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User Guide for SMART software

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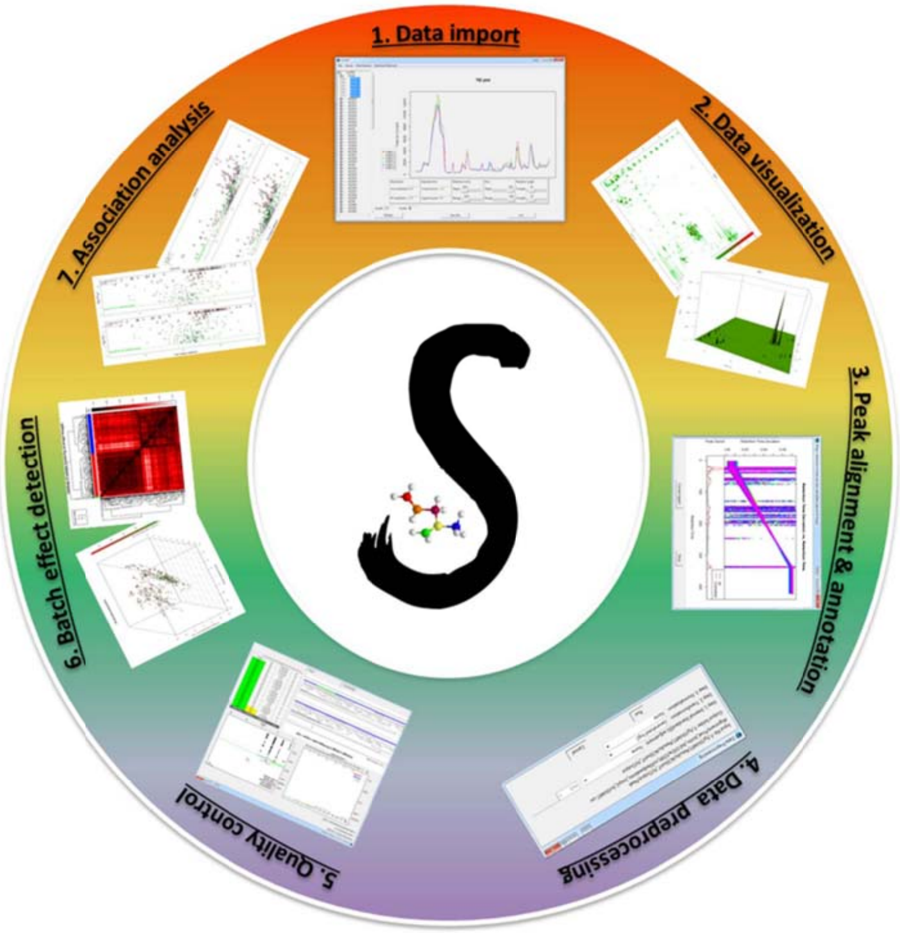

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1. SMART License

If publications are based on the results from the use of **SMART**, please cite the following reference:

Yu-Jen Liang, Yu-Ting Lin, Chia-Wei Chen, Chien-Wei Lin, Kun-Mao Chao, Wen-Harn Pan and Hsin-Chou Yang (2016). SMART: Statistical Metabolomics Analysis – An R Tool. Under revision in *Analytical Chemistry*.

2. Overview

SMART written in R and R GUI has been developed as user-friendly software for integrated analysis of metabolomics data. **SMART** streamlines the complete analysis flow from initial data preprocessing to downstream association analysis, consisting of analyzing different data file formats (e.g., .raw, .d, and mzXML), visually representing various types of data features (e.g., total ion chromatogram (TIC) and mass spectra), implementing peak alignment, conducting quality control for samples and peaks, exploring batch effects (e.g., known experimental conditions, unknown latent groups (LGs), or hidden substructures), and performing association analysis (**Figure 2.1**).

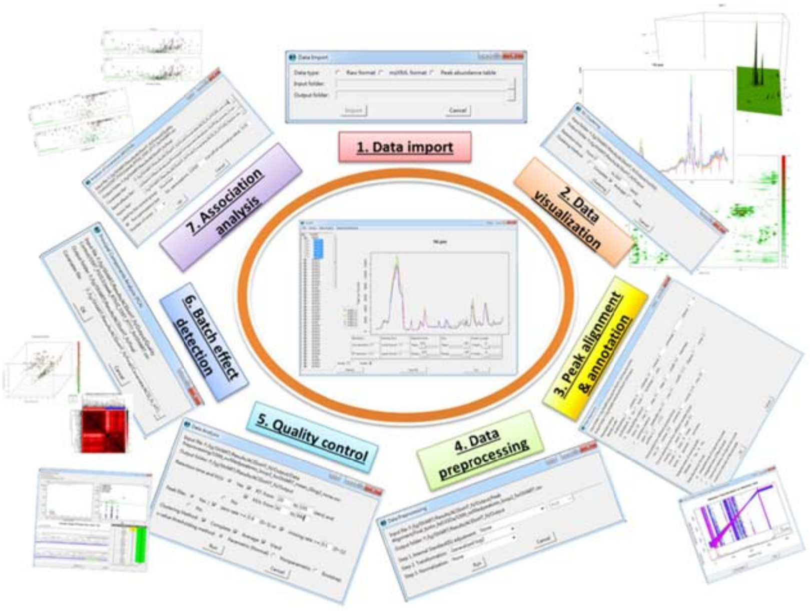


Figure 2.1. Overview of **SMART**.

3. Software Download and Installation

Execution of **SMART** requires installation of **SMART** program, R program, ActiveTcl, ProteoWizard, and some R packages.

3.1 SMART

We provide **SMART** programs for 32-bit and 64-bit Windows operating systems (Windows 7, Windows 8, and Windows 10) and Mac operating systems (OS X 10.6 or later). The programs, user guide, and examples can be downloaded from the **SMART** website at: http://www.stat.sinica.edu.tw/hsinchou/metabolomics/SMART.htm.

3.2 R program

Users can download R program from the website of “The R Project for Statistical Computing” at http://www.r-project.org/. Users click “CRAN” (Comprehensive R Archive Network) in the left of the page and then select a suitable mirror site to download R. Select a platform (Windows or Mac OS X) for R execution in your end. For Windows system users, click the hyperlink “base” and select “Download R 3.3.0 for Windows”. Then execute the file “R-3.3.0-win.exe” to install R to “C:\Program Files\R\R-3.3.0”. After finishing the installation of R, doubly click the icon “R i386 3.3.0” or “R x64 3.3.0” to initialize R in a 32-bit or 64-bit system, respectively. A window “RGui” with a sub-window “R Console” jumps up await for the subsequent analysis action. Users are suggested to update packages in R. They can select “Packages” in the tool bar, click “Update packages” and then select a suitable mirror site to update packages. A window “CRAN mirror” jumps up and the icon “OK” is clicked to update packages. For Mac system users, click the hyperlink “R-3.3.0.pkg” to download R-3.3.0.pkg for Mac version R and click the hyperlink “XQuartz” to download XQuartz.dmg for Mac version X. Execute XQuartz.dmg to obtain XQuartz.pkg. Execute R-3.3.0.pkg and XQuartz.pkg to initialize Mac version R and X, respectively.

3.3 ActiveTcl

Program ActiveTcl Community Edition can be downloaded from the website of ActiveState. For 32-bit Windows system users, please download ActiveTcl from the following hyperlink http://www.activestate.com/activetcl/downloads/thank-you?dl=http://downloads.activestate.com/ActiveTcl/releases/8.5.18.0/ActiveTcl8.5.18.0.298892-win32-ix86-threaded.exe.

For 64-bit Windows system users, please download ActiveTcl from the following hyperlink http://www.activestate.com/activetcl/downloads/thank-you?dl=http://downloads.activestate.com/ActiveTcl/releases/8.5.18.0/ActiveTcl8.5.18.0.298892-win32-x86_64-threaded.exe. For Mac OS, please download ActiveTcl from the following hyperlink http://www.activestate.com/activetcl/downloads/thank-you?dl=http://downloads.activestate.com/ActiveTcl/releases/8.5.18.0/ActiveTcl8.5.18.0.298892-macosx10.5-i386-x86_64-threaded.dmg.

3.4 ProteoWizard

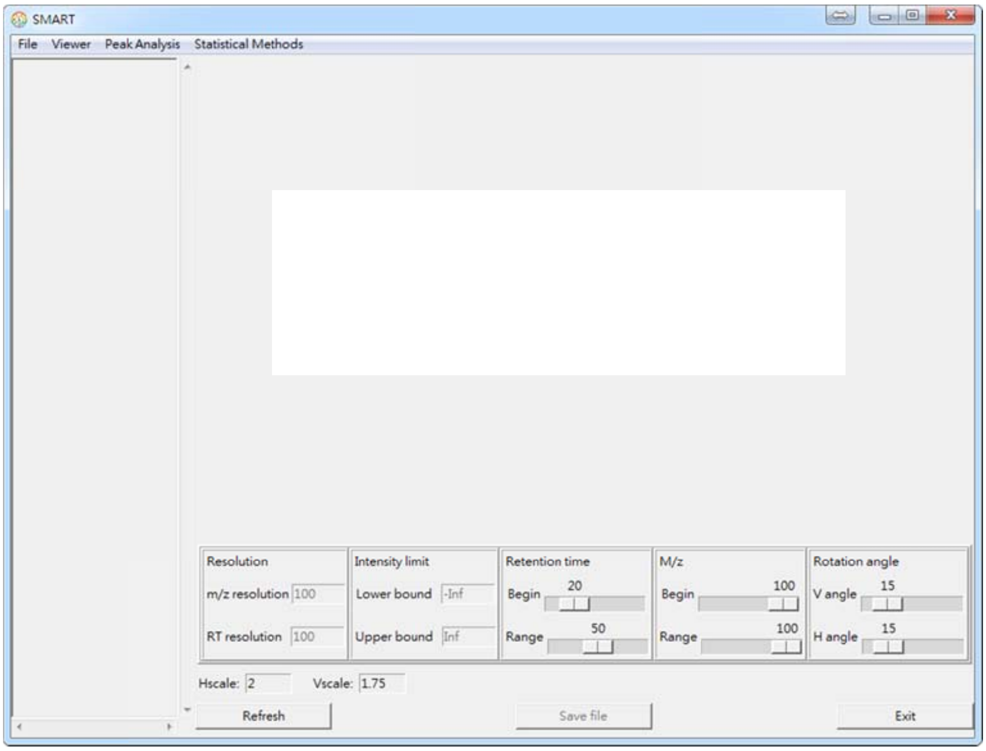
ProteoWizard can be downloaded from the website at http://proteowizard.sourceforge.net/downloads.shtml. Please select "Windows installer (able to covert vendor files)", choose a license agreement, and click "I agree to the licensing terms" to download and then install ProteoWizard. Note that ProteoWizard does not provide a version for Mac operating systems.

3.5 R packages

The analyses provided by **SMART** require several additional R packages, consisting of boot, car, fields, graphics, gtools, limma, mzR, png, tcltk2, tkrplot, scatterplot3d, snow, xcms, and CAMERA. **SMART** checks whether these R packages have been installed in R. If NOT, the packages will be automatically downloaded if users are using a latest version of R, e.g., R-3.3.0, in an environment with internet access. Note that program R-3.3.0 or a version of R program newer than version R-3.1.0 is recommended for running **SMART**.

4 SMART Initialization

For Windows system users, once all of the software and packages have been installed, **SMART** can be initialized by doubly clicking the executable file of **SMART.** The interface of SMART jumps up (see **Figure 4.1**).



Resolution: m/z resolution 100; RT resolution 100

Intensity limit: Lower bound -Inf; Upper bound Inf

Retention time: Begin 20; Range 50

M/z: Begin 100; Range 100

Rotation angle: V angle 15; H angle 15

Hscale: 2 Vscale: 1.75

Figure 4.1. Initial interface of **SMART** in Window systems.

For Mac users, please execute **SMART** by the following four steps:

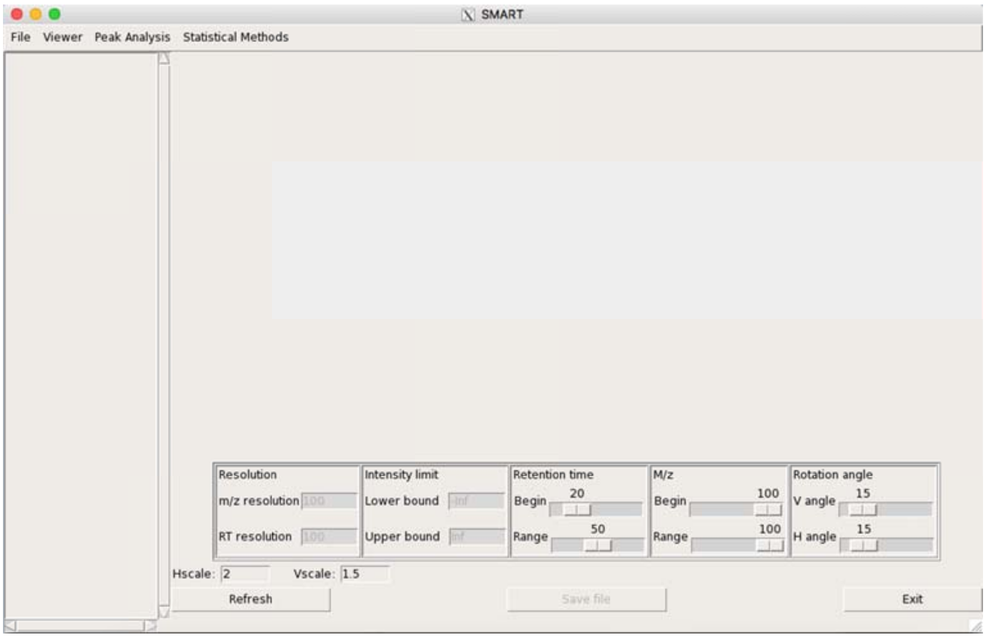
Step 1: Save three R programs, “SMART_V1.0_Mac.r”, “SMART_V1.0_Mac_Gui.r” and “SMART_V1.0_Mac_Sub.r”, in the same folder (e.g., ~/Documents/SMART).

