MS-CleanR tutorial:

Peak list cleaning, data concatenation and peak annotation

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1. Prerequisite :

Software installation

1.1. Downloading

MS-DIAL version up to 4.16:
http://prime.psc.riken.jp/Metabolomics_Software/MS-DIAL/index2.html

MS-FINDER version up to 3.30:
http://prime.psc.riken.jp/Metabolomics_Software/MS-FINDER/index2.html

R version up to 3.6.1 : https://cran.r-project.org/

R studio: https://rstudio.com/products/rstudio/

1.2. Installation

- **In R**, copy and paste the following command to update R version if necessary

```
if(!require(installr)) {
install.packages("installr"); require(installr)}
updateR()
```

- In **R studio**, update all your packages with the command

```
Setrepositories()
```

Select 1 and 2 for CRAN and BIOCONDUCTOR packages
Select the command Update on the right windows in the Package part

- Install MS-cleanR by copying and pasting the command :

```
devtools::install_github("SyrupType/mscleanr", auth_token =
"a29ed88006a073473a47cab074f9bc7e8dfacbac")
```

2. MS-CleanR workflow

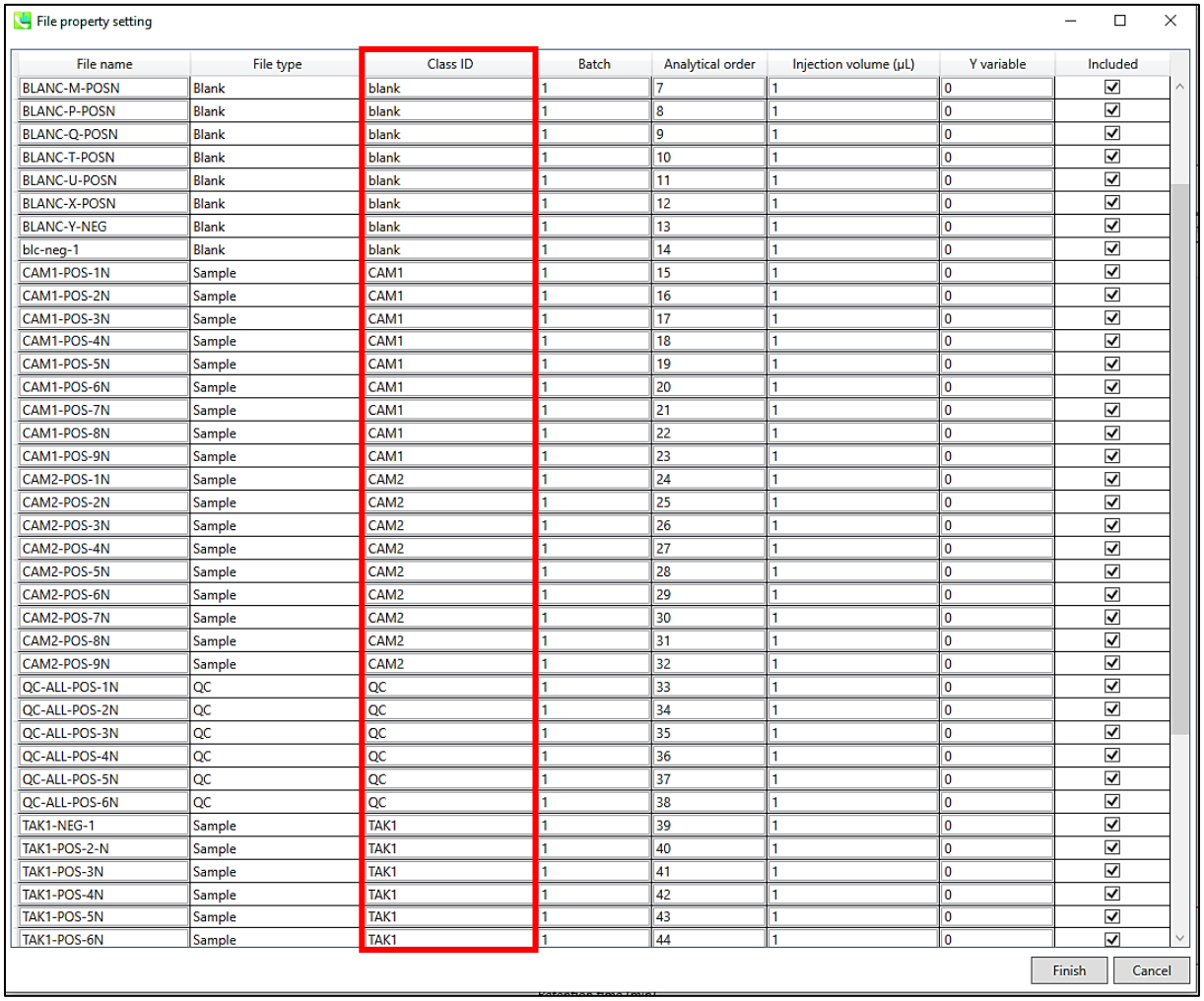
Within your project directory, create one subfolder for each ionization mode namely “pos” and “neg”. In each of this new directory, create another subfolder named “peaks”.

2.1. Process the data with MS-DIAL

Process data with MS-DIAL in either pos or neg mode or both according to the tutorial https://mtbinfo-team.github.io/mtbinfo.github.io/

Important notices:

A) During data importation, it is important to note the type (Blank, QC or Sample) and class of every sample in **Class ID column** (blank, sample class, QC)



