Comptes Rendus
Chimie
Supplementary material to the article available at:
https://doi.org/10.5802/crchim.250

Supplementary material: Effects of *Lagarosiphon major* extracts on the metabolome and photosynthesis of *Microcystis aeruginosa*

Juliette Tison-Rosebery [*, a], Sylvia Moreira [a], Mélissa Eon [a], Lucie Campillo [a], Samuel Maubert [a], Carmen Malepeyre [b], Julien Célerier [b], Vincent Sol [c], Jacky Vedrenne [a], Vincent Bertrin [a] and Nicolas Creusot [a, d]

[a] INRAE, UR EABX, F-33612 Cestas, France

[b] CVA, F-19100 Brive-la-Gaillarde, France

[c] Université de Limoges, LABCIS, F-87025 Limoges, France

[d] Plateforme Bordeaux Métabolome, France

E-mails: juliette.rosebery@inrae.fr (J. Tison-Rosebery), sylvia.moreira@inrae.fr (S. Moreira), melissa.eon@inrae.fr (M. Eon), lucie.c.campillo@gmail.com (L. Campillo), Samuel-Maubert@outlook.fr (S. Maubert), c.malepeyre@cva-ctt19.fr (C. Malepeyre), j.celerier@cva-ctt19.fr (J. Célerier), vincent.sol@unilim.fr (V. Sol), jacky.vedrenne@inrae.fr (J. Vedrenne), vincent.bertrin@inrae.fr (V. Bertrin), nicolas.creusot@inrae.fr (N. Creusot)

1. Material and methods

1.1. *Sampling and samples preparation*

Briefly, 10 mg of freeze-dried were added to a 2 mL microtube containing 150 mg of 0.5 mm silica beads. After the addition of 1 mL MeOH/MTBE (3:1; v:v) and 650 µL of UPW:MeOH (3:1, v/v), the microtubes were homogenized with a Fast Prep 5G (3 homogenization cycles at 15 s at 6 m/s) and then centrifugated at 4 °C at 12000 RPM during 5 min in order to separate the two phases. Thus, 600 µL of the lipophilic phase (upper phase) and 500 µL of the hydrophilic phases were collected in amber glass vials. Then a second round of extraction was performed according the same conditions. In total, 1.1 mL of lipophilic and 1.3 mL of hydrophilic were collected. In parallel of the sample, solvent alone (procedural blank) were also extracted. Following the extraction, 500 µL of both the hydrophilic and lipophilic stock solutions were evaporated using rotary evaporator (Speed-Vac, ThermoScientific) and gentle nitrogen stream respectively. They were then resuspended in adequate solvent: 100 µL of ACN/UW (50:50, v:v) for hydrophilic extract and 100 µL of ACN/ISO (50:50, v:v) for lipophilic extract. For both extracts, a pool of 5 µL of all the extracts were prepared as pool QC for further intensity and retention time drift correction (see part X).

In parallel to the cyanobacteria extracts, samples from LS extracts aging experiment were directly freeze-dried and resuspended in ACN/H20 in order to get an equivalent concentration to the exposure experiment.

* Corresponding author.

1.2. *UPLC-TOF HRMS analyses*

1.2.1. *Chromatographic separation*

For the chromatography, 2 different gradients were used:

For the **hydrophilic gradient**, the mobile phases consisted of ultrapure water supplemented with 0.1% formic acid (A), acetonitrile supplemented with 0.1% formic acid (B), and a mixture of acetonitrile and isopropanol (50:50, v:v) (Table S1a).

Supplementary Table S1. Detailed description of the gradient programs used in the chromatographic method for the analysis of the hydrophilic (a) and the lipophilic fraction(b), respectively

(a) Hydrophilic gradient (A: UW with 0.1% formic acid, B: ACN and 0.1% formic acid; C: ACN/ISO, 50:50, v:v)

Time (min)	Flow rate (mL/min)	A (%)	B (%)	C (%)
0.00	0.4	99	1	0
4.00	0.4	99	1	0
16.00	0.4	1	99	0
20.00	0.4	1	99	0
20.01	0.2	1	99	0
22.00	0.2	0	0	100
23.00	0.2	0	0	100
24.00	0.2	0	100	0
25.00	0.3	0	100	0
26.00	0.3	99	1	0
27.00	0.4	99	1	0
30.00	0.4	99	1	0

(b) Lipophilic gradient (A: ACN/UW with 0.1 formic acid; B ACN/ISOP with 0.1 formic acid)

Time (min)	Flow rate (mL/min)	A (%)	B (%)
0.00	0.3	70	30
5.0	0.3	55	45
14.0	0.3	30	70
21.0	0.3	1.0	99
24.0	0.3	1.0	99
24.10	0.3	70.0	30
30.00	0.3	70.0	30

1.2.2. *HRMS acquisition*

UPLC-TOF. Details on MSe acquisition parameters are provided in Table S2.

Supplementary Table S2. Sample and lockspray sources parameters

		Parameters	ESI+	ESI−
Sample source	Source	Capillary (kV)	0.7 kV	0.5 kV
		Sampling cone	30 V	30 V
		Source offset	80 V	80 V
	Temperature	Source (°C)	100 °C	100 °C
		Desolvation (°C)	550 °C	550 °C
	Gas flow	Cone gas (L/h)	50 L/h	50 L/h
		Desolvation gaz (L/h)	1000 L/h	1000 L/h
Lockspray source		Lockspray capill	3 kV	2.1 kV
		Collision	20 V	29 V

UPLC-QExactive+. Since the match between MSe mass spectra and in house library was quite low, additional injections of sample fractions were performed on UPLC-Qexactive+ instrument in order to get actual MS2 spectra. Details on source parameters are provided in Table S3.

