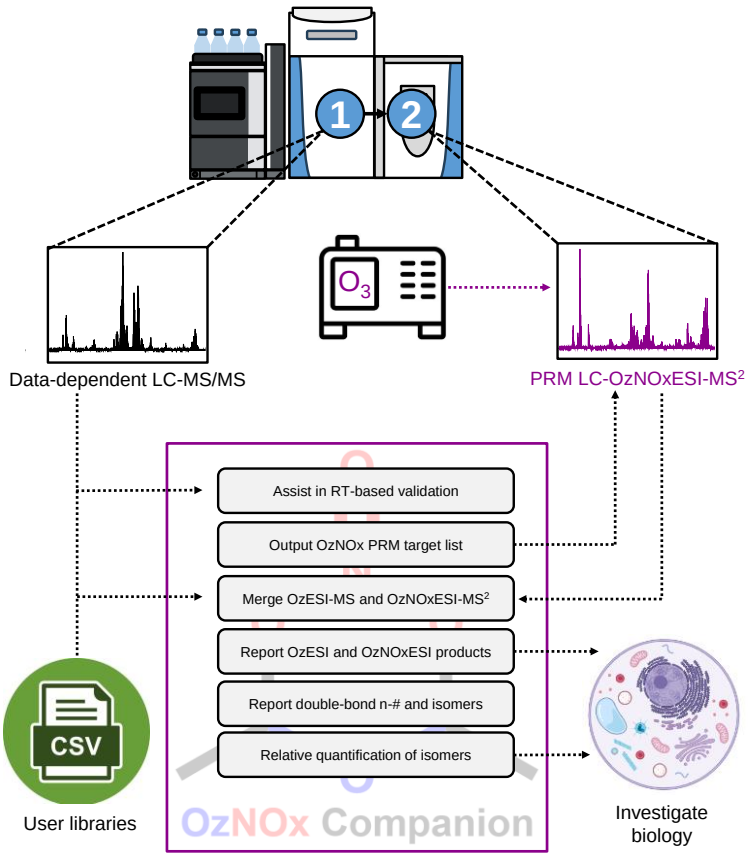
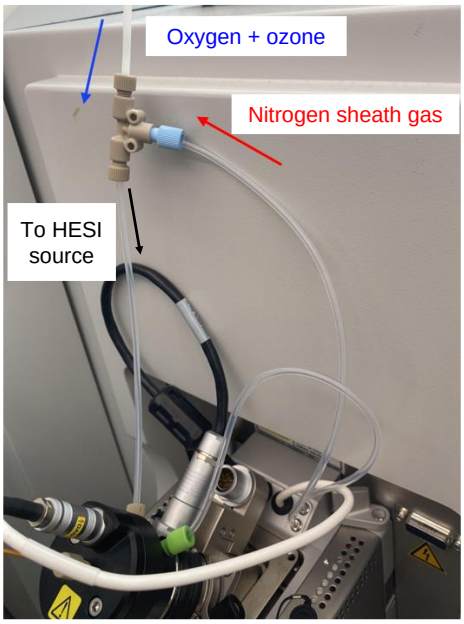




OzNOx Companion is a prototype software designed to assist in LC-OzNOxESI-MS/MS analysis of lipid double-bond positions. The OzNOxESI methodology requires an LC-MS/MS instrument with a HESI source where the sheath gas line can be accessed to introduce oxygen and ozone as pictured above. See the OzNOxESI publication for more details on this methodology and the underlying ion chemistry. There may well be other ways of achieving the OzNOx ion chemistry.

OzNOx Companion is available on Github as its Python source code. A Windows-compatible .exe version requiring no Python install or experience is available by request: q_zhang2@uncg.edu

OzNOx Companion is composed of 8 scripts:

- Script 1: Select project folder and load/update LC-MS annotations, parameter files
- Script 2: Process LC-MS annotations for manual RT-based validation/rejection
- Script 3: Output a PRM list for LC-OzNOxESI-MS^2 for your LC-MS annotations
- Script 4: Convert .txt LC-MS and PRM LC-MS^2 data to compatible .csv
- Script 5: Search for presumptive OzID MS1 and OzNOx MS2 double-bond product species
- Script 6: Unite and organize Script 5 output(s)
- Script 7: Double-bond annotation and regioisomer quantification with Script 6 output
- Script 8: Visualize a text-based spectrum from Script 5, 6, or 7 output

OzNOx Companion utilizes several imported .csv files that allow for non-coder changes to key algorithm parameters related to the OzNOx ion chemistry. Example files are on Github.

Parameter files.

	A	B	C	D	E	F	G	H	I	J	K
1	Class Key	Num Tail Chains	Num Fatty Acyls	Num Sphingoid Bases	OzNOx MS1 m/z Shift	OzNOx MS2 Collision Energy	OzNOx MS2 Product Ion Scheme 1	Scheme 1 Additional Mass Loss	OzNOx MS2 Product Ion Scheme 2	Scheme 2 Additional Mass Loss	Comment
2	AcCa	1	1	0	76.97491	15	TRUE	0	FALSE	0	OzNOx MS2 p
3	Cer	2	1	1	76.97491	16	TRUE	18.01056	FALSE	0	OzNOx MS2 p
4	DG	2	2	0	76.97491	25	TRUE	35.03712	TRUE	18.0343724	OzNOx MS2 p
5	FA	1	1	0	76.97491	10	TRUE	17.02655	FALSE	0	OzNOx MS2 p
6	GalCer	2	1	1	76.97491	22	TRUE	180.06339	FALSE	0	OzNOx MS2 p
7	Hex1Cer	2	1	1	76.97491	22	TRUE	180.06339	FALSE	0	GalCer is a re
8	Hex2Cer	2	1	1	76.97491	22	TRUE	342.11621	FALSE	0	Placeholder r
9	Hex3Cer	2	1	1	76.97491	22	TRUE	504.16903	FALSE	0	Placeholder r
10	LBPA	2	2	0	76.97491	15	TRUE	35.037112	FALSE	0	OzNOx MS2 p
11	LPA	1	1	0	76.97491	23	TRUE	115.00344	FALSE	0	Placeholder r
12	LPC	1	1	0	76.97491	15	TRUE	0	FALSE	0	Placeholder r
13	LPE	1	1	0	76.97491	16	TRUE	0	FALSE	0	OzNOx MS2 p
14	LPG	1	1	0	76.97491	29	TRUE	172.01368	FALSE	0	OzNOx MS2 p
15	LPI	1	1	0	76.97491	23	TRUE	277.05627	FALSE	0	Placeholder r
16	LPS	1	1	0	76.97491	23	TRUE	185.00892	FALSE	0	Placeholder r
17	MG	1	1	0	76.97491	30	TRUE	35.03712	FALSE	0	OzNOx MS2 p
18	PA	2	2	0	76.97491	23	TRUE	115.00344	FALSE	0	OzNOx MS2 p
19	PC	2	2	0	76.97491	15	TRUE	0	FALSE	0	OzNOx MS2 p
20	PE	2	2	0	76.97491	25	TRUE	141.01909	FALSE	0	OzNOx MS2 p
21	PG	2	2	0	76.97491	25	TRUE	189.04022	FALSE	0	OzNOx MS2 p
22	PI	2	2	0	76.97491	23	TRUE	277.05627	FALSE	0	OzNOx MS2 p
23	PS	2	2	0	76.97491	23	TRUE	185.00892	FALSE	0	OzNOx MS2 p
24	SM	2	1	1	76.97491	15	TRUE	0	FALSE	0	OzNOx MS2 p
25	SPH	1	0	1	76.97491	16	TRUE	18.01056	FALSE	0	Placeholder r
26	TG	3	3	0	76.97491	15	TRUE	0	TRUE	18.0343724	Further optin
27											

The core .csv parameter file, example above, is the class definitions file. Per class, various values are assigned for key algorithm parameters. In your project folder, this file must be titled some variation of "__OzNOx class definitions__.csv" (not case-sensitive). Examples:

