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METHOD FOR THE METABOLIC PROFILE OF PLANT TISSUES BY GAS CHROMATOGRAPHY COUPLED TO MASS SPECTROMETRY (GC/MS) V.2

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1 Works for me dx.doi.org/10.17504/protocols.io.8suhwew

Metabolomics Protocols & Workflows

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ABSTRACT

Plants produce a variety of compounds with diverse structure and abundance that play an important role in the development, growth and response to the environment. Metabolite profiles can be obtained from hundreds of samples by CG/MS (gas chromatography coupled to mass spectrometry). However, the large number of identify metabolites for each run and of treatments and replicates require automatic processing for both spectra (metabolite identification and quantification) and global analysis of the metabolome. Thus, this protocol describes in detail, step-by-step, the generation of the metabolite profiles from GC/MS data, as well as their Exploratory Statistical and Functional Enrichment analyzes.

THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

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MATERIALS TEXT

REAGENTS

- Methanol (LiChrosolv, Merck, Order n° 1.06001)
- Chloroform (LiChrosolv, Merck , Order n° 1.0244)
- Ribitol (Sigma)
- High pure water (18.2M Ω cm-1) provided by a Milli-Q system (Burlington, Massachusetts, USA)
- Methoxyamine hydrochloride (Sigma Part Number 226904)
- Pyridine (Sigma Part Number 270970)
- N-Methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA) (Sigma Part number 69479-10 X 1 mL)
- FAME ?????(retention time standard)
- Liquid Nitrogen.

EQUIPMENTS AND SUPPLIES

- Gaseous Chromatography System coupled to mass spectrometry (GC-MS) with the following specifications: Agilent 7890A GC System coupled to Mass Spectrometric TruTOF® HT TOFMS, Leco
- Capillary column 30 m (MDN-35)
- Thermomixer (Benchtop laboratory incubator)
- Benchtop centrifuge.
- Spedvac (vacuum centrifugation drier)

- Ultra-freezer.
- Benchtop balance
- Mortar and pestle.
- Vials, caps and septa (PTFE).
- Microtubes e tips (Axygem ou Sigma)

METABOLITE EXTRACTION

- 1) Collect samples of plant tissues, immediately freeze in liquid nitrogen and store them in freezer -80°C until use.
- 2) Macerate the samples in liquid nitrogen using mortar and pestle. Do not allow to thaw. Weigh approximately 100mg of each sample into microtubes (2ml) and annotate the weight (used for [normalization](#)).
Note: Always use microtubes and tips of good quality.
- 3) Add 700 µl of methanol and vortex for 10 seconds to stop the enzymatic activity
- 4) Add 30 µl ribitol from stock solution (0.2 mg/ml in water) as an internal quantitative standard for the polar phase. Vortex for 10 seconds.
- 5) Use a thermomixer to shake 15 minutes at 70°C, 1000 rpm . After 2 minutes of incubation, open the microtubes to relieve internal pressure.
- 6) Centrifuge the sample for 10 min at 14000 rpm and transfer supernatant to new microtubes.
- 7) Add 375µl of chloroform and 750µl ultrapure water. Vortex for 15 seconds and centrifuge at 4000rpm for 15 minutes.
- 8) Collect the upper phase (polar phase) to new microtubes (2ml).
Note: It is recommended to choose 2 contrasting samples for initial GC/MS tests. The goal is select the best sample volume for derivatization and GC injection. Test 3 aliquots for each sample (50, 100, 150 µl), evaluate the results in the GC/MS and define the best aliquote to derivatize the remaining samples.
- 9) Dry the aliquots in vacuum centrifugation drier (speedvac) overnight without heating.

DERIVATIZATION

- 1) Dissolve each dried aliquote with 40 µl methoxyamine hydrochloride (20 mg/ml in pyridin) and vortex for 30 seconds. Derivatize also one empty microtube as a 'blank'.
- 2) Shake in thermomixer for 2 hours at 37°C, 1000 rpm and then centrifuge fastly ("spin") to remove the drops of cover.
- 3) Add 70µl MSTFA containing retention time standard (Fatty Acid Methyl Esters, FAME) (20µl/ml) and shake in thermomixer for 30 minutes at 37°C.
- 4) Centrifuge fastly ("spin") to remove the drops of cover and transfer the sample into vials.