Step 2: Initialize R as mentioned in Section 3.2.

Step 3: Drag and drop “SMART_V1.0_Mac.r” to the window “R Console” to initialize **SMART**. Or you can type the command, **source(‘~/Documents/SMART/SMART_V1.0_Mac.r’)**, in the window “R Console”.

Step 4: Type the full path name (e.g., ~/Documents/SMART) and press the Enter key, and then the interface of **SMART** will jump up (see **Figure 4.2**).

There are four main functions **in SMART**: (1) File, (2) Viewer, (3) Peak Analysis, and (4) Statistical Methods. Descriptions about these functions will be introduced in Section 5.



m/z resolution 100
RT resolution 100
Lower bound -Inf
Upper bound Inf
Retention time Begin 20
Retention time Range 50
M/z Begin 100
M/z Range 100
V angle 15
H angle 15
Hscale: 2
Vscale: 1.5

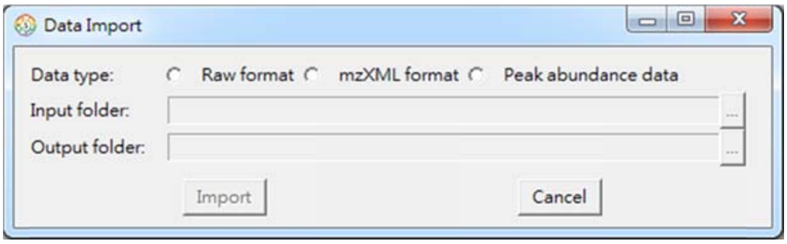
Figure 4.2. Initial interface of **SMART** in Mac systems.

5 SMART Interfaces, Tools, and Operating Procedures

The analysis procedures of **SMART** in Windows and Mac systems are almost the same. In this and next sections, we illustrate the operating environments and procedures in Windows systems.

5.1 Data import

SMART supports multiple input file formats (see **Figure 5.1.1**). The first format is the raw spectrum data format, such as .d files from Agilent Technologies (Santa Clara, CA, USA) and .raw files from Waters (Milford, MA, USA). The second format is the mzXML file format, which is an Extensible Markup Language (XML) file format for MS data. The third format is the comma-delimited peak abundance data of mass spectra. The peak abundance data file contains the mass-to-charge ratio (m/z), chromatographic retention time (RT, in seconds or minutes), and peak abundance or metabolite concentration of all replicate samples of a study subject.



Data type: ○ Raw format ○ mzXML format ○ Peak abundance data
Input folder:
Output folder:

Figure 5.1.1. Interface of data import.

- raw spectrum data file:

  Users follow the procedure to import data: *Click “File”* ➔ *Click “Data Import”* ➔ *Choose “Raw format”* ➔ *Specify the I/O path*. Note that only raw spectrum data files can be saved in the specified input folder. Note that this function is only available for Windows systems.

- mzXML data file:

  Users follow the procedure to import data: *Click “File”* ➔ *Click “Data Import”* ➔ *Choose “mzXML format”* ➔ *Specify the I/O path*. Note that only mzXML files can be saved in the specified input folder.

- comma-delimited peak abundance data file:

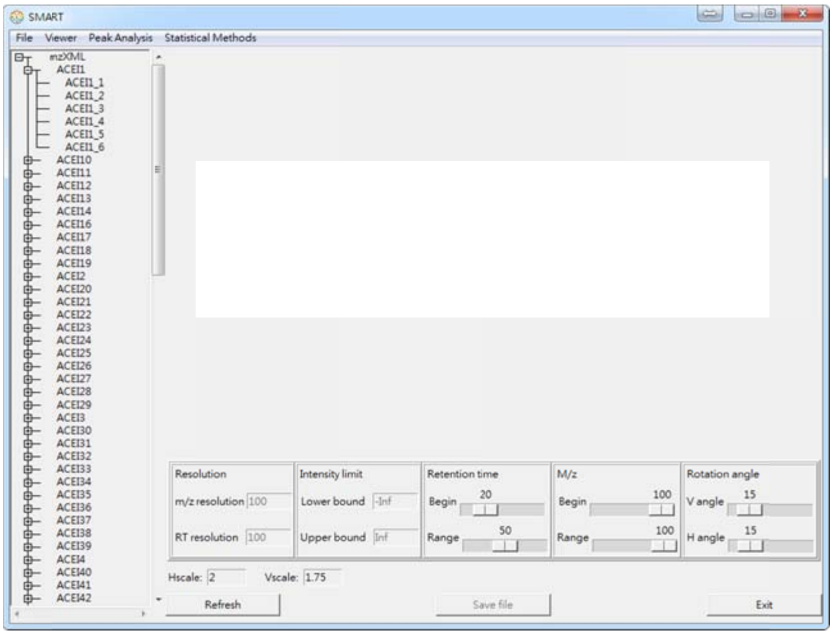
  Users follow the procedure to import data: *Click “File”* ➔ *Click “Data Import”* ➔ *Choose “Peak abundance data”* ➔ *Specify the I/O path*. An example of a comma-delimited peak abundance data file is provided (see **Table 5.1.1**). The first three columns are peak index, m/z, RT, and followed by peak abundance data of all replicate samples of all subjects. Each row indicates a peak/metabolite. The subject nomenclature consists of the subject name followed by the replicate sample name(s). For example, ACEI1_1 and ACEI1_2 represent the first and second replicate samples of subject ACEI1.

Table 5.1.1. The comma-delimited peak abundance data file.

Peak_		Ret_								
Index	mz	time.sec	ACEI1_1	ACEI1_2	ACEI2_1	ACEI2_2	N1_1	N1_2	N2_1	N2_2
1	22.98998	27.7059	7.0783	6.6935	6.5680	6.4190	7.0669	6.6445	7.0774	6.5764
2	55.93659	336.5655	6.9994	7.9379	NA	8.4114	8.3721	8.3248	8.0697	8.3277
3	56.96345	335.7621	6.3181	6.0349	6.8107	6.7456	6.4947	6.3940	6.2704	6.0403
4	57.93642	335.8248	6.2205	6.1592	6.8686	6.5870	6.5836	6.4859	6.4517	6.4039
5	60.07343	29.2071	6.2056	NA	6.6890	6.5365	6.2876	6.1709	6.1499	6.2357
6	68.98333	24.0914	6.4834	6.5592	7.4892	7.4233	6.4490	6.4999	6.4278	6.4466
7	69.07036	53.4821	NA	NA	7.4339	6.9873	5.6892	5.2228	5.4749	5.2144
8	70.06573	32.3258	NA	NA	8.3018	8.0467	6.0851	5.7031	NA	NA
9	72.08130	64.1186	NA	NA	7.8959	7.6318	7.1014	7.1676	NA	NA
10	72.08131	37.8573	7.7268	7.3779	10.2431	10.0360	8.3697	8.2444	8.2197	7.9276
11	74.93966	335.9213	6.7730	7.0727	7.6475	7.3816	7.1578	7.3676	7.3392	7.2886
12	77.03906	58.1514	6.7476	6.6204	9.4728	9.5908	6.8912	6.9771	6.8814	6.7502
13	79.05363	58.1659	6.0404	5.4665	8.3506	8.3917	5.9861	6.1343	5.7149	5.9090
14	82.01453	24.0855	5.9334	6.0697	7.2531	7.0337	5.8216	6.0698	5.9923	5.5052
15	82.01411	335.6980	NA	NA	NA	10.1490	NA	7.3956	NA	NA
16	84.04471	51.8347	6.5344	6.5024	7.6371	7.5025	7.3956	7.1509	6.2767	NA

17	84.04468	30.1110	7.4784	7.5663	10.1490	10.1662	8.7341	8.6003	7.9926	7.7599
18	84.08100	25.1120	NA	NA	9.2761	9.1194	7.0255	6.7111	5.2853	NA
19	84.96001	24.1023	9.7988	9.6337	10.8931	10.7249	9.6305	9.5511	9.5060	9.3717
20	84.95969	335.6133	10.0044	10.1116	10.8662	10.4914	10.4841	10.3043	10.4317	10.1453

After raw spectrum data or mzXML data import, a sample tree diagram will be shown in the left-hand panel of the interface of **SMART** (see **Figure 5.1.2**).

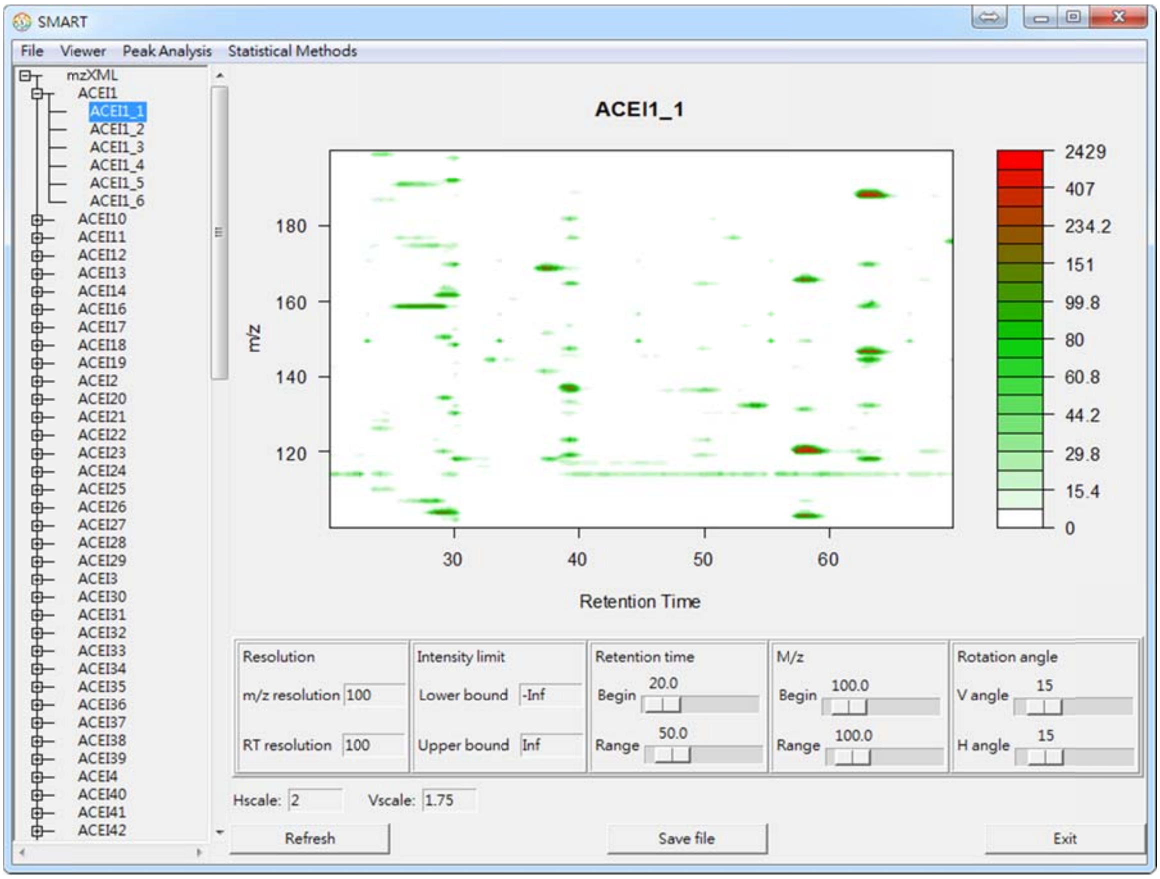


m/z resolution 100
RT resolution 100
Lower bound -Inf
Upper bound Inf
Begin 20
Range 50
Begin 100
Range 100
V angle 15
H angle 15
Hscale: 2
Vscale: 1.75

Figure 5.1.2. Interface after data import.

5.2 Data visualization

SMART supports visualization of mzXML files. First, users follow the procedure to visually represent two-dimensional (2D) spectrum data in any user-specified m/z and RT region for the replicate sample(s) of interest (See **Figure 5.2.1**): *Select one specific sample in sample tree diagram in the left-hand panel ➔ Click "Viewer" ➔ Choose "2D Plot"*.



