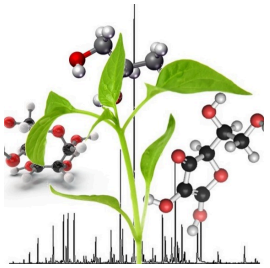
Oct 27, 2019 Version 2

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



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**Protocol status:** Working
**We use this protocol and it's working**

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**Keywords:** generation of the metabolite profile, metabolite profile, global analysis of the metabolome, metabolome, plant tissues by gas chromatography, metabolite, metabolites for each run, method for the metabolic profile, metabolic profile, mass spectrometry, gas chromatography, functional enrichment analyze, plant

## 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.

## Materials

**REAGENTS**

- Methanol (LiChrosolv, Merck, Order n° 1.06001 )
- Chloroform (LiChrosolv, Merck , Order n° 1.0244)
- Ribitol (Sigma)
- High pure water (18.2M Ωcm-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.
- Sppedvac (vacuum centrifugation drier)
- Ultra-freezer.
- Benchtop balance
- Mortar and pestle.
- Vials, caps and septa (PTFE).
- Microtubes e tips (Axygem ou Sigma )

## Troubleshooting

## METABOLITE EXTRACTION

1 **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 termomixer to shake 15 minutes at 70ºC, 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

2 **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 37ºC, 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 37ºC.
**4)** Centrifuge fastly (“spin”) to remove the drops of cover and transfer the sample into vials.

## METABOLIC PROFILE ANALYSIS BY GC/MS

3 **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

4 **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)

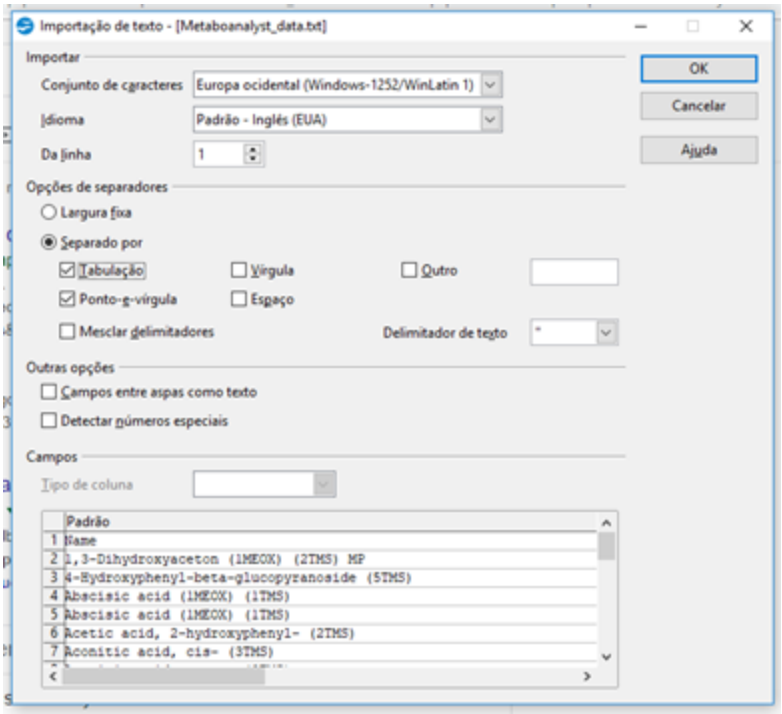
**8) Select the lines 110 to 121**, then click on "**Run**" or type "Ctrl+Enter"

**9)** Open the file **"Metaboanalyst_data.txt"** in a Text Editor or Spreadsheet Editor such as OpenOffice. Install fromhttps://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:**

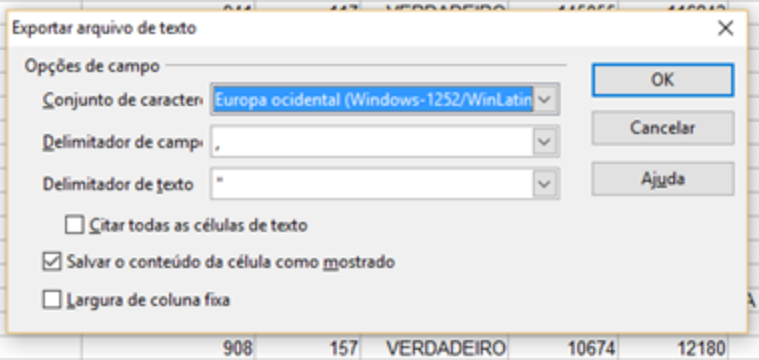
| Name | Azo_14_R3 | Azo_25_R2 | Azo_36_R1 | CTR_14_R1 | CTR_25_R2 | CTR_36_R3 | Herb_14_R2 | Herb_25_R1 | Herb_36_R3 |
|---|---|---|---|---|---|---|---|---|---|
| 1,3-Dihydroxyaceton (1MEOX) (2TMS) MP | NA | 70 | NA | 68 | 51 | 58 | 64 | NA | 66 |
| 4-Hydroxyphenyl-beta-glucopyranoside (5TMS) | 1212 | 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:**