METABOLIC PROFILE ANALYSIS BY GC/MS

- 1) The method were optimized for a GC/MS system equipped with a capillary column of 30m ((see specifications above).
- 2) Inject the samples (1µl) in splitless mode at 230 °C using a continuous flow of helium (2 mL/min).
- 3) Maintain the oven temperature initially at 80°C and increase 15°C per minute until it reaches 330°C. Keep this last temperature for 5 minutes.
- 4) Get the mass spectrum by full-scan method ranging from 33 to 600 m/z and use n-alkanes series (retention time standard) to calculate retention index.

DATA PROCESSING AND METABOLITE IDENTIFICATION

- Note:** For GC/MS TruTOF (Leco), the raw spectra are processed and converted to the CDF format (NetCDF) using the ChromaToF package and analyzed by TargetSearch algorithms (Cuadros-Inostroza et al. 2009). We use a script (Supplementary Material) for identification and quantification of metabolites designed to run on R package (Cuadros-Inostroza et al. 2009). The processing parameters and alignment utilized were optimized for our GC/TOF platform. The parameters used in the TargetSearch were: mass range of 85-500 Da; threshold 50; TopMasses 10; r thresh of 0.05; quality index threshold 600. The

compound identification were performed by database searches based on the combination of the mass spectrum and chromatographic retention indices (Cuadros-Inostroza et al. 2009). In this search were used a metabolites fragmentation library by electron impact (EI) and retention index (RI) GMDB_FAMELib_TS_20110228_IS.txt, generated by GMD Mass Spectrum Reference Library (<http://gmd.mpimp-golm.mpg.de/download/>). This library was generated using the same configuration of our GC/MS platform. A filtered identification table containing the identified compounds and their intensities were normalized by fresh mass of the leaves and internal standard ribitol and was used as input data for processing and statistical analysis by MetaboAnalyst platform (<http://www.metaboanalyst.ca/>).

1) Install the **R package** for the your operational system (**32 bits ou 64 bits**):

<https://cran.r-project.org/bin/windows/base/>

2) Install **OpenOffice**

<https://www.openoffice.org/download/>

3) Paste **all the spectra** exported as **.cdf files** from CromaTof (Leco TruTof users) in your work directory.

C:/myworkdirectory/

a) Download the files my.work.directory.rar from <https://figshare.com/s/dc13f7d8d8283e67ebdc>

b) Unpack in the "C:/myworkdirectory/". You will see the **R script ("MainScrip.r")**, the library downloaded from GMD Mass Spectrum Reference Library ("**GMDB_FAMELib_TS_20110228_IS.txt**") and the *txt files containing information for corrections of the FAMES retention times ("FAME_limist.txt" and "rimLimits.txt")*.

The file "**FAME_limist.txt**" and "**rimLimits.txt**" **must to be edited by the user** in accordance with the lower and upper limits of the **TICs for each alkanes**. This information is obtained using CromaTof package. This file must to be edited for each new analysis cycle.

- Exemple of the Information from **FAME_limist.txt** file:

LowerLimit UpperLimit Standard Mass

267 278 262320 87
328 330 323120 87
382 391 381020 87
486 495 487220 87
575 586 582620 87
662 666 668720 87
739 745 747420 87
810 817 819620 87
876 882 886620 87
937 945 948820 87
994 1000 1006900 87
1048 1054 1061700 87

4) The file "**MainScript.r**" must also **be edited**:

Open the file in **RStudio** [File > Open file...] (or type Ctrl+O). Edit the following lines of the script:

[Windows]

#Set working directory

setwd("C:\\my.work.directory/")

[Linux]

Set working directory

setwd("~/my.working.directory")

Save the modifications! [File > Save] (or type Ctrl+S)

5) **Install** the TargetSearch package: Select the lines 9 to 11, then click on "Run" or type "Ctrl+Enter". Wait the installation process to finish

6) **Load** the TargetSearch and **execute** the script: Select the lines 14 to 102, then click on "Run" or type "Ctrl+Enter"

7) **Export data to MetaboAnalyst**: Select the lines 107 and 108 of the script to install and load the package **dplyr**.

