

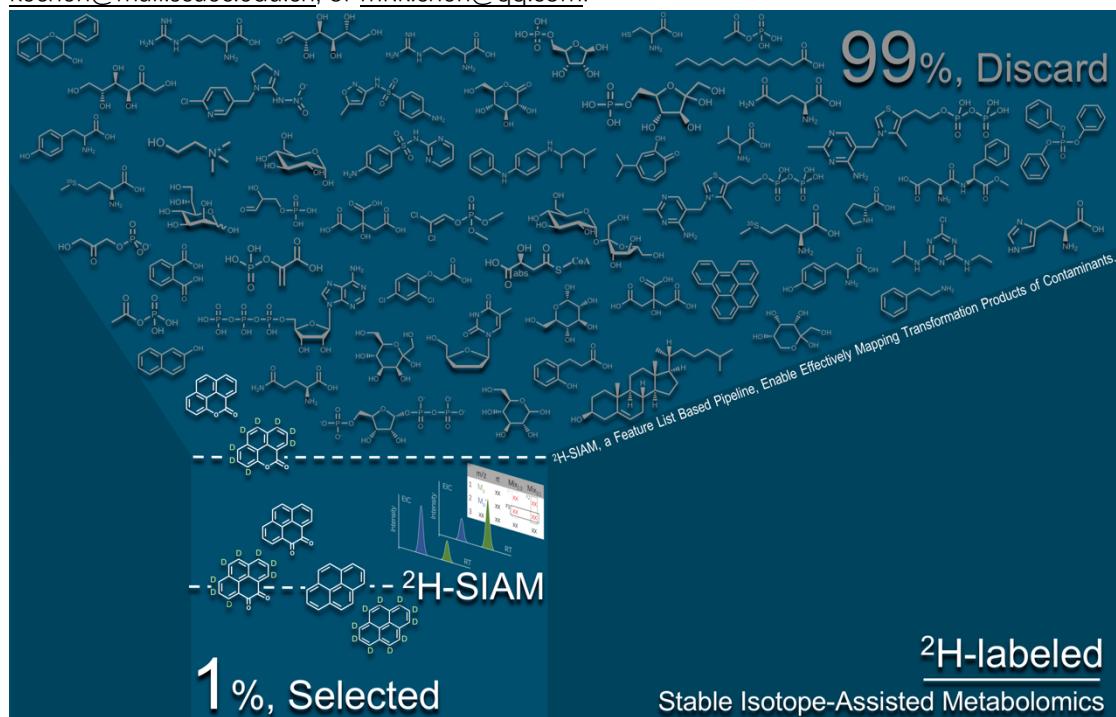
²H-SIAM Pipeline and ²H-SIAM(1.0) Algorithm

Ke Chen

²H-SIAM(V1.0) is a feature list-based algorithm developed for tracking possible isotopologue pairs of stable isotope-labeled organic matters. With the help of this algorithm, we developed a ²H-labeled Stable Isotope Assisted metabolomic (²H-SIAM) pipeline (Fig. 1) which facilitate the screening of transformation products (TPs) of the substrates with high confidence.

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Introduction

²H-SIAM(V1.0) is a feature list-based algorithm developed for tracking possible isotopologue pairs of stable isotope-labeled organic matters. With the help of this algorithm, we developed a ²H-labeled Stable Isotope Assisted metabolomic (²H-SIAM) pipeline (Fig. 1) which facilitate the tracking of transformation products (TPs) of the substrates in complex matrix with high confidence. Additionally, our proposed ²H-SIAM pipeline is equipped with full GUI interface which contribute to lower the skill barriers of its application in research field. We provided example data and a step-by-step protocol for evaluation.

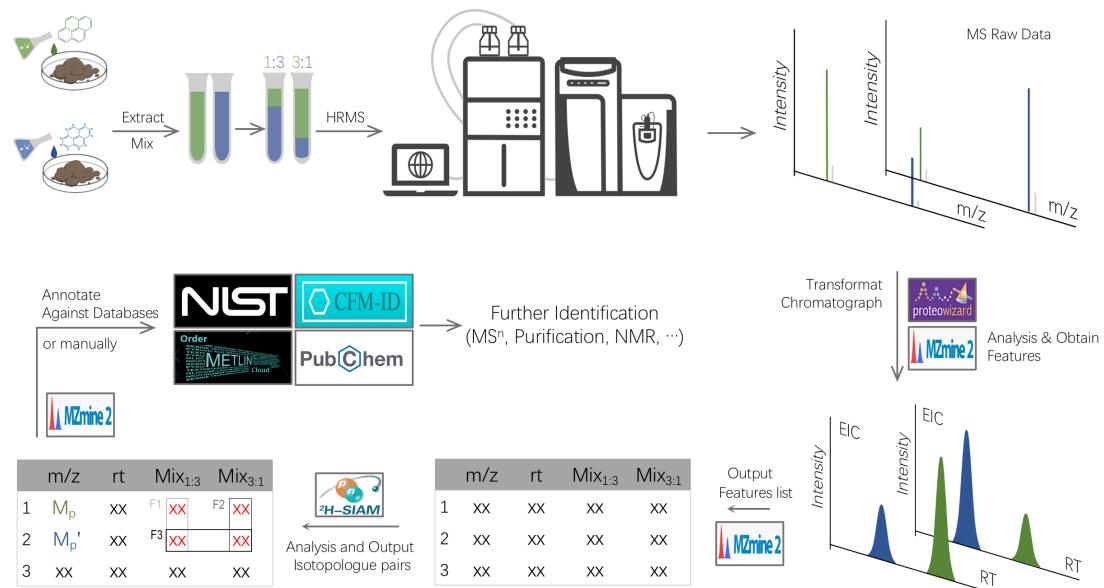


Figure 1 Proposed Pipeline for ²H-labeled Stable Isotope Assisted Metabolomic (²H-SIAM). Firstly, both natural and ²H-labeled parent contaminants are individually incubated in environment matrix for indicated duration; they are subsequently extracted and mixed with the ratio of 1:3 and 3:1 (Mix_{1:3} and Mix_{3:1} samples); the mixtures are determined by UPLC-ESI-HRMS or GC-EI-HRMS and thereafter obtained raw data are analyzed by MZmine2 to obtain a features list; it is imported into our developed algorithm ²H-SIAM(V1.0) to obtain possible isotopologue pairs as TPs of the substrate for further identification.