File name	File type	Class ID	Batch	Analytical order	Injection volume (µL)	Y variable	Included
BLANC-M-POSN	Blank	blank	1	7	1	0	☑
BLANC-P-POSN	Blank	blank	1	8	1	0	☑
BLANC-Q-POSN	Blank	blank	1	9	1	0	☑
BLANC-T-POSN	Blank	blank	1	10	1	0	☑
BLANC-U-POSN	Blank	blank	1	11	1	0	☑
BLANC-X-POSN	Blank	blank	1	12	1	0	☑
BLANC-Y-NEG	Blank	blank	1	13	1	0	☑
blc-neg-1	Blank	blank	1	14	1	0	☑
CAM1-POS-1N	Sample	CAM1	1	15	1	0	☑
CAM1-POS-2N	Sample	CAM1	1	16	1	0	☑
CAM1-POS-3N	Sample	CAM1	1	17	1	0	☑
CAM1-POS-4N	Sample	CAM1	1	18	1	0	☑
CAM1-POS-5N	Sample	CAM1	1	19	1	0	☑
CAM1-POS-6N	Sample	CAM1	1	20	1	0	☑
CAM1-POS-7N	Sample	CAM1	1	21	1	0	☑
CAM1-POS-8N	Sample	CAM1	1	22	1	0	☑
CAM1-POS-9N	Sample	CAM1	1	23	1	0	☑
CAM2-POS-1N	Sample	CAM2	1	24	1	0	☑
CAM2-POS-2N	Sample	CAM2	1	25	1	0	☑
CAM2-POS-3N	Sample	CAM2	1	26	1	0	☑
CAM2-POS-4N	Sample	CAM2	1	27	1	0	☑
CAM2-POS-5N	Sample	CAM2	1	28	1	0	☑
CAM2-POS-6N	Sample	CAM2	1	29	1	0	☑
CAM2-POS-7N	Sample	CAM2	1	30	1	0	☑
CAM2-POS-8N	Sample	CAM2	1	31	1	0	☑
CAM2-POS-9N	Sample	CAM2	1	32	1	0	☑
QC-ALL-POS-1N	QC	QC	1	33	1	0	☑
QC-ALL-POS-2N	QC	QC	1	34	1	0	☑
QC-ALL-POS-3N	QC	QC	1	35	1	0	☑
QC-ALL-POS-4N	QC	QC	1	36	1	0	☑
QC-ALL-POS-5N	QC	QC	1	37	1	0	☑
QC-ALL-POS-6N	QC	QC	1	38	1	0	☑
TAK1-NEG-1	Sample	TAK1	1	39	1	0	☑
TAK1-POS-2-N	Sample	TAK1	1	40	1	0	☑
TAK1-POS-3N	Sample	TAK1	1	41	1	0	☑
TAK1-POS-4N	Sample	TAK1	1	42	1	0	☑
TAK1-POS-5N	Sample	TAK1	1	43	1	0	☑
TAK1-POS-6N	Sample	TAK1	1	44	1	0	☑

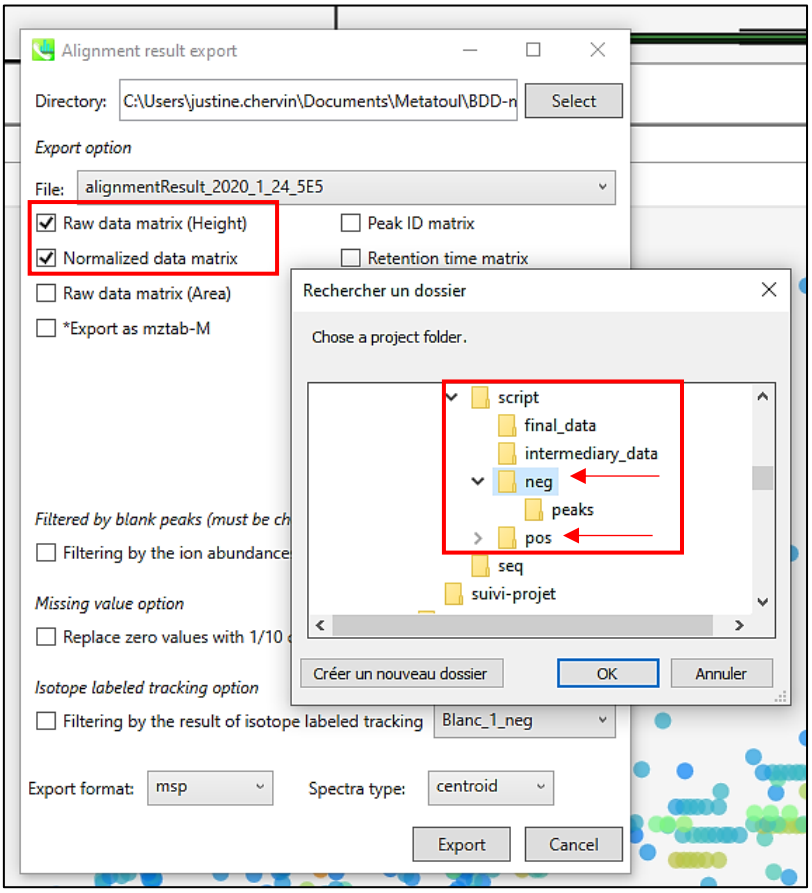
B) Be careful to have the **same number of samples** between pos and neg mode and in the **same order.**

2.2. Export peak list

After alignment process:

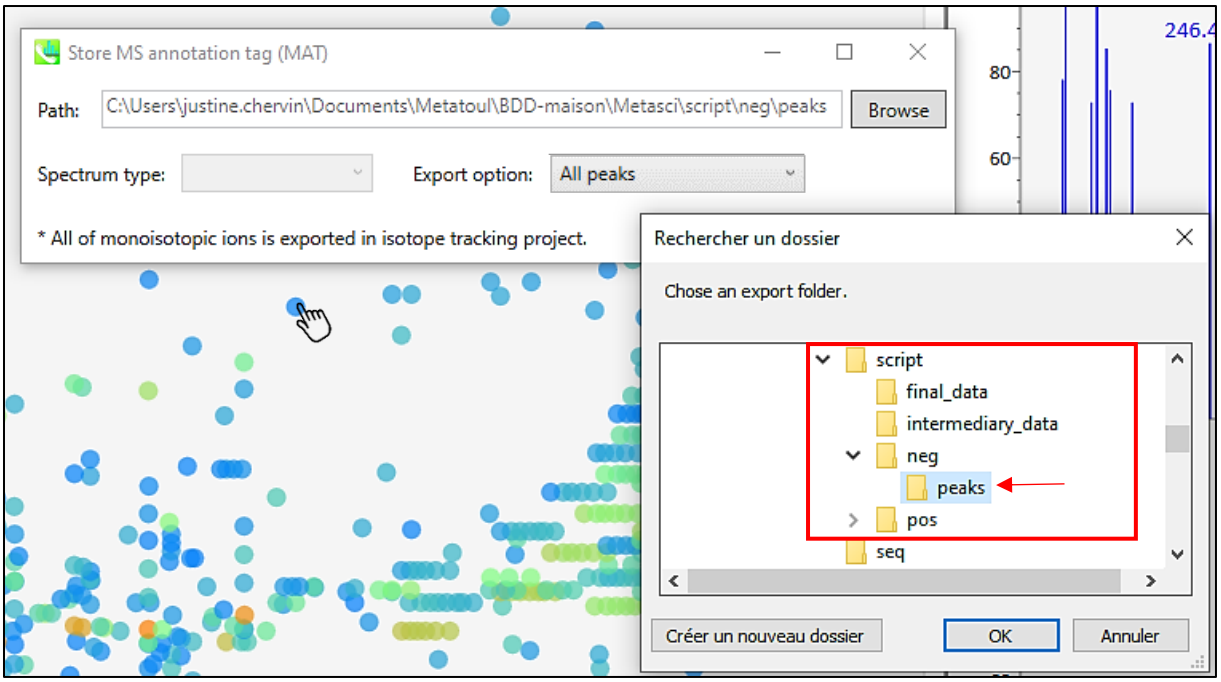
- **Normalized data** by Total ion chromatogram (TIC) or another method

- Export alignment results: both **Raw data matrix (Height)** and **Normalized data matrix** respectively in previously created folders named “pos” and “neg”.



