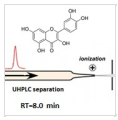

Mar 24, 2019

Working

FLAVONOID PROFILING BY LIQUID CHROMATOGRAPHY COUPLED TO MASS SPECTROMETRY (LC/MS)

Version 3

Forked from a private protocol

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ABSTRACT

Flavonoids play a variety of biological activities in plants, animals and bacteria range from physiological development to plant responses for abiotic and biotic stresses, as well as health-promoting effects of pharmaceutical interest. The following protocol describes the steps and details to generation of qualitative and quantitative broad-range profiles of flavonoids from plant tissues by ultra-high-performance liquid chromatograph (UHPLC) coupled to mass spectrometry

EXTERNAL LINK

Gómez JD, Vital CE, Oliveira MGA, Ramos HJO (2018) Broad range flavonoid profiling by LC/MS of soybean genotypes contrasting for resistance to Anticarsia gemmatalis (Lepidoptera: Noctuidae). PLoS ONE 13(10): e0205010. https://doi.org/10.1371/journal.pone.0205010

PROTOCOL STATUS

Working

We use this protocol in our group and it is working

MATERIALS TEXT

1. REAGENTS

- Acetonitrile LC/MS grade.
- Methanol LC-MS grade.
- Acetic Acid LC/MS grade.
- Formic Acid LC/MS grade.
- Crystalline reference substances of the flavonols (Kaempferol, Quercetin, Myricetin, Catechin, Epicatechin, and Morin), flavones (Luteolin, Apigenin, Oerientin, Isoorientin, Vitexin, Isovitexin), flavanones (Naringin, Hesperetin, Naringenin), chalcones (Phloretin), isoflavones (Daidzein and Genistein), and Glycoconjugates (Rutin, Hesperidin, Naringin).
- High pure water (18.2M Ωcm-1) provided by a Milli-Q system (Burlington, Massachusetts, USA).
- Liquid Nitrogen.

2. EQUIPMENTS AND SUPPLIES

- Ultra-High-Performance Liquid Chromatography (UHPLC) coupled online to a mass spectrometer QQQ (triple quadrupole). Agilent 1200 Infinity LC System coupled to Agilent 6430 Triple Quadrupole LC/MS System (Agilent Technologies, Santa Clara, California, USA)
- Column Zorbax Eclipse Plus C18 (1.8 μm, 2.1 x 50mm) and a guard column Zorbax SB-C18, 1.8 μm (Agilent Technologies, Santa Clara, California, USA).
- Ultrasonic cleaners.
- Benchtop centrifuge.
- Ultra-freezer.
- benchtop balance
- SpeedVac concentrator.
- Mortar and pestle.

- Vials, caps and septa.
- Polyvinyl Difluoride (PVDF) Syringe Filters 13mm and 0,2 μm.
- Softwares: Skyline Targeted Mass Spec Environment version 4.1 (MacCoss Lab Software), Agilent MassHunter Workstation Software and Microsoft Excel.

SAFETY WARNINGS

3.1 FLAVONOIDS 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 absolute quantifications)
3) Add 200 μl of a solution containing 75% methanol and 0.1% formic acid.
4) Sonicate the samples for 30 minutes and centrifuge for 14000g for 10 minutes at 4°C.
5) Collect the supernatants in new tubes. To the remaining pellet, repeat the procedures 3 and 4 (supernatant 2) and then pool the supernatants.
6) Filter the supernatant using disposable 0.2 ml PVDF membrane.
7) Dry the methanolic extracts in speed vac and resuspend in deionized water (50uL).
8) Store the samples in freezer -80°C until analyze them using LC-MS.