Meanwhile, we provided a data analysis workflow derived from our example data (Fig. 2). Raw data from LC-MS or GC-MS were transformed to mzXML files and imported into MZmine2 for feature detection, chromatogram build, deconvolution, align, peak filter and normalization. Feature lists were exported by MZmine2 and imported into ²H-SIAM(1.0) for isotopologue pair search and screening by triple filters. From Step3 to Step 6 could be replaced by the other algorithm, for instance, XCMS, MS-DIAL, et al.

Important tips: Although the proposed ²H-SIAM pipeline is designed for the screening of ²H-labeled TPs by UPLC-ESI-HRMS, data from any other types of isotope-labeled atoms, e.g., ¹³C, ¹⁵N and ¹⁸O, any other types of LC-MS or GC-MS platform, and any other mass spectrum data processing algorithm, e.g., XCMS, MetaboAnalyst and MS-DIAL, are compatible for our proposed ²H-SIAM pipeline.

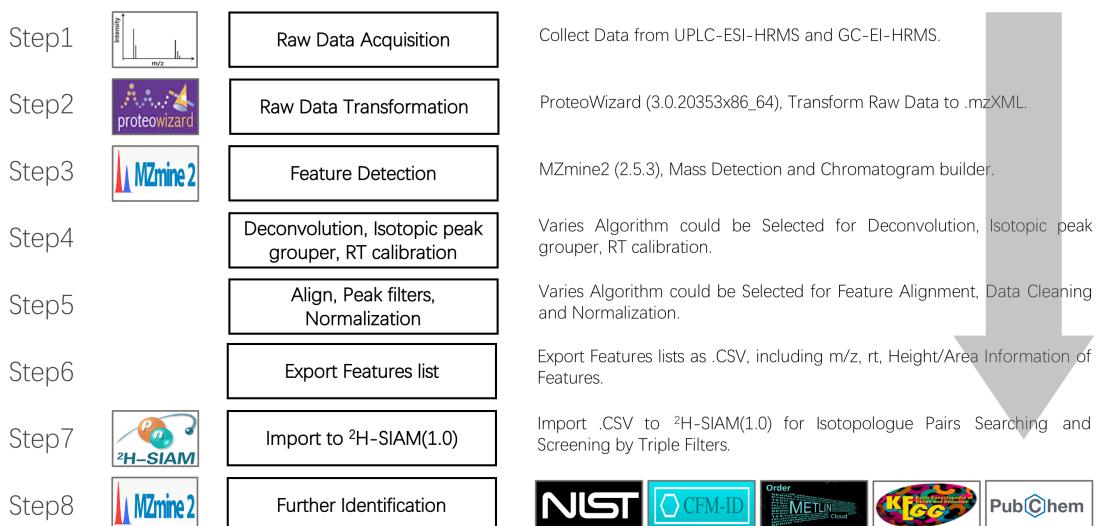


Figure 2 Proposed analysis workflow for a ²H-SIAM study. Raw data from UPLC-ESI-HRMS or GC-EI-HRMS are transformed to mzXML files and imported into MZmine2 for feature detection, chromatogram build, deconvolution, align, peak filter and normalization. A Features list is exported by MZmine2 and imported into 2H-SIAM for isotopologue pair search and screening by triple filters.

²H-SIAM pipeline

As shown in Fig. 1, a fully GUI-based pipeline is proposed, and it facilitate to lower the skill barriers of the application of ²H-SIAM in environment study. Parent isotopologue pairs of contaminants are added into environmental matrix respectively and incubated for indicated duration. Then, they are extracted and mixed with indicated ratio (e.g., 1:1 and 1:2, 1:3 and 3:1, 1:9 and 9:1). Here we propose a 1:3 and 3:1 ratio (Mix_{1:3} and Mix_{3:1} samples) which keep an appropriate equilibrium between accuracy and sensitivity of the mapping of isotopologue pairs.

Mixed samples are then determined by MS platform (e.g., UPLC-ESI-HRMS, GC-MS) for indicated replicates. MS raw data of Mix_{1:3} and Mix_{3:1} are transformed into mzXML format (by ProteoWizard 3.0.20353) and imported into MZmine2(2.53) to obtain feature list information. Then, Mix_{1:3} and Mix_{3:1} data in feature list was imported into ²H-SIAM(V1.0) for TPs tracking.

The key for efficient tracking isotopologue pairs is to acquire a high-quality features list, and then optimized parameters for the selected algorithm and selected data are important. Details information about how to effectively extract features by software XCMS, MZmine2, MS-DIAL or other data processing algorithm are available from websites.

How to use ²H-SIAM(V1.0)

In the cases readers have features lists, or prefer to check the performance of ²H-SIAM(1.0).exe, then read from here. Additionally, we provided a step-by-step workflow combined with the data processing by MZmine2 at the end of this

document.