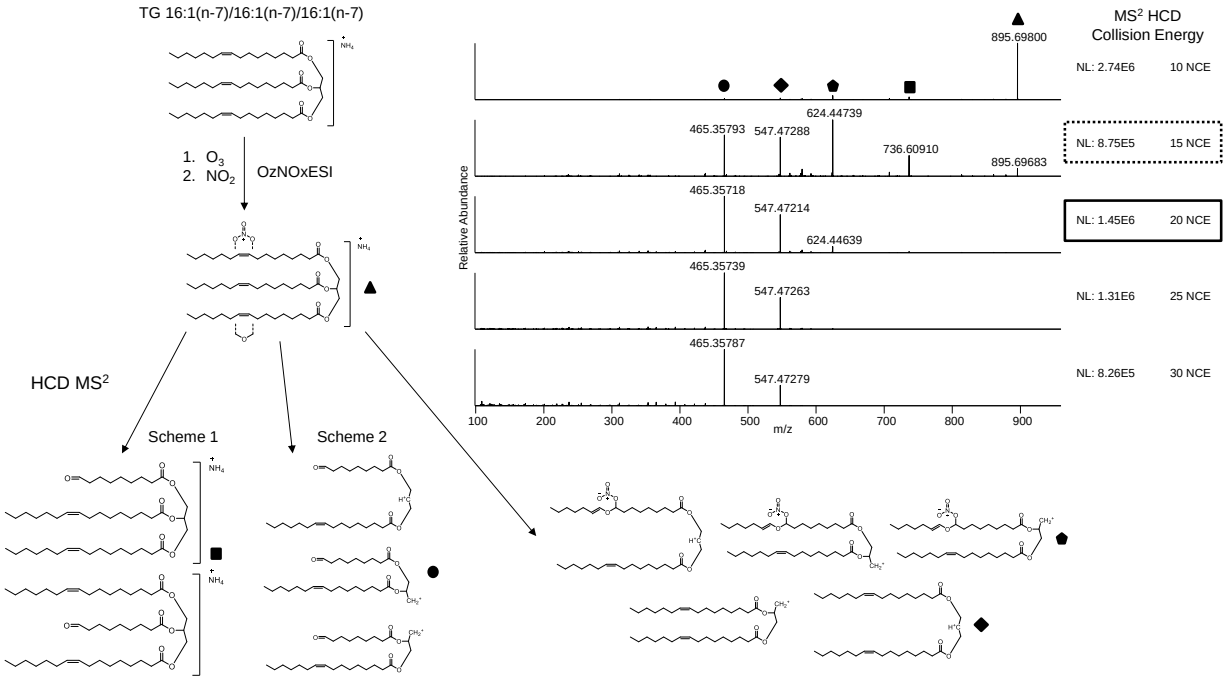
- oznox class definitions study2_plasma.csv
- Zhang Lab OzNOx Class Definitions 20240823.csv

If more than one .csv file meeting the naming requirement is present in the project folder, the most recently modified file will be selected by the program.
All the columns displayed above are required (case-sensitive) except for the 'Comment' field:

- Class Key: used to match these parameter rows to annotations with the same Class Key
- Num Tail Chains: the number of tail chains this class has
- Num Fatty Acyls: the number of fatty acyls this class has
- Num Sphingoid Bases: the number of sphingoid bases this class has
- OzNOx MS1 m/z Shift: the mass gain for the targeted OzNOxESI adduct. We have targeted the +77 Da adduct, but there are others you might consider.
- OzNOx MS2 Collision Energy: the MS^2 collision energy you intend for this class
- OzNOx MS2 Product Ion Scheme 1: True/False whether this class follows scheme 1. The product of this fragmentation scheme is analogous to OzESI/OzID[1-4] ozonolysis aldehydes. These products can be identical to ozonolysis aldehydes (additional mass loss of 0), or they could lose an additional moiety, such as the lipid head group. See example on next page.
- Scheme 1 Additional Mass Loss: additional mass loss in scheme 1 product (such as head group)
- OzNOx MS2 Product Ion Scheme 2: True/False whether this class follows scheme 2. This OzNOxESI-MS^2 scheme is similar to the first but involves the additional loss of a fatty acyl. See example on next page.
- Scheme 2 Additional Mass Loss: additional mass loss in scheme 2 product

Note that an error will arise if you have annotations defined with a Class Key not defined in this file. You can have classes that are 'FALSE' for both OzNOx MS2 Product Ion Schemes, meaning their MS^2 spectra will not be interrogated during data processing.

Example of Scheme 1 and Scheme 2 OzNOx-MS^2 product ions.



Pictured above is a direct infusion experiment with a TG standard. TG undergoes both Scheme 1 and Scheme 2 OzNOx-MS^2 fragmentation, in addition to a 3rd scheme currently not coded into OzNOx Companion. The Scheme 1 products are identical to OzESI products visible at the MS^1 level. The Scheme 2 products are the same species, with the additional loss of a fatty acyl. The unnamed 3rd scheme is similar in that there is the loss of a fatty acyl, but the derivatized C=C remains undissociated or is in fact in the lost acyl. In future method development, we will likely utilize all 3 of these schemes to fully interrogate TG isomerism and that of other similarly complex classes. Note in this example that the relative intensity of the different Schemes' products varies with collision energy. In your own analyses, you may consider a collision energy that maximizes one product scheme (20 NCE above), or you may consider a collision energy that gives a balanced spectrum (15 NCE above) with more products, more information.

Parameters files continued.

	A	B	C	D	E	F	G	H
1	Parent ID	Reject	Annotation	Class Key	Tail Key	m/z	RT	z
2	1	FALSE	AcCa(14:0)	AcCa	(14:0)	372.31049	4.362	1
3	2	FALSE	AcCa(24:3)_1	AcCa	(24:3)	506.42081	7.341	1
4	3	FALSE	AcCa(24:3)_2	AcCa	(24:3)	506.41965	8.051	1
5	4	FALSE	AcCa(26:3)	AcCa	(26:3)	534.4519	8.499	1
6	5	FALSE	Cer(d12:0_16:0)	Cer	(d12:0_16:0)	456.44086	7.774	1
7	15	FALSE	Cer(d18:1_23:0)	Cer	(d18:1_23:0)	636.62878	20.503	1
8	16	FALSE	Cer(d18:1_24:0)	Cer	(d18:1_24:0)	650.64331	20.785	1
9	18	FALSE	Cer(d19:1_16:1)	Cer	(d19:1_16:1)	550.51849	13.024	1
10	23	FALSE	Cer(d18:1_24:1)	Cer	(d18:1_24:1)	648.62781	20.158	1
11	89	FALSE	d5-LPC(15:0)	LPC	(15:0)	487.35541	4.807	1
12	90	FALSE	LPC(16:0)	LPC	(16:0)	496.33923	5.305	1
13	91	FALSE	d5-LPC(17:0)	LPC	(17:0)	515.38849	5.76	1
14	92	FALSE	LPC(18:0)_1	LPC	(18:0)	524.37006	6.054	1
15	93	FALSE	LPC(18:0)_2	LPC	(18:0)	524.37018	6.258	1
16	94	FALSE	d5-LPC(19:0)	LPC	(19:0)	543.41907	6.806	1
17	95	FALSE	LPC(18:2)	LPC	(18:2)	520.33948	4.671	1
18	96	FALSE	LPC(20:4)	LPC	(20:4)	544.33911	4.583	1
19	202	FALSE	d5-PE(17:0_20:3)	PE	(17:0_20:3)	761.58527	14.84	1
20	203	FALSE	d5-PE(17:0_22:4)	PE	(17:0_22:4)	787.59985	15.489	1
21	204	FALSE	PG(O-18:2)	PG	(O-18:2)	526.31641	5.342	1
22	205	FALSE	PG(O-20:2)	PG	(O-20:2)	554.34656	6.175	1
23	206	FALSE	PG(16:0_16:1)	PG	(16:0_16:1)	738.52808	10.519	1
24	286	FALSE	TG(O-15:0_18:0_3:0)	TG	(O-15:0_18:0_3:0)	642.60248	20.652	1
25	287	FALSE	TG(12:0_14:0_14:0)	TG	(12:0_14:0_14:0)	712.64465	21.212	1
26	288	FALSE	d5-TG(13:0_14:0_14:0)	TG	(13:0_14:0_14:0)	731.69202	21.357	1
27	289	FALSE	TG(12:0_14:0_16:0)	TG	(12:0_14:0_16:0)	740.67682	21.532	1
28	297	FALSE	d5-TG(14:0_14:0_15:1)	TG	(14:0_14:0_15:1)	757.70721	21.381	1
29	305	FALSE	d5-TG(16:0_16:0_17:1)	TG	(16:0_16:0_17:1)	841.80267	22.125	1
30	306	FALSE	TG(16:0_16:0_18:1)	TG	(16:0_16:0_18:1)	850.78595	22.28	1
31	316	FALSE	d5-TG(16:0_16:0_19:2)	TG	(16:0_16:0_19:2)	867.81635	22.158	1

The core .csv data file, example above, is the LC-MS annotations file. These are regular LC-MS(/MS) lipid annotations before double-bond analysis. In your project folder, this file must be titled some variation of "__OzNOx LC-MS annotations__.csv" (not case-sensitive). Examples:

- oznox lc-ms annotations study2_plasma.csv
- Zhang Lab OzNOx LC-MS Annotations 20240823.csv

All the columns displayed above are required (case-sensitive):

- Parent ID: indexing value between different files, must be unique here.
- Reject: whether or not to exclude this annotation. With 'TRUE', you can eliminate annotations from data processing without deleting them.
- Annotation: name of the annotation, must be unique.
- Class Key: key representation of the lipid class, must be represented in class definitions file
- Tail Key: key representation of the lipid tail(s). Parentheses are not required as pictured, but you may find these difficult to edit in Excel without parentheses due to automated conversion to time fields. Valid examples: d18:1_18:0, d18:1/18:0, d36:1, (d36:1), (d18:1/18:0-d5). Note that while deuterated Tail Keys can be interpreted for tail structure, the deuterium mass cannot be interpreted by OzNOx Companion at this time and can lead to erroneous outputs when dueterium is on an unsaturated tail as with d18:1-d7. This can be compensated for by making a deuterated class entry in the class definitions, but a more elegant solution is forthcoming.
- m/z, RT, and z: typical LC-MS values as understood and widely used in the field

Parameter files continued.