Edit the lines 110 and 111 of the script, including the correct names of "**profile**" and "**quantmatrix**" files which were created as **output of TargetSearch**:

```
profile_info<-read.delim("TargetSearch-XXXX-XX-XX.profile.info.txt", sep="\t", header=TRUE)
quant_matrix<-read.delim("TargetSearch-XXXX-XX-XX.profile.quantmatrix.txt", sep="\t", header=TRUE)
```

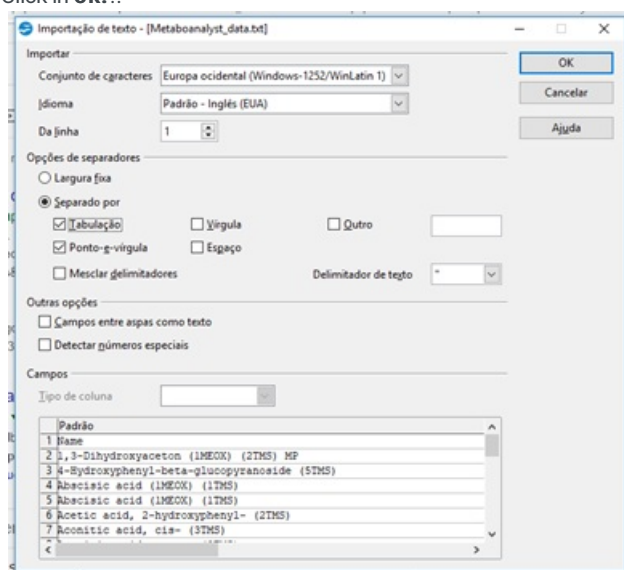
8) Select the lines 110 to 121, then click on "Run" or type "Ctrl+Enter"

9) Open the file "Metaboanalyst_data.txt" in a Text Editor or Spreadsheet Editor such as OpenOffice. Install from <https://www.openoffice.org/download/index.html>

Note: This file contain the **intensity for each compound identify from spectra GC/MS** with score higher than **600** and maintain only the compounds with **high confidence identification**.

Use the parameters below:

Click in **ok!!!**



Reorganize the columns to **identify the treatments and replicates**. Insert lines as "name". "sample" and "label" as in the figure below:

Before edition:

Name	Azo_14_R3	Azo_25_R2	Azo_36_R1	CTR_14_R1	CTR_25_R2	CTR_36_R3	Herb_14_R2	Herb_25_R1	Herb_36_R3
1,3-Dihydroxyacetone (1MEOX) (2TMS) MP	NA	70	NA	68	51	58	64	NA	66
4-Hydroxyphenyl-beta-glucopyranoside (5TMS)	1212	782	1561	488	643	662	520	515	1253
Abscisic acid (1MEOX) (1TMS)	NA	NA	61	58	54	NA	54	NA	67
Abscisic acid (1MEOX) (1TMS)	161	77	144	NA	71	87	1067	NA	404
Acetic acid, 2-hydroxyphenyl- (2TMS)	NA	55	73	60	63	78	54	69	NA
Aconitic acid, cis- (3TMS)	303464	309599	322020	255659	311819	319158	301315	243369	296809
Aconitic acid, trans- (3TMS)	19478	19376	20837	NA	11969	NA	NA	NA	18248
Acrylic acid, 4-imidazole- (2TMS)	65	NA	NA	60	65	70	60	NA	NA

After edition:

