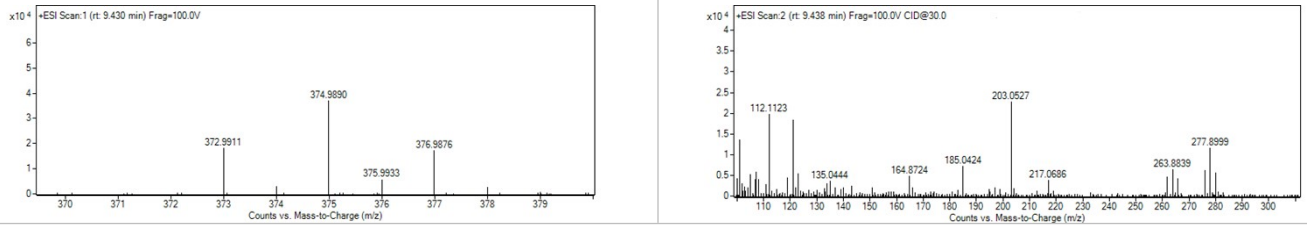


Figure S11 Spectrum of Structure 11_2,4-dibromo-6-((cyclohex-2-en-1-yl-(methyl)-amino)-methyl)-aniline (double bond in the cyclohexane ring position not defined). Accurate mass spectrum at m/z 374,9890 and 9.430 min (right) then the corresponding MS/MS fragmentation pattern at 30eV (left) with ions at m/z 263,8839 and 112,1123