2.3. Export all peaks

By clicking on one point, export « **all peaks »** to the “peaks” directory respectively created in “pos” and “neg” folders.



2.4. Open the shiny interface of MS-CleanR

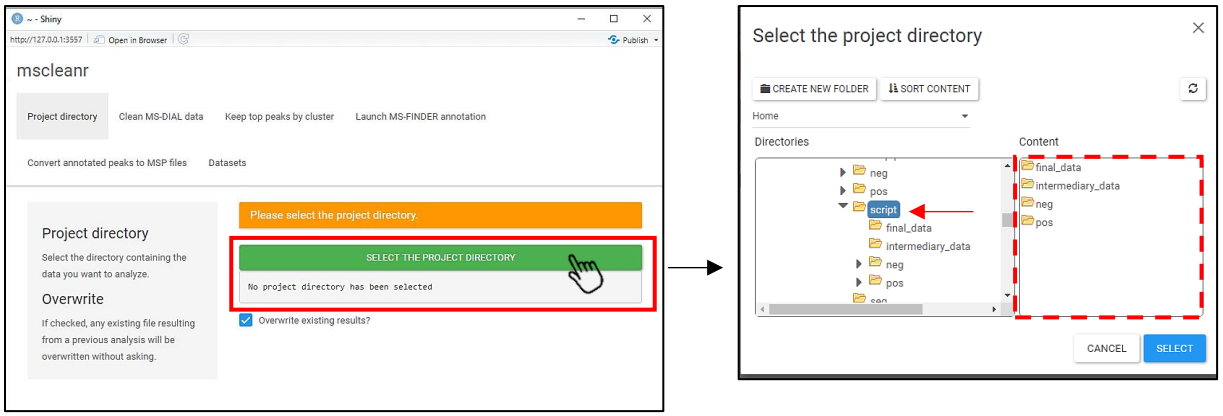
Select the MScleanR package in **Rstudio** and open the shiny interface using the following command:

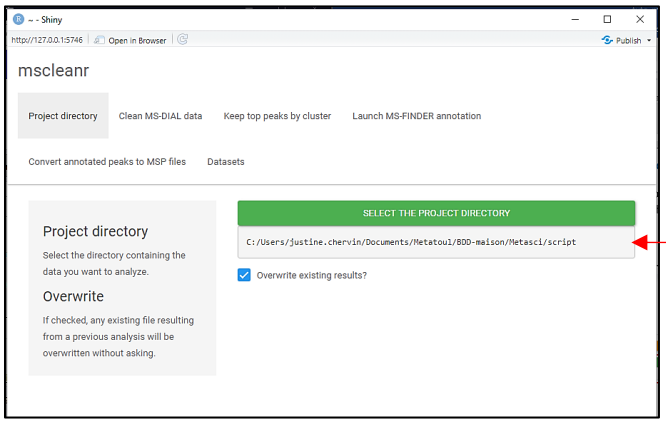
- *Note that if you encounter some issues, try to open the Shiny interface in internet browser.*
- *Sometimes Windows block file writing, close the shiny or R studio and run it again to solve the problem.*

```
runGUI()
```

2.4.1. Select the project directory

First step is to define the project directory on the first index called "**Project Directory**" by clicking on the green rectangle "*Select the project directory*" and by selecting the parent folder containing "pos and "neg" directories.





When your project directory is selected, it is written in the grey rectangle.

2.4.2. Define your parameter of filtration and Clean your data

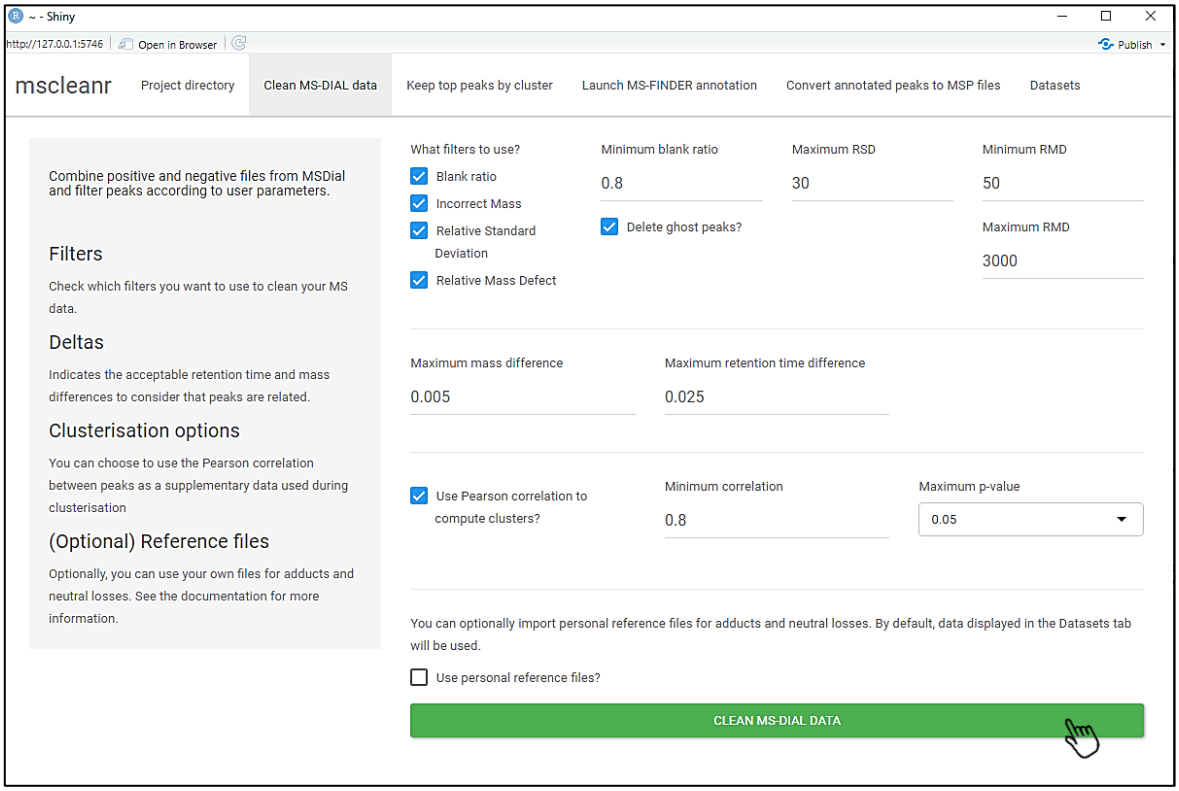
In the second index called "**Clean MS-DIAL data**" various parameters can be personalized to filter your data. You can decide to select any filter according to your goal and experimental design.