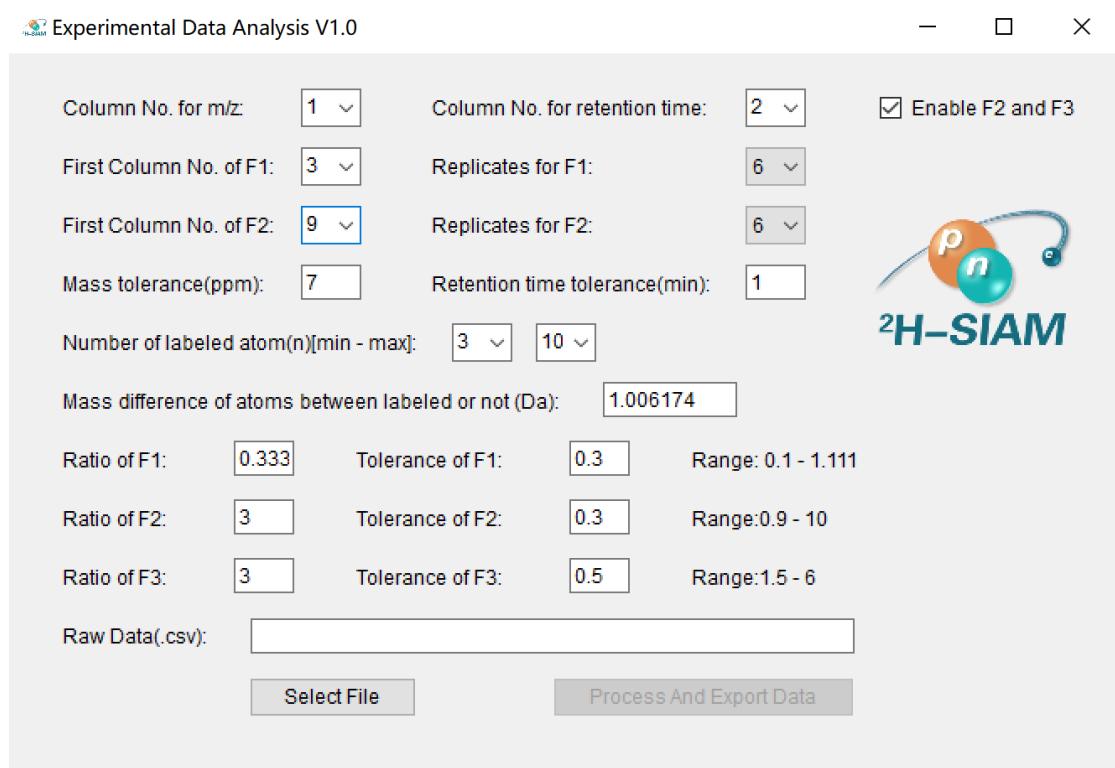


Figure 3 GUI interface of ^2H -SIAM(V1.0).

As shown in Fig. 3, ^2H -SIAM provided detailed setting for SIAM. People could define numbers of Filters, numbers of replicates, mass tolerances, RT tolerance, number of isotope labels, mass differences of atoms between labeled or not, Ratio of filters F1, F2 and F3, and tolerance of them.

Table 1 Functional Parameters in ^2H -SIAM(1.0)

Name	Explanation or Proposed Setting
Enable F2 and F3	enable the choose of 1 or 3 filters
Mass tolerance (ppm)	QExactive 10, Q-tof 40, Triple quadruple 1000
RT tolerance (min)	define as \pm RT tolerance, 1 or 0.5 min, depends on features
Number of labeled atoms	Indicate the numbers of isotope labels to be detected
Mass difference	define the labels to be used, e.g., ^{13}C , ^2H or ^{17}O . For ^2H , it is 1.006174
Ratio of F1, F2, F3	theoretic ratio for each filter
Tolerance of F1, F2, F3	for F1 and F2, set tolerance at 0.1-0.3; For F3, set tolerance at 0.3-0.5

In certain experiment regime, people may prefer to use only one filter. For example, mix natural and isotope labeled compounds with the ratio of 1:1, then you could remove tick before "Enable F1 and F2".

^2H -SIAM(V1.0) accept data in a .csv format as shown in Fig. 4. A features list with the name "**ImportFor2HSIAM(1.0).csv**" was provided in our example data, and it is ready for use for ^2H -SIAM(V1.0) by the setting as shown in Fig. 3.

The first row is titles, including m/z, RT and intensity of Mixture 1 and Mixture 2 with indicated replicates (defined by customers). The first and second columns define m/z and RT (minutes) of features obtained from your data processing algorithm. The following columns are intensities (height/area) of mixture samples

(e.g., Mix_{1:3} and Mix_{3:1} samples) measured by LC-MS or GC-MS. Replicates could be defined by customs.

Running ²H-SIAM(V1.0) and the GUI interface will appear (as shown in Fig. 3). Correlation between ²H-SIAM(V1.0) and the features list is shown in Fig. 5, contributing to define data frame. A typical setting for screening potential TPs of pyrene and pyrene-*d*10 Mix_{1:3} and Mix_{3:1} samples by UPLC-ESI-HRMS (Q Exactive, Thermo) is as following: Mass tolerance 7 ppm (or 10), RT tolerance 1 min, Number of labeled atoms 3-10, Mass difference of atoms between labeled or not (Da) 1.006174, Ratio for F₁, F₂ and F₃, 0.333, 3 and 3, Tolerance for F₁, F₂ and F₃, 0.3, 0.3 and 0.5.

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
1	row m/z	row retention	Mix1v3-1	PeMix1v3-2	PeMix1v3-3	PeMix1v3-4	PeMix1v3-5	PeMix1v3-6	PeMix3v1-1	PeMix3v1-2	PeMix3v1-3	PeMix3v1-4	PeMix3v1-5	PeMix3v1-6	Peak height
2	60.0534014	9.58938719	4.74179362	6.45391255	9.49996235	8.15031894	0	0	8.23417798	0	11.8223004	7.96992713	0	5.32542415	
3	60.2603882	15.4015725	3.32718716	3.77633901	4.86463585	2.3667658	0	7.104318716	5.11390236	2.62822296	5.28237039	0.95388082	0	4.39094929	
4	61.029299	6.3897052	25.5668419	10.2309765	32.7835548	24.7423277	35.1519265	18.3642296	20.210568	18.3737346	14.906701	10.9908504	6.33057595	3.19988118	
5	63.7893214	12.7523426	3.49104261	4.08821879	3.79601607	0	3.45806987	3.59996878	3.26550682	5.15946469	4.40171513	4.23488707	2.37686791		
6	64.0166295	3.9286561	3.24509871	4.05959006	5.32629956	3.59582403	5.21662408	4.92668156	4.44052733	4.69736776	3.91524268	0	3.4622314	2.9382202	
7	65.0268543	26.0290098	9.10849282	8.76738026	0	9.81963026	16.481173	0	10.8663454	11.6007272	0	6.39194742	0	0	
8	65.0390549	14.653747	16.379069	17.4432704	31.977666	19.712518	15.9246425	16.1614012	12.2313499	8.46969196	8.71387247	5.90039477	8.75594725		
9	68.0332565	19.8194984	6.1079396	9.7016414	11.6081521	9.19854263	0	10.6025977	0	6.65669658	6.68535866	0	3.84885641	0	
10	68.0641015	21.0101205	3.07815414	4.39381677	6.61407531	4.55310059	4.86219397	4.93400814	6.3109003	7.02843879	5.9900682	6.13532276	5.33166292	2.89130879	

Figure 4. A Typical features list in a .csv format used for ²H-SIAM(V1.0).

Then select your features list file and process, which will take several minutes depending on your PC and numbers of features.