Supplementary Table S3. Orbitrap-Qexactive parameters (DDA top5 mode)

Parameters	ESI+		ESI−	
	Full scan	MS/MS	Full scan	MS/MS
Scan range	75–1500	-	75–1500	-
Resolultion (FWHM)	70,000	17,500	70,000	17,500
Automated gain control (AGC)	500,000	500,000	500,000	500,000
Spray voltage (kV)	4	4	4	4
Sheath gas flow rate (µA)	40	40	40	40
Capillary temperature (°C)	350	350	350	350
NCE	-	50	-	50
Loop count	-	2	-	2

1.2.3. *Data processing (preprocessing and filtration)*

At a first step, all HRMS data acquired with the UPLC-TOF were processed by using W4M (Giacomoni *et al.* 2017), as previously reported in Creusot *et al.* 2021 with small modifications. Preprocessing step and parameters step are detailed below.

W4M. The FUNC003 (LockMass signal) was suppressed from the raw data prior to data conversion into mzML files by using MS-Convert (ProteoWizard) based on CWT algorithm in Peak-Picking filter. mzML files were pre-processed by using W4M (https://workflow4metabolomics.usegalaxy.fr/) based on XCMS scripts as described in Table S4.

Supplementary Table S4. W4M pre-processing parameters

	method	centWave
XcmsSet	ppm	15
	peakwidth	c(5, 25)
	snthresh	10
	mzdiff	0.01
	integrate	2
	prefilter	c(3, 1000)
	noise	0
	fitgauss	FALSE
	scanrange	numeric()
	sleep	0
	verbose.column	FALSE

	method	density
Group (2 times)	mzwid	0.1 then 0.05
	minfrac	0.5
	bw	5 then 2
	ignore sample	
	class	
	minsamp	1
	max	50
	sleep	0

	method	peakgroups
Retcor	smooth	c("loess")
	extra	1
	missing	0.9
	family	c("gaussian", "symmetric")
	span	0.2
	col	NULL
	plottype	c("none", "deviation", "mdevden")
	ty	NULL

fillPeaks	method	chrom
CAMERA annotate	mzabs	0.015
	ppm	5
	calcCaS	FALSE
	calcCiS	TRUE
	calcIso	FALSE
	cor_eic_th	0.75
	fc_th	NULL
	graphMetod	"hcs"
	intval	"into"
	max_peaks	100
	maxcharge	3
	maxiso	4
	minfrac	0.5
	multiplier	3
	nSlaves	1
	perfwhm	0.6
	polarity	"positive" / "negative"
	psg_list	NULL
	pval	0.05
	pval_th	NULL
	quick	FALSE
	rules	NULL
	sample	NA
	sigma	6

The obtained peak list were further filtrated for missing signal (NA > 75%), corrected for missing values (NA replaced by the minimal value of the entire dataset divided per 5), corrected for signal drift using all_loess_function based on QC samples. Features with CV above 30% between QC samples and with an intensity in the sample lower than in QC samples were removed from the dataset. The features were then filtrated against the procedural blank signal and injection blanks (i.e. only features with Sample/Blanks > 10 were conserved). The intensity of the features was then normalized by using the cyanobacteria dry weigh. The peak lists were then filtrated against the correlated signals at similar retention times by using between-table correlation and analytical correlation filtration functions. Finally, for hydrophilic datasets of the endometabolome, only the features not found in the macrophyte extract were kept. The processing allowed to get four datasets according to the fraction (i.e. hydrophilic vs lipophilic) and ionization mode (i.e. ESI+ vs ESI−) that were used to implement chemometrics analyses.

DROMICs. DROMICS tool (Shiny version, https://lbbe.univ-lyon1.fr/fr/dromics, Larras *et al.* 2018) was used in order to prioritize the metabolites annotation towards features that follow a quadratic trend (increase, decrease, U-shape, Bell) with increasing concentration of the macrophyte extract, according to five regression models (linear, hill, exponential, gauss-probit, log-Gauss-probit). This tool allows to encompass both monophasic (increase, decrease) and biphasic (U-shape, Bell) trends of each feature and further determination of benchmark dose (BMD) summarized as empirical cumulative distribution function (ECDF).

Step 1: the data were normalized (median), transformed (cube root) and scaled (Pareto).

Step 2: the relevant features (i.e. those displaying a significant change along the concentration gradient) were identified using a quadratic trend test with a False Discovery Rate (Benjamin-Hochberg test) of 0.001 because of the high number of metabolites, as suggested by Larras *et al.* (2018).

Step 3: concentration response modelling was performed for each of the selected features based on nominal concentrations. All these curves were manually curated to avoid further miscalculation and false modelling. In particular for U- and bell-shaped curves, the features for which the modelled curves did not fit the experimental values were removed from the dataset before reimplementation of steps 1–3.

Step 4: the model with the best fit was used to derive a benchmark dose (BMD-$_{1SD}$) for each feature, as recommended by EFSA (Committee 2017). The BMD_{1SD} values are the concentration corresponding to a Benchmark Response (BMR-zSD) defined as follows: BMR-zSD = $y_0 + / - z * \text{SD}$, where y_0 is the mean control response, and SD is the residual standard deviation of the considered concentration-response model and z is the SD factor (z fixed at 1 by default). These BMD values give an indication of the concentration leading to a significant level of change compared to the control (non-exposed in case of concentration-response).

MS-DIAL (v4.9.221218).