Command	Description
Blank ratio	Subtract blank peaks to samples based on the indicated "**Minimum blank ratio**" by default at 0.8. This operation is done on the **Height files** between Blanks and QCs.
Incorrect Mass	Delete all peaks with a mass defect in X.8 and X.9 which appear to be artifacts.
Relative standard Deviation (RSD)	Filter based on the **Maximum RSD** value set at 30 by default. The RSD is calculated on each defined class. If RSD of one feature is under 30 for all class, it is removed from the peak list.
Relative Mass Defect (RMD)[1]	RMD is calculated in ppm as ((mass defect/measured monoisotopic mass) × 10e6) Analysis of natural products from the DNP shows that 95 % of RMD are comprised between 50 and 3000 (values by default).
Delete ghost peaks	Delete variables with *m/z* values corresponding to blank peaks but with a different RT in samples.

[1] Ekanayaka EA, Celiz MD, Jones AD. Relative mass defect filtering of mass spectra: a path to discovery of plant specialized metabolites. *Plant Physiol*. 2015;167(4):1221–1232. doi:10.1104/pp.114.251165

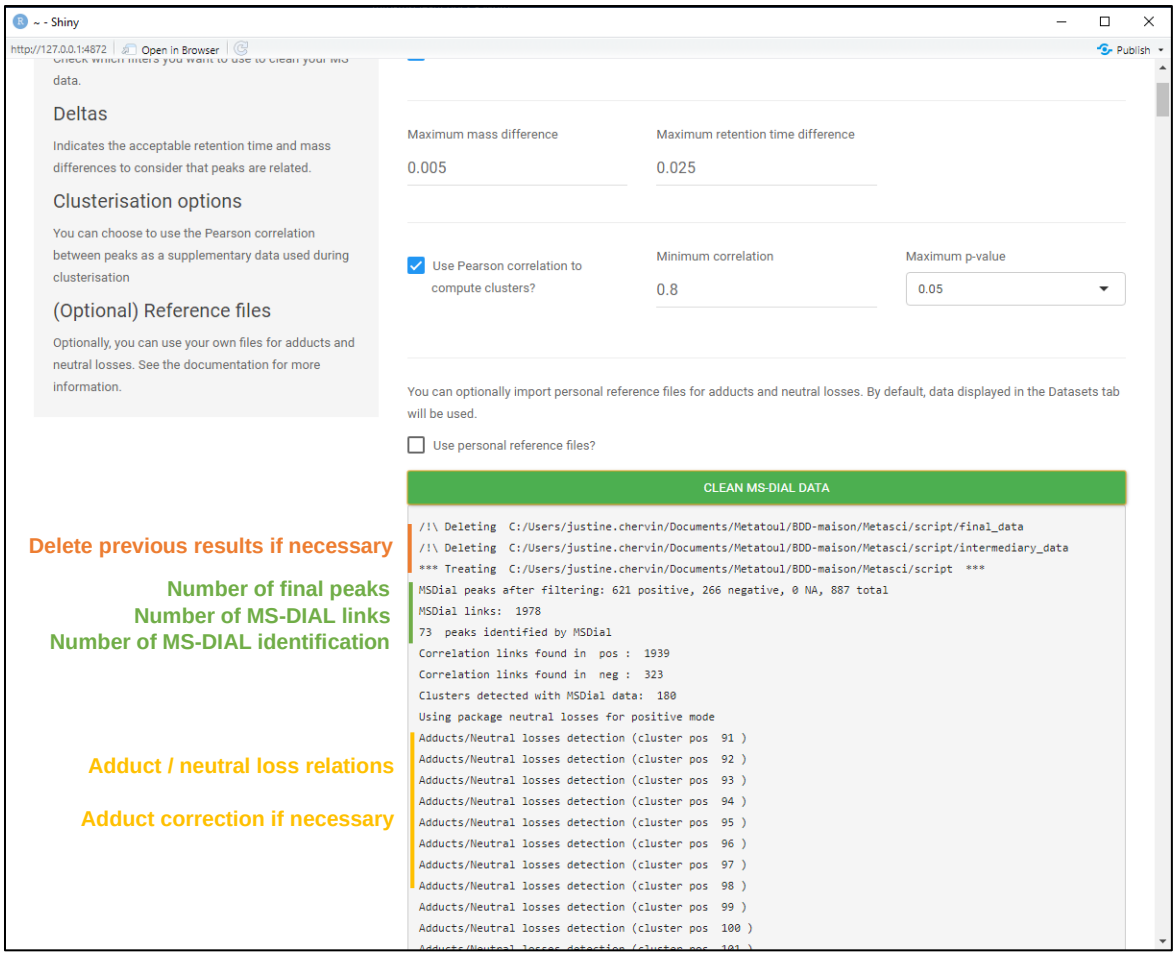
Maximum mass difference	m/z value tolerance set by default to 0.005 for Pearson correlation and pos/neg merging
Maximum retention time difference	RT value tolerance set by default to 0.025 (absolute value) for Pearson and pos/neg merging
Use Pearson correlation to compute clusters?	Extend MS-DIAL clusters with Pearson correlation. **Minimum correlation** and **maximum p-value** are respectively set by default to 0.8 and 0.05

Once your parameters are fixed, click on the green rectangle named "*Clean MS-DIAL data*". A green window appears with the writing "*Cleaning data…*".



During the cleaning:

- Clusters are formed based on MS-DIAL "post curation column", Pearson correlation, links such as adducts, neutral losses, dimers, …;
- Adducts are corrected based on previous found links;
- Pos and Neg clusters are concatenated if relational links are found (adducts mass difference)
- Once the cleaning is done, one new folder is created named "intermediary_data". Different information is obtained at the bottom of the index "Clean MS-DIAL data".



At this step, several files are created in the folder “intermediary_data”.