Resolution

m/z resolution 100

RT resolution 100

Intensity limit

Lower bound -Inf

Upper bound Inf

Retention time

Begin 20.0

Range 50.0

M/z

Begin 100.0

Range 100.0

Rotation angle

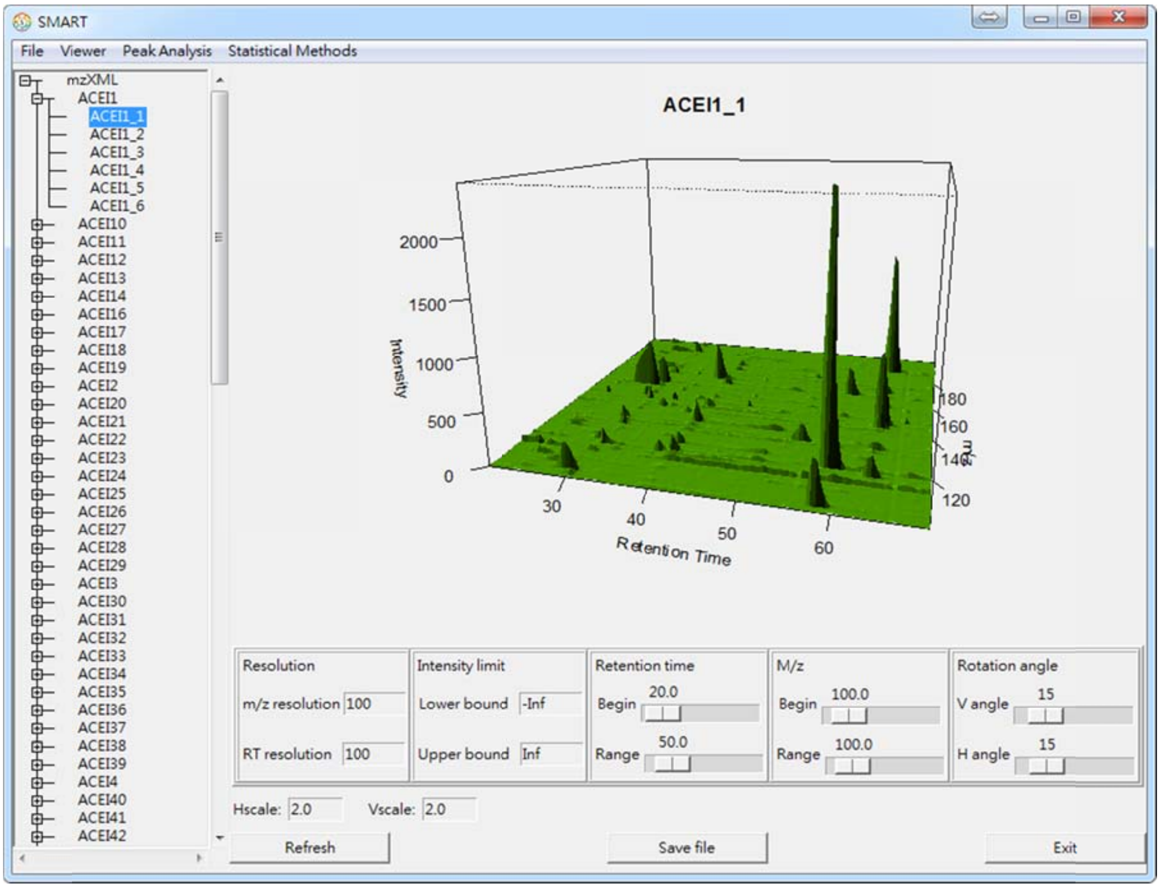
V angle 15

H angle 15

Hscale: 2 Vscale: 1.75

Figure 5.2.1. 2D spectrum plot.

Second, users follow the procedure to visually represent three-dimensional (3D) spectrum data in any user-specified m/z and RT region for the replicate sample(s) of interest (See **Figure 5.2.2**): *Select one specific sample in sample tree diagram in the left-hand panel ➔ Click “Viewer” ➔ Choose “3D Plot”*.



Resolution: m/z resolution 100; RT resolution 100

Intensity limit: Lower bound -Inf; Upper bound Inf

Retention time: Begin 20.0; Range 50.0

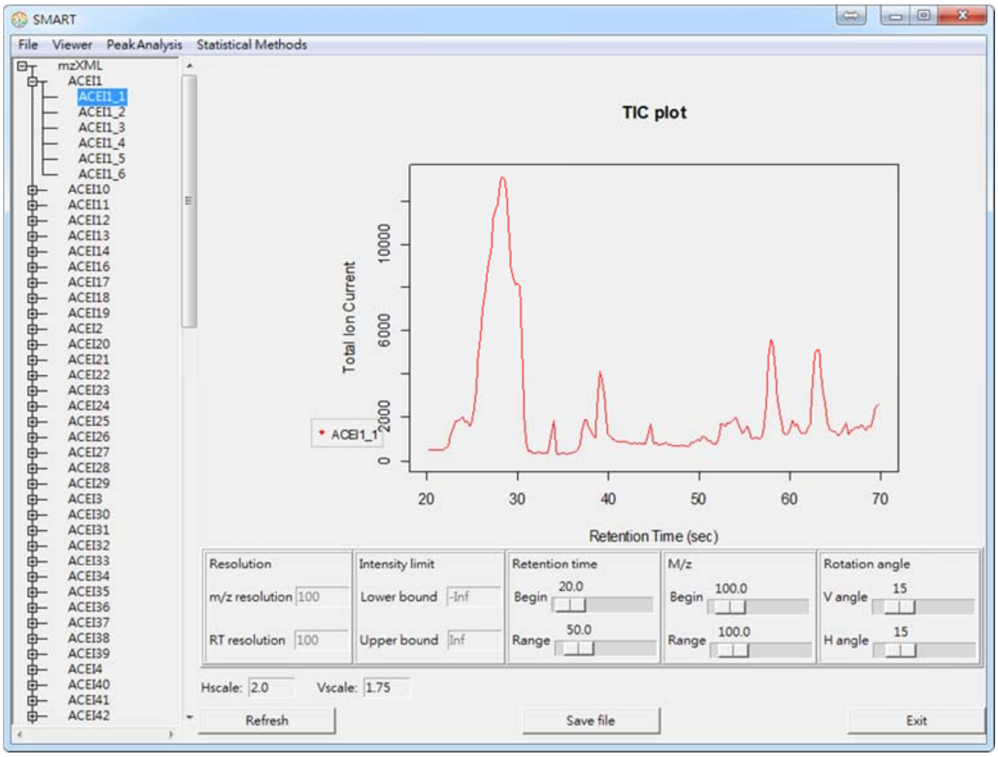
M/z: Begin 100.0; Range 100.0

Rotation angle: V angle 15; H angle 15

Hscale: 2.0 Vscale: 2.0

Figure 5.2.2. 3D spectrum plot.

Third, users follow the procedure to visually represent TIC in any user-specified m/z and RT region for the replicate sample(s) of interest (See **Figure 5.2.3**): *Select one specific sample ➔ Click "Viewer" ➔ Choose "TIC plot".* Note that users can doubly click any point on the TIC curve (i.e., at a fixed RT scan) to look into a spectrum plot (See **Figure 5.2.4**). Under a fixed RT, the spectrum plot displays peak intensities for peaks across all m/z in the data.



Resolution
m/z resolution 100
RT resolution 100

Intensity limit
Lower bound -Inf
Upper bound Inf

Retention time
Begin 20.0
Range 50.0

M/z
Begin 100.0
Range 100.0

Rotation angle
V angle 15
H angle 15

Hscale: 2.0 Vscale: 1.75

Figure 5.2.3. TIC plot.

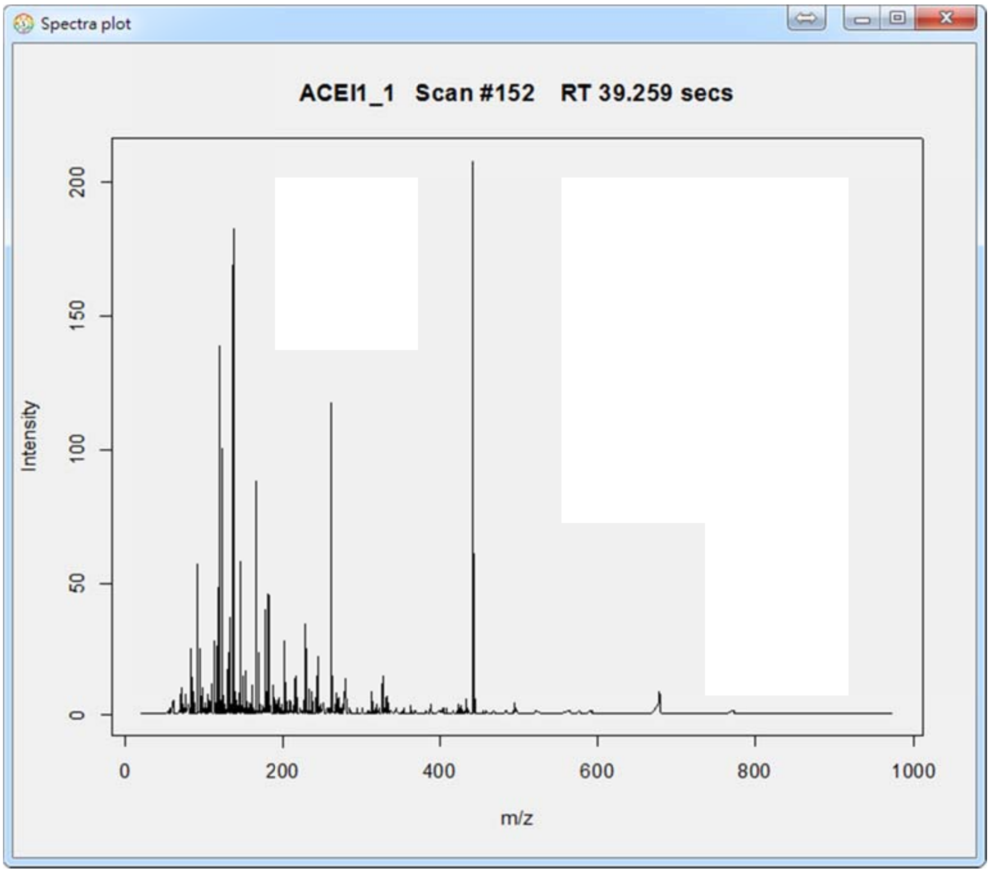
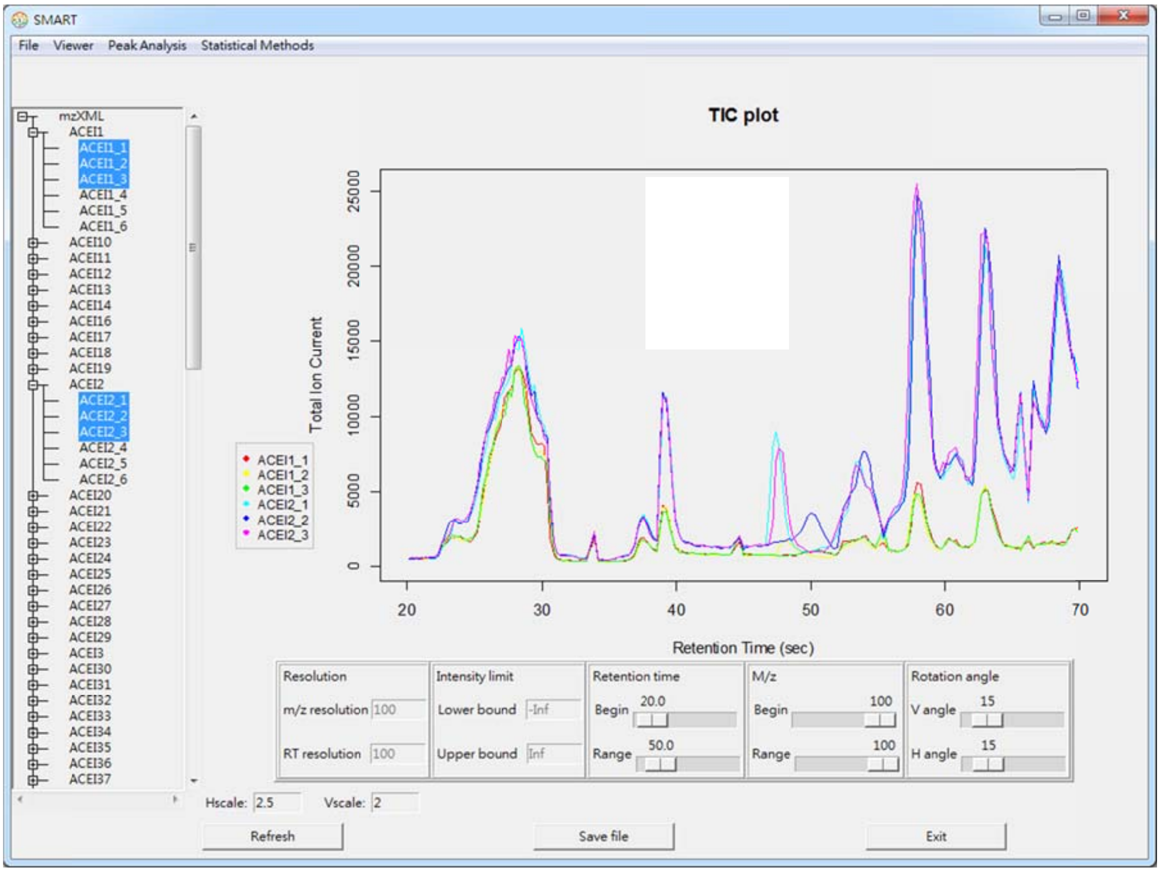


Figure 5.2.4. Spectrum plot at a fixed RT scan.

Fourth, users follow the procedure to visually represent TIC for multiple replicate samples and subjects (See **Figure 5.2.5**): *Select multiple replicate samples and subjects ➔ Click “Viewer” ➔ Choose “TIC plot”.*



Resolution: m/z resolution 100; RT resolution 100
Intensity limit: Lower bound -Inf; Upper bound Inf
Retention time: Begin 20.0; Range 50.0
M/z: Begin 100; Range 100
Rotation angle: V angle 15; H angle 15
Hscale: 2.5 Vscale: 2

Figure 5.2.5. Cross-replicate-sample and cross-subject TIC overlay plots.

SMART provides five figure display options, consisting of (1) “Resolution” – figure resolution for densities of m/z and RT, (2) “Intensity limit” – the lower and upper bounds of peak intensity, (3) “Retention time” – the beginning RT and the range of RT, (4) “M/z” – the beginning m/z and the range of m/z, and (5) “Rotation angle” – rotation angles for a 3D spectrum plot. Users can modify the parameters as their need. In addition, users can reset the height and width of the **SMART** interface by filling in numbers and clicking “Refresh”. Users can save figures in the SMART interface by clicking “Save file” and escape the **SMART** environment any time by clicking “Exit”.

Finally, users follow the procedure to visually represent a TIC cluster diagram of all replicate samples of all subjects (See **Figure 5.2.6**): *Click “Viewer” ➔ Choose “TIC*

Clustering” ➔ *Fill in retention time range of interest* ➔ *Choose clustering method (Complete linkage, Average linkage, or Ward’s method)* (See **Figure 5.2.7**).