	A	B	C	D
1	Sample Name	OzID (MS1)	OzNOx (MS2)	Sample Group
2	ddMS2 1	TRUE	TRUE	Plasma
3	ddMS2 2	TRUE	TRUE	Plasma
4	ddMS2 3	TRUE	TRUE	Plasma
5	PRM 1	FALSE	TRUE	Plasma
6	PRM 2	FALSE	TRUE	Plasma
7	PRM 3	FALSE	TRUE	Plasma
8	ddMS2 4	TRUE	TRUE	Liver
9	ddMS2 5	TRUE	TRUE	Liver
10	ddMS2 6	TRUE	TRUE	Liver
11	PRM 4	FALSE	TRUE	Liver
12	PRM 5	FALSE	TRUE	Liver
13	PRM 6	FALSE	TRUE	Liver
14				

The core .csv sample file, example above, is the sample definitions file. This file defines sample groups, replicates, and the type of analysis. In your project folder, this file must be titled some variation of "__OzNOx sample definitions__.csv" (not case-sensitive). Examples:

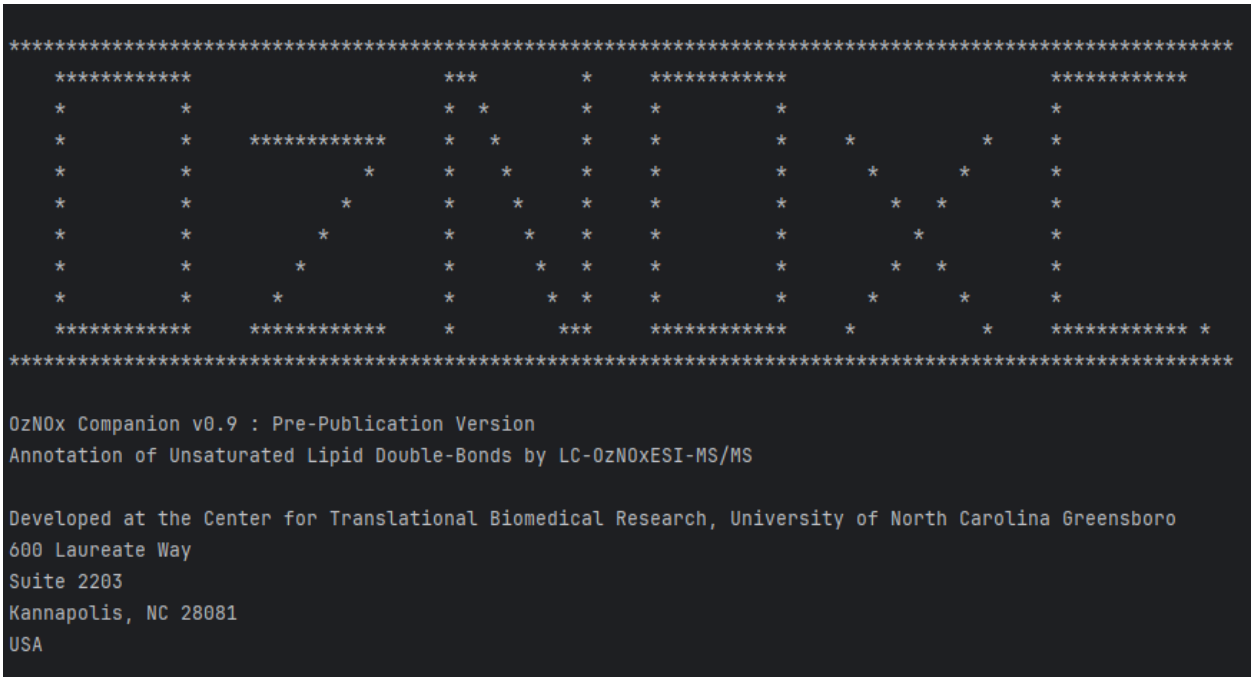
- oznox sample definitions study2_plasma vs liver.csv
- Zhang Lab OzNOx sample definitions 20240823.csv

All the column displayed above are required (case-sensitive):

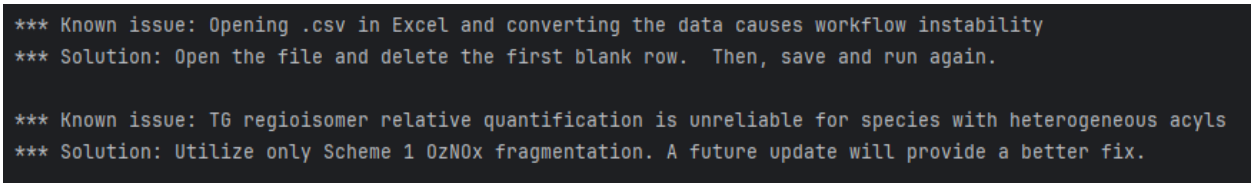
- Sample Name: used to reference the LC-MS(/MS) data files, which must bear matching names (PRM 1 to PRM 1.txt or PRM 1.csv). These must be unique.
- OzID (MS1): TRUE/FALSE whether this data file contains MS1 ozonolysis aldehydes
- OzNOx (MS2): TRUE/FALSE whether this data file contains OzNOxESI-MS^2
- Sample Group: used to group replicates and different analyses based on the sample group. Pictured above, there are two sample groups: Plasma and Liver. When analyzing OzNOxESI-MS^2 data for double-bonds in the Plasma set, OzNOx Companion will generate aligned spectra from the PRM 1, PRM 2, and PRM 3 samples and report the resulting analyses under a Plasma-derived heading. If there were no PRM datasets, OzNOx Companion would instead use ddMS2 1, ddMS2 2, and ddMS2 3.

Currently, OzNOx Companion reports OzESI product ions, present in the ddMS2 examples above, but does not utilize them in double-bond assignments. We plan to utilize both the MS1 and MS2 data of the same sample group in a future update.

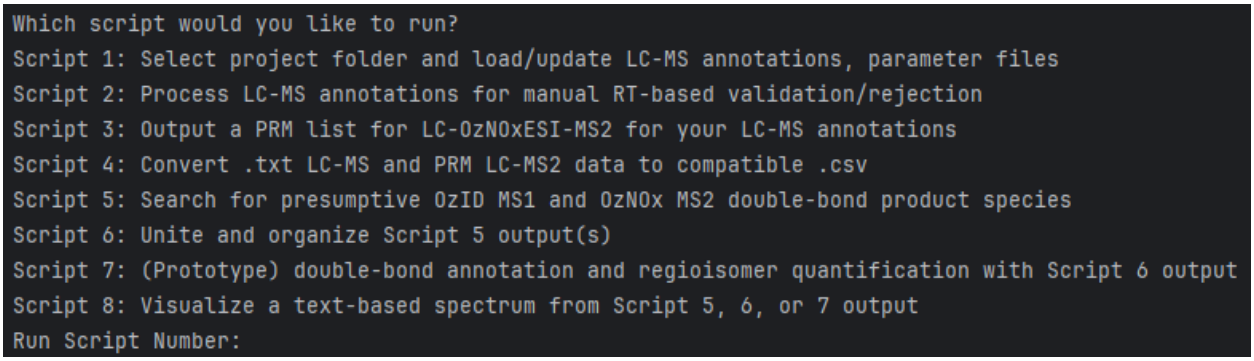
Main menu.



Upon launch, the program will initialize with a generic black window and print text about the program, its version, and documentation.



Check for known issues related to your version of OzNOx Companion.



The program then begins a loop where it will ask which script you want to run, it will run that script if the requirements are satisfied, and then it will return to the main menu. A script is selected by typing its number and hitting Enter, such as 3 for Script 3.

Script 1: Select project folder and load/update LC-MS annotations, parameter files

```
Which script would you like to run?
Script 1: Select project folder and load/update LC-MS annotations, parameter files
Script 2: Process LC-MS annotations for manual RT-based validation/rejection
Script 3: Output a PRM list for LC-OzNOxESI-MS2 for your LC-MS annotations
Script 4: Convert .txt LC-MS and PRM LC-MS2 data to compatible .csv
Script 5: Search for presumptive OzID MS1 and OzNOx MS2 double-bond product species
Script 6: Unite and organize Script 5 output(s)
Script 7: (Prototype) double-bond annotation and regioisomer quantification with Script 6 output
Script 8: Visualize a text-based spectrum from Script 5, 6, or 7 output
Run Script Number: 1

Beginning Script 1 ...

Enter project folder directory (copy from top of file explorer): C:\Users\rasmith12\OneDrive - UNCG\

Selecting OzNOx class definitions .csv file ...
OzNOx class definitions found: OzNOx class definitions.csv
Proceeding with newest OzNOx class definitions: OzNOx class definitions.csv
Checking OzNOx class definitions: OzNOx class definitions.csv
OzNOx Class Definitions file looks good.

Selecting OzNOx LC-MS annotations .csv file ...
OzNOx LC-MS annotations found: OzNOx LC-MS annotations.csv
OzNOx LC-MS annotations found: OzNOx LC-MS Annotations 2024_08_22_17_26_09.csv
Proceeding with newest OzNOx LC-MS annotations: OzNOx LC-MS Annotations 2024_08_22_17_26_09.csv
Checking OzNOx class definitions: OzNOx LC-MS Annotations 2024_08_22_17_26_09.csv
OzNOx LC-MS annotations file looks good.

Script 1 finished.  LC-MS annotations are ready for processing
```

Most of the scripts require that you first load the basic parameter files and assign a project folder. This is accomplished through script 1. This script will ask for the directory of your project folder, which you can obtain by opening the location in File Explorer and copying the directory from the address bar at the top of the window. The project folder must contain your class definitions and LC-MS annotations .csv files. Script 1 will check these files, report any issues, and then return to the main menu if there were no issues. When searching for parameter files, OzNOx Companion will always utilize the newest or most recently modified file found. Under OzNOx LC-MS annotations above, we can see that it found two different LC-MS annotations files, and it tells us which one was selected.