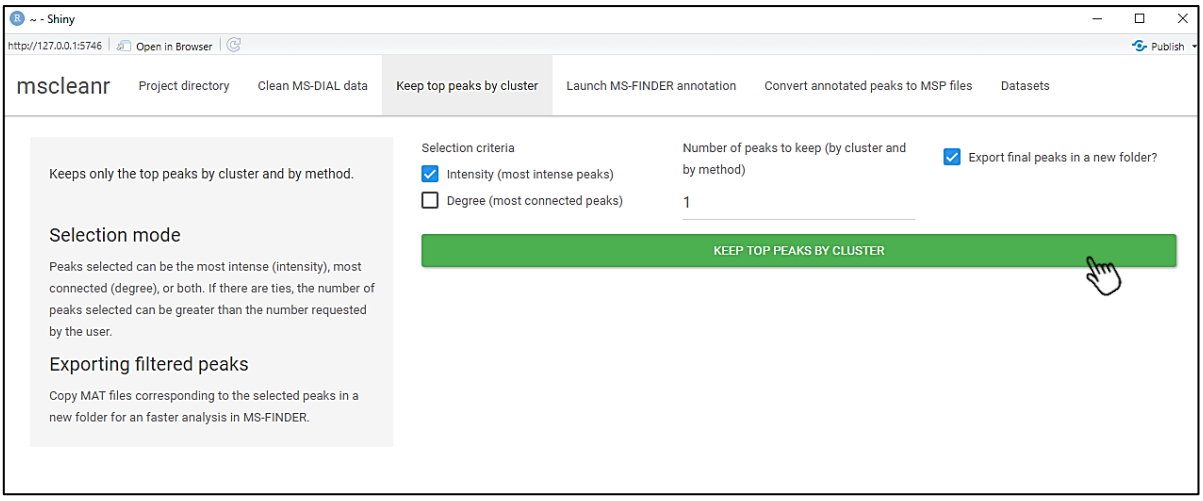
Files	Description
Adducts_massdiff_filtered	Reference file for mass difference between regular adducts
Adducts_massdiff_total	Reference file for mass difference between all possible adducts
Adducts_detected_by_MSDIAL	Reference file for adduct ponderation of regular adducts found by MS-DIAL
Adducts_filtered.graphml	A graph to display feature clusters based on adducts links
Adducts_final_selection	Final adducts resulting from MSdial and modified after pos/neg concatenation
Adducts_initial.graphml	A graph to display feature clusters based on MSdial data
Annotated_MS-peaks-MSDial	List of annotated peaks based on the database (msp file) imported in MS-DIAL
Deleted_blank_ghosts	List of peaks deleted with “delete ghost peaks”
Deleted_blanks	List of peaks deleted with the filter “blank ratio”
Deleted_mz	List of peaks deleted with the filter “incorrect mass”
Deleted_rmd	List of peaks deleted with the filter “RMD”

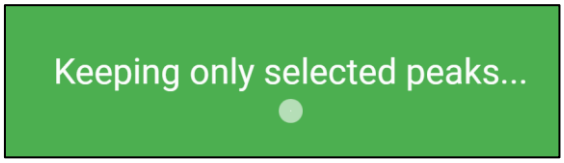
Deleted_rsd	List of peaks deleted with the filter “RSD”
Links_clusters_final	List of correlation (adduct, neutral loss, msdial) between peaks in neg and pos
Links_post_selection	Feature links after adduct prioritization process
Links_pre_selection	Feature links with all adducts possibilities
MS_peaks-clusters.graphml	A graph of final clusters (MS-DIAL + Pearson)
MS_peaks-clusters_final	List of final clusters (MS-DIAL + Pearson) in both pos and neg ionization
MS_peaks-clusters_msdial	List of MS-DIAL clusters in both pos and neg ionization
parameters	List of parameter used for the cleaning
samples	List of samples with indication of sample name, class, file type, script class and column name

2.4.3. Select number of peaks per cluster

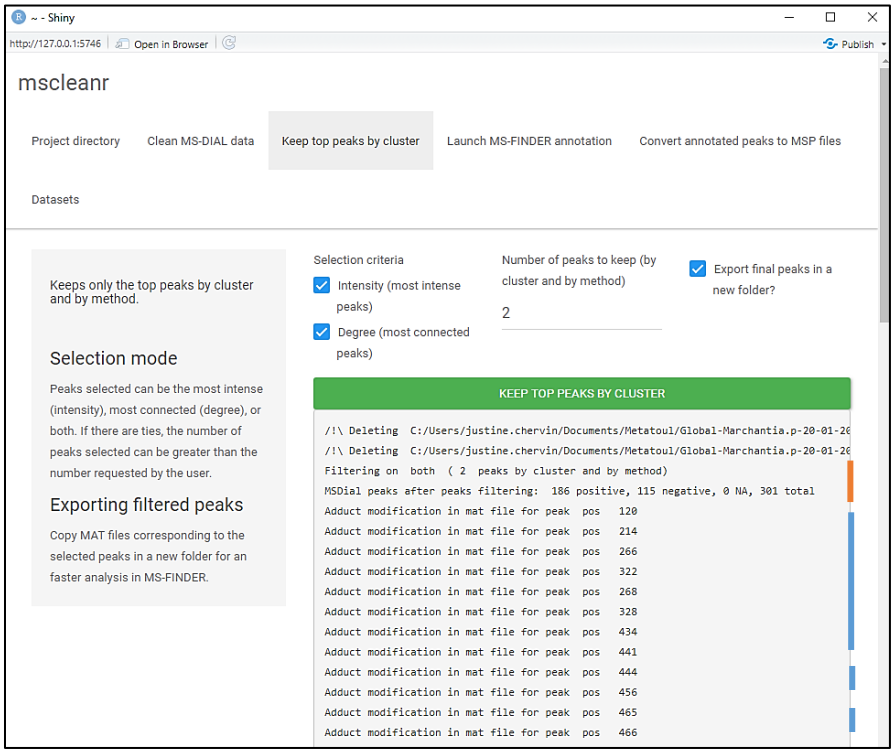
In the third index “**Keep top peaks by cluster**” you can select the number of features you want to keep in each cluster.
This step is based on the hypothesis that in one cluster, only one true metabolite is present. The other variables used to come from feature degeneration. Generally, this metabolite appears to be the **most intense** and/or **the most connected within the** graph (adducts, neutral loss, dimers…).

You can then choose to select as many peaks as you want and either the most intense(s) by clicking “**Intensity**”, the most connected by clicking “**Degree**” or both.
We advise to select both criteria and keep 2 top peaks by cluster for further MS-finder request.





At this step, a new folder is created in both “pos” and “neg” folders named “**filtered peaks**”. All .MAT files corresponding to kept peaks are copied from “peaks” folder and pasted in this new folder “filtered peaks”.



Number of kept peaks

Modification of adduct annotation directly in .MAT file for further MS-FINDER annotation

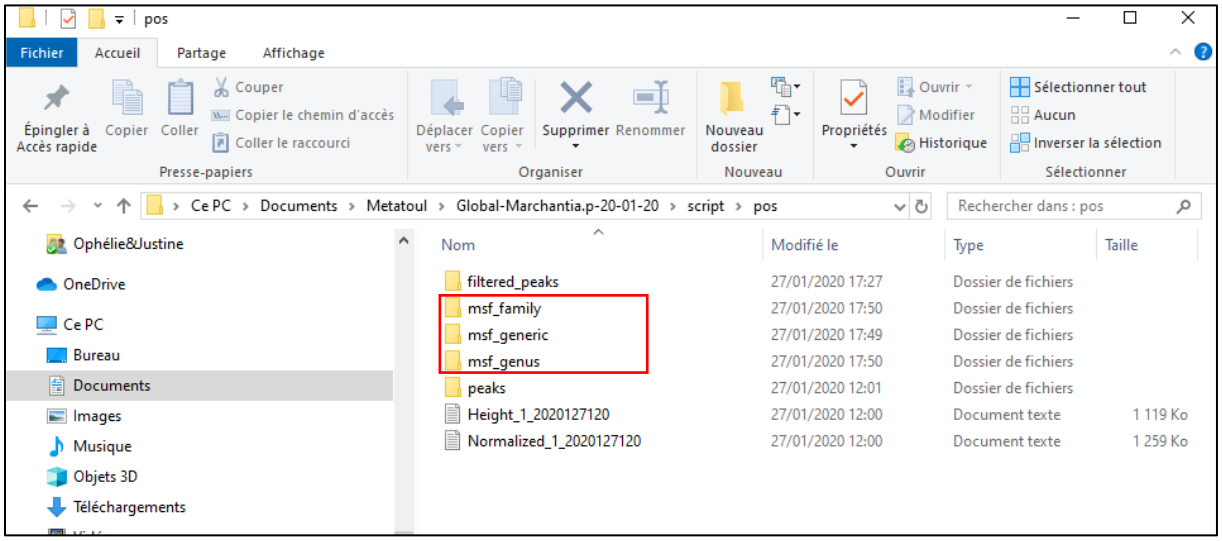
2.5. Interrogation of MS-FINDER

From « **filtered peaks** » folder, interrogate MS-FINDER based on several databases of your choice (for example plant genus, plant family, generic databases from MS-FINDER, …).