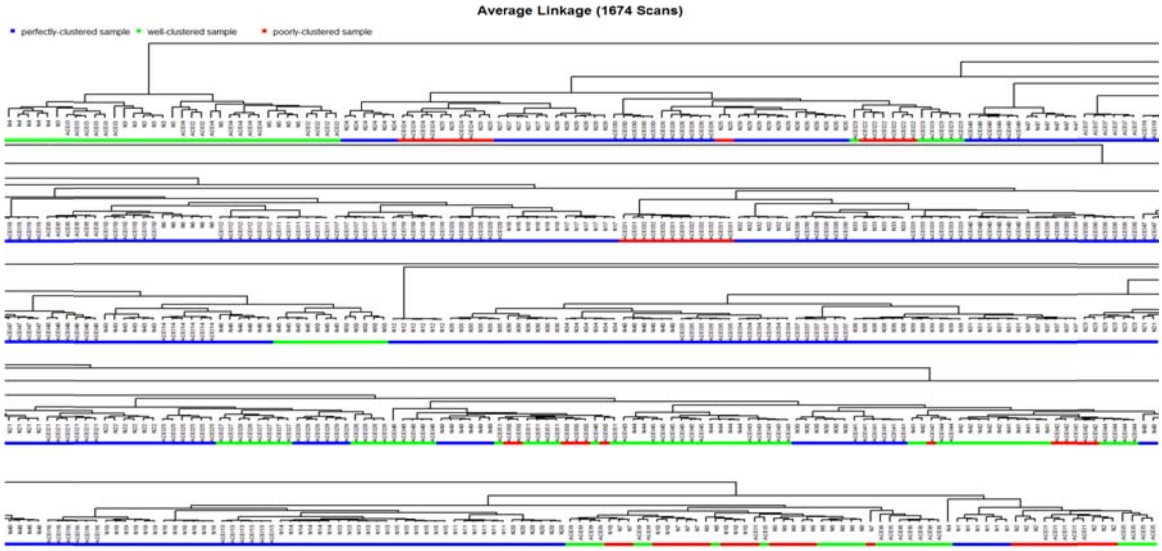
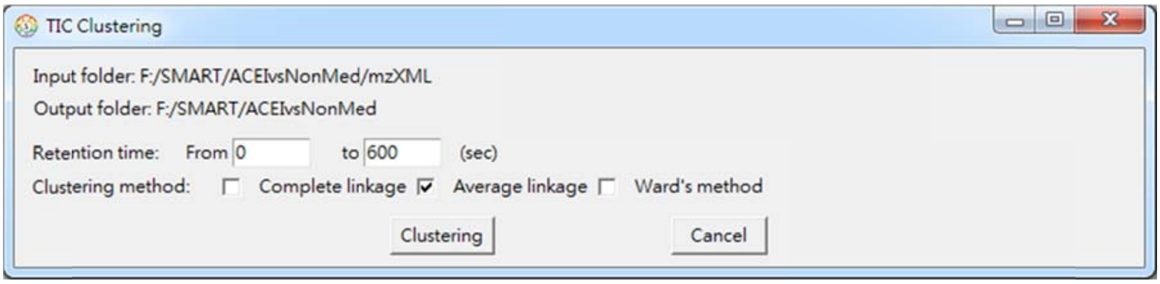


Figure 5.2.6. TIC cluster tree diagram.



Input folder: F:/SMART/ACEIvsNonMed/mzXML

Output folder: F:/SMART/ACEIvsNonMed

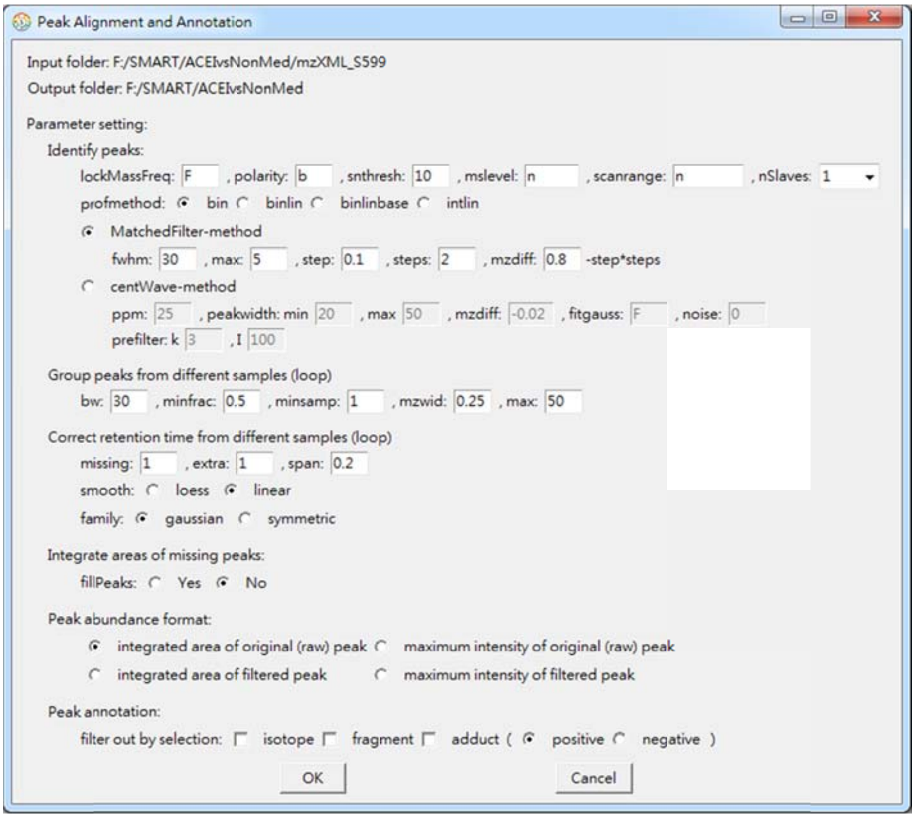
Retention time: From 0 to 600 (sec)

Clustering method: □ Complete linkage ☑ Average linkage □ Ward's method

Figure 5.2.7. Interface of TIC clustering.

5.3 Peak alignment and annotation

SMART carries out peak alignment (i.e., peak detection and RT alignment) by incorporating the matched filtration and centWave in XCMS [1] (see **Figure 5.3.1**) and provides peak annotation by incorporating package CAMERA [2]. After importing raw spectrum or mzXML data file(s), users follow the procedure to perform peak alignment: *Click “Peak Analysis”* ➔ *Choose “Peak Alignment and Annotation”*. Then a window about a citation of XCMS and CRAMEA will pop up.



Input folder: F:/SMART/ACEIvsNonMed/mzXML_S599

Output folder: F:/SMART/ACEIvsNonMed

Parameter setting:

Identify peaks:

lockMassFreq: F , polarity: b , snthresh: 10 , mslevel: n , scanrange: n , nSlaves: 1

profmethod: ● bin ○ binlin ○ binlinbase ○ intlin

● MatchedFilter-method

fwhm: 30 , max: 5 , step: 0.1 , steps: 2 , mzdiff: 0.8 -step*steps

○ centWave-method

ppm: 25 , peakwidth: min 20 , max 50 , mzdiff: -0.02 , fitgauss: F , noise: 0

prefilter: k 3 , I 100

Group peaks from different samples (loop)

bw: 30 , minfrac: 0.5 , minsamp: 1 , mzwid: 0.25 , max: 50

Correct retention time from different samples (loop)

missing: 1 , extra: 1 , span: 0.2

smooth: ○ loess ● linear

family: ● gaussian ○ symmetric

Integrate areas of missing peaks:

fillPeaks: ○ Yes ● No

Peak abundance format:

● integrated area of original (raw) peak ○ maximum intensity of original (raw) peak

○ integrated area of filtered peak ○ maximum intensity of filtered peak

Peak annotation:

filter out by selection: □ isotope □ fragment □ adduct (● positive ○ negative)

Figure 5.3.1. Interface and default settings of peak alignment and annotation.

After setting parameters and running peak alignment and annotation, **SMART** exports the comma-delimited peak abundance data, including the peak index, m/z, RT (seconds), and peak abundance of every replicate sample (see **Table 5.3.1**). In addition, **SMART** exports the peak annotation, including the peak index, m/z, RT (seconds), isotope, adduct, and peak group (pcgroup) (see **Table 5.3.2** as an example).

Table 5.3.1. Data after peak alignment

Peak_ Index	mz	Ret_ time.sec	ACEI1_1	ACEI1_2	ACEI2_1	ACEI2_2	N1_1	N1_2	N2_1	N2_2
1	22.98998	27.7059	7.0783	6.6935	6.5680	6.4190	7.0669	6.6445	7.0774	6.5764
2	55.93659	336.5655	6.9994	7.9379	NA	8.4114	8.3721	8.3248	8.0697	8.3277
3	56.96345	335.7621	6.3181	6.0349	6.8107	6.7456	6.4947	6.3940	6.2704	6.0403
4	57.93642	335.8248	6.2205	6.1592	6.8686	6.5870	6.5836	6.4859	6.4517	6.4039
5	60.07343	29.2071	6.2056	NA	6.6890	6.5365	6.2876	6.1709	6.1499	6.2357
6	68.98333	24.0914	6.4834	6.5592	7.4892	7.4233	6.4490	6.4999	6.4278	6.4466
7	69.07036	53.4821	NA	NA	7.4339	6.9873	5.6892	5.2228	5.4749	5.2144
8	70.06573	32.3258	NA	NA	8.3018	8.0467	6.0851	5.7031	NA	NA
9	72.08130	64.1186	NA	NA	7.8959	7.6318	7.1014	7.1676	NA	NA
10	72.08131	37.8573	7.7268	7.3779	10.2431	10.0360	8.3697	8.2444	8.2197	7.9276
11	74.93966	335.9213	6.7730	7.0727	7.6475	7.3816	7.1578	7.3676	7.3392	7.2886

12	77.03906	58.1514	6.7476	6.6204	9.4728	9.5908	6.8912	6.9771	6.8814	6.7502
13	79.05363	58.1659	6.0404	5.4665	8.3506	8.3917	5.9861	6.1343	5.7149	5.9090
14	82.01453	24.0855	5.9334	6.0697	7.2531	7.0337	5.8216	6.0698	5.9923	5.5052
15	82.01411	335.6980	NA	NA	NA	10.1490	NA	7.3956	NA	NA
16	84.04471	51.8347	6.5344	6.5024	7.6371	7.5025	7.3956	7.1509	6.2767	NA
17	84.04468	30.1110	7.4784	7.5663	10.1490	10.1662	8.7341	8.6003	7.9926	7.7599
18	84.08100	25.1120	NA	NA	9.2761	9.1194	7.0255	6.7111	5.2853	NA
19	84.96001	24.1023	9.7988	9.6337	10.8931	10.7249	9.6305	9.5511	9.5060	9.3717
20	84.95969	335.6133	10.0044	10.1116	10.8662	10.4914	10.4841	10.3043	10.4317	10.1453

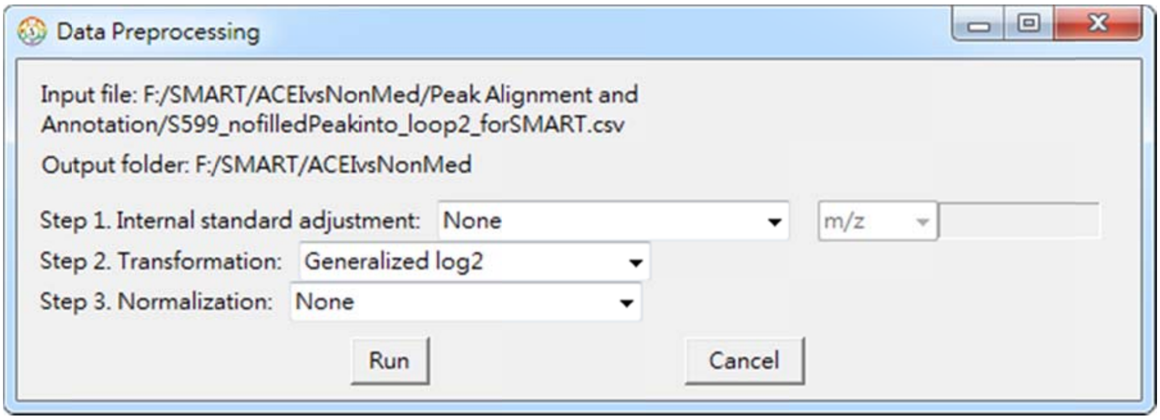
Table 5.3.2. Peak annotation

Peak_Index	mz	Ret_Time.sec	isotope	adduct	pcgroup
50	104.10134	149.0014			1
316	267.64494	149.0323			1
390	312.02142	149.0211			1
402	314.03463	149.0197			1
412	316.03100	149.0111			1
668	478.34350	149.0298	[67][M]+	[M+H-H20]+ 495.35	1
670	479.34696	149.0197	[67][M+1]+		1
699	496.35508	149.0359	[75][M]+	[M+H]+ 495.35	1
700	497.35911	149.0335	[75][M+1]+		1
703	499.36087	149.0323			1
731	518.34134	149.0651	[82][M]+	[M+Na]+ 495.35	1
734	519.34629	149.0789	[82][M+1]+		1
786	552.28134	149.0298			1
992	771.99371	148.9999			1
993	772.99560	148.9977			1

If users choose to filter out redundant isotopic peaks, unwanted adducts, and/or daughter ion fragments, SMART will export the files of the peak abundance data and annotation after the peak filter. We use the peaks in **Table 5.3.2** as examples. First, isotope information is contained in the fourth column “isotope”. Peaks 668 ([67][M]+) and 670 ([67][M+1]+) are isotopes, peaks 699 ([75][M]+) and 700 ([75][M+1]+) are isotopes, and peaks 731 ([82][M]+) and 734 ([82][M+1]+) are isotopes. If users choose to only filter out redundant isotopic peaks, peaks 670, 700, and 734 will be removed (i.e., peak with [M]+ will be remained). Second, adduct information is contained in the fifth column “adduct”. If users choose to filter out those adducts which are NOT M+H, then peaks 668 and 731 will be removed. Finally, fragment information is contained in the final column “pcgroup”. Parent and daughter ion fragments will be grouped together and assigned the same value in pcgroup by CAMERA [2]. All peaks in **Table 5.3.2** are in the same group (pcgroup = 1). If users choose to filter out fragments, then, except for the parent peak 699 which has the largest average abundance 25481.9445, adduct M+H and not an isotopic peak, other daughter peaks will be removed. Note that we do not suggest users to filter out the isotopes, adducts or fragments before the sample quality control procedure (see Section 5.5) because those peaks provide useful information for correlation estimation and quality control.