Script 2: Process LC-MS annotations for manual RT-based validation/rejection

```
Which script would you like to run?
Script 1: Select project folder and load/update LC-MS annotations, parameter files
Script 2: Process LC-MS annotations for manual RT-based validation/rejection
Script 3: Output a PRM list for LC-OzNOxESI-MS2 for your LC-MS annotations
Script 4: Convert .txt LC-MS and PRM LC-MS2 data to compatible .csv
Script 5: Search for presumptive OzID MS1 and OzNOx MS2 double-bond product species
Script 6: Unite and organize Script 5 output(s)
Script 7: (Prototype) double-bond annotation and regioisomer quantification with Script 6 output
Script 8: Visualize a text-based spectrum from Script 5, 6, or 7 output
Run Script Number: 2

Beginning Script 2 ...

Beginning Tail Key component analyses...
Tail Key component analyses complete.

Beginning RT curve modeling ...

On Class AcCa ...
RT = 0.40149999999995334 * TailCarbons + -0.2269999999999639 * TailDBs + -1.2589999999993466

On Class Cer ...
RT = 0.771790740176671 * TailCarbons + 1.2514950248756243 * TailDBs + -12.96792486546861
For 0 DB, RT = 0.040723214285714265 * TailCarbons^2 + -1.8088035714285708 * TailCarbons + 26.48299999999999
For 1 DB, RT = -0.07064276410998586 * TailCarbons^2 + 5.698071345875568 * TailCarbons + -94.25884023154897

On Class DG ...
RT = 0.7393297104725587 * TailCarbons + -1.027880271767242 * TailDBs + -5.733567150368508
For 0 DB, RT = 0.021169957046573692 * TailCarbons^2 + -0.33851584853388356 * TailCarbons + 7.152973174479434
For 1 DB, RT = -0.048976190476197674 * TailCarbons^2 + 4.237214285714776 * TailCarbons + -68.84666666667502
For 2 DB, RT = 0.00950000000000194 * TailCarbons^2 + 0.46769999999986867 * TailCarbons + -9.935199999997774
For 3 DB, RT = -0.2410543478260792 * TailCarbons^2 + 18.337271739129896 * TailCarbons + -329.3465217391212
For 4 DB, RT = -0.07946739130436946 * TailCarbons^2 + 6.866706521740807 * TailCarbons + -127.66513043481507
```

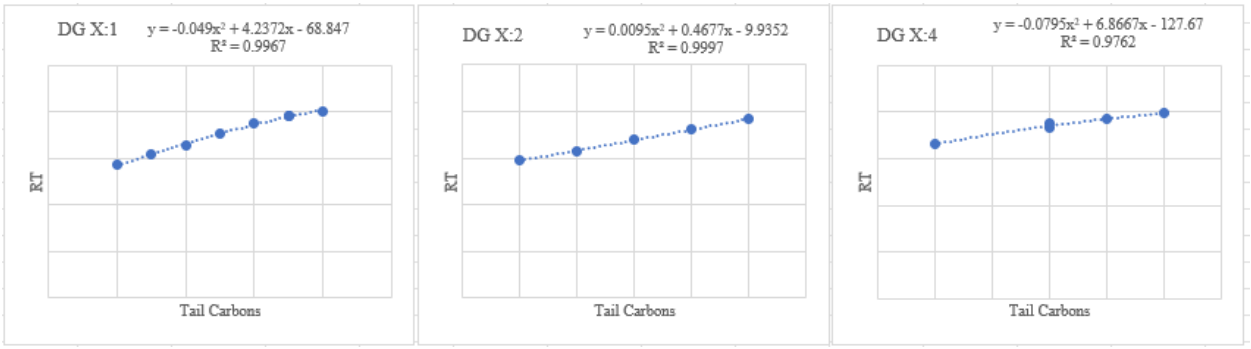
```
On Class TG ...
RT = 0.12717971789885257 * TailCarbons + -0.22426653696501386 * TailDBs + 16.138001945523534
For 0 DB, RT = -0.001900020021555133 * TailCarbons^2 + 0.292456487196855 * TailCarbons + 12.577847273873555
For 1 DB, RT = -0.0018961141950829168 * TailCarbons^2 + 0.3021724821569846 * TailCarbons + 11.896511498811282

Script 2 output available as OzNOx LC-MS Annotations 2024_08_23_10_31_59.csv

Script 2 finished.  Perform manual validation/rejection and rerun Script 1 to update the program dataframe.
```

Script 2 assists in RT-based validation of the initial LC-MS annotations. This is useful if you, like us, make your initial annotations with a third-party program that does not consider RT, just relying on MS^1 m/z and the MS^2 spectra. Script 2 makes a new LC-MS annotations file, leaving the original unedited, reorganized for convenient RT-based validation and has two new columns for Model RT and the discrepancy between RT and Model RT. To reject one or more annotation(s), change the Reject field to TRUE and rerun script 1 with the updated file. See the next page for an example of the script 2 output and how to perform RT-based validation.

Script 2: Process LC-MS annotations for manual RT-based validation/rejection continued

	A	B	C	D	E	F	G	H	I	J	K	L	M
1	Parent ID	Reject	Annotation	Class Key	Tail Key	m/z	z	Tail Carbons	Tail Double-Bonds	Tail Hydroxyls	RT	Model RT	Abs (Measured RT - Modeled RT)
2	46	FALSE	DG(6:0_10:0)	DG	(6:0_10:0)	362.28946	1	16	0	0	6.983	7.156	0.173
3	47	FALSE	DG(6:0_11:0)	DG	(6:0_11:0)	376.30472	1	17	0	0	7.736	7.516	0.220
4	48	FALSE	DG(12:0_16:0)	DG	(12:0_16:0)	530.47711	1	28	0	0	13.943	14.272	0.329
5	49	FALSE	DG(14:0_16:0)	DG	(14:0_16:0)	558.50818	1	30	0	0	16.152	16.050	0.102
6	50	FALSE	DG(15:0_16:0)	DG	(15:0_16:0)	572.52478	1	31	0	0	17.32	17.003	0.317
7	51	FALSE	DG(16:0_18:0)	DG	(16:0_18:0)	614.57172	1	34	0	0	19.98	20.116	0.136
8	52	FALSE	DG(14:0_16:1)	DG	(14:0_16:1)	556.49359	1	30	1	0	14.292	14.191	0.101
9	53	FALSE	d5-DG(17:0_14:1)	DG	(17:0_14:1)	575.53967	1	31	1	0	15.386	15.441	0.055
10	54	FALSE	DG(16:0_16:1)	DG	(16:0_16:1)	584.52527	1	32	1	0	16.443	16.593	0.150
11	55	FALSE	d5-DG(17:0_16:1)	DG	(17:0_16:1)	603.57178	1	33	1	0	17.579	17.646	0.067
12	56	FALSE	DG(16:0_18:1)	DG	(16:0_18:1)	612.55634	1	34	1	0	18.738	18.602	0.136
13	57	FALSE	DG(17:0_18:1)	DG	(17:0_18:1)	626.57214	1	35	1	0	19.555	19.460	0.095
14	58	FALSE	d5-DG(17:0_18:1)	DG	(17:0_18:1)	631.60522	1	35	1	0	19.563	19.460	0.103
15	59	FALSE	DG(18:0_18:1)	DG	(18:0_18:1)	640.58673	1	36	1	0	20.057	20.220	0.163
16	60	FALSE	DG(14:0_18:2)	DG	(14:0_18:2)	582.50812	1	32	2	0	14.779	14.759	0.020
17	61	FALSE	DG(15:0_18:2)	DG	(15:0_18:2)	596.5249	1	33	2	0	15.806	15.844	0.038
18	62	FALSE	DG(16:0_18:2)	DG	(16:0_18:2)	610.54224	1	34	2	0	16.945	16.949	0.004
19	63	FALSE	DG(17:0_18:2)	DG	(17:0_18:2)	624.55585	1	35	2	0	18.115	18.072	0.043
20	64	FALSE	DG(18:0_18:2)	DG	(18:0_18:2)	638.57214	1	36	2	0	19.193	19.214	0.021
21	65	FALSE	DG(16:1_18:2)	DG	(16:1_18:2)	608.52386	1	34	3	0	15.406	15.462	0.056
22	66	FALSE	DG(16:0_20:3)	DG	(16:0_20:3)	636.55603	1	36	3	0	17.55	18.389	0.839
23	67	FALSE	DG(17:0_19:3)	DG	(17:0_19:3)	636.55786	1	36	3	0	19.563	18.389	1.174
24	68	FALSE	d5-DG(17:0_20:3)	DG	(17:0_20:3)	655.60455	1	37	3	0	18.682	19.129	0.447
25	69	FALSE	DG(18:0_20:3)	DG	(18:0_20:3)	664.58716	1	38	3	0	19.555	19.387	0.168
26	70	FALSE	DG(16:0_20:4)	DG	(16:0_20:4)	634.54016	1	36	4	0	16.545	16.547	0.002
27	71	FALSE	DG(16:0_22:4)	DG	(16:0_22:4)	662.57239	1	38	4	0	18.248	18.519	0.271
28	72	FALSE	DG(18:0_20:4)	DG	(18:0_20:4)	662.57031	1	38	4	0	18.799	18.519	0.280
29	73	FALSE	d5-DG(17:0_22:4)	DG	(17:0_22:4)	681.61896	1	39	4	0	19.254	19.267	0.013
30	74	FALSE	DG(18:0_22:4)	DG	(18:0_22:4)	690.60229	1	40	4	0	19.86	19.855	0.005
31	75	FALSE	DG(16:0_22:5)_1	DG	(16:0_22:5)	660.55542	1	38	5	0	16.717	17.222	0.505
32	76	FALSE	DG(16:0_22:5)_2	DG	(16:0_22:5)	660.55554	1	38	5	0	17.429	17.222	0.207
33	77	FALSE	DG(18:0_22:5)_3	DG	(18:0_22:5)	688.58728	1	40	5	0	18.992	18.700	0.292
34	78	FALSE	DG(18:0_22:5)_4	DG	(18:0_22:5)	688.5871	1	40	5	0	19.508	18.700	0.808