Our example feature list contains ~4000 features, and it takes around 1 minutes for isotopologue pairs screening in a PC equipped with 3.7GHz Intel Core i9. Additionally, if you have more than 10000 features, time may be significant prolonged, e.g., ~30 min. After that, a table will appear, and you could double click the table and save the result as a .csv file.

	A	B	C	D	E	F	G	H	I	J	K	L	M	N
1	row m/z	row retention	Mix1v3-1	Mix1v3-2.m	Mix1v3-3.m	Mix1v3-4.m	Mix1v3-5.m	Mix1v3-6.m	Mix3v1-1.m	Mix3v1-2.m	Mix3v1-3.m	Mix3v1-4.m	Mix3v1-5.m	Mix3v1-6.m
2	212.007851	6.59656888	21881.4843	29001.8058	42398.9702	31600.5508	10459.7907	16873.3077	5704.42398	7290.09305	2640.29675	5350.86999	3659.77262	3986.93254
3	308.021273	6.59683965	21295.5722	12891.3152	17205.0222	14271.6044	5562.09338	8410.91647	2924.68407	4166.23862	1528.28068	2438.56358	1647.86639	1716.9767
4	111.092053	28.5429268	3015.1929	2885.37528	0	3516.26316	8954.65005	0	5515.47124	5829.48038	2591.55209	2210.35298	3880.92619	4330.10526
5	415.211532	13.0057648	1175.5007	14258.1956	20768.8915	14183.4311	15434.9417	14415.6302	12661.4739	13780.5762	13261.5049	11090.7536	9600.78849	9733.09389
6	129.018443	6.6039747	7916.23719	6328.80072	7849.10701	6692.96206	9605.93616	7580.14075	6196.91314	6434.71874	5809.076	3284.66957	2087.17498	2026.13641
7	87.0922991	28.5331484	2895.89506	2499.13513	0	2080.34761	7336.77042	3711.73406	4774.50008	4591.43206	1885.9586	2098.38735	3384.88313	4026.10568
8	149.023371	14.6446289	1871.0291	20900.2991	27267.0355	21469.854	21728.7044	17704.2084	13125.521	11685.2197	11433.4764	9410.56069	8778.86049	7812.93981
9	126.102773	28.4772714	2477.03559	1846.5915	1597.62774	2227.37276	6383.57504	2401.80354	4072.52481	3838.01506	1833.58646	1845.28836	2222.79623	0

Experimental Data Analysis V1.0

Column No. for m/z: 1 Column No. for retention time: 2 Enable F2 and F3:

First Column No. of F1: 3 Replicates for F1: 6 First Column No. of F2: 9 Replicates for F2: 6

Mass tolerance(ppm): 7 Retention time tolerance(min): 1

Figure 5. Correlation between ²H-SIAM(V1.0) and the features list.

Algorithm for ²H-SIAM(V1.0)

When a features list (.csv format) is imported into ²H-SIAM(V1.0), it is start with the calculation of means of replicates. Then, it screens isotopologue pairs with indicated isotope label, m/z and RT tolerance. the potential natural compounds were denoted as M, and the potential isotope-labeled compounds were denoted as M'. The mean intensities of the features M and M' from the Mix_{1:3}

and Mix_{3:1} samples, that is M_{Mix1:3}, M_{Mix3:1}, M'_{Mix1:3} and M'_{Mix3:1}, were used for the calculation of f_n. Then 3 defined quantitative filters F₁, F₂ and F₃ are used to select potential natural TPs with a isotopologue. In the case of R₁ = 0.33, Tol.₁ = 0.3, Filter F₁ will constitute a range between 0.11 and 1.1. When the calculated f₁ follows into this range, the feature pairs will pass this filter, and recorded in the output file of 2H-SIAM(1.0), and further filters will be further evaluated.

Make sure that your features list **DO NOT CONTAIN ANY FEATURE WITH THE MEAN OF INTENSITY OF "0".**

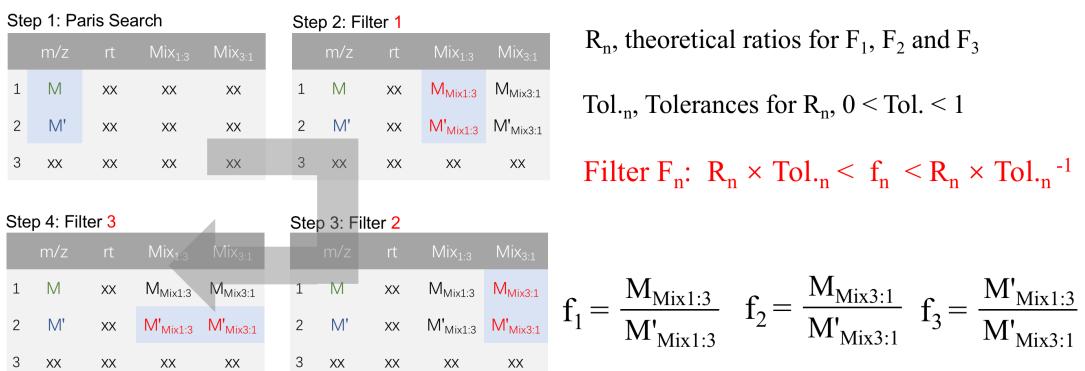


Figure 6 Algorithm for ²H-SIAM(1.0). When a features list (.csv format) is imported into ²H-SIAM(V1.0), it is start with the calculation of means of replicates. Then, it screens isotopologue pairs with indicated isotope label, m/z and RT tolerance. Then 3 defined quantitative filters F₁, F₂ and F₃ are used to select potential natural TPs with a isotopologue.

Illustration of the Result

As shown in Fig. 7, ²H-SIAM(V1.0) will output a features list as a .csv file (Fig. 7). The result contain Means of F1, Means of F2, Tentative match, Filter 1, Filter 2 and Filter 3. Isotopologue pairs data are given with the natural compounds, for example, pyrene with m/z 202.077737 at 18.38 min. The features passing through the indicated filters will provide information, and they follow the sequences of F1, F2 and F3. Our example data selected around 50 features from ~4000 features, and 14 of them were annotated as pyrene or its TPs.