3.2 LC/MS CONDICTIONS

2 **1)** UHPLC system containing vials for 50 μl and loop for injection of 5 μl.
2) Use a mass spectrometer triple quadrupole that enable product, precursor and MRM scans. The methods were optimized for an Ultra-High-Performance Liquid Chromatography (UHPLC) coupled online to a mass spectrometer QQQ (triple quadrupole).
3) Chromatographic separation is performed by reverse phase columns, such as an analytical Zorbax Eclipse Plus C18 (1.8 μm, 2.1 x 50mm) and a guard column Zorbax SB-C18, 1.8 μm.
4) The mobile phase consists of buffers A (water acetic acid 0.02%) and B (acetonitrile acetic acid 0.02%) and a gradient of %B: 5% x 0 min^{-1}; 60% x 11 min^{-1}; 95% x 13 min^{-1}; 95% x 17 min^{-1}; 5% x 19 min^{-1}; and 5% x 20 min^{-1}. The solvent flow rate is 0.3ml x min^{-1} in a column at 30°C. The mass spectrometer is operated by positive mode according to method for flavonoids detection.
5) The ionization method used in the mass spectrometry was an ESI (Electrospray Ionization) under the conditions: gas temperature of 300 °C, nitrogen flow rate of 10 L x min^{-1}, nebulizer pressure of 35 psi and capillary voltage of 4000 V.

3.3 FLAVONOID PROFILE

3 The flavonoid profiles are obtained through a three-step process:
1) Analysis of target phenolic compounds **(Table 1)**.
2) Analysis of flavonoid classes using a non-target method with insource fragmentation ISCID (**Table 2**), following Abrankó and Szilvássy (2015).
3) Determination of glycoconjugates flavonoid m/z values and putative structures using precursor ions.

Table 1. Transition list and MS parameters used for analysis of twenty-one target phenolic compounds

Molecule Name	Precursor Charge	Product m/z	Product charge	Precursor RT	Precursor CE	Precursor m/z	Polarity
Hesperidin	1	303	1	6.8	30	611	Positive
Rutin	1	303	1	6.2	30	611	Positive
Naringin	1	273	1	6.8	30	581	Positive
Orientin	1	329	1	5.8	30	449	Positive
Isoorientin	1	299	1	5.6	30	449	Positive
Vitexin	1	313	1	6.2	30	433	Positive
Isovitexin	1	283	1	6.2	30	433	Positive
Myricetin	1	153	1	7.2	30	319	Positive
Morin	1	153	1	8	30	303	Positive
Hesperetin	1	153	1	6.8	30	303	Positive
Quercitin	1	153	1	8	30	303	Positive

Epicatechin	1	139	1	5	30	291	Positive
Catechin	1	139	1	4.2	30	291	Positive
Kaempferol	1	153	1	8.6	30	287	Positive
Luteolin	1	153	1	7.9	30	287	Positive
Phloretin	1	107	1	7.1	30	275	Positive
Narigenin	1	153	1	8.4	30	273	Positive
Genistein	1	153	1	8.4	30	271	Positive
Apigenin	1	153	1	8.4	30	271	Positive
Daidzein	1	137	1	9.6	30	255	Positive
Chalcone	1	104	1	11.2	30	210	Positive

Table 2. LC/MS conditions to analyze the flavonoid classes and used as input in the Skyline analysis.