If your LC-MS annotations file looked like the example on page 3, the new LC-MS annotations file generated by script 2 will look something like the example above, minus the color-coding which was added in Excel. Highlighted in green, these columns organized at the end are essential for RT-based validation of lipid annotations in RPLC LC-MS. Highlighted in blue and red are sections of annotations belonging to the same class, DG, with increasing degree of unsaturation, number of tail double-bonds. When generating Tail Carbons vs RT curves within these sections, the curve should readily indicate if an annotation is erroneous, along with the Model RT and RT discrepancy columns after. Note that the quality of the Model RT prediction is a function of the dataset: how many annotations there are and with what degree of unsaturation diversity. Again, to reject an annotation, you can either delete it or simply change the Reject field to TRUE. After finishing validation/rejection, save the file and rerun script 1 to update the internal DataFrame.



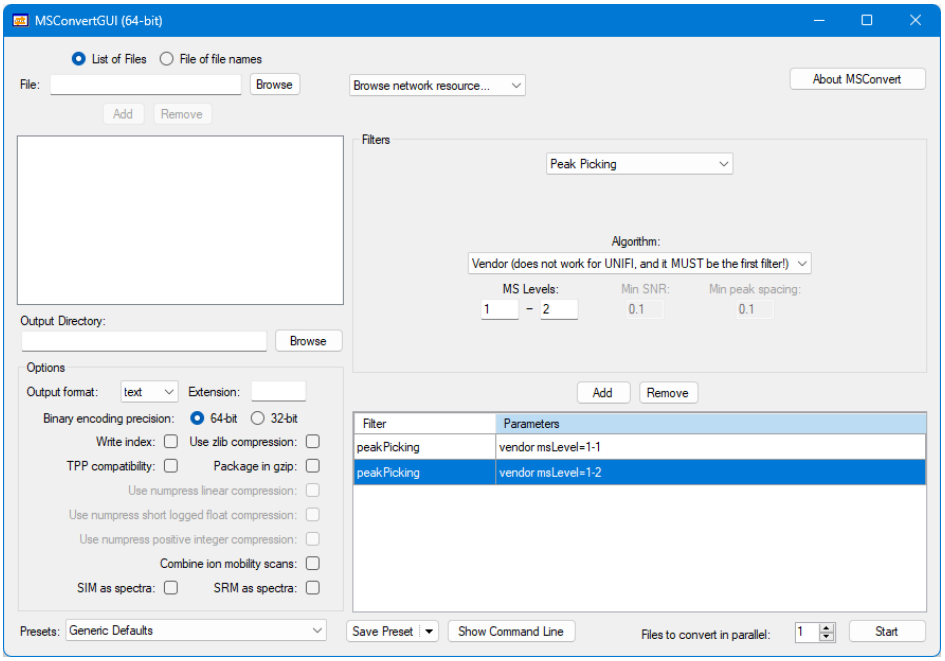
Script 3: Output a PRM list for LC-OzNOxESI-MS^2 for your LC-MS annotations

```
Which script would you like to run?
Script 1: Select project folder and load/update LC-MS annotations, parameter files
Script 2: Process LC-MS annotations for manual RT-based validation/rejection
Script 3: Output a PRM list for LC-OzNOxESI-MS2 for your LC-MS annotations
Script 4: Convert .txt LC-MS and PRM LC-MS2 data to compatible .csv
Script 5: Search for presumptive OzID MS1 and OzNOx MS2 double-bond product species
Script 6: Unite and organize Script 5 output(s)
Script 7: (Prototype) double-bond annotation and regioisomer quantification with Script 6 output
Script 8: Visualize a text-based spectrum from Script 5, 6, or 7 output
Run Script Number: 3

Beginning Script 3 ...

Enter PRM RT window width: 0.5

Script 3 output available as OzNOx PRM list 2024_08_23_10_58_40.csv

Script 3 finished.  Use the PRM list generated to conduct LC-MS2 analyses.
```

	A	B	C	D	E	F	G	H	I	J	K
1	Mass [m/z]	Formula [M]	Formula type	Species	CS [z]	Polarity	Start [min]	End [min]	(N)CE	MSX ID	Comment
2	583.39572				1	Positive	7.091	7.591	15		
3	583.39456				1	Positive	7.801	8.301	15		
4	611.42681				1	Positive	8.249	8.749	15		
5	573.44622				1	Positive	14.182	14.682	16		
6	601.47863				1	Positive	16.464	16.964	16		
7	629.50878				1	Positive	18.798	19.298	16		
8	699.58654				1	Positive	19.939	20.439	16		
9	713.60369				1	Positive	20.253	20.753	16		
10	727.61822				1	Positive	20.535	21.035	16		
11	627.4934				1	Positive	12.774	13.274	16		
12	725.60272				1	Positive	19.908	20.408	16		
13	696.59021				1	Positive	21.684	22.184	35		
14	724.62158				1	Positive	21.973	22.473	35		
15	717.57678				1	Positive	21.99	22.49	35		
16	731.59301				1	Positive	22.142	22.642	35		
17	752.65271				1	Positive	22.274	22.774	35		

From the currently loaded LC-MS annotations DataFrame and the class definitions file, script 3 will generate a PRM target list compatible with Thermo instruments. Script 3 queries the user for a PRM RT window width. For example, if an LC-MS feature annotation had an RT of 4.00 and a window width of 0.50 was provided, the resulting PRM acquisition would run from 3.75 to 4.25. Additional editing may be needed depending on your instrument's manufacturer or software version.

Script 4: Convert .txt LC-MS and PRM LC-MS^2 data to compatible .csv



OzNOx Companion utilizes instrument data files in its own generic .csv format, which it converts from .txt format. To arrive at .txt format, please use the freely available MSConvert[5], which can be used to convert a variety of proprietary vendor formats, Thermo's .raw in our work. The recommended settings are pictured above. To save space, you can optionally do MS Levels 2 – 2, eliminating MS1 data, but this will prevent reporting of OzESI aldehydes.

```
Beginning Script 4 ...

Enter LC-MS(/MS) .txt data folder directory (copy from top of file explorer): C:\Users

Data found: PRM 1.txt
Data found: PRM 2.txt
Data found: PRM 3.txt

Enter LC-MS(/MS) .csv data folder directory (copy from top of file explorer): C:\Users

Converting PRM 1.txt ...
Finished converting: C:\Users\rasmith12\OneDrive - UNC6\Desktop\OzNOx manuscript\data

Converting PRM 2.txt ...
Finished converting: C:\Users\rasmith12\OneDrive - UNC6\Desktop\OzNOx manuscript\data

Converting PRM 3.txt ...
Finished converting: C:\Users\rasmith12\OneDrive - UNC6\Desktop\OzNOx manuscript\data

Script 4 finished.  The LC-MS and LC-MS2 .csv files created can be used by Script 5.
```

Script 4 will ask for the folder directory of your .txt data files as well as the folder directory that you would like the new .csv data files deposited in. It is best practice to have this be a dedicated folder apart from or nested inside of your project folder to avoid overwriting other .csv files.