1	name	A	B	C	D	E	F	G	H	I	J	K
2	sample	Azo_36_R1	Azo_25_R2	Azo_14_R3	CTR_14_R1	CTR_25_R2	CTR_36_R3	Herb_25_R1	Herb_14_R2	Herb_36_R3		
3	label	Azo	Azo	Azo	CTR	CTR	CTR	Herb	Herb	Herb		
4	Hexadecanoic acid methyl ester, n-	868146	794995	799669	696700	840948	699469	700343	862915	837617		
5	Butanoic acid, 2-amino- (2TMS)	1534	1972	1942	878	1903	841	895	1723	1860		
6	Acetic acid, cis- (3TMS)	322020	309599	303464	256659	311819	319158	243369	301315	296809		
7	Psicose (1MEOX) (5TMS) MP	354466	323384	319754	116400	245922	145650	220858	256255	322623		
8	Fructose (1MEOX) (5TMS) BP	289571	196266	189848	91836	150543	96678	146917	190988	226461		
9	Heptadecanoic acid (1TMS)	55333	46911	62057	29115	37929	53146	36689	46878	62731		
10	Tetradecanoic acid (1TMS)	113490	116243	145055	66731	84669	113444	73906	98891	120072		
11	Succinic acid (2TMS)	33824	22165	28455	19039	23802	23035	17643	31121	29710		
12	Itaconic acid (2TMS)	30900	28396	27489	22189	25931	25868	11651	27155	24806		
13	Pregn-4-ene-11beta-17alpha-diol-3-2>	145	124	142	123	85	128	85	144	138		

Save the TXT edited file, such as **"Metaboanalyst_data.txt"**.

Save as **text CSV** "Metaboanalyst_data.csv">>> **select filter configuration**

	B	C	D	E	F	G
	Score_cor_mases	quantMass	isSelectiveMass	Azo_14_R3	Azo_25_R2	Azo_36_R1
	960	87	VERDADEIRO	799669	794995	868146
	949	130	VERDADEIRO	1942	1972	1534
	949	229	VERDADEIRO	303464	309599	322020
	948	217	VERDADEIRO	319754	323384	354466
	945	217	VERDADEIRO	189848	196266	289571
	945	117	VERDADEIRO	62057	46911	55333
						113490
						33824
						30900
						145
						792342
						8217
						20287
						80418
						770492
						1140
						313051
						25225
						248464
	908	157	VERDADEIRO	10674	12180	11429

Exportar arquivo de texto

Opções de campo

Conjunto de caracteres: Europa ocidental (Windows-1252/WinLatin)

Delimitador de campo: ,

Delimitador de texto: "

☐ Citare todas as células de texto
 ☒ Salvar o conteúdo da célula como mostrado
 ☐ Largura de coluna fixa

OK

Cancelar

Ajuda

Note: This file contain the **intensity** for each compound identify from spectra **GC/MS** , that could be used for the **statistical and pathways analyzes, compatible by the *MetaboAnalyst* plataform**. It provide a user-friendly, web-based analytical pipeline for high-throughput metabolomics studies. In particular, MetaboAnalyst aims to offer a variety of commonly used procedures for metabolomic data processing, normalization, multivariate statistical analysis, as well as data annotation.

METABOLITE DATA ANALYSIS

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1) Go to <https://www.metaboanalyst.ca/faces/ModuleView.xhtml>

Click in **Statistical Analysis** >>> **"Select "Peak Intensity Table" and format as "sample in columns (unpaired)".**
Select the file exported from OpenOffice such as **"TargetSearch-2016-06-06.profile.quantmatrixYourData.txt"**
 Click in **submit ...** You will see **error message** such as:

ErrorFailed to read in the CSV file.

Possible causes of error (last one being the most relevant):

Duplicate feature names are not allowed!

Abscisic acid (1MEOX) (1TMS)

The duplicate name **must be renamed**. Go back to the spreadsheet **"TargetSearch-2016-06-06.profile.quantmatrixYourData.txt"** and **edit** using the OpenOffice:

Use CTRL+F

Find Absciscic acid (1MEOX) (1TMS)
Replace Absciscic acid_2 (1MEOX) (1TMS)
Repeat for **all duplicate names** indicated in the error message!!!

Save again as before.