5.4 Data preprocessing

SMART provides three procedures for data transformation and normalization (see **Figure 5.4.1**). Users follow the procedure to perform data preprocessing: *Click "Peak Analysis" ➔ "Data Preprocessing"*. The first procedure is abundance adjustment by using an internal standard. Users can choose "None", "Scaling to the mean of internal standard", or "Scaling to the median of internal standard". Users must provide the m/z or RT of the internal standard in users' peak abundance data if an international standard adjustment is requested (see **Figure 5.4.2**).



Input file: F:/SMART/ACEIvsNonMed/Peak Alignment and Annotation/S599_nofilledPeakinto_loop2_forSMART.csv

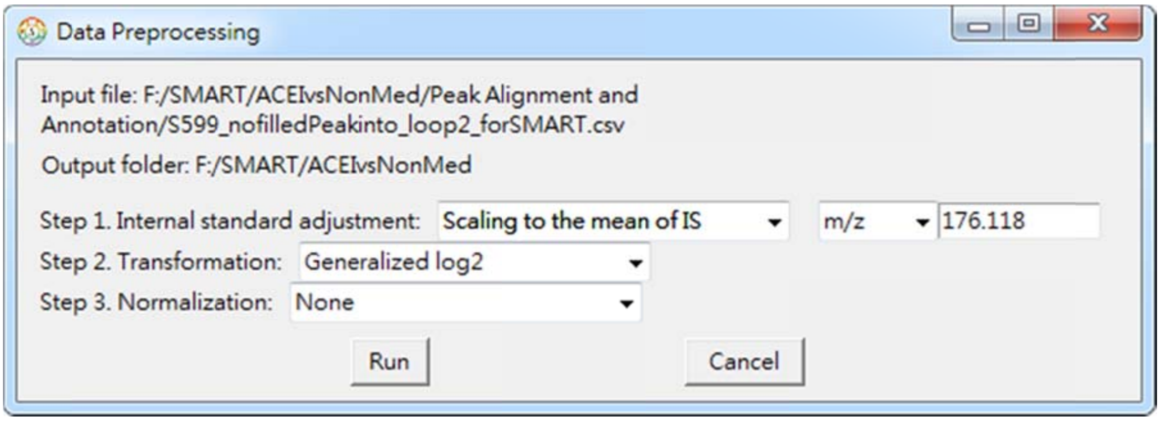
Output folder: F:/SMART/ACEIvsNonMed

Step 1. Internal standard adjustment: None m/z

Step 2. Transformation: Generalized log2

Step 3. Normalization: None

Figure 5.4.1. Interface of data preprocessing.



Input file: F:/SMART/ACEIvsNonMed/Peak Alignment and Annotation/S599_nofilledPeakinto_loop2_forSMART.csv

Output folder: F:/SMART/ACEIvsNonMed

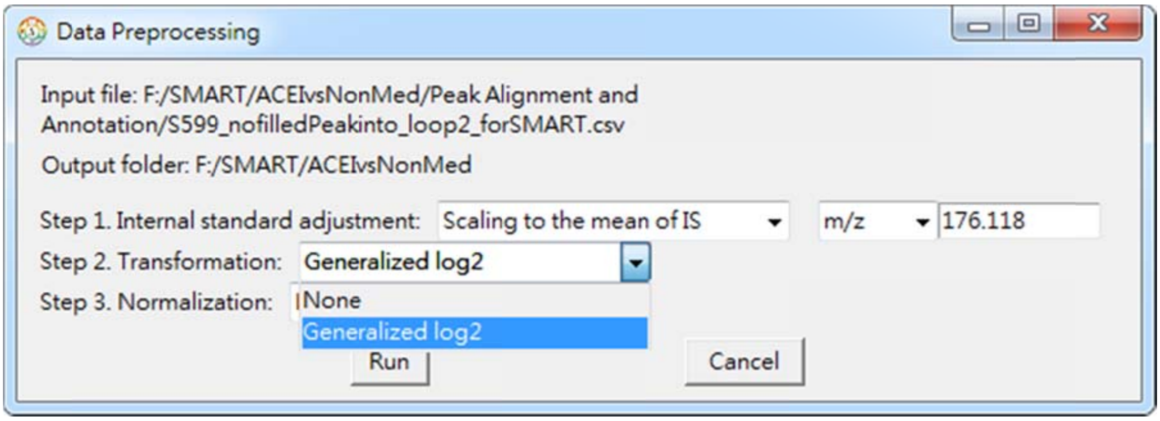
Step 1. Internal standard adjustment: Scaling to the mean of IS m/z 176.118

Step 2. Transformation: Generalized log2

Step 3. Normalization: None

Figure 5.4.2. Internal standard adjustment.

The second procedure is to consider variable transformation. Users can choose “None” or “Generalized log2” [3]. The latter transformation is a variance-stabilization procedure and can avoid the problem of log transformation of a zero value (see **Figure 5.4.3**). Note that if generalized log2 was performed then a zero value in the original data will be transformed as 2.



Data Preprocessing

Input file: F:/SMART/ACEIvsNonMed/Peak Alignment and Annotation/S599_nofilledPeakinto_loop2_forSMART.csv

Output folder: F:/SMART/ACEIvsNonMed

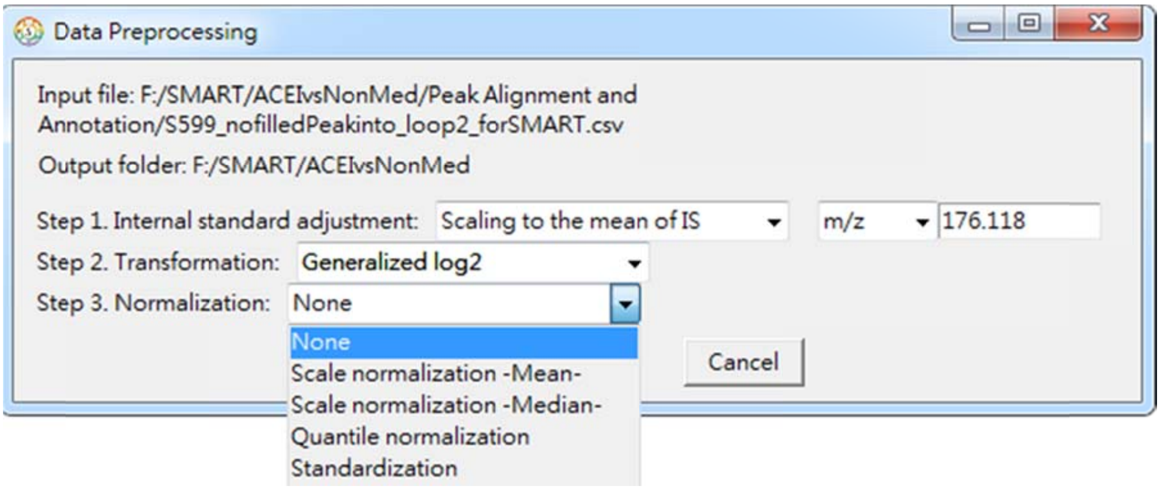
Step 1. Internal standard adjustment: Scaling to the mean of IS | m/z | 176.118

Step 2. Transformation: Generalized log2

Step 3. Normalization:

Figure 5.4.3. Transformation.

The third procedure is sample-based data normalization that includes “None”, “Scale normalization – Mean”, “Scale normalization – Median”, “Quantile normalization”, and “Standardization” (see **Figure 5.4.4**). Users choose one(s) of the aforementioned three preprocessing procedures according to their data properties. If data preprocessing has been done before, users can skip the transformation or normalization step in **SMART**.



Data Preprocessing

Input file: F:/SMART/ACEIvsNonMed/Peak Alignment and Annotation/S599_nofilledPeakinto_loop2_forSMART.csv

Output folder: F:/SMART/ACEIvsNonMed

Step 1. Internal standard adjustment: Scaling to the mean of IS | m/z | 176.118

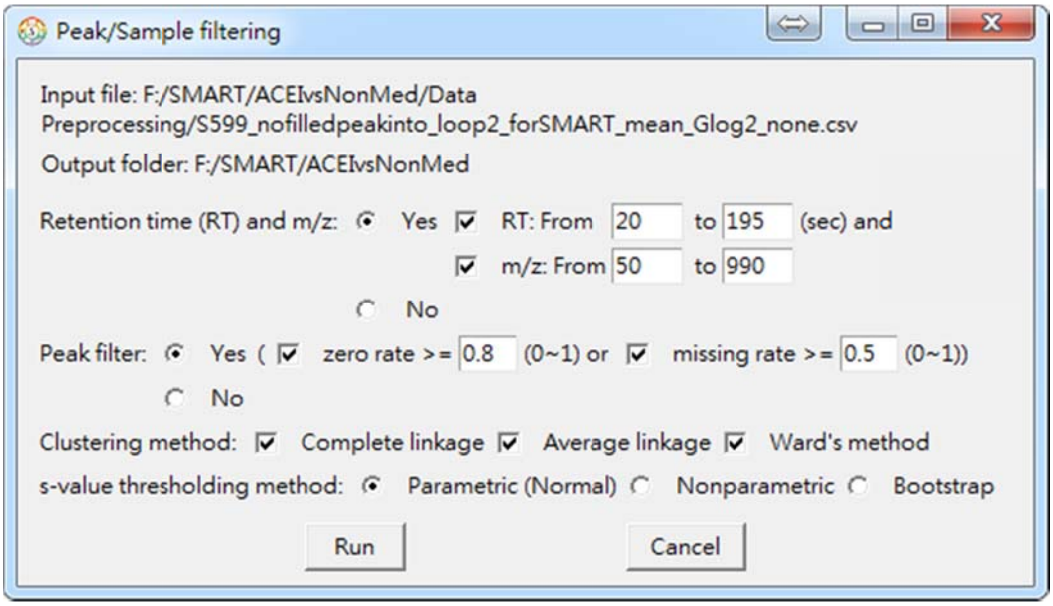
Step 2. Transformation: Generalized log2

Step 3. Normalization: None

Figure 5.4.4. Normalization.

5.5 Quality control

SMART provides quality control for peak filtering and sample filtering (see **Figure 5.5.1**). Users follow the procedure to perform quality control: *Click "Peak Analysis" ➔ Choose "Quality Control" ➔ Choose "Peak/Sample Filtering"*. The first step is to specify the ranges of RT and m/z for quality control.



Input file: F:/SMART/ACEIvsNonMed/Data Preprocessing/S599_nofilledpeakinto_loop2_forSMART_mean_Glog2_none.csv

Output folder: F:/SMART/ACEIvsNonMed

Retention time (RT) and m/z: ● Yes ☑ RT: From 20 to 195 (sec) and
☑ m/z: From 50 to 990
○ No

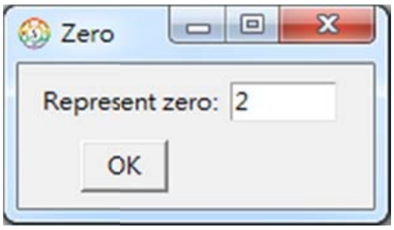
Peak filter: ● Yes (☑ zero rate >= 0.8 (0~1) or ☑ missing rate >= 0.5 (0~1))
○ No

Clustering method: ☑ Complete linkage ☑ Average linkage ☑ Ward's method

s-value thresholding method: ● Parametric (Normal) ○ Nonparametric ○ Bootstrap

Figure 5.5.1. Peak and sample filtering.

The second step is to specify cutoffs for the zero rate (0 ~ 1) and missing rate (0 ~ 1). If users check the option to filter out peaks with a high zero rate, **SMART** requires users to input what value corresponds to "zero" in the data (see **Figure 5.5.2**). In other words, if users did not perform generalized log2 transformation, please input 0; if users performed generalized log2 transformation in data preprocessing, please input 2. For peak filtering, if the missing or zero rate of a peak is higher than the cutoff, the peak is removed.

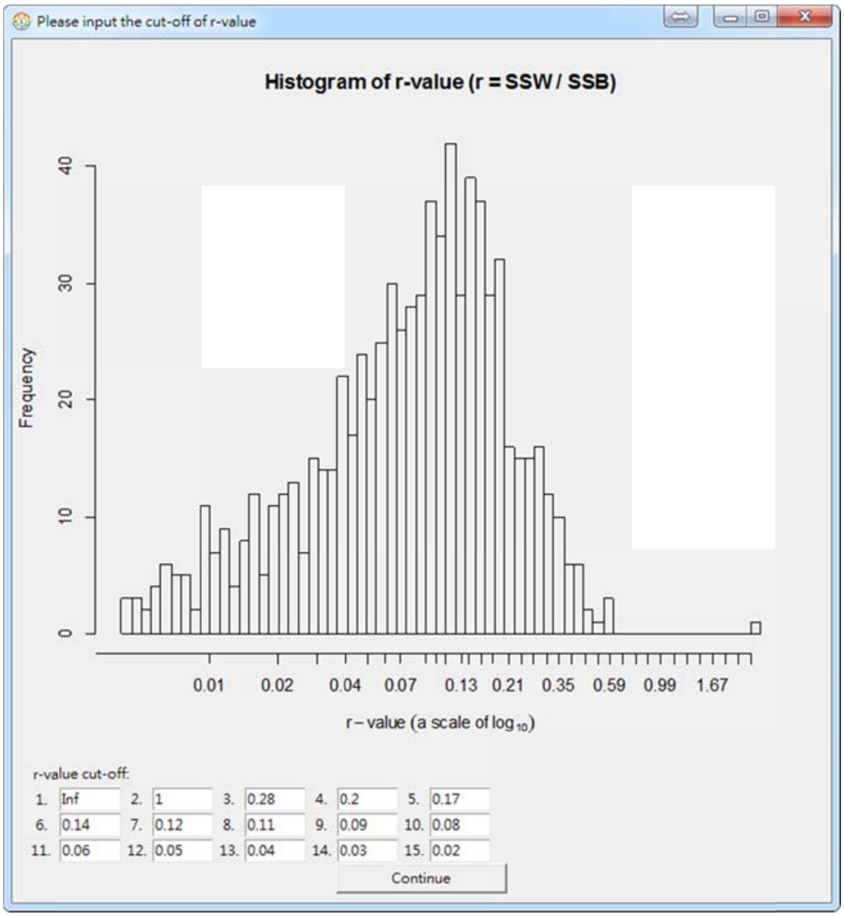


Represent zero: 2

Figure 5.5.2. Interface of zero-value indication.