Script 5: Search for presumptive OzID MS1 and OzNOx MS2 double-bond product species

```
Beginning Script 5 ...

Enter LC-MS(/MS) .csv data folder directory (copy from top of file explorer): C:\Users\rasmith12\

Found compatible .csv data: PRM 1.csv
Found compatible .csv data: PRM 2.csv
Found compatible .csv data: PRM 3.csv

Enter 0 to select a different folder.
Enter 1 to proceed with MS1 OzID aldehyde m/z detection.
Enter 2 to proceed with MS2 OzNOx product ion detection.
Enter 3 to proceed with both MS1 OzID aldehyde and OzNOx product ion detection.

Enter 0, 1, 2, or 3: 3

Enter maximum OzID reactions per molecule: 1

Enter MS1 m/z tolerance (such as 0.003): 0.003

Enter MS1 RT tolerance (such as 0.10): 0.10

Enter MS2 m/z tolerance (such as 0.005): 0.005

Enter MS2 RT tolerance (such as 0.10): 0.10

Beginning processing at time stamp 2024_08_23_11_10_01
Seeking OzID aldehyde signals: True
Seeking OzNOx product ion signals: True
Maximum OzID reactions per molecule: 1
OzID aldehyde m/z tolerance: 0.003
OzID aldehyde RT tolerance: 0.1
OzNOx precursor m/z tolerance: 0.003
OzNOx precursor RT tolerance: 0.1
OzNOx product ion m/z tolerance: 0.005
```

Script 5 searches through .csv data for OzESI and OzNOxESI-MS^2 product ions based on your loaded LC-MS annotations and class definitions. First, it will ask for the directory of your .csv data files. Then, it will report what compatible .csv data files were found in that directory. Then it will ask whether you wish to select a different folder, proceed with OzESI product detection, proceed with OzNOxESI-MS^2 product detection, or proceed with both product detections. For these calculations, several additional parameters must be provided:

- Maximum OzID reactions per molecule: relevent for OzESI products, the maximum number of OzID cleavages. For example, TG 16:1/18:1/20:1 can undergo up to 3 cleavages. Allowing for more than one theoretical cleavage generates interesting data, but it is difficult to interpret.
- MS1/2 m/z tolerance: the max MS m/z difference between detected and theoretical feature
- MS1/2 RT tolerance: the max LC RT difference between detected and annotation feature

Script 5: Search for presumptive OzID MS1 and OzNOx MS2 double-bond product species continued

```
On data file 1 of 3: PRM 1.csv

On LC-MS annotation 1 of 269: AcCa(14:0)
On LC-MS annotation 2 of 269: AcCa(24:3)_1
On LC-MS annotation 3 of 269: AcCa(24:3)_2
On LC-MS annotation 4 of 269: AcCa(26:3)
On LC-MS annotation 5 of 269: Cer(d12:0_16:0)
On LC-MS annotation 6 of 269: Cer(d14:0_16:0)
On LC-MS annotation 7 of 269: Cer(d16:0_16:0)
On LC-MS annotation 8 of 269: Cer(d16:0_18:0)
On LC-MS annotation 9 of 269: Cer(d18:0_18:0)
On LC-MS annotation 10 of 269: Cer(d20:0_18:0)
On LC-MS annotation 267 of 269: d5-TG(16:0_16:0_17:1)
On LC-MS annotation 268 of 269: TG(16:0_16:0_18:1)
On LC-MS annotation 269 of 269: d5-TG(16:0_16:0_19:2)

Script 5 output available as: C:\Users\rasmith12\OneDrive - UNC6\Desktop\OzNOx manuscript\data\20240719\Pla

Script 5 finished.  The newly created files in the project folder can be used for double-bond annotation.
```

	A	B	C	D	E	F	G	H	I	J	K	L	M	N
1	Parent ID	Is Parent	Annotation	Class Key	Tail Key	Oz(NOx) Key	Scheme 2 FA Loss	MS Level	Precursor m/z	m/z	User RT	Max Counts RT	Counts	RT:Counts Spectrum
2	16	TRUE	Cer(d18:1_24:0)	Cer	(d18:1_24:0)			1		650.6433	20.79			
3	16	FALSE	Cer(d18:1_24:0) n-14 OzID aldehyde with 0 DBs, 0 hydroxyls in neutral loss		(d18:1_24:0)	AN14DB0OH0		2	727.6182	452.4081		20.78	1.54E+04	20.686561:0.0 20.695281:0
4	23	TRUE	Cer(d18:1_24:1)	Cer	(d18:1_24:1)			1		648.6278	20.16			
5	23	FALSE	Cer(d18:1_24:1) n-9 OzID aldehyde with 0 DBs, 0 hydroxyls in neutral loss		(d18:1_24:1)	AN9DB0OH0		2	725.6027	520.4710		20.17	8.94E+04	20.07272:0.0 20.109461:0.0
6	53	TRUE	d5-DG(17:0_14:1)	DG	(17:0_14:1)			1		575.5397	15.39			
7	53	FALSE	d5-DG(17:0_14:1) n-5 OzID aldehyde with 0 DBs, 0 hydroxyls in neutral loss - FA 17:0		(17:0_14:1)	AN5DB0OH0	(17:0)	2	652.5146	234.1732		15.40	1.03E+06	15.300238:60365.359375 1
8	53	FALSE	d5-DG(17:0_14:1) n-5 OzID aldehyde with 0 DBs, 0 hydroxyls in neutral loss		(17:0_14:1)	AN5DB0OH0		2	652.5146	486.4191		15.40	9.70E+05	15.300238:47002.28125 15
9	54	TRUE	DG(16:0_16:1)	DG	(16:0_16:1)			1		584.5253	16.44			
10	54	FALSE	DG(16:0_16:1) n-7 OzID aldehyde with 0 DBs, 0 hydroxyls in neutral loss - FA 16:0		(16:0_16:1)	AN7DB0OH0	(16:0)	2	661.5002	229.1431		16.48	1.04E+05	16.357401:8436.40625 16.3
11	54	FALSE	DG(16:0_16:1) n-7 OzID aldehyde with 0 DBs, 0 hydroxyls in neutral loss		(16:0_16:1)	AN7DB0OH0		2	661.5002	467.3733		16.44	7.57E+04	16.357401:5646.53369141
12	54	FALSE	DG(16:0_16:1) n-9 OzID aldehyde with 0 DBs, 0 hydroxyls in neutral loss		(16:0_16:1)	AN9DB0OH0		2	661.5002	439.3419		16.39	3.96E+04	16.357401:7448.90673828
13	55	TRUE	d5-DG(17:0_16:1)	DG	(17:0_16:1)			1		603.5718	17.58			
14	55	FALSE	d5-DG(17:0_16:1) n-7 OzID aldehyde with 0 DBs, 0 hydroxyls in neutral loss - FA 17:0		(17:0_16:1)	AN7DB0OH0	(17:0)	2	680.5467	234.1740		17.58	3.62E+06	17.498253:262821.03125 1
15	55	FALSE	d5-DG(17:0_16:1) n-7 OzID aldehyde with 0 DBs, 0 hydroxyls in neutral loss		(17:0_16:1)	AN7DB0OH0		2	680.5467	486.4198		17.58	2.62E+06	17.498253:189334.234375
16	55	FALSE	d5-DG(17:0_16:1) n-8 OzID aldehyde with 0 DBs, 0 hydroxyls in neutral loss - FA 17:0		(17:0_16:1)	AN8DB0OH0	(17:0)	2	680.5467	220.1583		17.60	1.01E+04	17.498253:0.0 17.526862:4
17	56	TRUE	DG(16:0_18:1)	DG	(16:0_18:1)			1		612.5563	18.74			
18	56	FALSE	DG(16:0_18:1) n-9 OzID aldehyde with 0 DBs, 0 hydroxyls in neutral loss - FA 16:0		(16:0_18:1)	AN9DB0OH0	(16:0)	2	689.5313	229.1428		18.73	1.82E+06	18.655569:120633.265625
19	56	FALSE	DG(16:0_18:1) n-9 OzID aldehyde with 0 DBs, 0 hydroxyls in neutral loss		(16:0_18:1)	AN9DB0OH0		2	689.5313	467.3730		18.73	1.71E+06	18.655569:90639.609375 1
20	56	FALSE	DG(16:0_18:1) n-7 OzID aldehyde with 0 DBs, 0 hydroxyls in neutral loss		(16:0_18:1)	AN7DB0OH0		2	689.5313	495.4044		18.68	1.44E+05	18.655569:26253.3046875
21	56	FALSE	DG(16:0_18:1) n-7 OzID aldehyde with 0 DBs, 0 hydroxyls in neutral loss - FA 16:0		(16:0_18:1)	AN7DB0OH0	(16:0)	2	689.5313	257.1742		18.71	1.16E+05	18.655569:24025.7617188
22	58	TRUE	d5-DG(17:0_18:1)	DG	(17:0_18:1)			1		631.6052	19.56			
23	58	FALSE	d5-DG(17:0_18:1) n-9 OzID aldehyde with 0 DBs, 0 hydroxyls in neutral loss - FA 17:0		(17:0_18:1)	AN9DB0OH0	(17:0)	2	708.5801	234.1760		19.56	2.64E+06	19.472494:21854.953125 1
24	58	FALSE	d5-DG(17:0_18:1) n-9 OzID aldehyde with 0 DBs, 0 hydroxyls in neutral loss		(17:0_18:1)	AN9DB0OH0		2	708.5801	486.4219		19.59	2.34E+06	19.472494:14239.7558594

Script 5 iterates through each LC-MS annotation for each data file and creates one output .csv per data file like the example above. Note that these are generated in the script 1-assigned project folder with the same names as your .csv data files, which ideally are in a separate or nested folder. There will be one line per parent LC-MS annotation and then lines beneath for any double-bond related products found. The products will be in descending order by detector counts (intensity). Interpretation examples:

- For Cer d18:1/24:0, the n-14 sphingoid base double-bond was identified.