Repeat the process in the **MetaboAnalyst** and **verify** if it was **fixed**.

2) The next module is **"Data Normalization"**.

Note: The sample normalization allows general-purpose adjustment for differences among your sample; data transformation and scaling are two different approaches to make individual features more comparable. You can use one or combine them to achieve better results. For details, see documentation on the MetaboAnalyst site.

Here is also possible normalize the data using the fresh mass of the tissues used for metabolite extraction (Sample-specific normalization). However, for better results proceed it in the spreadsheet "TargetSearch-2016-06-06.profile.quantmatrixYourData.txt". Use the table containing the fresh tissue masses to generate a correction factor for each sample. Localize the sample showing highest fresh weight and call 1.0 (100%), then generated the relative factor for the fresh weight of the others samples that will be relative and higher than 1.0. Thus, differences in the weight between samples are corrected without modified the magnitude of the peak intensity from the GC/MS data. Now, the "corrected" data by fresh weight can be normalized using different statistical methods.

During normalization process the "Data transformation" and "data scaling" have been recommended when some requirement of the parametric statistic, such as normality of the error distribution, homogeneity of variance and etc., are not met. The MetaboAnalyst pipeline enable to **choice the better normalization options**, indicating the result after and before the procedure. Thus, it is necessary that you data be in normal distribution.

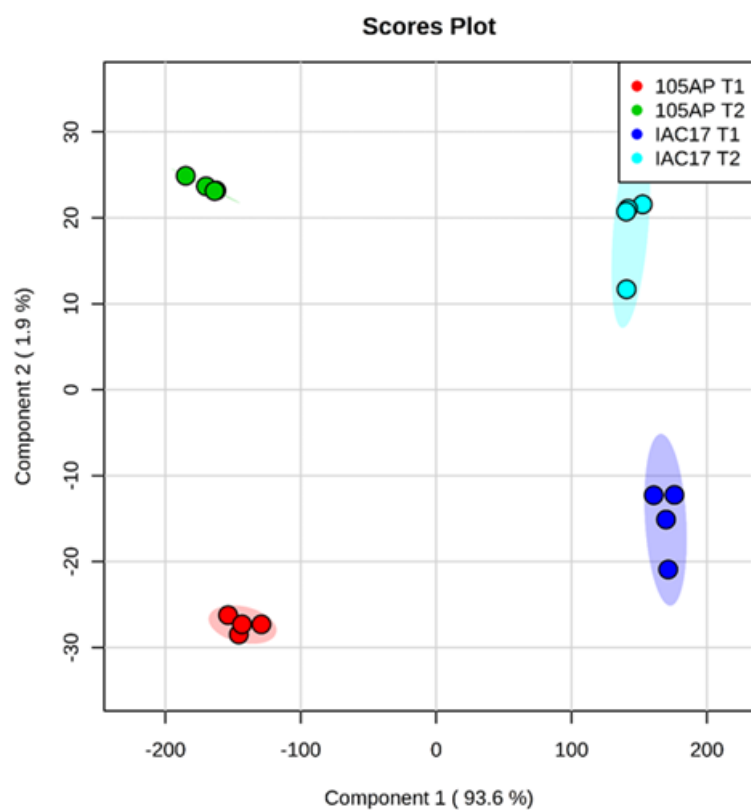
The normalization using an internal control is also possible. For this, use **"Normalization by reference feature"**.

Find and select as normalizer, in the **tab of the compound list**, the internal control **Ribitol** that was added in the extraction procedure. Data normalization by media or median also show satisfactory results and could be used when normalization by Ribitol is not efficient. See the results before and after normalization to verify if the data is under normal distribution and if the variability were reduced.