Supplementary Table S5. MS-Dial parameters

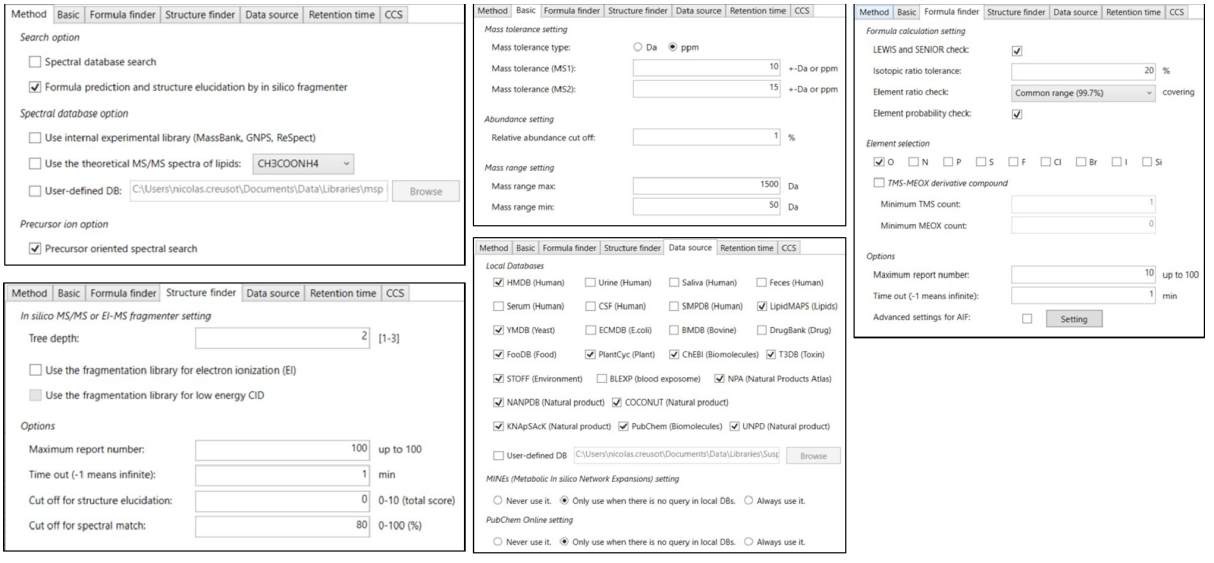
	TOF	Qex
Data collection	MS1: 0.015 MS2: 0.05 *Advanced* Isotope: Max: 2 Cl&Br: checked	MS1: 0.01 MS2: 0.05 *Advanced* Isotope: Max: 2 Cl&Br: checked
Peak detection	Peak heigt: 5000 Mass slice width: 0.05 *Advanced* Linear weighted moving average Smoothing: 3 Minimum peak width: 5	Peak heigt: 25000 Mass slice width: 0.1 *Advanced* Linear weighted moving average Smoothing: 3 Minimum peak width: 5
MS2Dec	Sigma 0.5 MS/MS 10 *Advanced* Exclude after: checked Keep the isotopic: 3 Da Keep the isotopic ions w/o: checked	Sigma 0.5 MS/MS 10 *Advanced* Exclude after: checked Keep the isotopic: 3 Da Keep the isotopic ions w/o: checked
Identification	MSP file Rt: 100 min MS1 0.01 Da MS2 0.05 Da Cut off: 80% *Advanced* Rt: 2 min Accurate mass: 0.015 Score cut off: 85% Relative abundance: 0%	MSP file Rt: 100 min MS1 0.01 Da MS2 0.05 Da Cut off: 80% *Advanced* Rt: 2 min Accurate mass: 0.015 Score cut off: 85% Relative abundance: 0%

(continued on next page)

Supplementary Table S5. (continued)

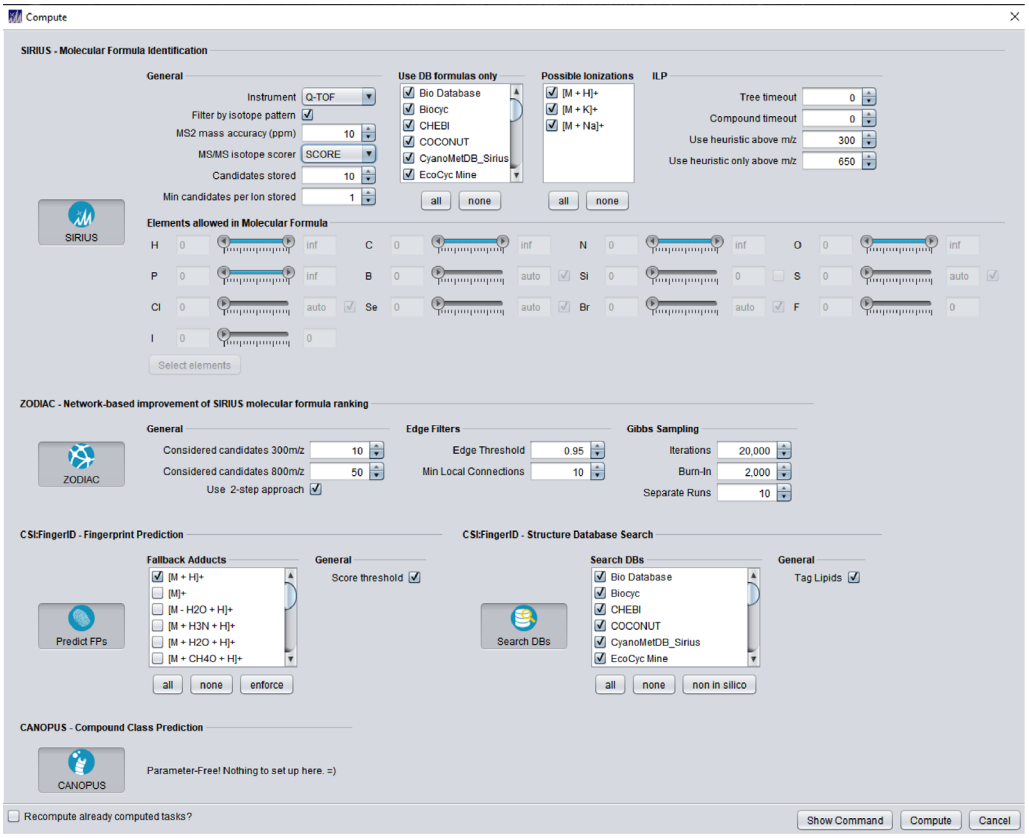
	TOF	Qex
Adduct	[M+H]+, [M+Na+], [M+K]+, [M+Li]+, [M+ACN+H]+, [M+H–H_20]+, [M+H–$_2H_20$]+, [M+ACN+Na]+, [2M+H]+, [2M+Na]+, [2M+K]+, [2M+ACN+H]+, [2M+CAN+Na]+	[M+H]+, [M+Na+], [M+K]+, [M+Li]+, [M+ACN+H]+, [M+H–H_20]+, [M+H–$_2H_20$]+, [M+ACN+Na]+, [2M+H]+, [2M+Na]+, [2M+K]+, [2M+ACN+H]+, [2M+CAN+Na]+
Alignment	Rt: 0.1 mn MS1: 0.025 *Advanced:* Rt factor 0.1 MS1 factor 0.5 Peak 0% N%: 0% Remove feature: checked Sample max / blank: 10 fold change Keep reference matched: checked Keep suggested (w/o MS): checked Keep removable features: checked Gap filling: checked	Rt: 0.1 mn MS1: 0.025 *Advanced:* Rt factor 0.1 MS1 factor 0.5 Peak 0% N%: 0% Remove feature: checked Sample max / blank: 10 fold change Keep reference matched: checked Keep suggested (w/o MS): checked Keep removable features: checked Gap filling: checked