- For DG 16:0/18:1, we can see there is clearly n-9 / n-7 regioisomerism because both the scheme 1 and scheme 2 OzNOxESI-MS^2 products were identified for these regioisomers. The counts reported for these products can be used to determine the relative abundance of regioisomers.

This is PRM data, and so only OzNOxESI-MS^2 products could be queried. If it was ddMS^2 or otherwise contained OzESI data, then the parents would also have Counts and RT:Counts spectra and there would be OzESI products reported. The RT:Counts spectra are in a text format used by other tools in the industry and can be visualized by OzNOx Companion using script 8.

Script 6: Unite and organize Script 5 output(s)

```
Which script would you like to run?
Script 1: Select project folder and load/update LC-MS annotations, parameter files
Script 2: Process LC-MS annotations for manual RT-based validation/rejection
Script 3: Output a PRM list for LC-OzNOxESI-MS2 for your LC-MS annotations
Script 4: Convert .txt LC-MS and PRM LC-MS2 data to compatible .csv
Script 5: Search for presumptive OzID MS1 and OzNOx MS2 double-bond product species
Script 6: Unite and organize Script 5 output(s)
Script 7: (Prototype) double-bond annotation and regioisomer quantification with Script 6 output
Script 8: Visualize a text-based spectrum from Script 5, 6, or 7 output
Run Script Number: 6

Beginning Script 6 ...

Enter folder with Script 5 .csv outputs (copy from top of file explorer): C:\Users\rasmith12\OneDr

Found compatible .csv data: PRM 1.csv
Found compatible .csv data: PRM 2.csv
Found compatible .csv data: PRM 3.csv

Proceed? Enter Y for Yes or N for No: Y

Importing data ...

Importing data from file 1 of 3 ...
Importing data from file 2 of 3 ...
Importing data from file 3 of 3 ...

Merging data ...

Merging is 0% finished
Merging is 1% finished
Merging is 2% finished
Merging is 3% finished
```

```
Merging is 96% finished
Merging is 97% finished
Merging is 98% finished
Merging is 99% finished
Merging is 100% finished

Organizing merged data ...

Output available as: OzNOx Script 6 output 2024_08_23_11_44_58.csv

Script 6 finished.  See the newly created .csv file combining your prior Script 5 outputs.
```

Script 6 merges the script 5 outputs, one output per data file, into a single .csv that can be interpreted by script 7. The RT, Counts, and RT:Counts Spectrum fields are included per sample. Script 7 will use this data to create aligned spectra and averaged values.

M	N	O	P	Q	R	S
All Samples Max Counts	Max Counts RT (PRM 1)	Counts (PRM 1)	Max Counts RT (PRM 2)	Counts (PRM 2)	Max Counts RT (PRM 3)	Counts (PRM 3)
1.96E+04	20.78	1.54E+04	20.77	1.96E+04	20.78	1.20E+04

Script 7: Double-bond annotation and regioisomer quantification with script 6 output

```
Beginning Script 7 ...

Script 7 requires three .csv files: Script 6 output, sample definitions, and class definitions.
Enter folder with the necessary .csv files (copy from top of file explorer): C:\Users\rasmith12\

Selecting OzNOx class definitions .csv file ...
OzNOx class definitions found: OzNOx class definitions.csv
Proceeding with newest OzNOx class definitions: OzNOx class definitions.csv
Checking OzNOx class definitions: OzNOx class definitions.csv
OzNOx Class Definitions file looks good.

Selecting OzNOx sample definitions .csv file ...
OzNOx sample definitions found: OzNOx sample definitions.csv
Proceeding with newest OzNOx sample definitions: OzNOx sample definitions.csv
Checking OzNOx sample definitions: OzNOx sample definitions.csv
OzNOx sample definitions file looks good.

Selecting OzNOx Script 6 output .csv file ...
OzNOx Script 6 output found: OzNOx Script 6 output 2024_08_22_17_31_59.csv
OzNOx Script 6 output found: OzNOx Script 6 output 2024_08_23_11_44_58.csv
Proceeding with newest OzNOx Script 6 output: OzNOx Script 6 output 2024_08_23_11_44_58.csv
Checking OzNOx Script 6 output: OzNOx Script 6 output 2024_08_23_11_44_58.csv
OzNOx Script 6 output file looks good.

For sample group: Example PRM
To assign double-bond locations, the following samples will be queried:
PRM 1
PRM 2
PRM 3

Proceed? Enter Y for Yes or N for No: Y
Performing group-wise sample spectrum alignment ...
Group-wise sample spectrum alignment complete.

On parent compound 1 of 214: AcCa(24:3)_1
On parent compound 2 of 214: AcCa(24:3)_2
```

```
On parent compound 213 of 214: TG(16:0_16:0_18:1)
On parent compound 214 of 214: d5-TG(16:0_16:0_19:2)

Output available as OzNOx Script 7 output 2024_08_23_11_51_31.csv

Script 7 finished.
```

Script 7 does not utilize the script 1-assigned project folder and parameter files. Rather, it asks for the essential files independently. You must provide a folder with the script 6 output, sample definitions, and class definitions files. If you have been using one project folder all along, that folder will already contain these files. Note that the LC-MS annotations file is no longer used; all its necessary information is now contained within the script 6 output, so take care in making any edits to the script 5 and 6 outputs. You may find it necessary to manually curate (delete) the occasional erroneous product in the script 6 output to assist script 7. We sometimes did this on the basis of % detection, a filter not yet implemented in the software. If a product species was detected in fewer than 3 out of 4 replicates, we deleted it from the script 6 output before running script 7.

Script 7: Double-bond annotation and regioisomer quantification with script 6 output continued

	A	B	C	D	E	F	G	H	I
1	Parent ID	Parent Annotation	Class Key	Tail Key	Double-Bond n-#	Isomer Quantification Factor (Example PRM)	Corrected Counts (Example PRM)	Max Log Score (Example PRM)	RT:Log Score Spectrum (Example PRM)
2	16	Cer(d18:1_24:0)	Cer	(d18:1_24:0)	(n-14)	100%	1.57E+04	3.634867852	20.686561:0.0 20.695281:0.0 20.703997:0
3	23	Cer(d18:1_24:1)	Cer	(d18:1_24:1)	(n-9_n-9)	96.10%	9.17E+04	8.351597211	20.072597:0.0 20.10935:7.126353247069
4	23	Cer(d18:1_24:1)	Cer	(d18:1_24:1)	(n-14_n-9) | (n-9_n-14)	3.90%	3.76E+03	7.751426435	20.072597:0.0 20.10935:0.0 20.141418:7.:
5	23	Cer(d18:1_24:1)	Cer	(d18:1_24:1)	(n-14_n-14)	< 1%		5.947135677	20.072767:0.0 20.1095:0.0 20.14155:5.94:
6	53	d5-DG(17:0_14:1)	DG	(17:0_14:1)	(n-5)	100%	2.29E+06	10.8692621	15.309279:10.050575564490103 15.3441
7	54	DG(16:0_16:1)	DG	(16:0_16:1)	(n-7)	81.50%	1.83E+05	8.786015841	16.362786:8.151385947602064 16.40010
8	54	DG(16:0_16:1)	DG	(16:0_16:1)	(n-9)	18.00%	4.03E+04	6.789972156	16.362786:3.8512163632172016 16.4001
9	54	DG(16:0_16:1)	DG	(16:0_16:1)	(n-11)	0.50%	1.22E+03	3.087186532	16.359083:0.0 16.396397:0.0 16.438351:0

The script 7 output is rich with data. For sake of simplified discussion, 4 parent species are presented with numerous columns removed. Each species is reported with double-bond assignments, regioisomer relative quantification, corrected detector counts, a maximum logarithmic probability score, and a RT:Score spectrum.

- ID 16 Cer d18:1/24:0: Only one double-bond product was detected, leading to only one reported regioisomer here. There may be others, but they are below the limit of detection. The double-bond detected is the n-14 double-bond of the sphingoid base, an expected annotation.