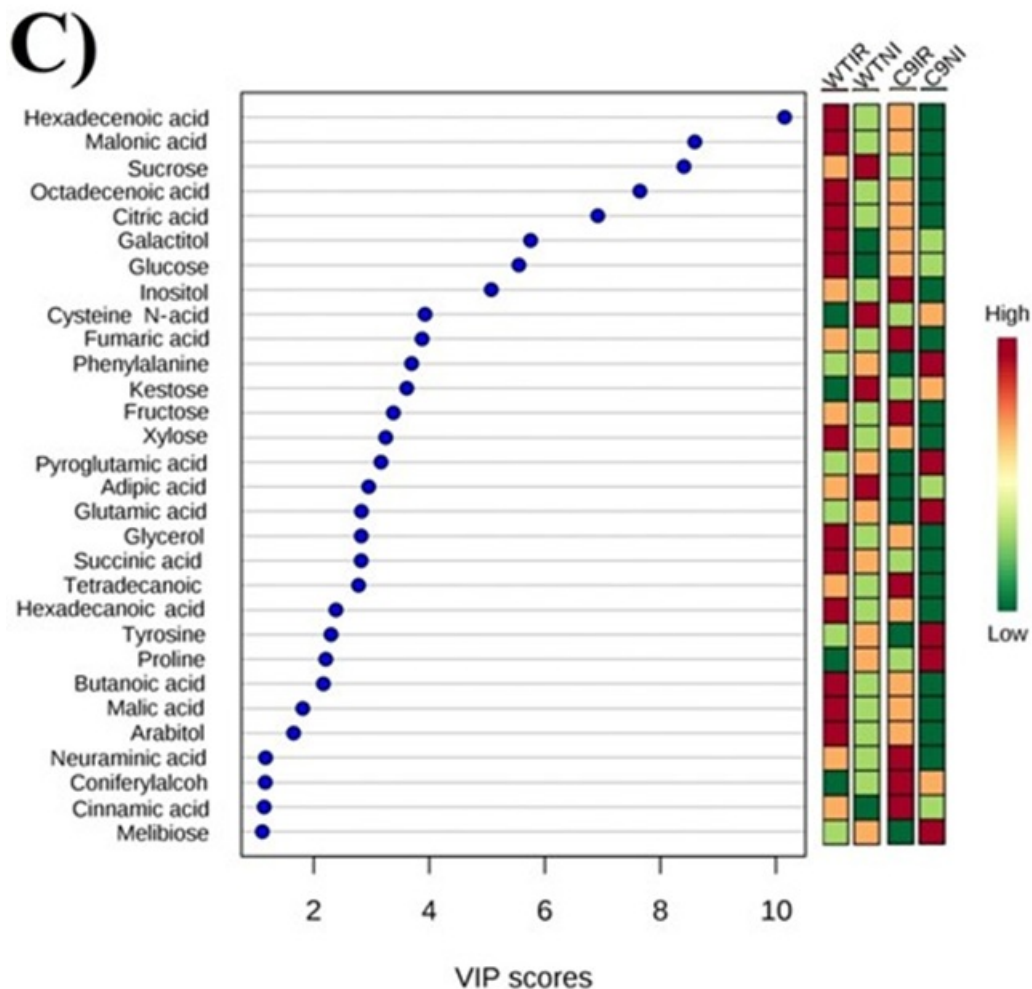
After the selecting of normalization method, click in **"Normalize"** and in **"view results"**. If the normalization was efficient, click in **"proceed"**.