MS-Finder (v3.52).



Supplementary Figure S1. MS-Finder parameters.

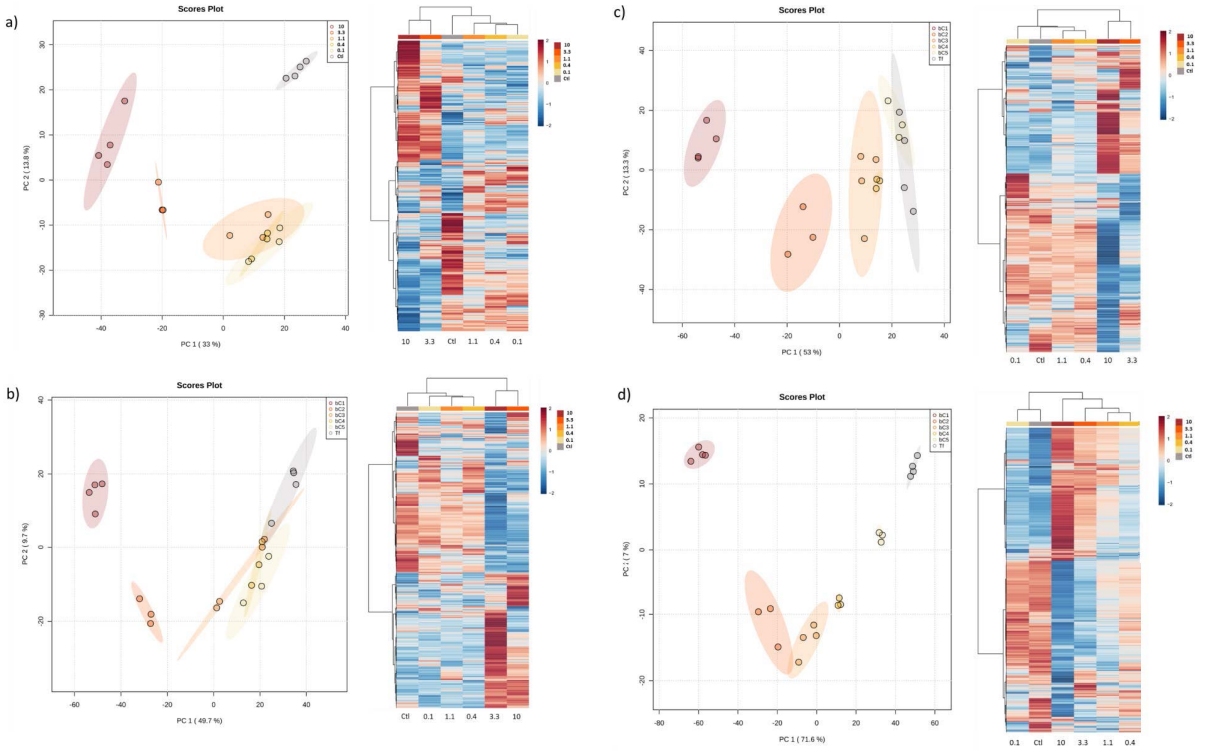
SIRIUS (v5.6.3).