Optional: Add a "Compound_level" column within your in-house database for MSfinder. This level will be used for annotation ranking in the next step.

The most **important thing** to do is to create respectively in "pos" and "neg" directories, new folders named "**msf_X**" (for example msf_genus) which correspond to the name of each database used for feature annotation. The msf_generic is mandatory and correspond to internal database in MS-Finder.

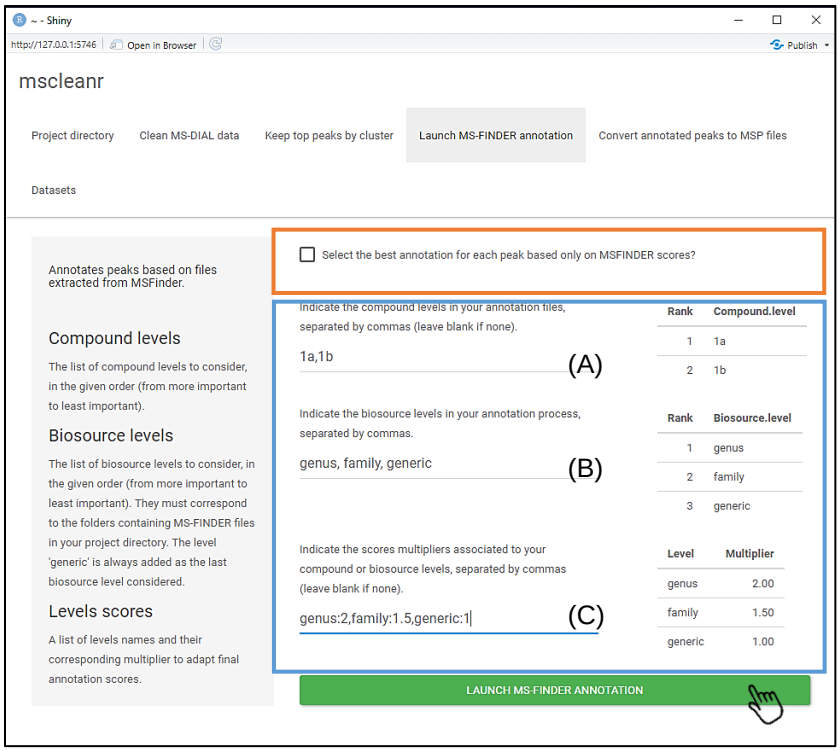
For each database used, export "structure" and "formula" as a single file in the corresponding folder.



2.6. Launch MS-FINDER annotation

Once all your MS-FINDER interrogations are done and your folder "msf_X" filled with "**structure**" and "**formula**" files, go to the fourth index called "**Launch MS-FINDER annotation"**.

This step will permit the annotation of the variables based either only on the score of MS-FINDER or on the prioritization of the different databases, used to indicate the more pertinent annotation.



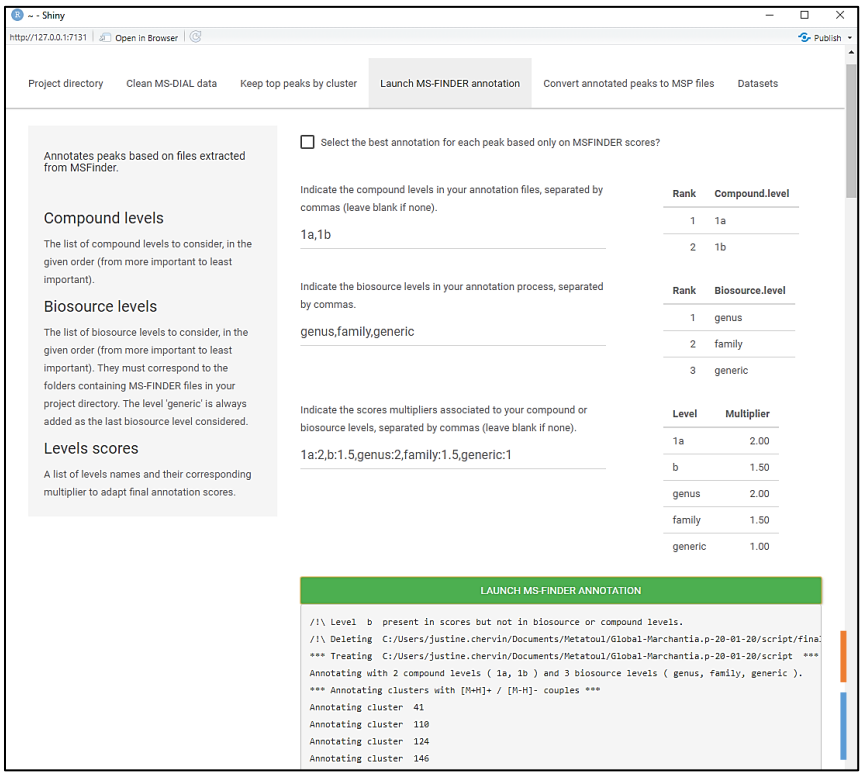
This option is used to report the identification with the best MS-FINDER score

This option is used when you want to prioritize some databases.

In (A) you have to indicate the compound level within your database

In (B) you have to order your database

In (C) you can dedicate to your database levels a multiplier to calculate new scores from MS-FINDER ones.