Molecule Name	Precursor Charge	Product m/z	Product charge	Precursor CE	Precursor m/z
Daidzein Class 1	1	137	1	30	255
Daidzein Class 1	1	181	1	30	255
Daidzein Class 1	1	153	1	30	255
Daidzein Class 1	1	91	1	30	255
Apigenin Class 2	1	153	1	30	271
Apigenin Class 2	1	145	1	30	271
Apigenin Class 2	1	91	1	30	271
Apigenin Class 2	1	69	1	30	271
Genistein Class 2	1	153	1	30	271
Genistein Class 2	1	145	1	30	271
Genistein Class 2	1	91	1	30	271
Genistein Class 2	1	69	1	30	271
Phloretin Class 3	1	150.7	1	30	275
Phloretin Class 3	1	107	1	30	275
Phloretin Class 3	1	79	1	30	275
Phloretin Class 3	1	77	1	30	275
Luteolin Class 4	1	153	1	30	287
Luteolin Class 4	1	135	1	30	287
Luteolin Class 4	1	121	1	30	287
Luteolin Class 4	1	69	1	30	287
Kaempferol Class 4	1	153	1	30	287
Kaempferol Class 4	1	135	1	30	287
Kaempferol Class 4	1	121	1	30	287
Kaempferol Class 4	1	69	1	30	287
Catechin Class 5	1	161	1	30	291
Catechin Class 5	1	123	1	30	291
Catechin Class 5	1	119.2	1	30	291
Catechin Class 5	1	69	1	30	291
Epicatechin Class 5	1	161	1	30	291
Epicatechin Class 5	1	123	1	30	291
Epicatechin Class 5	1	119.2	1	30	291
Epicatechin Class 5	1	69	1	30	291
Quercetin Class 6	1	229.2	1	30	303
Quercetin Class 6	1	153	1	30	303
Quercetin Class 6	1	137	1	30	303
Quercetin Class 6	1	89	1	30	303
Hesperentin Class 6	1	229.2	1	30	303
Hesperentin Class 6	1	153	1	30	303
Hesperentin Class 6	1	137	1	30	303

Hesperentin Class 6	1	89	1	30	303
Morin Class 6	1	229.2	1	30	303
Morin Class 6	1	153	1	30	303
Morin Class 6	1	137	1	30	303
Morin Class 6	1	89	1	30	303
Myricetin Class 7	1	245	1	30	319
Myricetin Class 7	1	217	1	30	319
Myricetin Class 7	1	164.8	1	30	319
Myricetin Class 7	1	153	1	30	319
Naringenin Class 8	1	153	1	30	273
Naringenin Class 8	1	147	1	30	273
Naringenin Class 8	1	119	1	30	273
Naringenin Class 8	1	91	1	30	273

3.4 TARGET METHOD

4 **Note:** Inthis method, aflavonoid compound should be specified to detection and absolute quantification (ug/g fresh tissue) using standard curves.

1) Prepare a standard solution containing 1.0 ug/mL of each compound in methanol 50% and transfer it to vials. (foi usado essa concetração para injetar neste momento?).
2) Setup a product ion scan method for each compound and optimize the transmission and fragmentation parameters. This procedure may be executed manually or automatically.
3) Select the higher intensity fragment ions to compose the transition list used in the scan mode by MRM (Multiple Reaction Monitoring) as illustrated in the Table 1.
4) Prepare serial dilutions of 0.1 ng/mL up to 1.0 ng/mL in according with the mass spectrometer sensitivity and linearity. Two replicate is enough to prepare the standard curves.
5) Inject 5.0 ul and perform the MRM method as setup in step 3.
6) Generate the area from XICs (extracted ion chromatograms) for each dilution using Skyline software. Note: A complete tutorial for processing of the mass spectra data using Skyline software is described in the Supplementary Material 1.
7) Export the XICs to Microsoft Excel following the instructions of the Skyline tutorial (Supplementary Material 1).
8) Prepare the standard curves for each compound in ng/mL of fresh tissue. Use the XIC area versus flavonoid concentration (ng/mL) to generated linear curves (**Figure 1**).