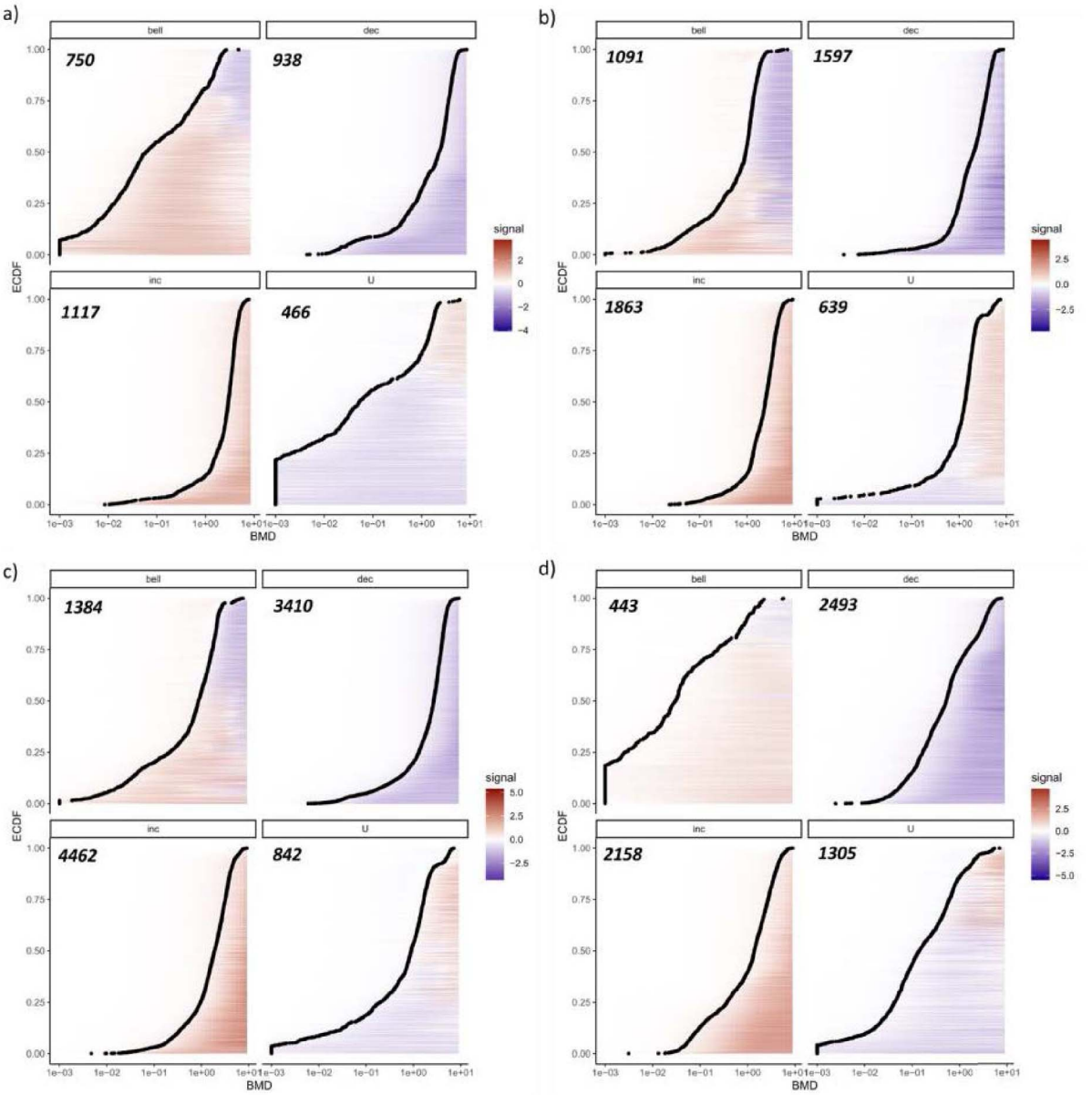
Supplementary Figure S2. SIRIUS parameters.

2. Results

2.1. *Metabolomic response of Microcystis aeruginosa exposed to Lagarosiphon major extracts*



Supplementary Figure S3. Metabolomics fingerprint summarized as PCA score plot and HCA heatmap of HydroPOS (a), HydroNeg (b), LipoPOS (c) and LipoNEG (d).

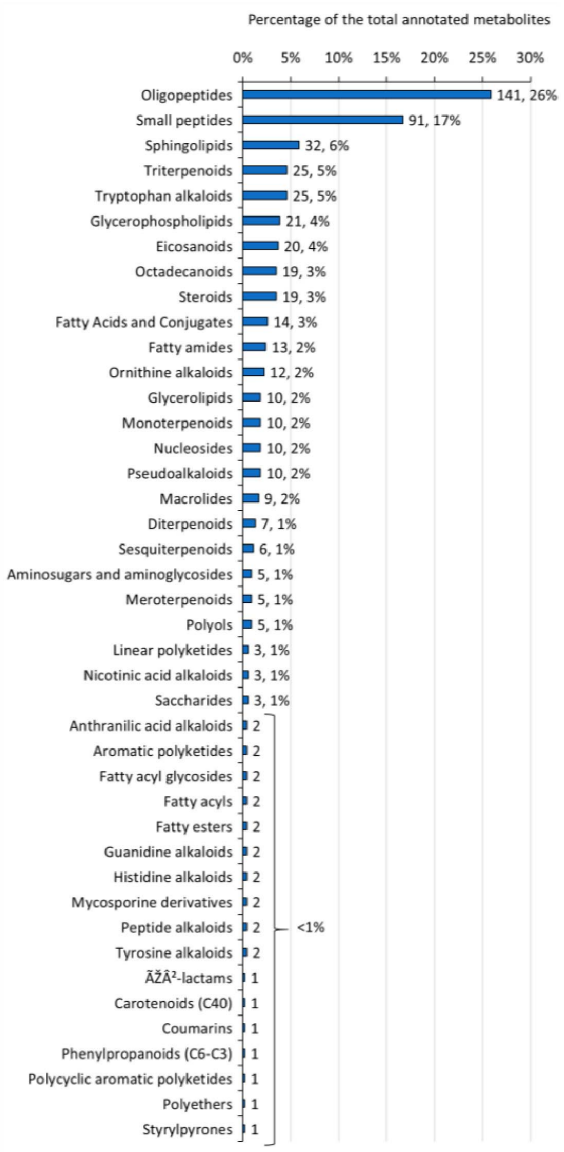


Supplementary Figure S4. ECDF trends (bell, dec, inc, U) of hydrophilic (a, ESI+; b, ESI−) and lipophilic (c, ESI+; d, ESI−) fractions of the metabolome.