Summary of compounds and biosource levels used

Paste of annotation in the final peak list

Two files are created in the "**final-data**" folder:

- **Annotated MS peaks cleaned** = the final peak list with annotation from MS-FINDER

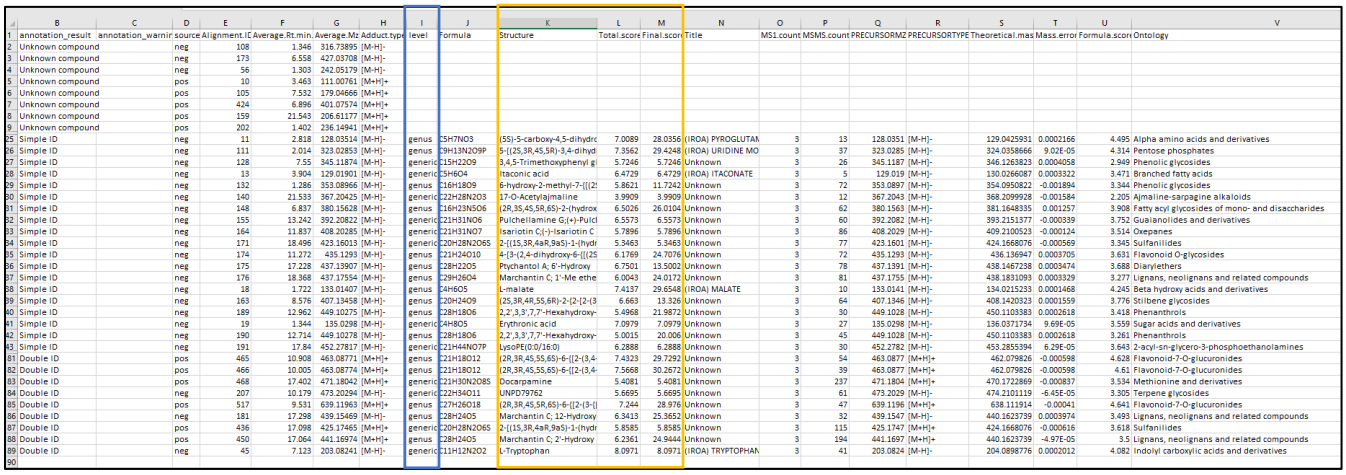
- **Annotated MS peaks normalized** = the final peak list renormalized based on total peak area

The final peak list looks like as follow. Different information are available such as:

- The average m/z value;
- The average RT value;
- The annotation based on MS-FINDER interrogation on the "**Structure**" column with the associated **Total score** of MS-FINDER and **Final score** calculated from the indicated multipliers.
- The source of the annotation in the "level" column;
- The ontology of the compound; ...

The variable are also identified as:

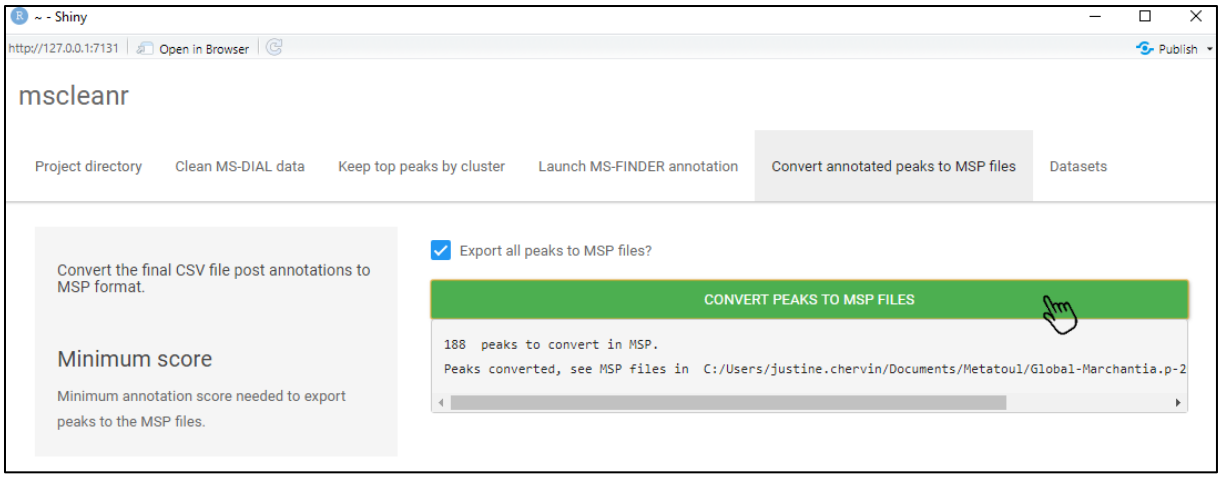
- Unknown compound = variable with no annotation
- Simple ID = based on a single feature in pos or neg mode
- Double ID =based on annotation retrieve in pos and neg mode