SMART calculates an *r-value* as the sum of squares within a subject (SSW) divided by the sum of squares between subjects (SSB) based on the aligned peak abundance data.

The *r-value* is a quality index for peaks; the higher the *r-value* is, the poorer the peak quality. **SMART** calculates quantiles of the *r-value* (see **Figure 5.5.3**). Sample accuracy at the quantiles will be calculated and shown later. Users can also specify preferred (nonnegative) values as cutoffs of the *r-value*.

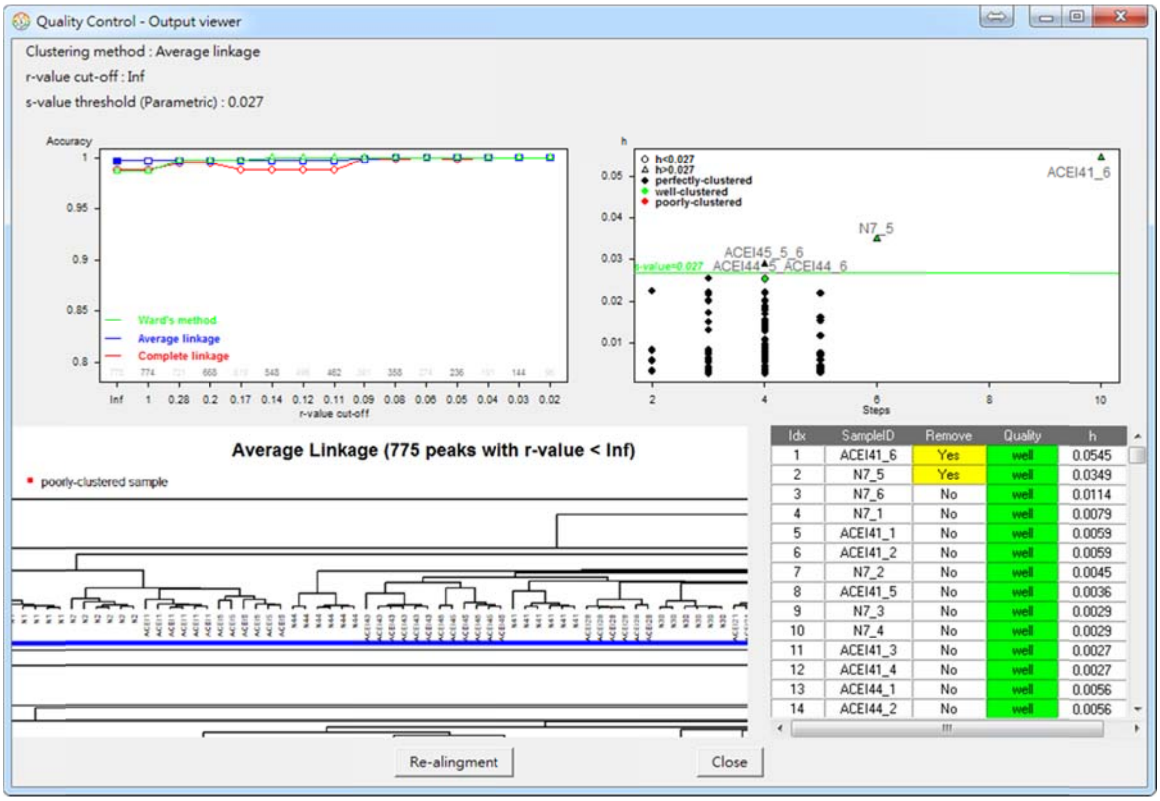

Please input the cut-off of r-value
Histogram of r-value (r = SSW / SSB)
Frequency
0
10
20
30
40
0.01
0.02
0.04
0.07
0.13
0.21
0.35
0.59
0.99
1.67
r − value (a scale of log10)
Continue

r-value cut-off:

1. Inf 2. 1 3. 0.28 4. 0.2 5. 0.17
6. 0.14 7. 0.12 8. 0.11 9. 0.09 10. 0.08
11. 0.06 12. 0.05 13. 0.04 14. 0.03 15. 0.02

Figure 5.5.3. Distribution and quantiles of the *r-value*.

For sample filtering, quality of the replicate samples and subjects is evaluated only relying on the remaining high-quality peaks that have an *r-value* lower than a specified cutoff. Interactive window of the sample filtering comprises four plots (**Figure 5.5.4**). The upper-left plot is a sample accuracy plot. Note that users can click the legend of a clustering method (green: Ward's method; blue: Average-linkage method; red: Complete-linkage method) to hide the results of the cluster method and doubly click the legend to show the results again. Users follow the procedures to perform quality control: Users follow the procedure to start quality control: *Move the cursor to a point on a sample accuracy curve* ➔ *Click to specify the cluster method and the cutoff of r-value*. For example, users may choose the average linkage method and *r-value* cutoff = Inf because the setting attains the highest sample accuracy (**Figure 5.5.4**).



Quality Control - Output viewer

Clustering method : Average linkage

r-value cut-off : Inf

s-value threshold (Parametric) : 0.027

Average Linkage (775 peaks with r-value < Inf)

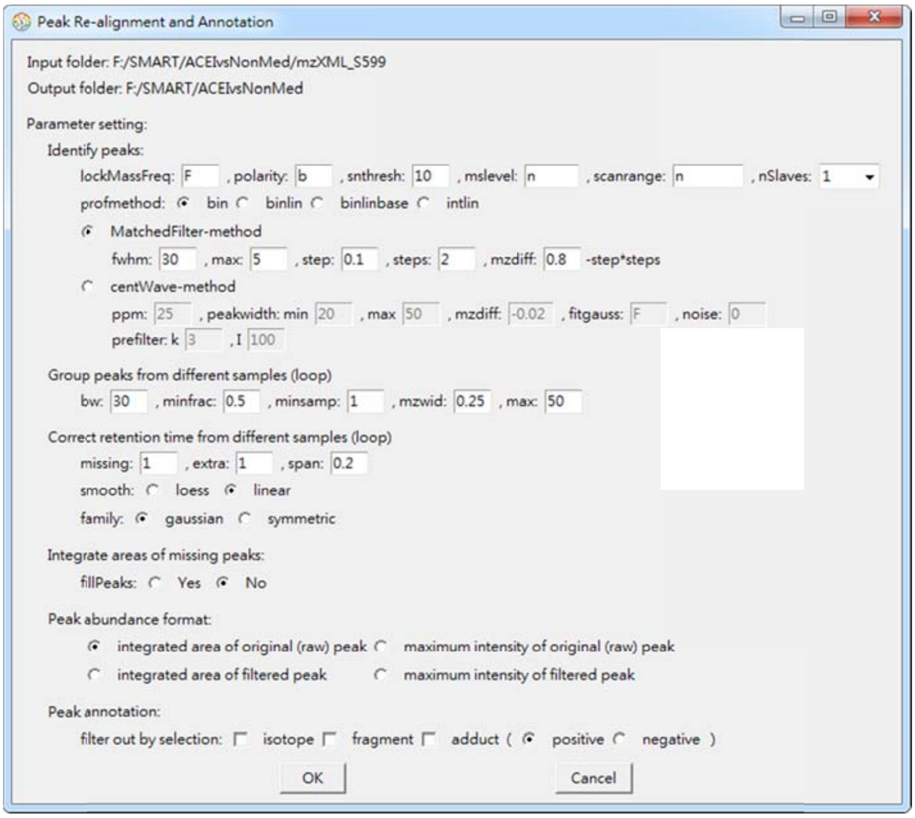
Idx	SampleID	Remove	Quality	h
1	ACEI41_6	Yes	well	0.0545
2	N7_5	Yes	well	0.0349
3	N7_6	No	well	0.0114
4	N7_1	No	well	0.0079
5	ACEI41_1	No	well	0.0059
6	ACEI41_2	No	well	0.0059
7	N7_2	No	well	0.0045
8	ACEI41_5	No	well	0.0036
9	N7_3	No	well	0.0029
10	N7_4	No	well	0.0029
11	ACEI41_3	No	well	0.0027
12	ACEI41_4	No	well	0.0027
13	ACEI44_1	No	well	0.0056
14	ACEI44_2	No	well	0.0056

Figure 5.5.4 Output of quality control.

The lower-left plot is a cluster tree diagram of all replicate samples. Users can click the right button in mouse to zoom out and doubly click the left button to zoom in the tree diagram. The upper-right plot displays the measures of the replicate sample quality and *s-value*. The lower-right table is a summary result table for sample quality control. Users can drag and drop the label of sample to avoid labels overlapped. The fourth column lists the categories of replicate samples: perfectly clustered (red), well clustered (green), and poorly clustered (black). The third column lists the replicate samples that must be removed. The results will change according to the parameters setting in the sample accuracy plot. After quality control, users can click “Re-alignment” to realign the data or click “Close” to finish the quality control. The results after quality control will be exported as an R data file. After the output window is closed, if users would like to see the output again, users can follow the procedure to view the outputs again: *Click “Peak Analysis” ➔ Choose “Quality Control” ➔ Choose “Output Viewer” ➔ Specify the output R data file*.

5.6 Re-alignment and annotation

If users decide to do realignment (**Figure 5.6.1**) after removing poor-quality peaks and samples, users just specify the folder where they saved their mzXML files, and other procedures are the same as introduced in Section 5.3. Users can optionally choose to filter out the redundant isotopic peaks, unwanted adducts, and daughter ion fragments from the subsequent analysis. This can be done by checking "isotope", "fragment", and "adduct" and specifying "positive" and/or "negative" ion mode(s).



Peak Re-alignment and Annotation

Input folder: F:/SMART/ACEIvsNonMed/mzXML_S599
Output folder: F:/SMART/ACEIvsNonMed

Parameter setting:

Identify peaks:

lockMassFreq: F , polarity: b , snthresh: 10 , mslevel: n , scanrange: n , nSlaves: 1

profmethod: ● bin ○ binlin ○ binlinbase ○ intlin

● MatchedFilter-method

fwhm: 30 , max: 5 , step: 0.1 , steps: 2 , mzdiff: 0.8 -step*steps

○ centWave-method

ppm: 25 , peakwidth: min 20 , max 50 , mzdiff: -0.02 , fitgauss: F , noise: 0

prefilter: k 3 , I 100

Group peaks from different samples (loop)

bw: 30 , minfrac: 0.5 , minsamp: 1 , mzwid: 0.25 , max: 50

Correct retention time from different samples (loop)

missing: 1 , extra: 1 , span: 0.2

smooth: ○ loess ● linear

family: ● gaussian ○ symmetric

Integrate areas of missing peaks:

fillPeaks: ○ Yes ● No

Peak abundance format:

● integrated area of original (raw) peak ○ maximum intensity of original (raw) peak

○ integrated area of filtered peak ○ maximum intensity of filtered peak

Peak annotation:

filter out by selection: □ isotope □ fragment □ adduct (● positive ○ negative)

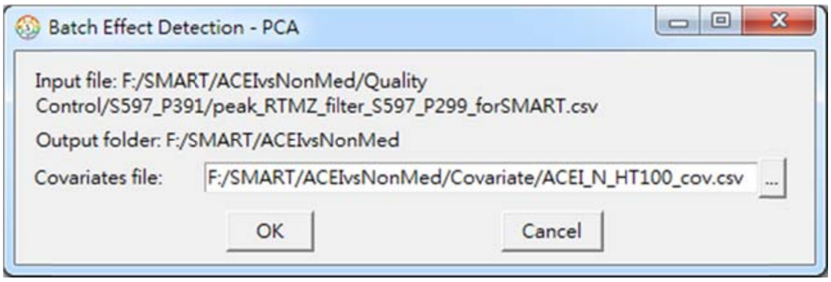
Figure 5.6.1. Interface of re-alignment and annotation

5.7 Batch effect detection

SMART can evaluate batch effects caused by known experimental conditions, unknown latent groups (LGs), or hidden substructures. Users follow the procedure to perform batch effect detection: *Click "Statistical Methods" ➔ Choose "Batch Effect Detection" ➔ Choose "Principal Component Analysis (PCA)" or "Latent Group (LG)"*. Because **SMART** will check collinearity of batch effect variables and covariates in the subsequent analysis of covariance (ANCOVA), a covariate file (see **Table 5.7.1**) should be provided if some covariate(s) will be adjusted for (see **Figure 5.7.1** for a PCA and **Figure 5.7.2** for an LG analysis). In a covariate file, the first column is sample id and followed by covariate data with comma delimited. Name of a discrete covariate should be prefixed with "D" and a continuous covariate with “C”. For example, there are four discrete covariates (Dataset, Gender, Date, and Month) and two continuous covariates (Age and BMI) in this example (see **Table 5.7.1**).

Table 5.7.1. Covariate table.