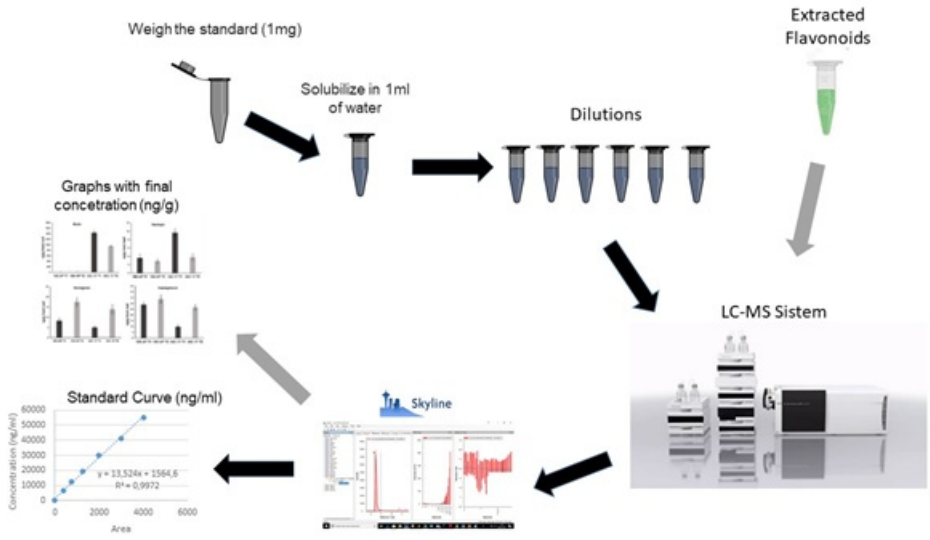

Figure 1. Schematic diagram of the steps used for prepare of the standard curves.

SAMPLE ANALYSIS

5 **1)** Inject all sample randomly using the MRM method, which was used to generate the standard curves. Use the raw data to analyze mass spectra using Skyline software (section 3.3; step 6).

2) For each compound, use the XICs area exported to Microsoft Excel and convert to ng/mL using the standard linear curves (section 3.3; step 6).

Quantification of Kaempferol for a Sample 1 with a XIC area of 7250:

Kaempferol ; transition 287 >153; retention time 8.6 (Table 1).

Conversion to ng/mL:
Standard curve for Kaempherol:
y=13.524x + 1564.6; where x is the sample concentration (ng/mL) and y is the XIC area (arbitrary units)
Kaempherol concentration in the sample 1: 420.4 ng/mL

Conversion of ng/mL to ng/g of fresh tissue:
420,4 ---- 1000ul
x------ 50ul
Then:21 ng of Kaempherol from 100mg of fresh tissue, 210 ng/g fresh leaves

3.5 NON TARGET METHOD

6 **Note:** In this method, glyconjugate flavonoids are fragmented in-source and the respective aglycones are monitored by MRM using four specific transitions. It allows the characterization of the flavonoid classes of samples as well as their relative abundances. The relative abundances of the fragment ions (FRI%, fragment relative intensity) is used to characterize the glyconjugates from different flavonoid classes that show same nominal mass (for instance: Kaempherol from Luteolin; isomers with m/z = 286).

1) Inject in LC/MS system the standards of glycoconjugates, such as rutin, hesperidin, naringin to optimize the in-source energy for better release the aglycone core before the first quadrupole.
2) Inject also in the LC/MS system the standards (such as dadzein, apigenin, genistein, etc) for each aglycone belonging to the flavonoid classes. Use the product ion scan to optimize the fragmentation conditions to generate the fragment ions. Do not use high energy in the source,just use the optimal transmission setting. Verify the fragments which show intensities distinct from aglycones of the same class (same nominal mass, isomers), as reference see Abrankó and Szilvássy (2015) and Gómez et al. (2018) prepare a MRM method (**Table 2**).
3) Inject again all the standards using MRM scan for the selected transitions in the step 2. Use four transitions by compound (Table 2). Use the raw spectrum as input of Skyline software and export the XICs areas to generate the FRI%, which will used as signature for identification of each compound from each class using the Microsoft Excel. For instance, despite kaempferol and luteolin show the same nominal mass of m/z 287, the FRI% of fragments (153, 135, 121 and 69) enable a undoubted differentiation of the aglycone (**Figure 2**).