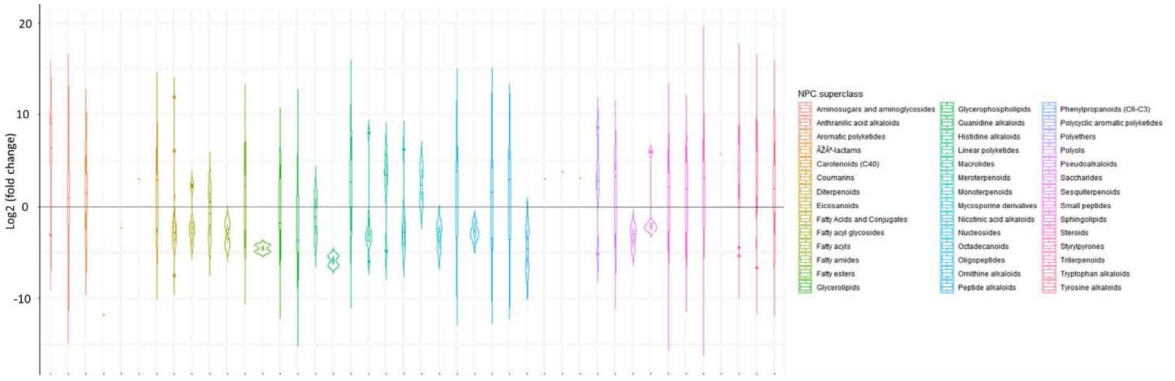
Supplementary Table S6. List of annotated metabolites, pathways and classes in the endometabolome (hydrophilic phase) (Tabular file)

Supplementary Table S7. List of annotated metabolites, pathways and classes in the endometabolome (lipophilic phase) (Tabular file)

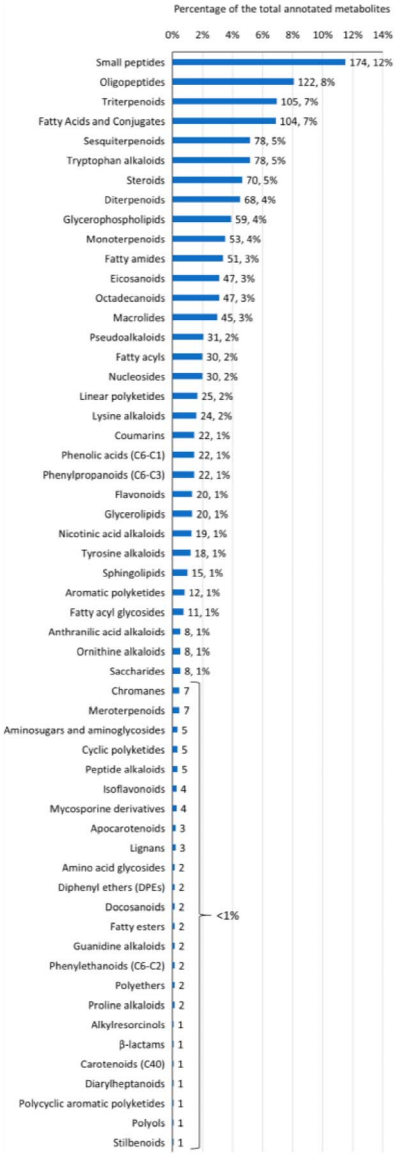
2.2. *Biomolecules in L. major extracts*



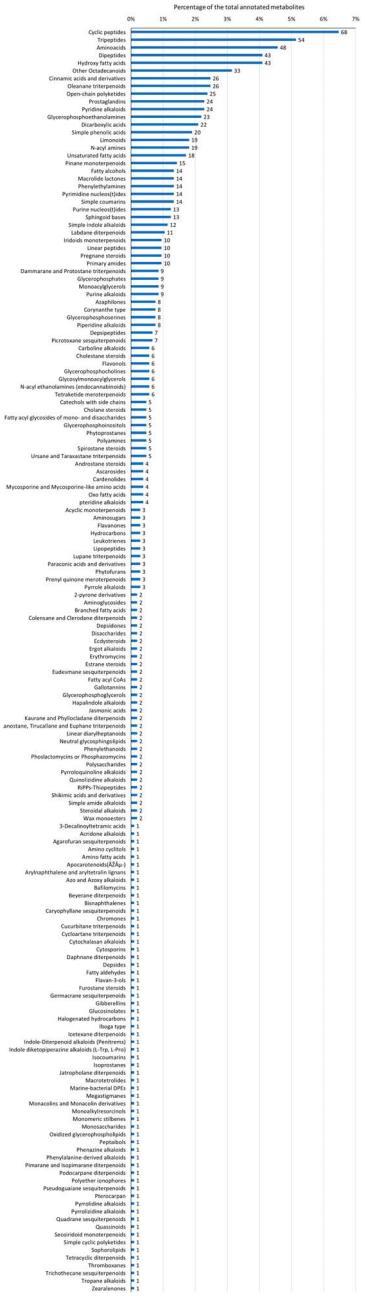
Supplementary Figure S5. NPC_superclass of the up/down regulated endo-metabolome (both hydrophilic and lipophilic phase).