| | A | B | C | D | E | F | G | H | I | J | K |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | name | 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 | |
| 2 | sample | Azo | Azo | Azo | CTR | cTR | CTR | Herb | Herb | Herb | |
| 3 | label | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 | |
| 4 | Hexadecanoic acid methyl ester, n- | 868146 | 794995 | 799669 | 695700 | 840948 | 699469 | 700343 | 862915 | 837617 | |
| 5 | Butanoic acid, 2-amino- (2TMS) | 1534 | 1972 | 1942 | 878 | 1903 | 841 | 895 | 1723 | 1860 | |
| 6 | Aconitic acid, cis- (3TMS) | 322020 | 309599 | 303464 | 255659 | 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_masses | 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 |
| ) MP | | | | | | 145 |
| | | | | | | 792342 |
| | | | | | | 8217 |
| | | | | | | 20287 |
| | | | | | | 80418 |
| | | | | | | 770492 |
| | | | | | | 1140 |
| | | | | | | 313051 |
| | | | | | | 25225 |
| | | | | | | 248464 |
| | 908 | 157 | VERDADEIRO | 10674 | 12180 | 11429 |




**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

5

**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** Abscisic acid (1MEOX) (1TMS)
**Replace** Abscisic 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.

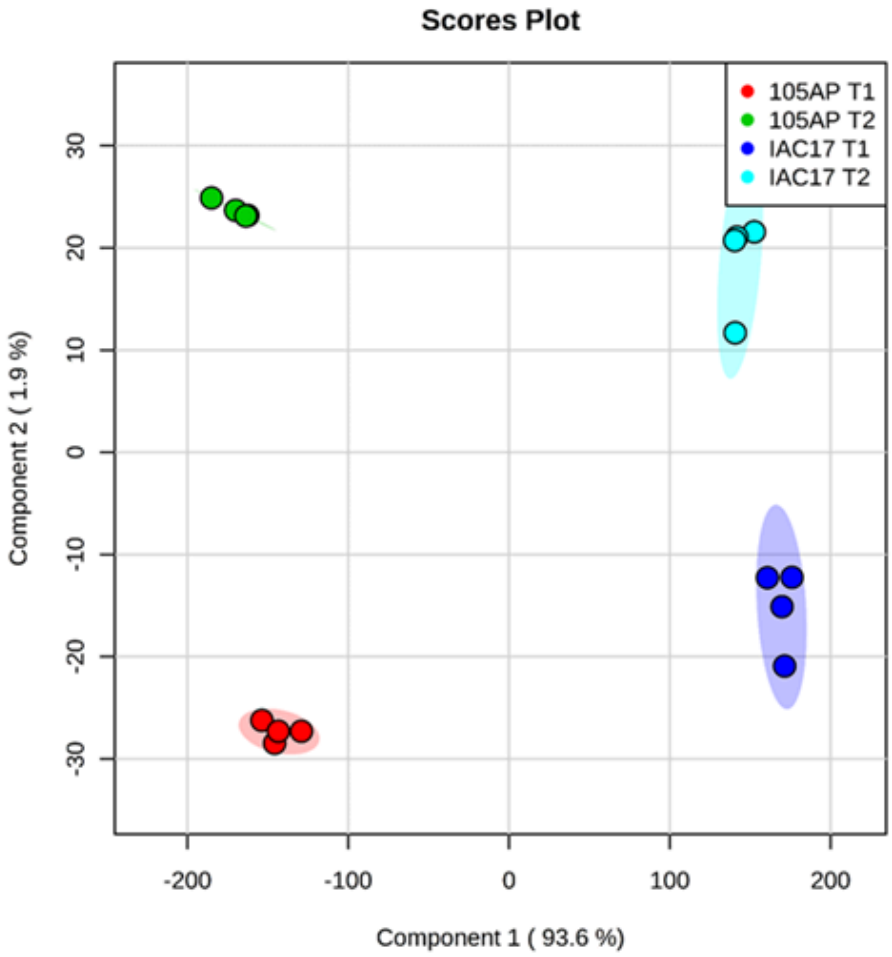
Here is also possible normalize the data using the fresh mass of the tissues used for metabolite extraction (Sample-specific normalization). However, for better results proceed it in the spreadsheet "TargetSearch-2016-06-06.profile.quantmatrixYourData.txt". Use the table containing the fresh tissue masses 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.