SampleID	DDataset	DGender	CAge	CBMI	DDate	DMonth
ACEI1	1	1	46	26.08	D0914	M09
ACEI2	1	2	32		D0914	M09
ACEI3	2	2	46	28.36	D1005	M10
ACEI4	2	1	32	23.79	D1005	M10
ACEI5	2	1	39	23.66	D1005	M10
N1	1	2	37	25.23	D0914	M09
N2	1	1	38	25.35	D0914	M09
N3	2	2	39	24.20	D1005	M10
N4	2	1	32	25.60	D1005	M10
N5	2	1	23	22.69	D1005	M10

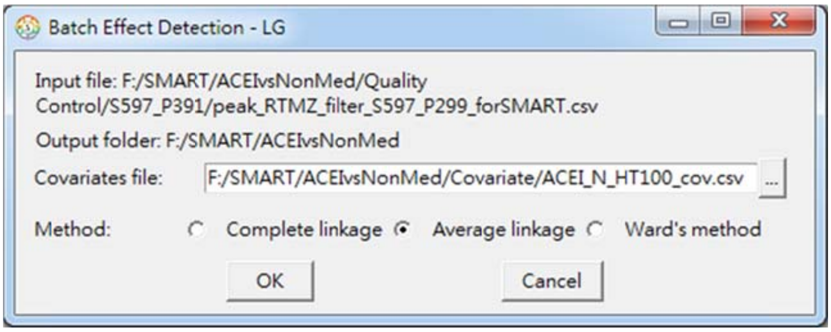

Batch Effect Detection - PCA

Input file: F:/SMART/ACEIvsNonMed/Quality Control/S597_P391/peak_RTMZ_filter_S597_P299_forSMART.csv

Output folder: F:/SMART/ACEIvsNonMed

Covariates file: F:/SMART/ACEIvsNonMed/Covariate/ACEI_N_HT100_cov.csv

Figure 5.7.1. Interface of principal component analysis.


Batch Effect Detection - LG

Input file: F:/SMART/ACEIvsNonMed/Quality Control/S597_P391/peak_RTMZ_filter_S597_P299_forSMART.csv

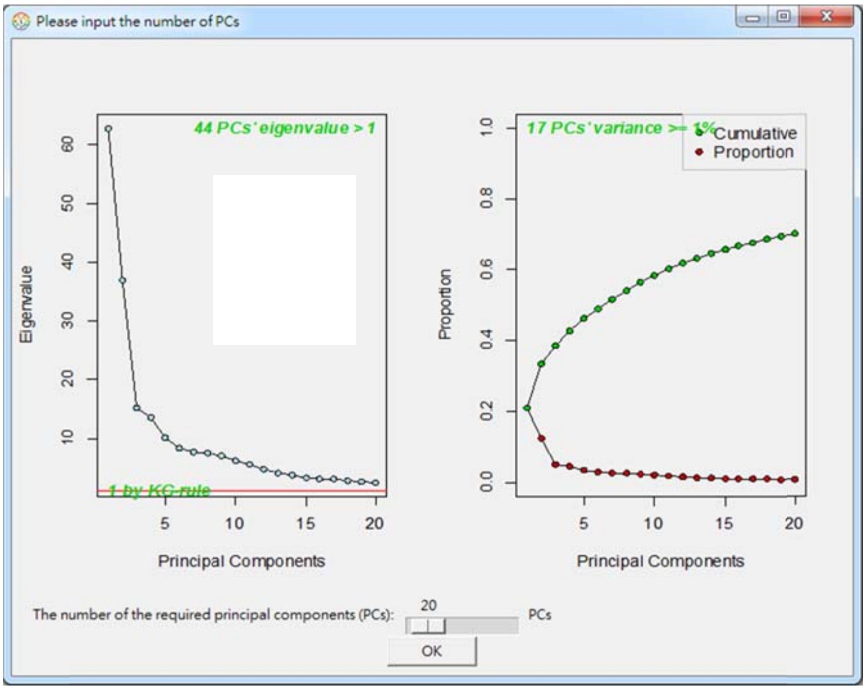
Output folder: F:/SMART/ACEIvsNonMed

Covariates file: F:/SMART/ACEIvsNonMed/Covariate/ACEI_N_HT100_cov.csv

Method: ○ Complete linkage ● Average linkage ○ Ward's method

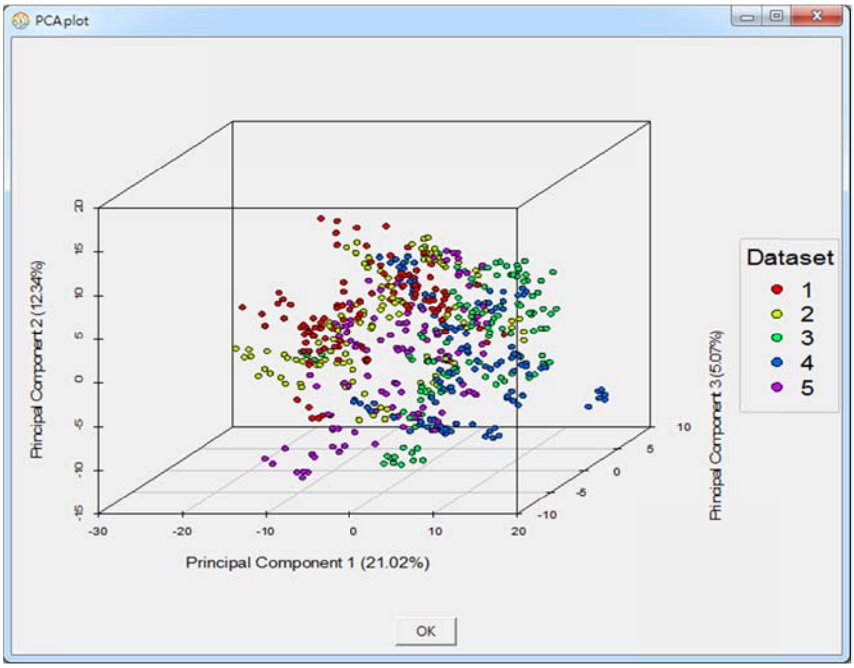
Figure 5.7.2. Interface of latent group analysis.

For a PCA, users can determine the number of PCs by a scree plot (left) or according to the number of PCs with an eigenvalue of ≥ 1 or with a proportion of variance explained of >1% in a variation-explained plot (right) (**Figure 5.7.3**). Finally, users can drag and drop the button “Number of PCs” in the bottom of **Figure 5.7.3** and click the button OK to assign the number of PCs. **SMART** provides PCA plots, where the PCs are colored according to known covariates and used to evaluate the relationship between the known covariates and the PCs (**Figure 5.7.4**). For example, if the pattern of the experiment date is matching to that of the pattern of LGs, then it reveals that the experiment date is one of the major batch effects. After a PCA, **SMART** exports PC scores for all replicate samples of all subjects for adjusting for the batch effects in the subsequent association analysis.


Please input the number of PCs

The number of the required principal components (PCs): 20 PCs

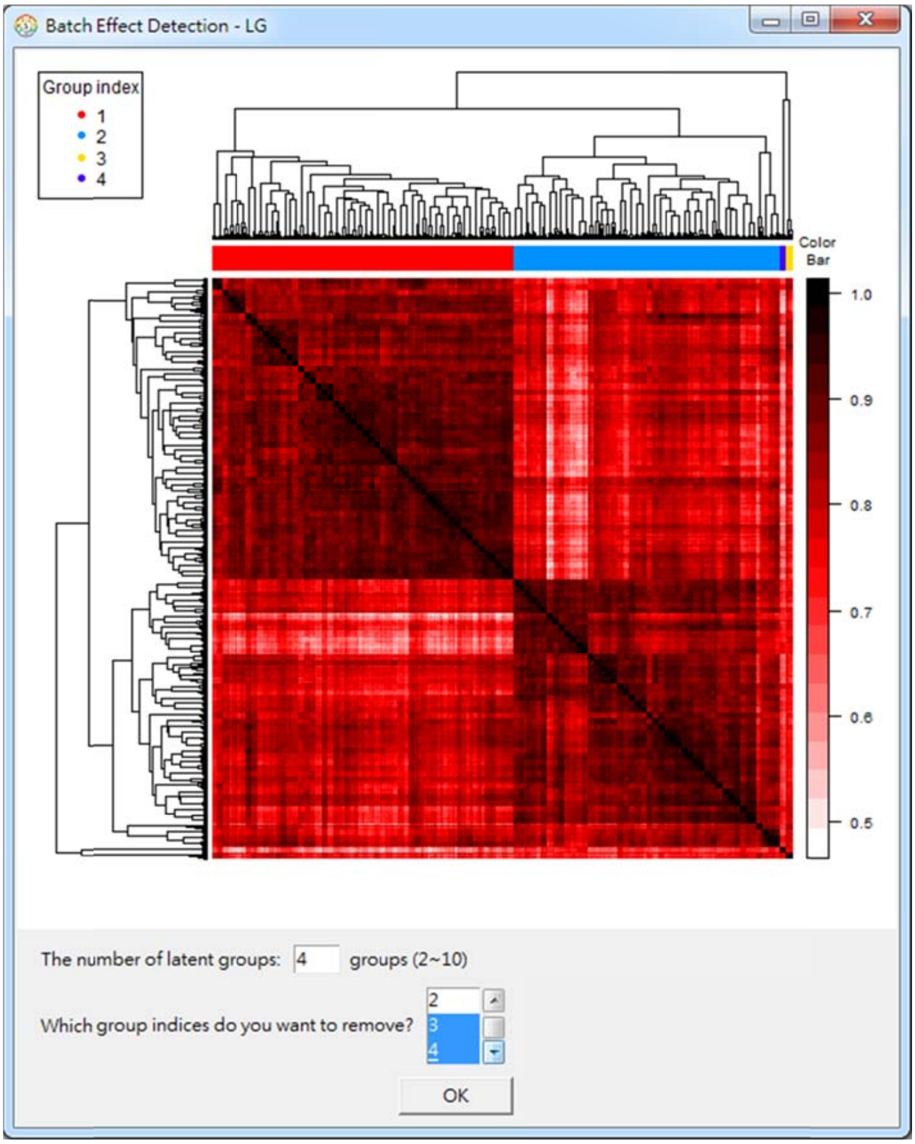
Figure 5.7.3. Interface of scree plot (left) and a variation-explained plot (right).


PCAplot

Principal Component 2 (12.34%)
20
15
10
5
0
-5
-10
-15
-30
-20
-10
0
10
20
Principal Component 1 (21.02%)
10
5
0
-5
-10
Principal Component 3 (5.07%)
Dataset
1
2
3
4
5
OK

Figure 5.7.4. PCA plot.

For an LG analysis, users can determine the number of LGs and evaluate the patterns between known covariates and LGs by a heat map and cluster tree diagram (**Figure 5.7.5**). For example, a heat map and cluster tree diagram showed a substructure of three LGs. Then users key in the number of LGs in the bottom of the interface of heat map and cluster tree diagram. Because the third LG contains only two patients, **SMART** can remove the third LG by specifying the index of LG in the bottom of the interface. After an LG analysis, **SMART** exports index of LG for all replicate samples of all subjects for adjusting for batch effects in the subsequent association analysis.



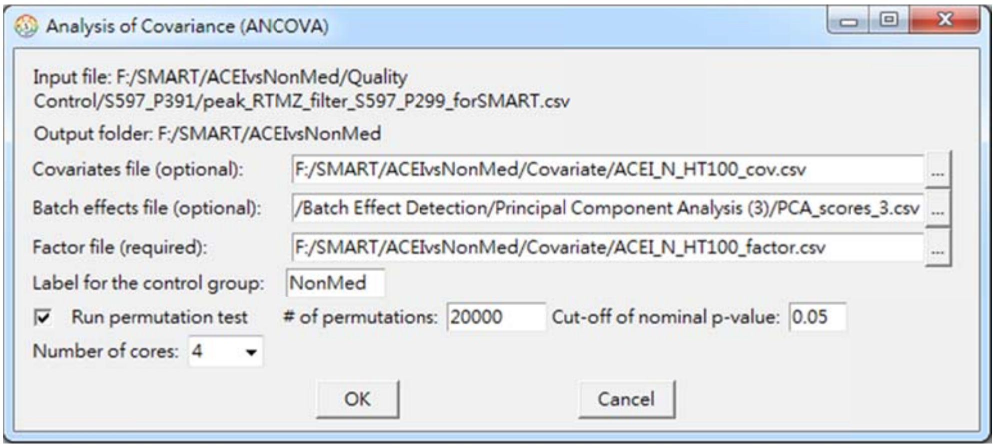
The number of latent groups: 4 groups (2~10)

Which group indices do you want to remove? 2 3 4

Figure 5.7.5. Heat map and cluster tree diagram.

5.8 Association analysis

To discover the association between metabolites and variables of interest, **SMART** provides a general Analysis of Covariance (ANCOVA) model for MWASs. Users follow the procedure to perform association analysis: *Click "Statistical Methods" ➔ Choose "Analysis of Covariance (ANCOVA)"*. In this model, the dependent variable is the peak abundance and the independent variables include the factor groups (e.g., case vs. control) or quantitative traits of interest (e.g., blood pressure), covariates, and batch effects. Users should specify their covariate file (optional), batch effect file (optional), and factor file (required) (see **Figure 5.8.1**). Covariate data format has been introduced in **Table 5.7.1**. In a batch effect file, the first column is sample id and followed by the data of batch effect variables (e.g., index of LG or PC scores). In a factor file, the first column is sample id and followed by a multinomial variable (i.e., factor group) or a continuous variable (i.e., quantitative trait(s)).



Input file: F:/SMART/ACEIvsNonMed/Quality Control/S597_P391/peak_RTMZ_filter_S597_P299_forSMART.csv

Output folder: F:/SMART/ACEIvsNonMed

Covariates file (optional): F:/SMART/ACEIvsNonMed/Covariate/ACEI_N_HT100_cov.csv

Batch effects file (optional): /Batch Effect Detection/Principal Component Analysis (3)/PCA_scores_3.csv

Factor file (required): F:/SMART/ACEIvsNonMed/Covariate/ACEI_N_HT100_factor.csv

Label for the control group: NonMed

☑ Run permutation test # of permutations: 20000 Cut-off of nominal p-value: 0.05

Number of cores: 4

Figure 5.8.1. Interface of analysis of covariance (ANCOVA).