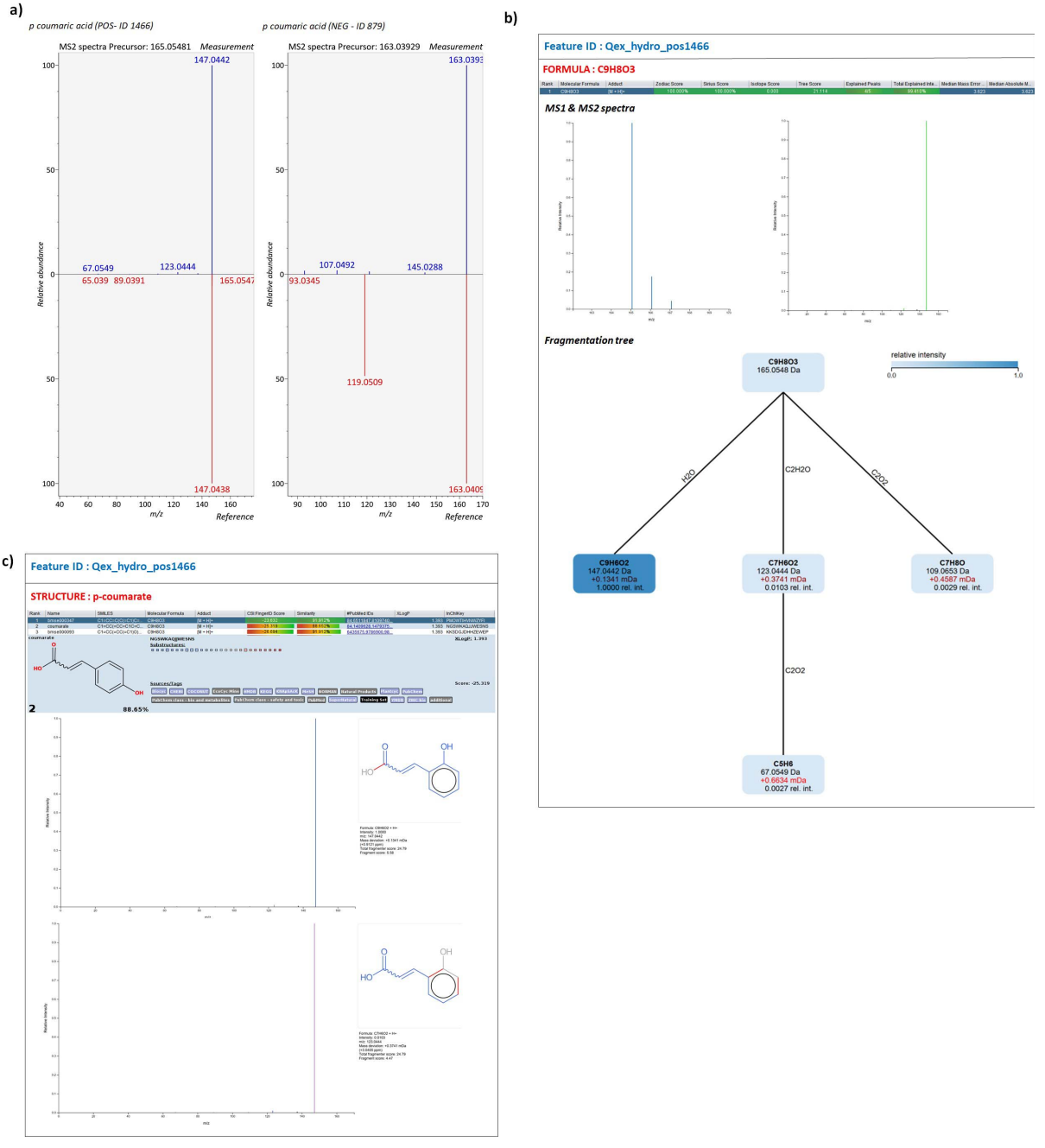
Supplementary Figure S6. Distribution of significant fold change (between control and 10 mg/L) among the NPC superclass of the annotated putative metabolites for both hydrophilic and lipophilic phases. The fold change values were from VolcanoPlot analyses based on T.test anova (p value < 0.01) following DROMICs processing.



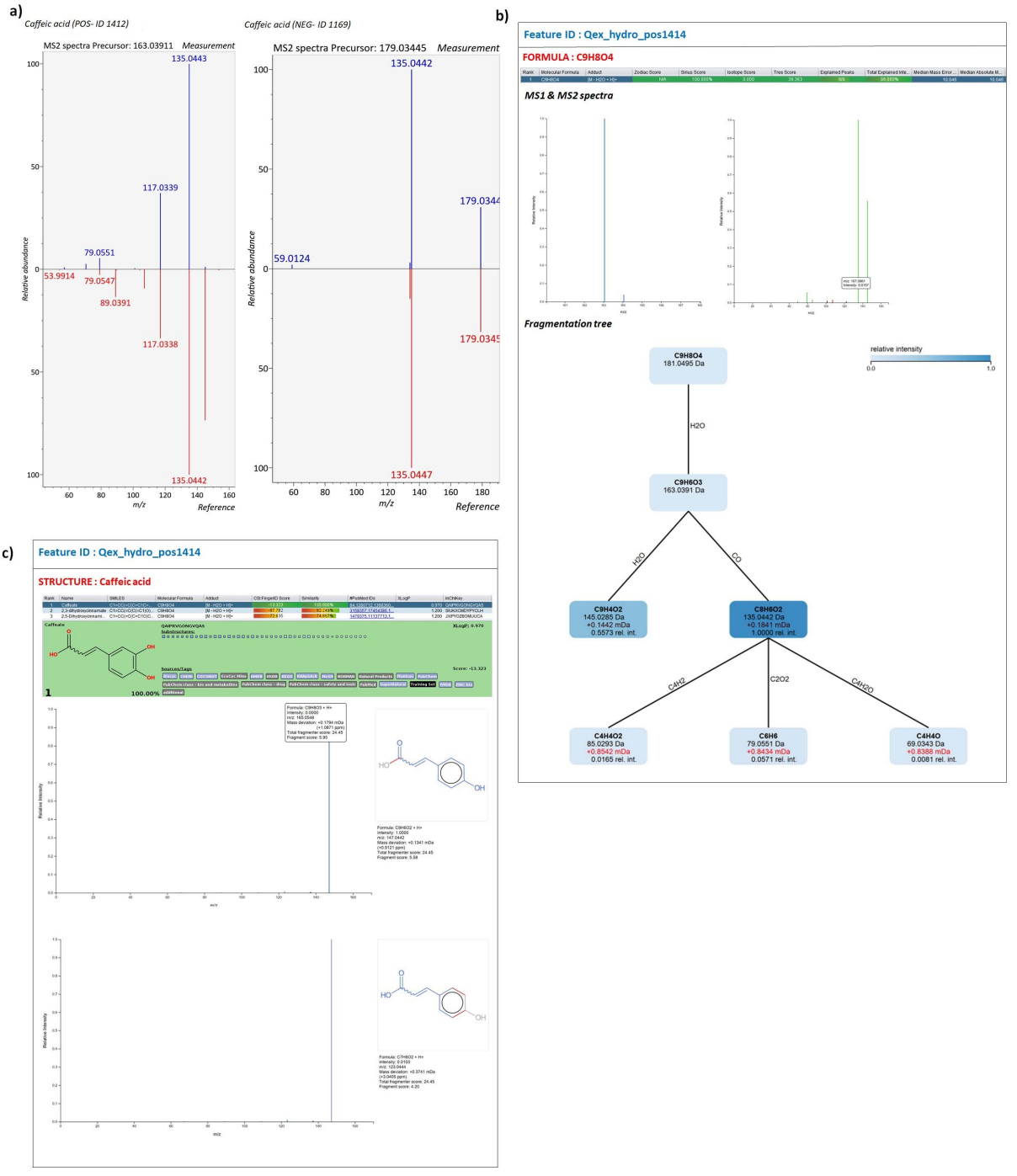
Supplementary Figure S7. NPC superclass of the aged macrophyte extracts from UPLC-HRMS-analyses. Probability > 0.5, $n = 1518$.