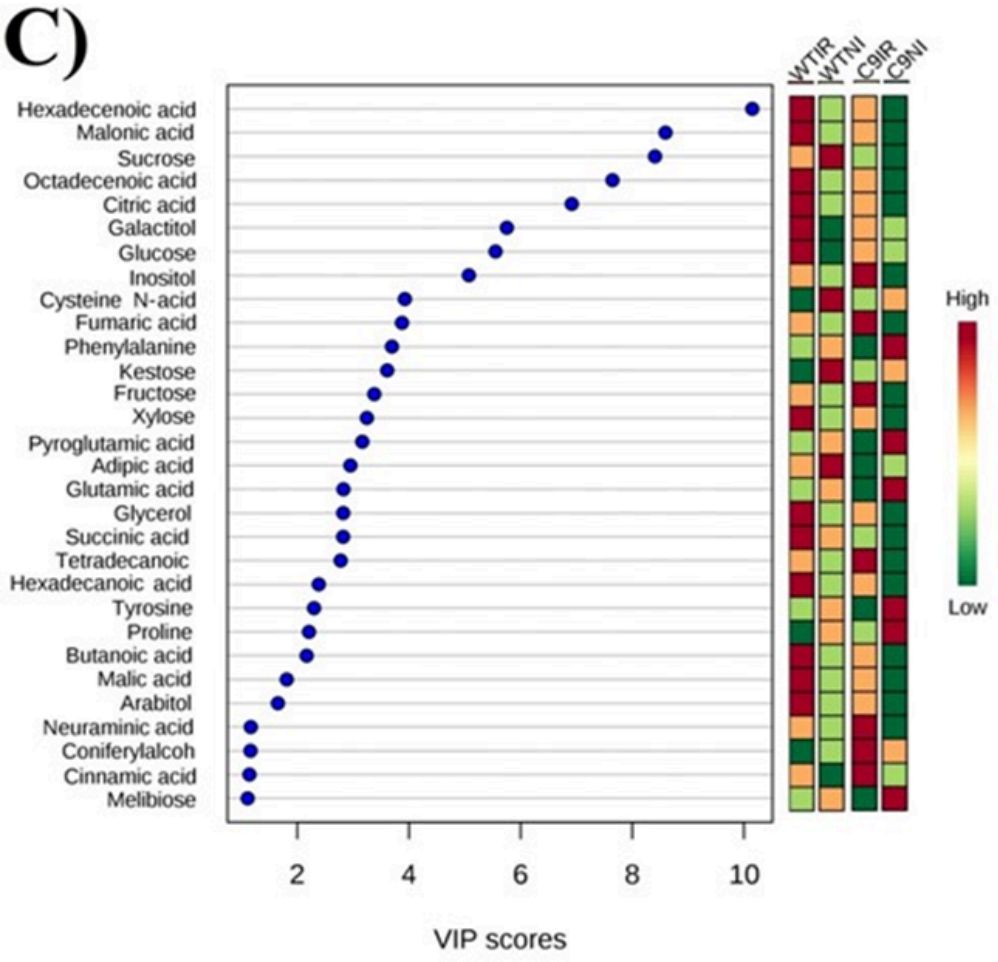
The normalization using an internal control is also possible. For this, use "***Normalization by reference feature".***
**Find and select as normalizer,** in the **tab of the compound list**, the internal control **Ribitol** that was added in the extraction procedure. Data normalization by media or median 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**".

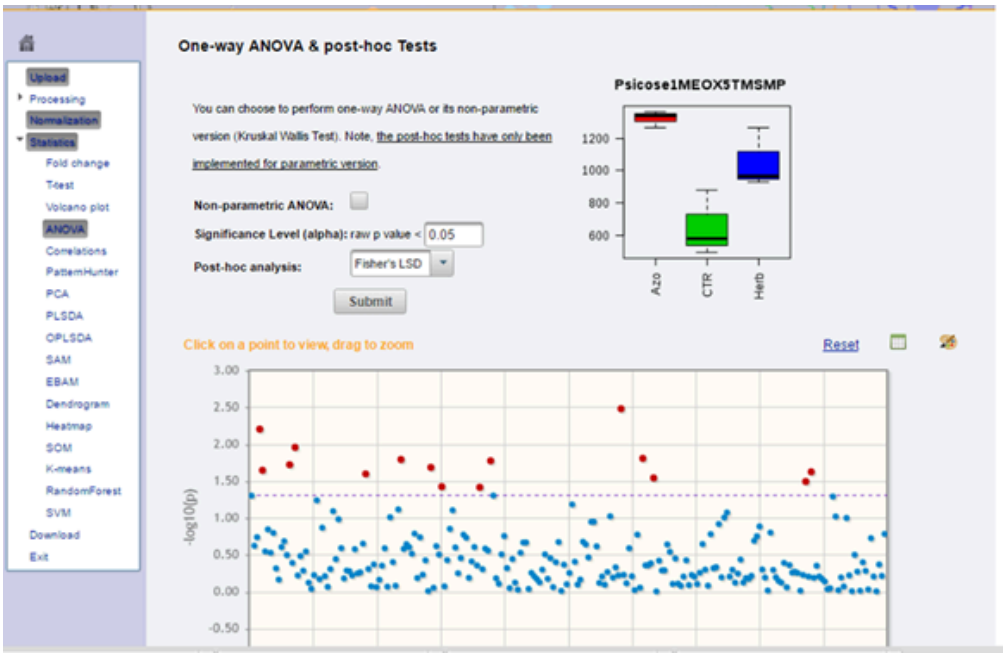
**3) Analysis by MetaboAnalyst**
Now, you can choice the analysis according with your experimental design and hypothesis. It convenient start with **"Cluster Analysis**" such as PCA enable view the general behaviors of the treatments and replicates, identifying the effect of specific treatments or genotype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