Next, "Label for the control group:" is used to specify a control group. If it is specified, **SMART** performs an ANCOVA analysis that the dependent variable is peak abundance and the main independent variable is a factor group variable. Here we use an antihypertensive pharmacometabolomics study as an example to demonstrate the operation. We are interested in association between peak abundance and a multinomial factor group (*X* = 1, 2, 3, 4, and 5 indicates "no medication", "ACEi medication", "ARB medication", "CCB medication", and "Diuretics" respectively). There are six covariates: Dataset, Gender, Age, BMI, Date, and Month. The control group is treated as a reference and other groups will be compared with the control group individually. For example, if

“NonMed” is specified in “Label for the control group:”, then **SMART** performs four ANCOVA analyses to compare means of peak abundance for ACEi vs. NonMed, ARB vs. NonMed, CCB vs. NonMed, and Diuretics vs. NonMed, separately. If “Label for the control group:” is not specified, then **SMART** performs a regression analysis, where the dependent variable is peak abundance and the main independent variable is a quantitative trait of interest (e.g., blood pressure).

Each of the ANCOVA analyses in the aforementioned example can consider different covariates. **SMART** automatically generates six covariates for each factor group. For example, in the ACEi group, the six covariates are named as “Dataset (ACEi vs. NonMed)”, “Gender (ACEi vs. NonMed)”, “Age (ACEi vs. NonMed)”, “BMI (ACEi vs. NonMed)”, “Date (ACEi vs. NonMed)”, and “Month (ACEi vs. NonMed)” (see **Figure 5.8.2**). Users can click some or all of the six covariates in the left-hand side window and press the >> button to include the covariates into the ANCOVA model in a comparison of ACEi vs. NonMed (see **Figure 5.8.2**). Similar procedures are applied to comparisons between NonMed and other medications.

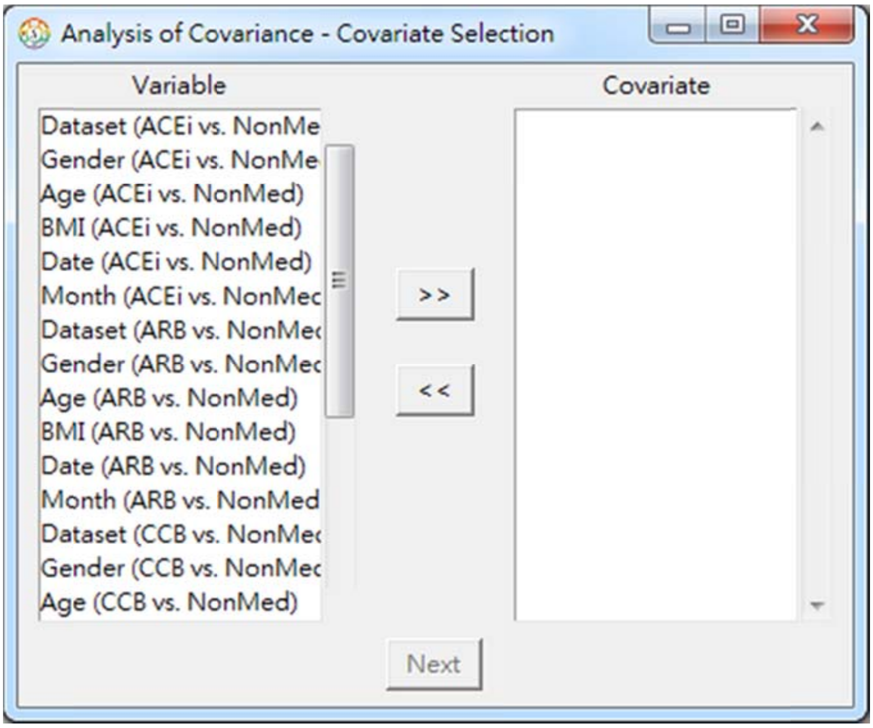
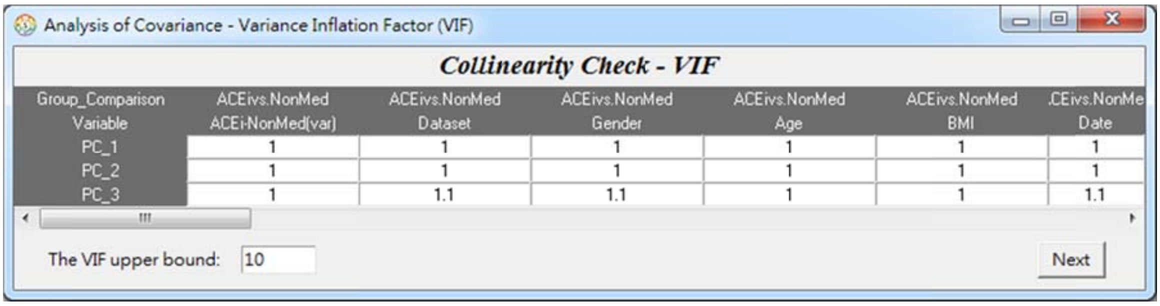


Figure 5.8.2. Interface of covariate selection in ANCOVA.

To avoid multicollinearity among the independent variables in ANCOVA, **SMART** calculates the variance inflation factor (VIF) for evaluating multicollinearity between batch effects and factor groups and between batch effects and covariates. Users can input a VIF upper bound (see **Figure 5.8.3**). The default cutoff of VIF is 10. An LG or PC is removed from the ANCOVA model if its VIF is greater than the cutoff.



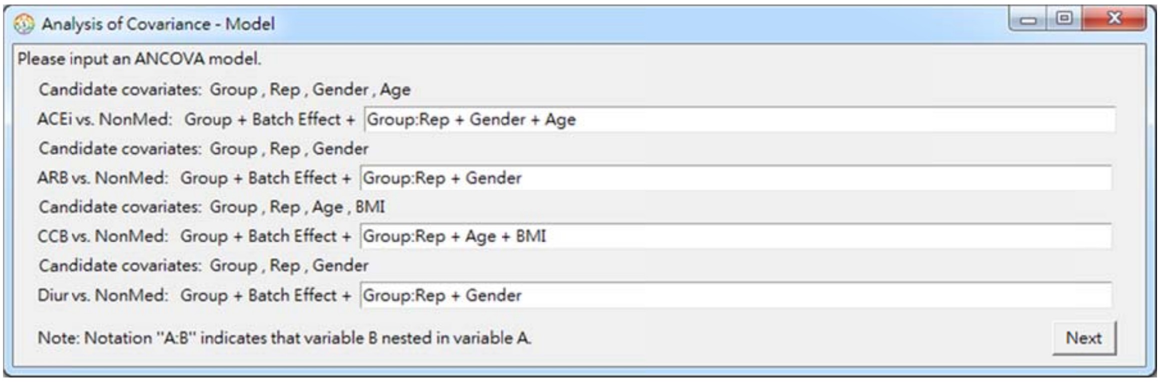
Collinearity Check - VIF

Group_Comparison Variable	ACEivs.NonMed ACEi-NonMed(var)	ACEivs.NonMed Dataset	ACEivs.NonMed Gender	ACEivs.NonMed Age	ACEivs.NonMed BMI	.CEivs.NonMe Date
PC_1	1	1	1	1	1	1
PC_2	1	1	1	1	1	1
PC_3	1	1.1	1.1	1	1	1.1

The VIF upper bound: 10

Figure 5.8.3. Interface of collinearity check.

Finally, users should specify the fitted ANCOVA models (see **Figure 5.8.4**). For example, “ACEi vs. NonMed: Group + Batch Effect + Group:Rep + Gender + Age” is the first ANCOVA model. This model is fitted to examine the difference of peak abundance in ACEi medication group and NonMed group. Group variable and batch effect variable are forced to include. Term “Group:Rep” considers replicate samples nested in the factor group under a nested study design. Term “Gender + Age” means that covariates gender and age are adjusted for.



Please input an ANCOVA model.

Candidate covariates: Group , Rep , Gender , Age

ACEi vs. NonMed: Group + Batch Effect + Group:Rep + Gender + Age

Candidate covariates: Group , Rep , Gender

ARB vs. NonMed: Group + Batch Effect + Group:Rep + Gender

Candidate covariates: Group , Rep , Age , BMI

CCB vs. NonMed: Group + Batch Effect + Group:Rep + Age + BMI

Candidate covariates: Group , Rep , Gender

Diur vs. NonMed: Group + Batch Effect + Group:Rep + Gender

Note: Notation "A:B" indicates that variable B nested in variable A.

Figure 5.8.4. Interface of ANCOVA model construction.

SMART provides two methods for evaluating the statistical significance of the association between the metabolites and the factor groups. When the peak abundance follows a normal distribution, the F test is used; otherwise, a permutation test, which randomly shuffles the values of the factor group(s) or quantitative trait, can be used (see **Figure 5.8.1**). Nominal *p*-values (pv), adjusted *p*-values after false discovery rate (FDR) adjustment (pFDR), empirical *p*-values (epv), and empirical *p*-values after FDR adjustment (epFDR) are exported. A volcano plot is generated to present the results (see **Figure 5.8.5**).

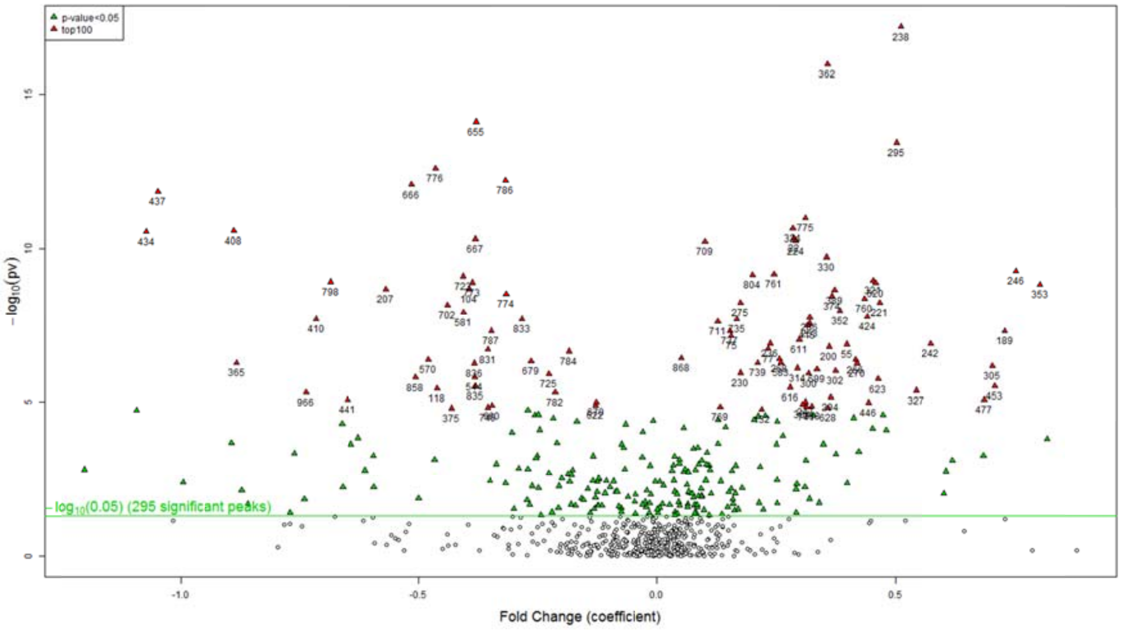


Figure 5.8.5. Volcano plot of nominal *p*-values.

To reduce the computational time of permutations, **SMART** supports parallel computing by applying the snow package. Users can specify the number of computation cores in the analysis (see **Figure 5.8.1**).

6. Example

SMART provides a real example from our antihypertensive pharmacometabolomics study. The data can be downloaded from the **SMART** website. In this example, there are 10 young-onset hypertensive patients from the Academia Sinica Multi-Center Young-Onset Hypertension Study [4]. The patients are divided into two groups: the medication group comprises 5 patients who were treated with ACEis and the nonmedication group (NonMed) comprises 5 patients who were not treated with antihypertensive medicine. Six technical replicate samples were used for each of the 10 patients.

7. Application Programming Interface

This section provides brief description about the application programming interface (API) that users can use them to develop their own packages easily (see **Table 7.1**).

Table 7.1. Main APIs in SMART.

Function (no arguments and no return value)	Input		Output	
	Variable	Description	Variable	Description
Quality control QualityControl()	QCtable	data.frame: peak abundance data	QCtable_temp	data.frame: post-QC peak abundance data
	method	numerical vector: 1 = complete linkage 2 = average linkage 3 = ward method	r	numerical vector: peak’s r-value
	height_method	a numerical value: 1 = parametric method 2 = nonparametric method 3 = bootstrap	acc	list: $height = heights for different linkage methods $height_thres = s-value for different linkage methods
Batch effect detection (PCA) pcaStat()	pcatable	data.frame: peak abundance data	pcainfo	list: $scores = PC scores $scree = eigenvalues $varprop = proportion of explained variation $varpropcum = cumulative proportion of explained variation
	Covariate	data.frame: covariate data		
Batch effect detection (LG) LatentGroup()	BEtable	data.frame: peak abundance data	hr	cluster object: hierarchical clustering result

	Covariate	data.frame: covariate data		
ANCOVA ANCOVA()	peakabun	data.frame: peak abundance data	pvalue	matrix: p-values of factor, covariates, and batch effects (per row a peak)
	Factor	data.frame: factor information	pfdr	matrix: FDR-adjusted p-values of factor, covariates, and batch effects (per row a peak)
	ctrl_label	a character value: control label	beta_temp	matrix: estimates of beta of factor, covariates, and batch effects (per row a peak)
	ncpu	a numeric value: number of threads		
	Covariate	data.frame: covariate data		
	batch_effect	data.frame: batch effect data		

8. References

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