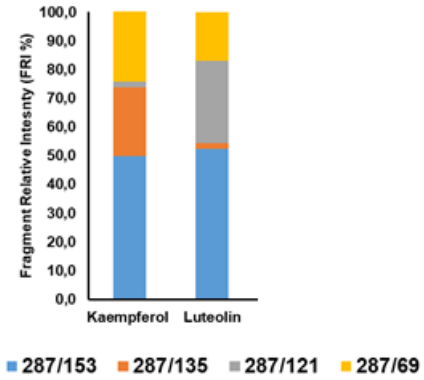

Figure 2. Fragment relative intensity (FRI%) from Kaempferol and Luteolin:

SAMPLE ANALYSIS

7 **1)** Inject all sample randomly in LC/MS system using the in-source/MRM setup (**section 3.4**)
2). Use the raw spectra as input of Skyline software to obtain the XIC area for each transition (**Figure 3A**) and the FRI% for each retention time (RT) (**Figure 3B**).

Note: The eluted compounds that shown the same FRI% of the specific standard will be considerate as containing the same aglycone (**Figure 3B**).

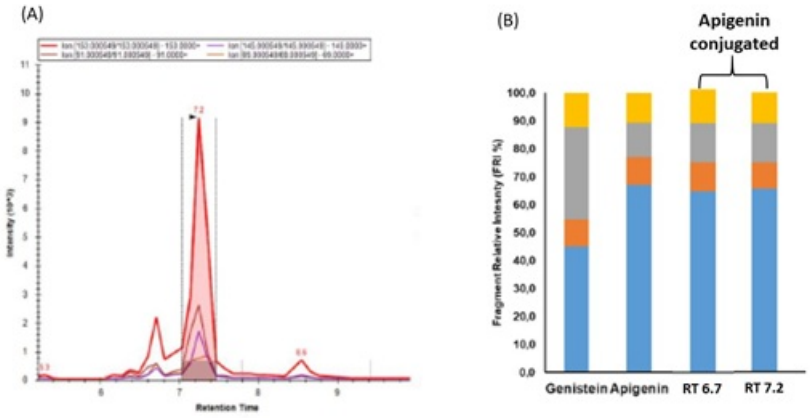

Figure 3. Nontarget analysis of flavonoids. Analysis of Apigenin and genistein results in the Skyline software (**A**) and fragment relative intensity (FRI %) in the standard and plant sample (**B**). Compound with RT 6.7 and 7.2 are characteristic of apigenin core.

3.6 DETERMINATION OF THE GLUCOCONJUGATED MASS

8 **Note:** In this method, a precursor ion scan is used to determinate the m/z of the glycoconjugates from eluted compounds in different RTs which match with the FRI% from the flavonoid standards (section 3.5).

1) Inject again the all sample randomly in the LC/MS system. Setup a *precursor ion scan* for each class using the same parameters of the section 3.5.
2) Use the mass spectrometermanufacturer software to proces the spectrum raw data. The **Figure 4** shows the layout of Mass Hunter software (Agilent). For each XIC, annotate the m/z values of the precursor ions eluted in each RT.
3) Search each m/z value (deprotonated value, subtracted of 1.0 Da)in a mass spectrum database, such as MassBank (https://massbank.eu/MassBank/), by "Quick Source module" using the mass tolerance of 0.3 Da. Additionally, search for scientific reports which describe flavonoids with the same nominal mass containing the same aglycone core. Then subtract the nominal mass of glycoconjugate of the aglycone core to obtain the sugar moiety.