A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U					
1 m/z	A	Retention	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11	A12	A13	A14	A15	A16	A17						
2 219.060464	12.5309568	12.517803	17.2425444	14.6076232	12.5028075	12.8362985	32.9616059	36.3612127	28.5803994	29.3407491	27.0562105	26.4390584	13.6	30.1	7L @ 226.12	10L	0.788	1.10L	2.79	12.10L	1.60	12.09			
3 247.070351	12.160351	4.4747603	9.4378659	9.9845436	7.8624374	9.4945436	16.5002749	17.7532118	18.1720731	12.1373311	6.89	16.6	10L	@ 250.12	10L	1.00	1.10L	1.10L	1.10L	1.10L	1.10L	1.10L	1.10L		
4 202.077737	18.3803609	18.3803609	18.3803609	154.28316	149.19474	149.19474	198.96172	221.81173	389.45454	173.065	173.065	1439.1914	1318.77886	100	100	100	100	100	100	100	100	100	100	100	100
5 130.05576	1.274375	5.07562071	0	0.63565126	0	0	1.7701265	9.0564921	7.46703039	3.3089496	6.12563242	4.6690014	0	5.09	4.22	10L	@ 140.1	10L	0.355	1.10L	0.945	1.10L	3.20	1.140	
6 235.076371	18.347362	20.6910238	30.8945488	40.9837791	42.943882	40.702629	82.0171931	91.650145	82.9807621	32.3935227	71.2037382	57.8287029	36.9	88.8	10L	@ 245.1	10L	-1.07	17.10L	9.09	17.10L	3.77	17.92		
7 194.052742	13.0380351	5.03326366	6.25097905	0	6.134634986	6.9204917	6.07451746	5.91873794	9.49303081	2.48061825	7.56760772	7.91155232	5.06	8.19	3L	@ 107.07	3L	0.541	12.3L	1.34	13.	3L	1.53	13.73	

Figure 7 Typical result indicates features with their isotopologue.

The meaning of the result file was provided in Table 2. We provide an explanation of the result of the feature m/z 202.07774 at 18.38, corresponding to pyrene, as shown in Table 3.

Table 2 Meaning of the result file (.csv) from ²H-SIAM(1.0)

Title	Explanation
A1-A6	intensities of Replicates of the first mixture sample
B1-B6	intensities of Replicates of the second mixture sample
Means of F1	means of the first mixture sample, in our data it is Mix _{1:3} sample
Means of F2	means of the second mixture sample, in our data it is Mix _{3:1} sample
Tentative match	tentative match isotopologue with the numbers of labels and its m/z in

	the indicated duration
Filter 1	indicate the numbers of isotope labels to be detected
Filter 2	depends on which isotope to be used, e.g., ^{13}C , ^2H or ^{18}O .
Filter 3	For F1 and F2, set tolerance at 0.1-0.3; For F3, set tolerance at 0.3-0.5.

As shown in Table 3, the column "Tentative match" provide possible isotopologue features with the numbers of ^2H labels, m/z and RT. There were three isotopologue features detected within the indicated RT tolerance, 210.12797 at 18.03 min, 211.13424 at 17.98 min and 212.14049 at 17.92 min. There were separated by ";". However, only the feature 212.14049 at 17.92 min (pyrene- d_{10}) go through triple filters. For Filter 1, the data provide the numbers of ^2H labels of isotopologue (10), the ratio of the calculated filter ($r_n = 1.01$, follows into the range of filter F1 0.11-1.1, theoretic Ratio is 0.333), and the RT of the isotopologue (17.92). The numbers of ^2H labels of isotopologue (10) and its RT (17.92) restrict the feature's m/z as 202.14049.

Table 3 Illustration of the feature m/z 202.07774 at 18.38

Title	Data
Tentative match	8L @ 210.12797 @ 18.03 ; 9L @ 211.13424 @ 17.98 ; 10L @ 212.14049 @ 17.92
Filter 1	10L : 1.01 17.92;
Filter 2	10L : 3.5 17.92;
Filter 3	10L : 2.63 17.92;

Data availability

We offer our example data, features lists, software for practice of ^2H -SIAM pipeline as following:

- README 2H-SIAM.docx
- 18 mzXML files from the pyrene soil experiment
- OutputFromMZmine2.csv
- ImportFor2HSIAM(1.0).csv
- ResultFrom2HSIAM(1.0).csv
- BatchModelForC18PyrSoil1T3&3T1.xml
- 2H-SIAM(V1.0).exe
- pwiz3.0.20353x86_64.msi
- MZmine2_2.53 for win10 64.zip

Description of the data:

18 mzXML files of the experiment, contain mzXML data from UPLC-ESI-HRMS (Q Exactive, Thermo) including blank, Mix_{1:3} and Mix_{3:1} with 6 replicates.

OutputFromMZmine2.csv, feature list obtained by MZmine2 by batch model using the script offered here. Standard compound normalizer and export to .csv is accomplished manually. Before the use for 2H-SIAM(1.0), the blank data in the list should be deleted, and the sequence should be adjusted. Additionally, if you follow the protocol at the end of this document, the output file from your running may have little difference with ours. It is the result of the execute of MZmine2 with different batches.

ImportFor2HSIAM(1.0).csv, typical ready for use .csv file for 2H-SIAM(1.0).

ResultFrom2HSIAM(1.0).csv, 2H-SIAM(V1.0) analysis output file.

BatchModelForC18PyrSoil1T3&3T1.xml, MZmine2 analysis script for example data.

2H-SIAM(V1.0).exe, windows software (VB-based) for isotope-label metabolomics.

pwiz3.0.20353x86_64.msi, software for format raw data from mass spectrum.

MZmine2_2.53 for win10 64.zip, data processing software.

Description of the example experiment:

pyrene and pyrene-*d*10 were dissolved in acetone (ACE) and mixed respectively with one quarter of blank soil. After solvent evaporation, they were mixed with the rest unspiked soils to obtain 100 ppm pyrene and pyrene-*d*10 contaminated soil respectively. Then, blank and contaminated soils were placed in room temperature for 60 days and watered weekly to keep wet. After 60 days incubation, soil samples from blank, pyrene and pyrene-*d*10 treatment were harvested and air dried at 60 °C until to constant weight. 1 gram of contaminated soils were extracted by 10 mL of 1:3 acetone and Hexane by microwave extraction, and anhydrous NaSO₄ were used to remove residual water. After that solvent was replaced by acetonitrile by solvent exchange, and extracts were concentrated to 1 mL under nitrogen flow.

