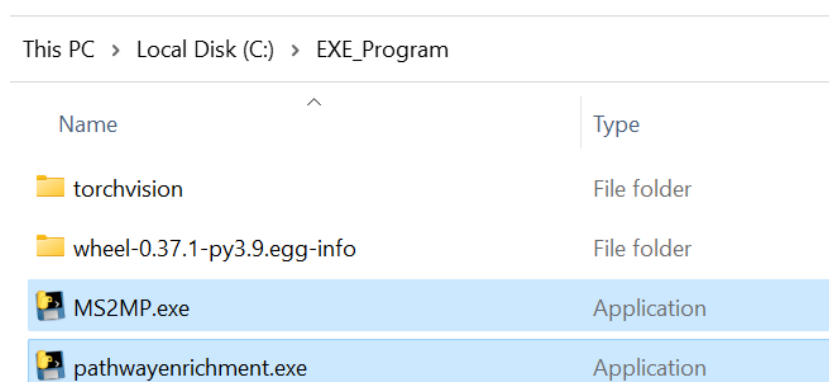


0 Recommended workflow for untargeted LC-MS/MS acquisition

Full-scan acquisition is performed in Full MS/ddMS2 mode, with resolution set to 120,000 for full scans and 30,000 for MS/MS. The scan range is set from 85 to 1250 m/z. The top 10 ions from each full scan are selected for fragmentation. Ion fusion is conducted to eliminate redundancy arising from ions that originate from the same metabolites, including isotopic ions and adduct ions. In order to match MS1 and MS2, deviation thresholds of 0.005 and 12 seconds are selected for m/z and retention time, respectively. If multiple candidates remain, the MS2 spectrum with the lowest sum deviation of m/z and retention time is selected. In this way, a metabolic feature with MS1 and matched MS2 can be obtained. As for subsequent analysis, pathway labels can be predicted using MS2(“MS2MP.exe”), and statistically significant differences can be calculated using relative abundance of MS1 (“pathwayenrichment.exe”).

1 Overview

Download the file “EXE_Program.7z” and extract it. Inside, you will find two executable files, “MS2MP.exe” and “pathwayenrichment.exe” (**Figure 1**). “MS2MP.exe” is used for predicting the metabolic pathways of MS/MS spectra, while “pathwayenrichment.exe” is used for pathway enrichment analysis. The usage procedures for these two executable files will be explained separately using example files.

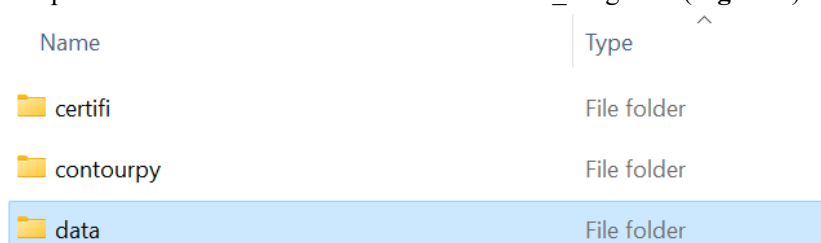


This PC > Local Disk (C:) > EXE_Program	
Name	Type
torchvision	File folder
wheel-0.37.1-py3.9.egg-info	File folder
MS2MP.exe	Application
pathwayenrichment.exe	Application

Figure 1

2 Usage Procedure for “MS2MP.exe”

2.1 Locate and open the folder “data” within the folder “EXE_Program” (**Figure 2**).



This PC > Local Disk (C:) > EXE_Program > data	
Name	Type
certifi	File folder
contourpy	File folder
data	File folder

Figure 2

2.2 Inside the folder “data”, locate and open the folder “jsontest” (**Figure 3**).

Name	Type
jsontest	File folder
test.csv	Microsoft Excel Com...

Figure 3

2.3 Place the fragmentation tree files (JSON format), generated by the software of SIRIUS, into the folder “jsontest”. The file names should only contain numbers without the “.json” extension (**Figure 4**).

Name	Type
23456789.json	JSON File
93692851.json	JSON File

Figure 4

2.4 Go back to the folder “data” and find the file “test.csv”, open it using the software of “Excel” (**Figure 5**).

Name	Type
jsontest	File folder
test.csv	Microsoft Excel Com...

Figure 5

2.5 In the “jsoninput” column of the file “test.csv”, enter the file names from step 2.3 without the “.json” extension. Do not modify data in other cells (**Figure 6**).

	A	B
1	jsoninput	NO INPUT
2	23456789	0,1,2,3,4
3	93692851	0,1,2,3,4

Figure 6

2.6 Go back to the folder “EXE_Program” and run the file “MS2MP.exe” (**Figure 7**).

Name	Type
torchvision	File folder
wheel-0.37.1-py3.9.egg-info	File folder
MS2MP.exe	Application

Figure 7

2.7 The initial interface of “MS2MP.exe” is shown in **Figure 8**. Follow the prompts and input the number of predictions required, which corresponds to the number of JSON files from step 2.3. After “MS2MP.exe” finishes running, the interface will appear as shown in **Figure 9**, and “Please input any key to exit” will be displayed. The prediction results will be shown within the red box (**Figure**

9) and can also be viewed in the file “Results.txt” (Figure 10-11).