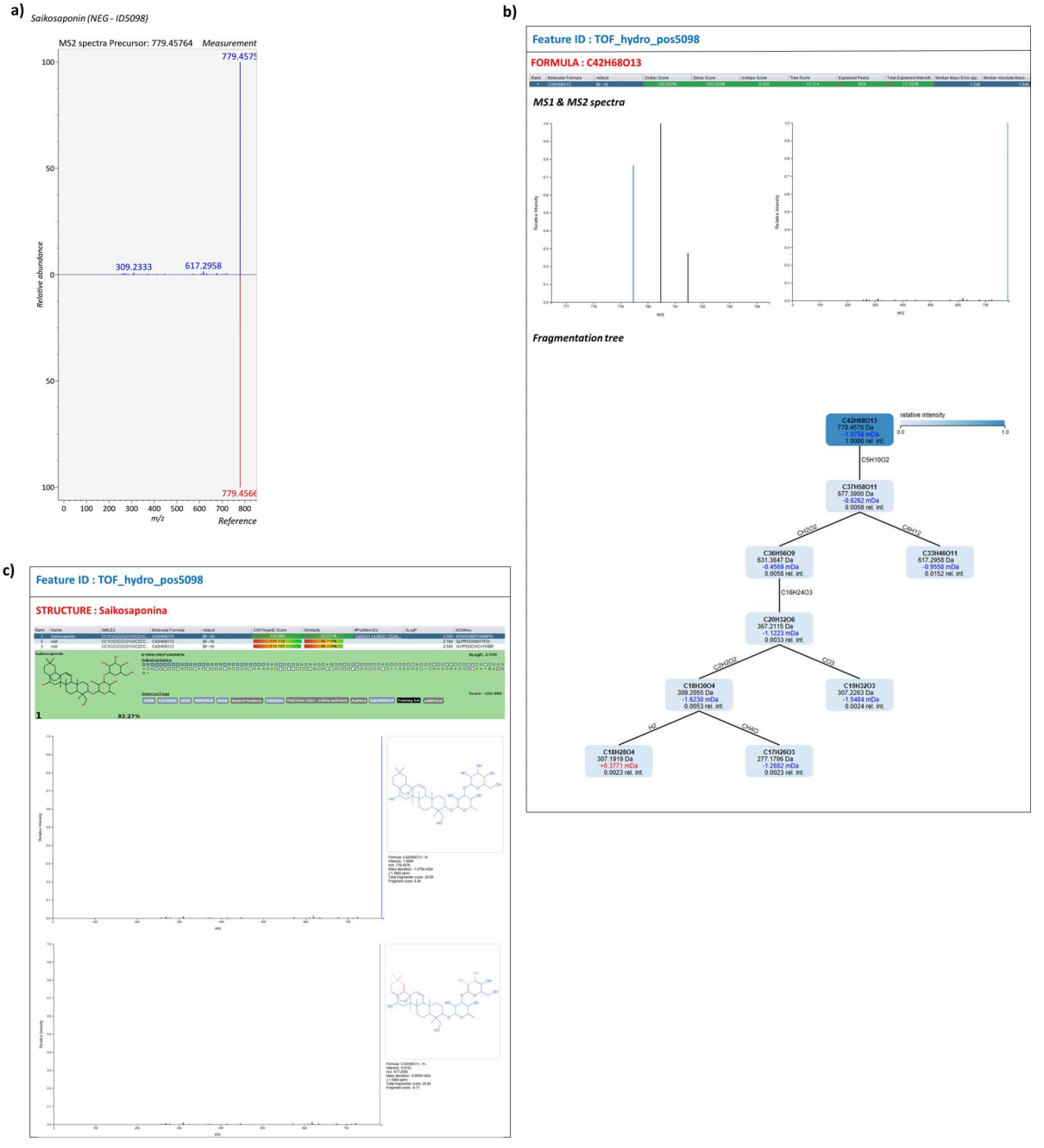
Supplementary Figure S8. NPC class of the aged macrophyte extracts from UPLC-HRMS-analyses. Probability > 0.5, $n = 1054$.



Supplementary Figure S9. Weight of evidence elements related to p-coumaric acid annotation, as experimental vs in house databasis (a), formula (b) and structural (c) elucidation from SIRIUS.



Supplementary Figure S10. Weight of evidence elements related to caffeic acid annotation, as experimental vs in house databasis (a), formula (b) and structural (c) elucidation from SIRIUS.



Supplementary Figure S11. Weight of evidence elements related to saikosaponin a annotation, as experimental vs in house databasis (a), formula (b) and structural (c) elucidation from SIRIUS.

Supplementary Table S8. List of annotated metabolites, pathways and classes in the *L. major* extract (Tabular file)