Extracts from pyrene and pyrene-*d*10 were mixed with the ratio of 1:3 and 3:1 to obtain Mix_{1:3} and Mix_{3:1} samples. Anthracene-*d*10 were added into blank and mixed samples as internal standard. Then, blanks and mixed samples were subjected to UPLC-ESI-HRMS analysis (6 replicates).

5 μL of samples was injected into UPLC-ESI-HRMS system. UPLC solvents were A, water in 0.1% formic acid, and B, acetonitrile with 0.1% formic acid. UPLC were performed at 1 mL/min at 25 °C with the following linear gradient (minutes, %B): 0, 5%; 4, 5%; 8, 95%; 26, 95%; 28, 5%; 30, 5%.

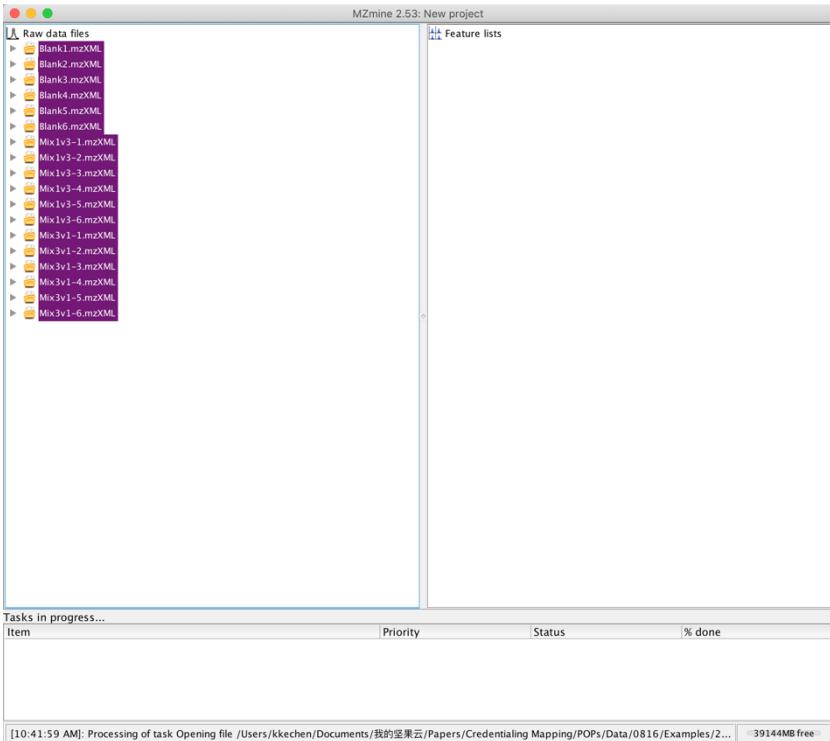
Mass spectrometry was detected via profile mode (scan range, m/z 60–900) with a resolving power of 140 000 fwhm (at m/z 200) and automatic gain control setting of 3×10^6 with a maximum injection time of 200 ms. The heated electrospray ionization (ESI) source was operated using the following settings: sheath gas flow rate, 40 au; auxiliary gas flow rate, 20 au; spray voltage, 3.8 kV; capillary temperature, 325 °C.

STEP BY STEP of a ²H-SIAM Study by the Provided Example Data

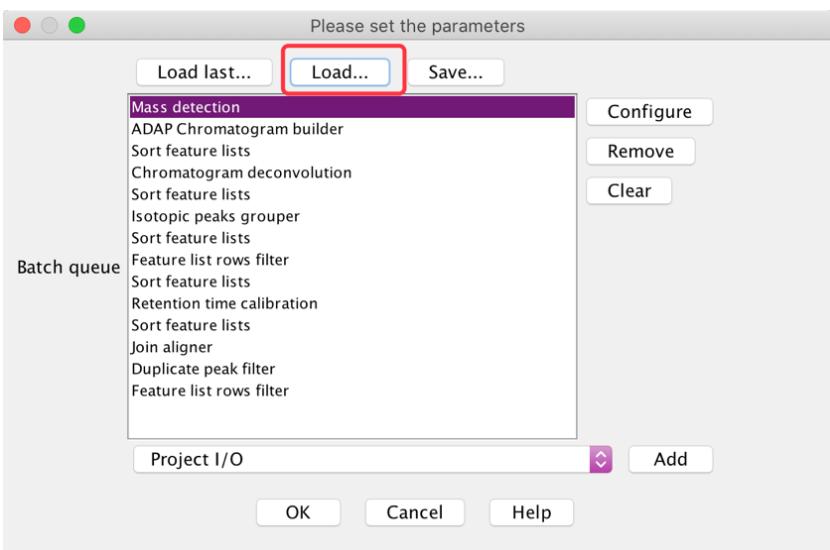
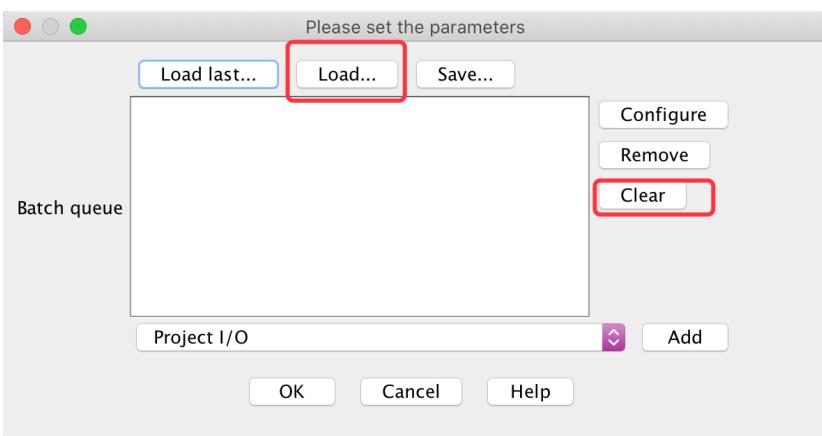
1. Preparing a Features List by MZmine2

1.1 Download and install MZmine2 2.53. For the first time use of MZmine2, it should be better to find some videos from YouTube.

1.2 Start MZmine2 2.53, Use **Raw data import**, import 18 mzXML, sort, select them.



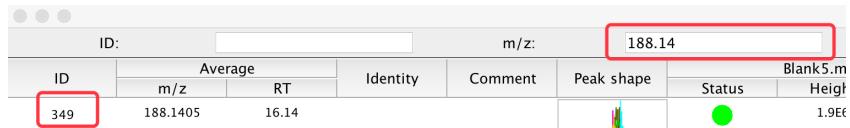
1.3 Use Batch Model, Clear, load the script "BatchModelForC18PyrSoil1T3&3T1.xml", Replace, and run.