- ID 23 Cer d18:1/24:1: Two double-bond products were detected, n-9 and n-14, resulting in three possible combinations of regioisomers: n-9 and n-9, n-9 and n-14, and n-14 and n-14. The truth is that Cer d18:1(n-14)/24:1(n-9) is the most abundant species by a significant margin. However, OzNOx Companion reports that Cer d18:1(n-9)/24:1(n-9) is the most abundant. It is important to know that this software is a prototype and will report inaccurate regioisomer information for certain classes, including classes with sphingoid bases (SPH, Cer, SM, HexCer, etc). This is due to the steric hindrance and so low reactivity of the sphingoid base double-bond.[6] Glycerophospholipids and glycerolipids do not suffer from this issue. A future update will provide an algorithm for correction of the asymmetry in product intensity for sphingoid base double-bonds. In the meantime, users should utilize the script 6 and script 5 outputs when investigating sphingoid base-containing classes or at least tread carefully if utilizing the script 7 output.

- ID 53 d5-DG 17:0/14:1: One product was reported, and so one regioisomer is in the output.

- ID 54 DG 16:0/16:1: Three products were reported, and so three regioisomers are in the output with relative quantification based on the product intensities in spectra alignment.

The Max Log Score reported is the maximum logarithmic probability score found for that regioisomer in any one scan, either of the sample file or the aligned spectrum in the case of replicate sampling. Within a scan, a regioisomer is scored by multiplying all its requisite product ion intensities. If a product ion is missing, a score of 0 is given. In the case where a product ion represents more than one theoretical double-bond, the algorithm divides and multiplies accordingly. In the case where a double-bond is represented by both scheme 1 and scheme 2 products, they are both multiplied.

Note that OzNOx Companion performs some significant figure -based rounding of regioisomer percentages. For example, you might see two regioisomers with 53.00% and 47.00% in Excel. This is an artifact of Excel data formatting; the values were very likely written as 53% and 47%, which you may see in other spreadsheet applications if they do not similarly format the data.

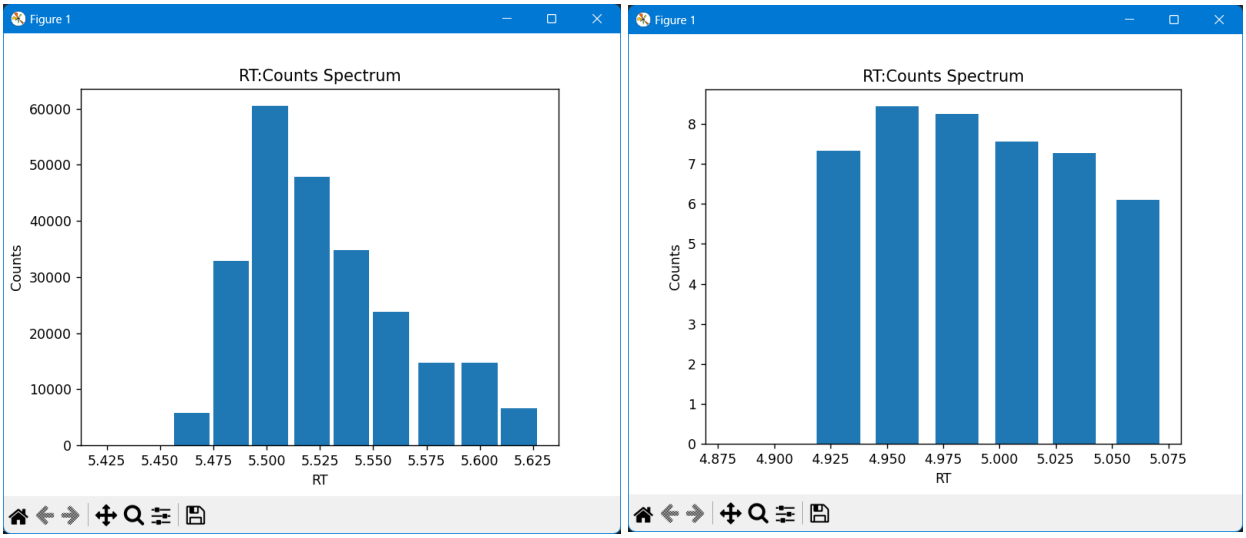
Script 7: Further elaboration of how regioisomers are computed and quantified

From the list of OzNOxESI-MS^2 product species, OzNOx Companion generates a list of all possible regioisomers given the fatty acyl content and combinations of product species. For monounsaturated lipids, the list of regioisomers is the same length as the list of double-bonds elucidated by product ions, while polyunsaturated lipids may have numerous theoretical combinations of double-bond positions. Many combinations can be eliminated based on incompatibility. For example, FA 22:4(*n*-6,9,12,15) must produce four unique products, and each product indicates the *n*-# of its position but also the number of up- or downstream double-bonds. The *n*-9 product *m/z* suggests that there is one double-bond at lower *n*-# and by subtraction two double-bonds at higher *n*-#. This provides a logic for putting double-bonds together and invalidating some combinations of products. Theoretical regioisomers are scored on a scan-by-scan basis, utilizing aligned spectra when there is replicate sampling, and are reported with logarithmic probability scores and text-based RT:score spectra. These probability scores reflect the combined observed intensities of the necessary product species to build the theoretical regioisomer and so the likelihood that the given regioisomer is a legitimate sample species. OzNOx Companion subsequently uses the probability scores and several mathematical assumptions to divide product ion detector counts between theoretical regioisomers, providing the basis for relative quantification.

These assumptions include neglecting species projected to be below 1% relative abundance if necessary to solve the logical puzzle (reported as < 1%) and that all product ions belonging to the same regioisomer have the same intensity due to that regioisomer. This second assumption has been shown to be reasonable in some species and unreasonable in others and will be the subject of future algorithm development when the necessary authentic standards can be run to better understand the *n*-# dependence of product ion intensity. This may also be improved by altering collision energy, as collision energy is the dominant factor in product ion intensity.

Script 8: Visualize a text-based spectrum from Script 5, 6, or 7 output

```
Which script would you like to run?
Script 1: Select project folder and load/update LC-MS annotations, parameter files
Script 2: Process LC-MS annotations for manual RT-based validation/rejection
Script 3: Output a PRM list for LC-OzNOxESI-MS2 for your LC-MS annotations
Script 4: Convert .txt LC-MS and PRM LC-MS2 data to compatible .csv
Script 5: Search for presumptive OzID MS1 and OzNOx MS2 double-bond product species
Script 6: Unite and organize Script 5 output(s)
Script 7: (Prototype) double-bond annotation and regioisomer quantification with Script 6 output
Script 8: Visualize a text-based spectrum from Script 5, 6, or 7 output
Run Script Number: 8

Beginning Script 8 spectrum visualization ...
Enter spectrum to visualize (copy-paste from .csv file): 5.4313843:0.0 5.448149:0.0 5.4649029:5750.41699219 5.4832845:32883.6875 5.5016225:60518.5976562 5.5212926:47848.45

Spectrum visualized.  Close the spectrum to return to main menu.
```



OzNOxESI-MS^2 product RT:Counts spectrum left, RT:Log Score spectrum right.

Script 8 will visualize copy-pasted text spectra from script 5, 6, or 7 output files. The visualization code is minimal and will not give you a manuscript-ready figure, but you can zoom and edit some of the visualization parameters and finally save as a high-resolution image if desired. The intent here is to allow for checking in with what OzNOx Companion is "seeing" in the data. It is very likely that your instrument manufacturer has furnished you with software that provides superior spectra visualization in the native format.

Other known issues:

- Workflow instability has been observed when running off of or utilizing parameter files on a shared network drive. It is best practice to have OzNOx Companion and all necessary files on the computer where the program is being run.
- If you receive an error saying that one of your .csv files could not be accessed, reported with a name you do not recognize including a $ character, this is an artifact of Excel. When you open a file in Excel, a duplicate file is made and nominally only exists while you have the file open, represented as the same file name led by a $ character. Close all iterations of Excel, and make a new version of your parameter file by copy-and-pasting the old one. This issue is again more prevalent when operating from a shared network drive.

References

1. Thomas, M.C., et al., *Elucidation of double bond position in unsaturated lipids by ozone electrospray ionization mass spectrometry.* Anal Chem, 2007. **79**(13): p. 5013-22.
2. Thomas, M.C., T.W. Mitchell, and S.J. Blanksby, *OnLine ozonolysis methods for the determination of double bond position in unsaturated lipids.* Methods Mol Biol, 2009. **579**: p. 413-41.
3. Poad, B.L., et al., *Ozone-induced dissociation on a modified tandem linear ion-trap: observations of different reactivity for isomeric lipids.* J Am Soc Mass Spectrom, 2010. **21**(12): p. 1989-99.
4. Pham, H.T., et al., *Structural characterization of glycerophospholipids by combinations of ozone- and collision-induced dissociation mass spectrometry: the next step towards "top-down" lipidomics.* Analyst, 2014. **139**(1): p. 204-14.
5. Chambers, M.C., et al., *A cross-platform toolkit for mass spectrometry and proteomics.* Nat Biotechnol, 2012. **30**(10): p. 918-20.
6. Barrientos, R.C., N. Vu, and Q. Zhang, *Structural Analysis of Unsaturated Glycosphingolipids Using Shotgun Ozone-Induced Dissociation Mass Spectrometry.* J Am Soc Mass Spectrom, 2017. **28**(11): p. 2330-2343.