	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V
1	annotation_result	annotation_warnir	source	Alignment.ID	Average.Rt	min.	Average.Mz	Adduct.type	level	Formula	Structure	Total.score	Final.score	Title	MS1.count	MSMS.count	PRECURSORMZ	PRECURSORTYPE	Theoretical.mass	Mass.error	Formula.score	Ontology
2	Unknown compound		neg	108	1.346		316.73895	[M-H]-														
3	Unknown compound		neg	173	6.558		427.03708	[M-H]-														
4	Unknown compound		neg	56	1.303		242.05179	[M-H]-														
5	Unknown compound		pos	10	3.463		111.00761	[M+H]+														
6	Unknown compound		pos	105	7.532		179.04666	[M+H]+														
7	Unknown compound		pos	424	6.896		401.07574	[M+H]+														
8	Unknown compound		pos	159	21.543		206.61177	[M+H]+														
9	Unknown compound		pos	202	1.402		236.14941	[M+H]+														
25	Simple ID		neg	11	2.818		128.03514	[M-H]-	genus	C5H7NO3	(5S)-5-carboxy-4,5-dihydrc	7.0089	28.0356	(IROA) PYROGLUTAM	3	13	128.0351	[M-H]-	129.0425931	0.0002166	4.495	Alpha amino acids and derivatives
26	Simple ID		neg	111	2.014		323.02853	[M-H]-	genus	C9H13N2O9P	5-[(2S,3R,4S,5R)-3,4-dihyd	7.3562	29.4248	(IROA) URIDINE MO	3	37	323.0285	[M-H]-	324.0358666	9.02E-05	4.314	Pentose phosphates
27	Simple ID		neg	128	7.55		345.11874	[M-H]-	genericC15H22O9		3,4,5-Trimethoxyphenyl gl	5.7246	5.7246	Unknown	3	26	345.1187	[M-H]-	346.1263823	0.0004058	2.949	Phenolic glycosides
28	Simple ID		neg	13	3.904		129.01901	[M-H]-	genericC5H6O4		Itaconic acid	6.4729	6.4729	(IROA) ITACONATE	3	5	129.019	[M-H]-	130.0266087	0.0003322	3.471	Branched fatty acids
29	Simple ID		neg	132	1.286		353.08966	[M-H]-	genus	C16H18O9	6-hydroxy-2-methyl-7-[[(2	5.8621	11.7242	Unknown	3	72	353.0897	[M-H]-	354.0950822	-0.001894	3.344	Phenolic glycosides
30	Simple ID		neg	140	21.533		367.20425	[M-H]-	genericC22H28N2O3		17-O-Acetylajmaline	3.9909	3.9909	Unknown	3	12	367.2043	[M-H]-	368.2099928	-0.001584	2.205	Ajmaline-sarpagine alkaloids
31	Simple ID		neg	148	6.837		380.15628	[M-H]-	genus	C16H23N5O6	(2R,3S,4S,5R,6S)-2-(hydrox	6.5026	26.0104	Unknown	3	62	380.1563	[M-H]-	381.1648335	0.001257	3.908	Fatty acyl glycosides of mono- and disaccharides
32	Simple ID		neg	155	13.242		392.20822	[M-H]-	genericC21H31NO6		Pulchellamine G;(+)-Pulcl	6.5573	6.5573	Unknown	3	60	392.2082	[M-H]-	393.2151377	-0.000339	3.752	Guaianolides and derivatives
33	Simple ID		neg	164	11.837		408.20285	[M-H]-	genericC21H31NO7		Isariotin C;(-)-Isariotin C	5.7896	5.7896	Unknown	3	86	408.2029	[M-H]-	409.2100523	-0.000124	3.514	Oxepanes
34	Simple ID		neg	171	18.496		423.16013	[M-H]-	genericC20H28N2O6S		2-[(1S,3R,4aR,9aS)-1-(hydr	5.3463	5.3463	Unknown	3	77	423.1601	[M-H]-	424.1668076	-0.000569	3.345	Sulfanilides
35	Simple ID		neg	174	11.272		435.1293	[M-H]-	genus	C21H24O10	4-[3-[2,4-dihydroxy-6-[(2S	6.1769	24.7076	Unknown	3	72	435.1293	[M-H]-	436.136947	0.0003705	3.631	Flavonoid O-glycosides
36	Simple ID		neg	175	17.228		437.13907	[M-H]-	genus	C28H22O5	Ptychantol A; 6'-Hydroxy	6.7501	13.5002	Unknown	3	78	437.1391	[M-H]-	438.1467238	0.0003474	3.688	Diarylethers
37	Simple ID		neg	176	18.368		437.17554	[M-H]-	genus	C29H26O4	Marchantin C; 1'-Me ethe	6.0043	24.0172	Unknown	3	81	437.1755	[M-H]-	438.1831093	0.0003329	3.277	Lignans, neolignans and related compounds
38	Simple ID		neg	18	1.722		133.01407	[M-H]-	genus	C4H6O5	L-malate	7.4137	29.6548	(IROA) MALATE	3	10	133.0141	[M-H]-	134.0215233	0.0001468	4.245	Beta hydroxy acids and derivatives
39	Simple ID		neg	163	8.576		407.13458	[M-H]-	genus	C20H24O9	(2S,3R,4R,5S,6R)-2-[2-[2-(3	6.663	13.326	Unknown	3	64	407.1346	[M-H]-	408.1420323	0.0001559	3.776	Stilbene glycosides
40	Simple ID		neg	189	12.962		449.10275	[M-H]-	genus	C28H18O6	2,2',3,3',7,7'-Hexahydroxy-	5.4968	21.9872	Unknown	3	30	449.1028	[M-H]-	450.1103383	0.0002618	3.418	Phenanthrols
41	Simple ID		neg	19	1.344		135.0298	[M-H]-	genericC4H8O5		Erythronic acid	7.0979	7.0979	Unknown	3	27	135.0298	[M-H]-	136.0371734	9.69E-05	3.559	Sugar acids and derivatives
42	Simple ID		neg	190	12.714		449.10278	[M-H]-	genus	C28H18O6	2,2',3,3',7,7'-Hexahydroxy-	5.0015	20.006	Unknown	3	45	449.1028	[M-H]-	450.1103383	0.0002618	3.261	Phenanthrols
43	Simple ID		neg	191	17.84		452.27817	[M-H]-	genericC21H44NO7P		LysoPE(0:0/16:0)	6.2888	6.2888	Unknown	3	30	452.2782	[M-H]-	453.2855394	6.29E-05	3.643	2-acyl-sn-glycero-3-phosphoethanolamines
81	Double ID		pos	465	10.908		463.08771	[M+H]+	genus	C21H18O12	(2R,3R,4S,5S,6S)-6-[[2-(3,4-	7.4323	29.7292	Unknown	3	54	463.0877	[M+H]+	462.079826	-0.000598	4.628	Flavonoid-7-O-glucuronides
82	Double ID		pos	466	10.005		463.08774	[M+H]+	genus	C21H18O12	(2R,3R,4S,5S,6S)-6-[[2-(3,4-	7.5668	30.2672	Unknown	3	39	463.0877	[M+H]+	462.079826	-0.000598	4.61	Flavonoid-7-O-glucuronides
83	Double ID		pos	468	17.402		471.18042	[M+H]+	genericC21H30N2O8S		Docarpamine	5.4081	5.4081	Unknown	3	237	471.1804	[M+H]+	470.1722869	-0.000837	3.534	Methionine and derivatives
84	Double ID		neg	207	10.179		473.20294	[M-H]-	genericC22H34O11		UNPD79762	5.6695	5.6695	Unknown	3	61	473.2029	[M-H]-	474.2101119	-6.45E-05	3.305	Terpene glycosides
85	Double ID		pos	517	9.531		639.11963	[M+H]+	genus	C27H26O18	(2R,3R,4S,5R,6S)-6-[[2-[3-[(	7.244	28.976	Unknown	3	47	639.1196	[M+H]+	638.111914	-0.00041	4.641	Flavonoid-7-O-glucuronides
86	Double ID		neg	181	17.298		439.15469	[M-H]-	genus	C28H24O5	Marchantin C; 12-Hydroxy	6.3413	25.3652	Unknown	3	32	439.1547	[M-H]-	440.1623739	0.0003974	3.493	Lignans, neolignans and related compounds
87	Double ID		pos	436	17.098		425.17465	[M+H]+	genericC20H28N2O6S		2-[(1S,3R,4aR,9aS)-1-(hydr	5.8585	5.8585	Unknown	3	115	425.1747	[M+H]+	424.1668076	-0.000616	3.618	Sulfanilides
88	Double ID		pos	450	17.064		441.16974	[M+H]+	genus	C28H24O5	Marchantin C; 2'-Hydroxy	6.2361	24.9444	Unknown	3	194	441.1697	[M+H]+	440.1623739	-4.97E-05	3.5	Lignans, neolignans and related compounds
89	Double ID		neg	45	7.123		203.08241	[M-H]-	genericC11H12N2O2		L-Tryptophan	8.0971	8.0971	(IROA) TRYPTOPHAN	3	41	203.0824	[M-H]-	204.0898776	0.0002012	4.082	Indolyl carboxylic acids and derivatives
90																						

2.7. Export peaks as msp files

In the fifth index "**Convert annotated peaks to MSP files**", you will be able to create two msp files named "peaks-neg" and "peaks-pos" in the folder "final_data". All peaks can be converted, or user can choose a scoring threshold based on multiplied MSfinder score.



These two files could then be imported in MetGem software or GNPS facility to create mass spectral similarity networks.