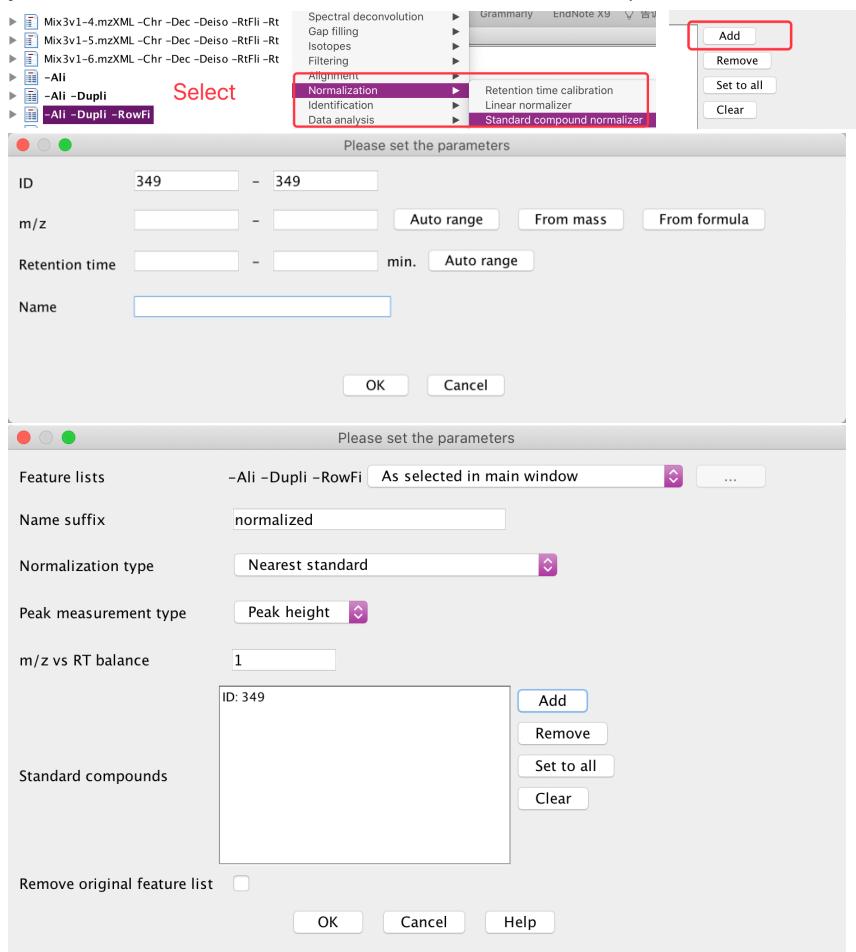
It will take few hours depending on your PC. An PC equipped with i9 11900K and 128 GB ram took ~70 minutes.

1.4 Double click align features list "-Ali -Dupli -RowFi", FIND ID of Internal Standard anthracene-*d*10 with m/z 188.1405 at 16.14. You could input 188.14 to find it quickly.

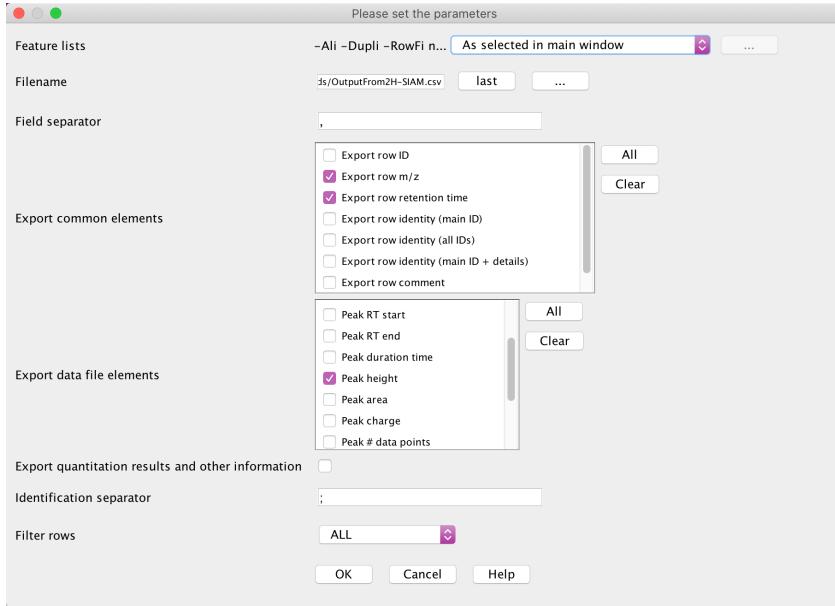
Then you will find the ID of m/z 188.1405 at 16.14 is 349 (**ID 349 MAY CHANGE CASE BY CASE, USE THE ID YOU FIND**).



1.5 Select align features list "-Ali -Dupli -RowFi", Use Normalization, Standard compound normalizer, and add 349 as standard compounds as following, and run.



1.6 Select align features list "-Ali -Dupli -RowFi normalized", Export/Import, Export to CSV file, tick m/z, RT and Peak height, save the file to an indicated pathway.



1.7 Open the output file by EXCEL, delete columns with the name of "BlankX.mzXML".

They are useless for tracking isotopologue pairs.