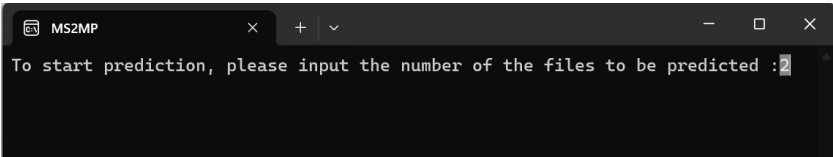


Figure 8

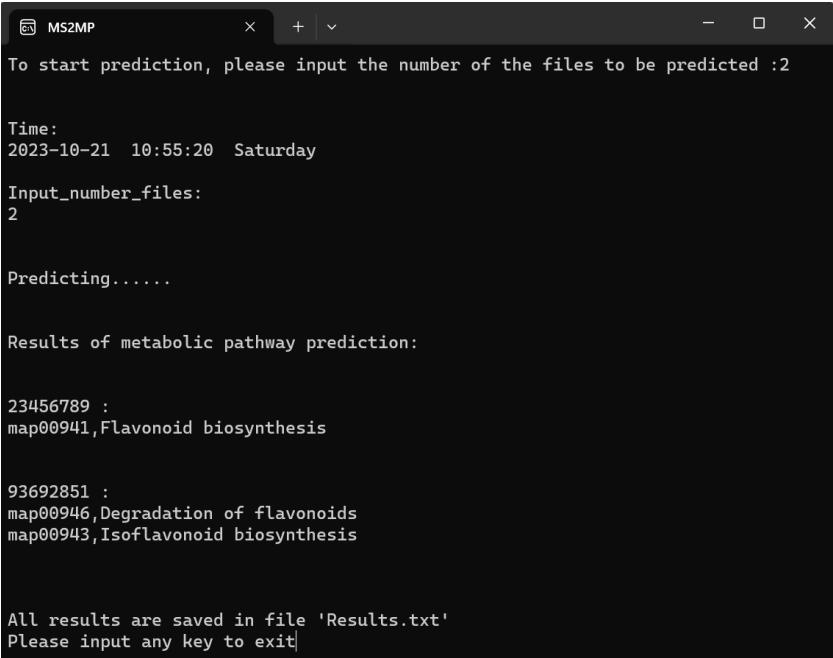


Figure 9




Name	Type
 python3.dll	Application extension
 python310.dll	Application extension
 Results.txt	Text Document

Figure 10

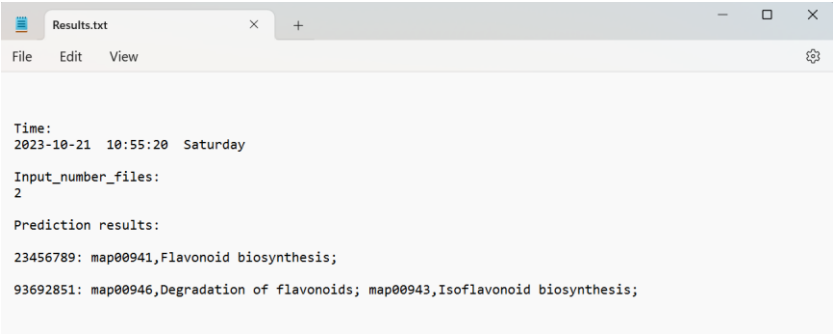


Figure 11

3 Usage Procedure for “pathwayenrichment.exe”

3.1 Locate and open the folder “data_pathwayenrichment” within the folder “EXE_Program” (Figure 12).

Name	Type
data	File folder
data_pathwayenrichment	File folder
kiwisolver	File folder

Figure 12

3.2 Inside the folder “data_pathwayenrichment”, find and open the file “input_pathwayenrichment.csv” using the software of “Excel” (**Figure 13**).

Name	Type
input_pathwayenrichment.csv	Microsoft Excel Com...

Figure 13

3.3 Enter the required data in the file “input_pathwayenrichment.csv” (**Figure 14**). For pathway enrichment analysis based on compound identification, data in column A should be the pathway names, data in column B should be the total number of compounds in the selected pathway background, data in column C should be the total number of compounds in this pathway, data in column D should be the number of significantly different metabolites in the experimental sample, and data in column E should be the number of significantly different metabolites in this pathway. For MS2 spectra-based pathway enrichment analysis, column A represents the pathway names, column B represents the total number of MS2MP-predicted metabolic features, column C represents the total number of MS2MP-predicted metabolic features in a specific pathway, column D represents the significantly differential number of MS2MP-predicted metabolic features, and column E represents the significantly differential number of MS2MP-predicted metabolic features in a specific pathway.

	A	B	C	D	E
1	mapXXXXXX	5000	13	500	10
2	mapYYYYYY	5000	29	500	16
3	mapZZZZZZ	5000	51	500	24

Figure 14

3.4 Go back to the folder “EXE_Program” and run the file “pathwayenrichment.exe” (**Figure 15**).

Name	Type
pathwayenrichment.exe	Application
pyexpat.pyd	PYD File
python3.dll	Application extension

Figure 15

3.5 The interface of “pathwayenrichment.exe” during runtime is shown in **Figure 16**. When “Please input any key to exit” appears, it indicates that the program has finished running. The prediction results can be found in the file “result_pathwayenrichment” (**Figure 17**) within the folder “data_pathwayenrichment” (**Figure 13**). Data in the last column (F) are the p-values for pathway enrichment.

```
pathwayenrichment
data loading.....
['mapXXXXXX', 'mapYYYYYY', 'mapZZZZZZ'] ['5000', '5000', '5000'] ['500', '500', '500'] ['13', '29', '51'] ['10', '16', '24']

results:
mapXXXXXX 5000 13 500 10 1.9915537663450777e-08
mapYYYYYY 5000 29 500 16 1.5710897027787496e-09
mapZZZZZZ 5000 51 500 24 1.0270007066992548e-11

All results are saved in file 'result_pathwayenrichment.csv'
Please input any key to exit
```

Figure 16

	A	B	C	D	E	F
1	mapXXXXXX	5000	13	500	10	1.99E-08
2	mapYYYYYY	5000	29	500	16	1.57E-09
3	mapZZZZZZ	5000	51	500	24	1.03E-11

Figure 17