3) Analysis by MetaboAnalyst

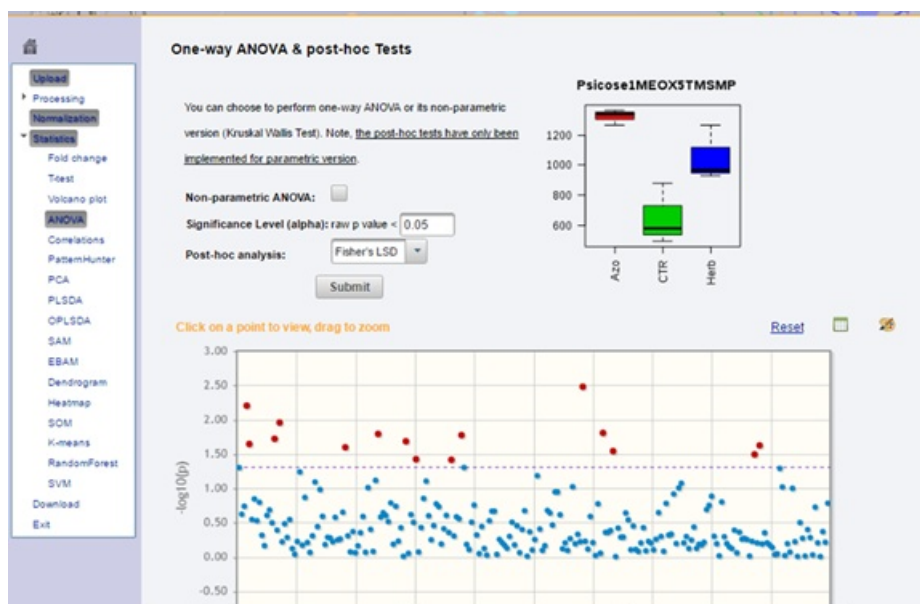
Now, you can choice the analysis according with your experimental design and hypothesis. It convenient start with **"Cluster Analysis"** such as PCA enable view the general behaviors of the treatments and replicates, identifying the effect of specific treatments or genotype relative to the metabolite abundances.



“Cluster Analysis” by PSDLA enable also identify which **metabolites** showed **higher variations** for each group (the output indicate the relative concentrations of the corresponding metabolite in each group under study).



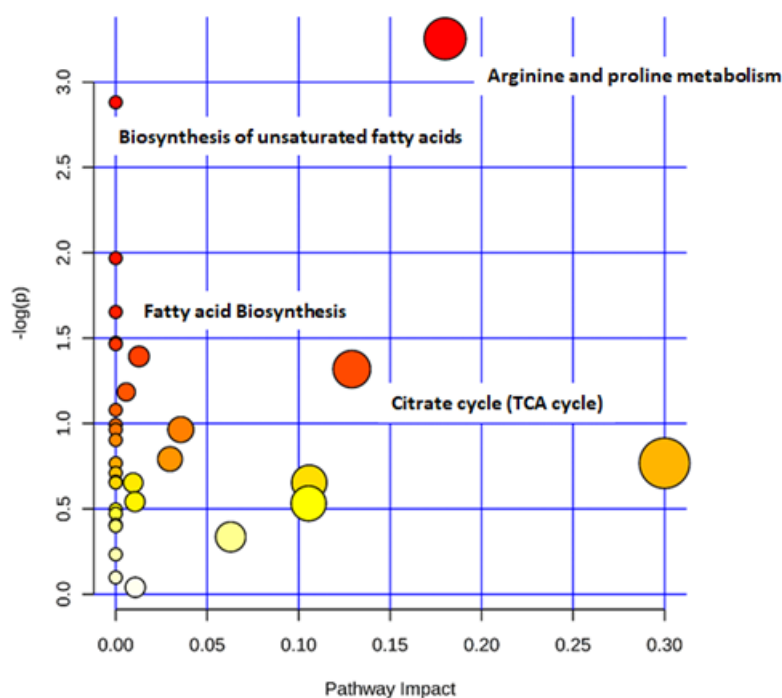
Analysis of variance (**ANOVA**) is very useful to detect which compounds showed significantly variation related to treatments.



Note: MetaboAnalyst containing a total of 12 modules organized into four general categories: **Exploratory statistical analysis, Functional enrichment analysis, Data integration & systems biology and Data processing & utility functions.**

Pathway Analysis generate the **metabolome views** from a list of compounds for each treatment (with or without intensity). The list is submitted to pipeline, however is necessary altered the compound synonymous in your compound list extracted from the "*TargetSearch-2016-06-06.profile.quantmatrixYourData.txt*", **in order to use the names compatible with the pathway library. The pipeline will suggest synonymous, for example, from KEGG and PubChen.** This analyzes shows all matched pathways according to the p values from the pathway enrichment analysis and pathway impact values from the pathway topology analysis.

Overview of Pathway Analysis



Finally. The result tables and the figures can be **downloaded** with high **resolution**.

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