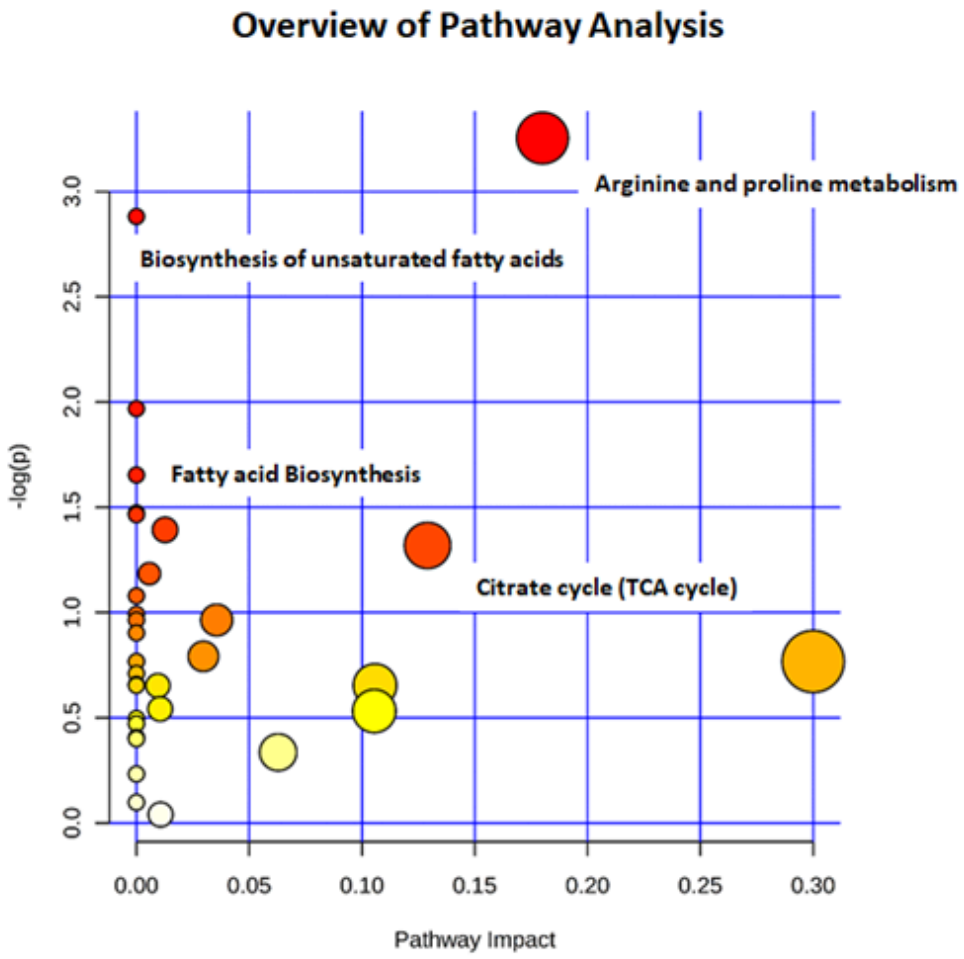

**"Cluster Analysis"** by **PSDLA** enable also identify which **metabolites** showed **higher variations** for each group (the output indicate the relative concentrations of the corresponding metabolite in each group under study).




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




**Note**: MetaboAnalyst containg 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.




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

## ACKNOWLEDGMENTS

6 The authors would like to thank the Núcleo de Análises de Biomoléculas (NuBioMol) of the Universidade Federal de Viçosa for providing the facilities for the data analysis. The authors also acknowledge the financial support provided by the following Brazilian agencies: Fundação de Amparo à Pesquisa do Estado de Minas Gerais (Fapemig), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES – Finance code 001), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Financiadora de Estudos e Projetos (Finep), and Sistema Nacional de Laboratórios em Nanotecnologias (SisNANO)/Ministério da Ciência, Tecnologia e Informação (MCTI).

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