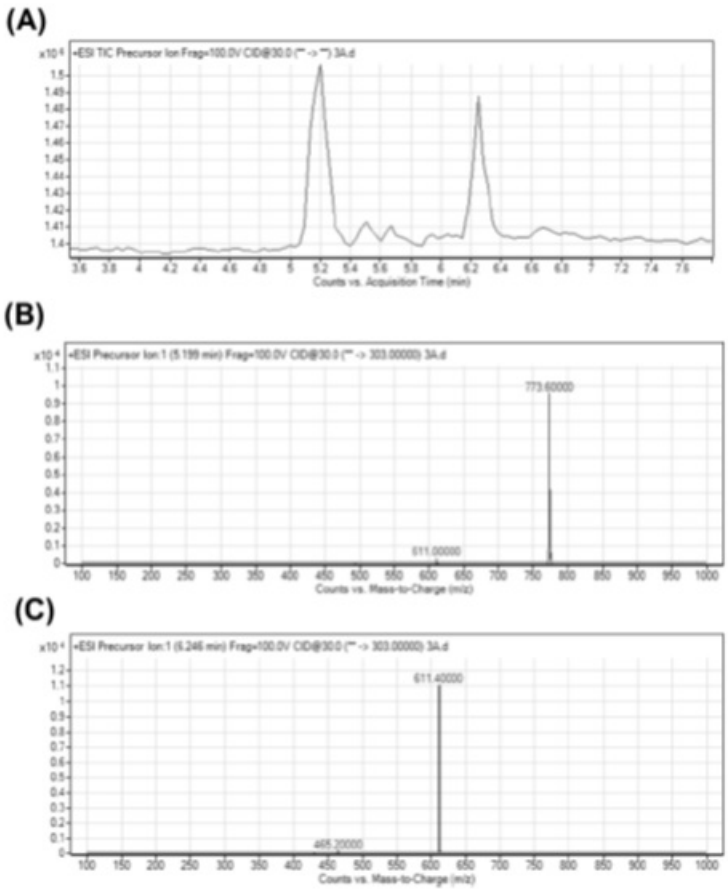

Figure 4. Determination of the m/z values for the detected Glucoconjugated flavonoids in the step 3.5.

ACKNOWLEDGMENTS

9 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), 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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Gómez JD, Vital CE, Oliveira MGA, Ramos HJO (2018) Broad range flavonoid profiling by LC/MS of soybean genotypes contrasting for resistance to *Anticarsia gemmatalis* (Lepidoptera: Noctuidae). PLoS ONE 13(10): e0205010. https://doi.org/10.1371/journal.pone.0205010

SUPPLEMENTARY MATERIAL: Skyline Tutorial

11 **Note:** Tutorial for analysis of mass spectra from small molecules by skyline software. Adapted from tutorial " Skyline Small Molecule Targets" [*https://skyline.ms/_webdav/home/software/Skyline/@files/tutorials/SmallMolecule-3_6.pdf*]

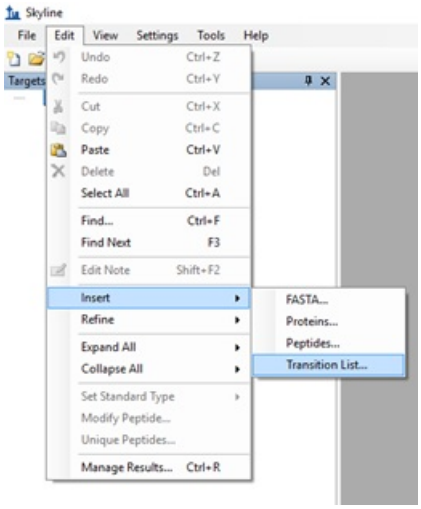
1) Install the Skyline Package (32 or 64 bits):
https://skyline.ms/wiki/home/software/Skyline/page.view?name=SkylineInstall_64_4-1&submit=false or
https://skyline.ms/project/home/software/Skyline/begin.view

2) Generate a *transition list table* in accordance with the MRM parameters such as in the **Table 1**.