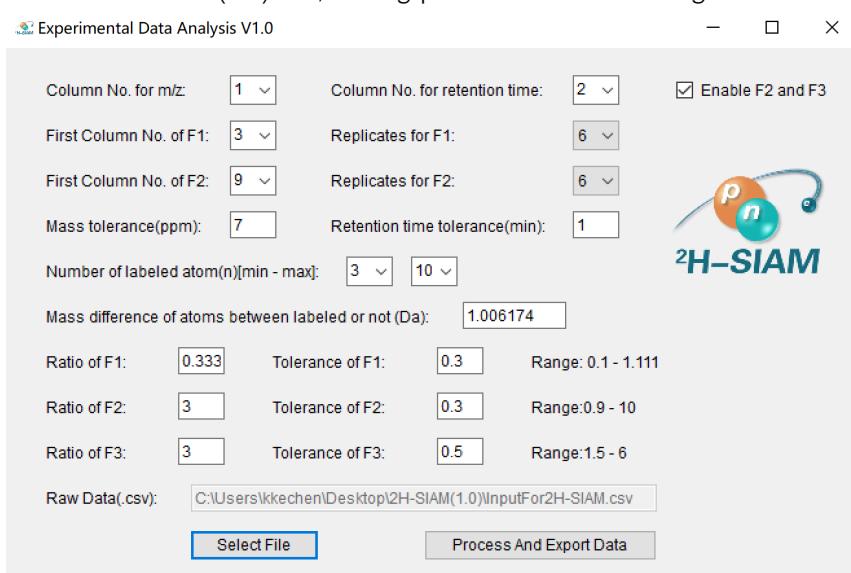
A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	
row m/z	row retention	Blank5.mzXML	Blank4.mzXML	Blank6.mzXML	Blank7.mzXML	Blank8.mzXML	Blank9.mzXML	Blank10.mzXML	Blank11.mzXML	Blank12.mzXML	Blank13.mzXML	Blank14.mzXML	Blank15.mzXML	Blank16.mzXML	Blank17.mzXML	Blank18.mzXML	Blank19.mzXML	Blank20.mzXML	Blank21.mzXML	Blank22.mzXML	
212.00781	6.59883680	1311.03407	3631.12041	48853.4597	3998.02654	21881.4840	18873.3077	42398.9702	10459.207	7200.0305	10450.7007	20901.8058	3659.77262	5350.88998							
308.02177	12.59883965	8731.09347	1105.03349	8419.43382	1716.9767	12195.5723	8410.91647	17205.0222	574.37521	394.221296	14771.6044	2924.68407	5962.03938	4166.23882	1528.28067	12891.3151	1647.86639	0	2438.56358		
111.090294	28.3792996	531.9771	2609.76984	0.89395278	4330.10526	1463.40904	0.40389328	409.485798	6515.27879	9738.7051	0	321.347611	8574.38645	7455.34911	0	8394.00956	4765.59581	6584.10229	4446.68202	2474.98183	
85.076613	28.8926055	6767.23986	3988.02568	3037.3729	4517.20068	5333.79367	6515.27879	1222.47209	14415.27879	1222.47209	14415.27879	1222.47209	14415.27879	1222.47209	14415.27879	1222.47209	14415.27879	1222.47209	14415.27879		
415.211532	13.039748	6117.06605	5061.73091	5551.28996	9733.09380	11755.007	14415.27879	20768.8914	4881.5774	5774.6798	14183.4311	12661.4739	15434.9417	13780.5768	13261.5049	14258.1956	9600.7884	3503.08625	11090.7536		
129.018443	6.60399747	6039.76102	1253.97862	5437.01255	2026.13641	7916.23719	57580.14075	7849.10704	3204.55956	1473.46861	6691.96263	6196.91314	9605.93616	6434.71874	5809.076	6328.80071	2087.17495	0	3284.66956		
87.0922991	28.5333484	4643.03038	2956.52006	1235.47920	4026.10594	1865.89906	3711.73406	0	5059.71689	459.652	2068.34761	4774.50008	7336.77042	4591.43203	1884.5986	2499.13513	3384.88313	869.95067	2098.38735		
149.00777	12.59883965	4463.03038	4625.27689	4555.37526	7812.93968	1870.50008	1787.12041	27267.05826	2992.20554	3701.221296	2139.655	1374.521	2139.655	2139.655	2139.655	2139.655	2139.655	2139.655	2139.655		
186.10777	28.4772714	1105.03349	1862.38916	5485.37526	0	1461.80354	1579.62301	1749.13101	0	2407.63839	2407.63839	2407.63839	2407.63839	2407.63839	2407.63839	2407.63839	2407.63839	2407.63839	2407.63839		
150.154009	13.5655631	3744.01028	2620.01036	3465.37526	4305.45236	5544.36318	6749.13101	0	1609.71128	2499.61247	25764.862	2338.69794	5759.65465	4844.5015	2242.89263	734.70741	1845.26838				
82.049846	12.4809998	3625.26352	2608.06932	3875.0522	11120.8278	15194.5155	1622.4247	4831.5138	0	249.32615	8930.61764	1038.25677	1322.45855	1432.28472	468.495162	8004.3516	753.698008	2578.15936	1606.63236		
423.008932	6.51794459	2921.31755	0	3096.44928	871.713407	5283.64624	3144.0519	13683.4564	0	249.32615	4831.5138	0	977.313409	4287.93447	3930.72161	2424.76706	3582.84557	3620.0235	3798.876		
230.099228	6.37810134	2921.31755	0	6.559195231	1626.01979	0	4108.38876	4831.5138	3211.21864	1207.46932	2172.29024	2061.42404	2981.36027	2123.64634	717.939202	0	2216.79014				
210.069865	1.14897619	2764.00513	3884.5021	3146.5169	0	2631.81946	736.853403	3522.98346	3065.3424	4471.65307	1534.7951	659.571893	3508.62892	1965.1088	3065.86536	2252.46945	1798.675	3329.32083	764.41506	0	
215.024622	1.14897619	2763.26832	0	2631.81946	2843.08804	6265.35888	7716.03338	10890.5316	13363.7829	0	1232.6027	12444.0742	9659.05673	1237.57477	8541.66566	8974.17588	9207.41253	7655.39932	0	8441.38151	
99.0899782	11.5981552	2658.85949	1776.31717	2484.08804	0	2631.81946	736.853403	3522.98346	3065.3424	4471.65307	1534.7951	659.571893	3508.62892	1965.1088	3065.86536	2252.46945	1798.675	3329.32083	764.41506	0	1195.72401

Sort sequences of the data as following and saving.

A	B	C	D	E	F	G	H	I	J	K	L	M	N
row m/z	row retention	Mix1v3-1.m	Mix1v3-2.m	Mix1v3-3.m	Mix1v3-4.m	Mix1v3-5.m	Mix1v3-6.m	Mix3v1-1.m	Mix3v1-2.m	Mix3v1-3.m	Mix3v1-4.m	Mix3v1-5.m	Mix3v1-6.m

2. Run ²H-SIAM(1.0)

2.1 Start ²H-SIAM(1.0).exe, setting parameters as following



2.2 Select the features list, wait a second for loading data, and then click "Process And