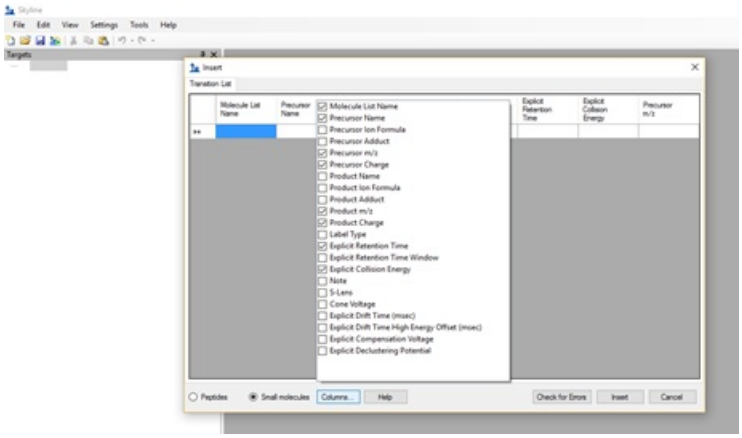
3) Open the *transition list* file in the OpenOffice software (Avoid language incompatibly).
Select and Copy all lines, except the column heading.

4) Open Skyline package and click in *blank document*

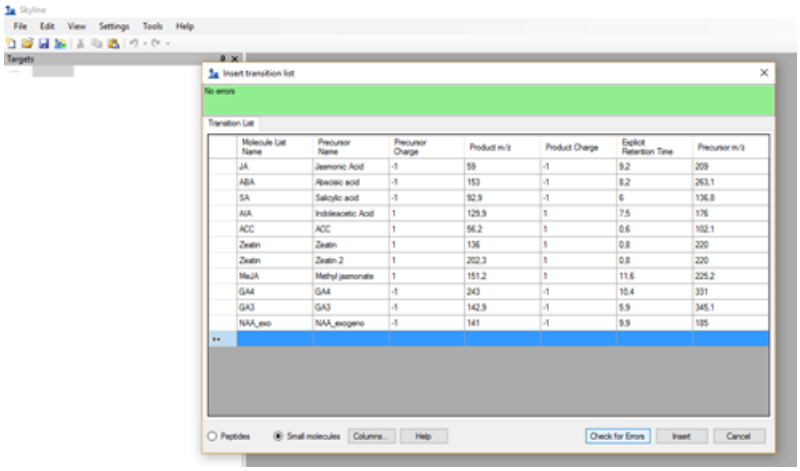
5) Proceed *edit>>>>insert>>>>transition list*



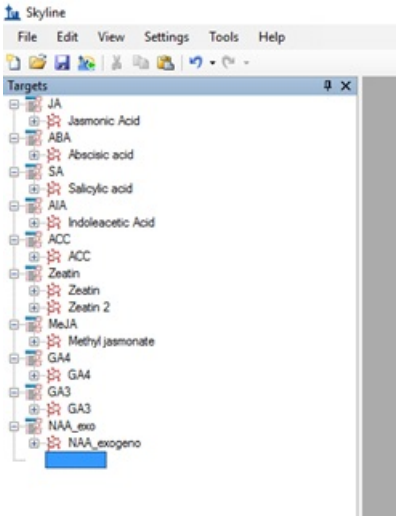
6) Click in "*columns"* to edit. Select in accordance with the transition list create before. Use the mouse to dragging and to change the column order.



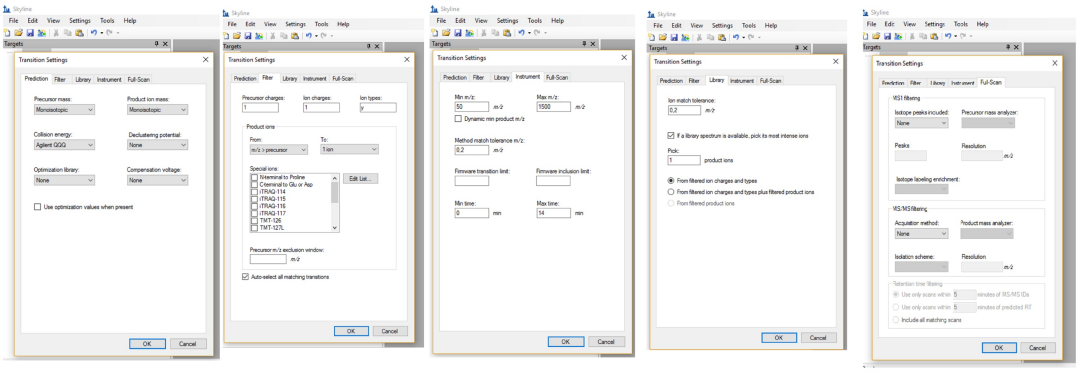
7) Paste the transition list information. Click in *check for errors*



8) Click in "*insert*".

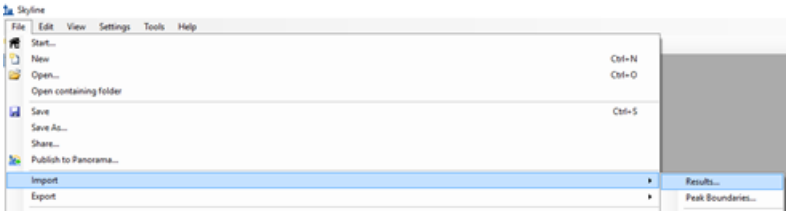


9) Open *Setting* >>> *transitions setting* and configure to processing the raw data from the QqQ mass spectrometry



10) Open *Setting*>>> *Save Current* .

11) Open *File*>> *import* >> *Results*

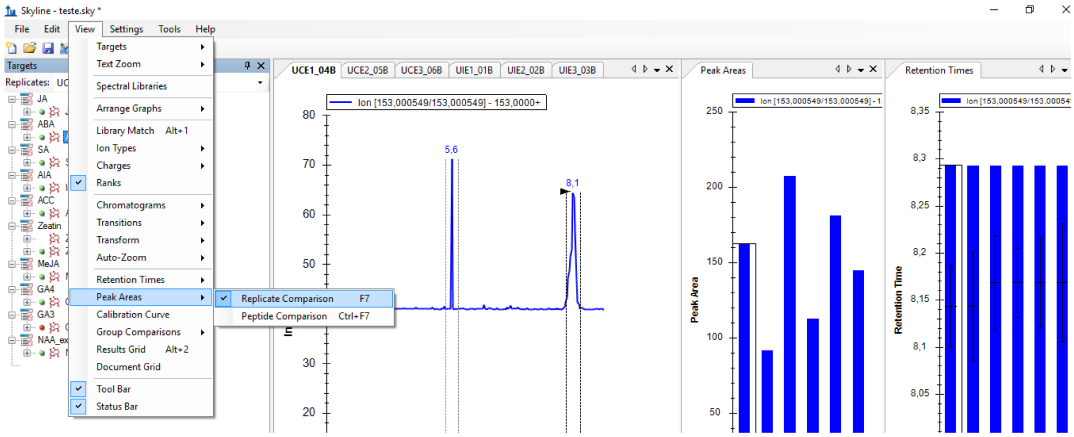


12) Click in *Ok* to import and *save* the Project as *myprojectname. Sky*

13) In the windown *importResults* >> click in *OK*!! Select and import all data from files *.d,* generated by LC/MS QqQ for all the samples.

14) After the data uploading, open *View* >>>> *retention times* >>> and select *ReplicateComparison*

15) Open *View*>>>> *Peak Areas* >>> and select *replicate comparison*



16) Proceed a *double-click* in the tabs *"Peak Areas" and "Retention Times"*.
17) Click over the retention time (RT) bar to edit and correct the selected chromatogram area that was generated automatically. The dashed line could be moved!! Repeat for all compound and samples!!!
18) Save the project.
19) Open *File*>>> *Export* >> *report* >> select *transition result* >>>> OK, to export the peak areas of the all XICs as spreadsheet file. Open this file in the OpenOffice and use the XIC area for each compounds to obtain the quantitative information.