

Mar 24, 2019

Version 2

PHYTOHORMONE PROFILING BY LIQUID CHROMATOGRAPHY COUPLED TO MASS SPECTROMETRY (LC/MS) V.2

DOI

<https://dx.doi.org/10.17504/protocols.io.zgff3tn>

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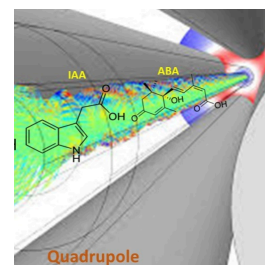
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Protocol Citation: Camilo E. Vital, Jenny D. Gómez, Pedro M. Vidigal, Edvaldo Barros, Claudia S.L. Pontes, Nívea . Vieira, Humberto Ramos 2019. PHYTOHORMONE PROFILING BY LIQUID CHROMATOGRAPHY COUPLED TO MASS SPECTROMETRY (LC/MS). **protocols.io** <https://dx.doi.org/10.17504/protocols.io.zgff3tn>

Manuscript citation:

Coutinho, FS; Santos, DS; Lima, LL; Vital, CE; Santos, LA; Pimenta, MR; Silva, JC; Soares-Ramos, JRL; Mehta, A; Fontes, EPB; Ramos, HJO. Mechanism of the Drought Tolerance of a Transgenic Soybean Overexpressing the Molecular Chaperone BiP. Physiology and Molecular Biology of Plants 2019. DOI : 10.1007/s12298-019-00643-x

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Protocol status: Working

We use this protocol and it's working

Created: March 24, 2019

Last Modified: March 24, 2019

Protocol Integer ID: 21735

Keywords: Phytohormones, liquid chromatograph, mass, spectrometry, (cytokinin), abscisic acid, indoleacetic acid , salicylic acid, gibberellins , jasmonic acid, quantitative profiles of phytohormone, phytohormone, mass spectrometry, performance liquid chromatograph, plant response, plant tissue

Abstract

Phytohormones play a key role in regulating development and growth, as well as acting on plant responses to biotic and abiotic stresses. The following protocol describes a target specific methodology to obtaining quantitative profiles of phytohormones from plant tissues by ultra-high-performance liquid chromatograph (UHPLC) coupled to mass spectrometry.

Materials

REAGENTS

- Methanol LC-MS grade.
- Acetic Acid LC/MS grade.
- Isopropanol LC/MS grade.
- Acetonitrile LC/MS grade.
- Crystalline reference substances of the phytohormones -zeatin(cytokinin), 1-aminocyclopropane-1-carboxylic acid (ACC) (ethylene precursor), abscisic acid (ABA), 3-indoleacetic acid (IAA), salicylic acid (SA), gibberellins (GA₃ and GA₄), jasmonic acid (JA), methyl jasmonate (MeJA)
- High pure water (18.2M Ω cm⁻¹) provided by a Milli-Q system (Burlington, Massachusetts, USA).
- Liquid Nitrogen.

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 μ m, 2.1 $\times$ 50mm) and a guard column Zorbax SB-C18, 1.8 μ m (Agilent Technologies, Santa Clara, California, USA).
- Ultrasonic cleaners.
- Benchtop centrifuge.
- Ultra-freezer.
- Benchtop balance
- Mortar and pestle.
- Vials, caps and septa.
- Polyvinyl Difluoride (PVDF) Syringe Filters 13mm and 0,2 μ m.
- Softwares: Skyline Targeted Mass Spec Environment version 4.1 (MacCoss Lab Software) and Microsoft Excel.

PHYTOHORMONES 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 110mg of each sample into microtubes (2ml) and annotate the weight (used for absolute quantifications). Alternatively, the samples may be weighed into 1.5 ml tubes (eppendorff - round bottom) and macerate using glass or tungsten beads at 25 Hz / s for 3 minutes.
 - 3) Add 400 μl of a solution containing methanol:isopropanol:acetic acid (20:79:1).
 - 4) Vortex samples 4 times for 20 seconds (keep on ice) and then sonicate for 10 minutes. Let the samples stand on ice for 30 minutes and again sonicate for 10 minutes.
 - 5) Centrifuge for 13000g for 10 minutes at 4°C and collect the supernatants in new tubes.
 - 6) To the remaining pellet, repeat the procedures 3, 4 and 5 (supernatant 2) and then pool the supernatants.
 - 7) Centrifuge the samples a 20000g for 5 minutes at 4°C to remove debris and collect about 600 μl for new tubes
 - 8) Filter the supernatant using disposable 0.2ml PVDF membrane.
 - 9) Store the samples in freezer -80°C until analyze them using LC-MS.

LC/MS CONDICTIONS

- 2
 - 1) UHPLC system containing vials for 1mL and loop for injection of 5 μl .
 - 2) Use a mass spectrometer triple quadrupole which enables product and MRM (multiple reaction monitoring) 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 , $2.1 \times 50\text{mm}$) 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 min^{-1} in a column at 30°C .
 - 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 10L min^{-1} , nebulizer pressure of 35psi and capillary voltage of 4000V. The mass spectrometer is operated by positive or negative mode according to method for phytohormone detection.

6) INSERIR UMA TABELA OU COMO FOTO DO SETUP DO QQQ OU IDICANTO OS SEGMENTOS E O USO DO MRM DYNAMIC!!!

STANDARD CURVES

3 Note: For absolute quantification of phytohormones, a standard curve with known concentrations should be prepared.

1) Prepare a standard solution containing 1.0 ug/mL of each compound in methanol:isopropanol:acetic acid (20:79:1) and transfer it to vials.

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. A second transition can be used to confirm the first transition used for the quantification, especially for low intensity chromatogram signals.

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.

	Molecule List Name	Precursor Name	Precursor Charge	Product m/z	Product charge	Precursor or RT	Precursor CE	Precursor or m/z	Polarity
	JA	Jasmonic Acid	-1	59	-1	9,2	7	209	negative
	ABA	Absciscic acid	-1	153	-1	8,2	7	263	negative
	SA	Salicylic acid	-1	92,9	-1	6	7	136,8	negative
	IAA	Indoleacetic Acid	1	129,9	1	7,5	15	176	positive
	ACC	ACC	1	56,2	1	0,6	7	102,1	positive
	Zeatin	Zeatin	1	202,3	1	0,8	7	220	positive
	MeJA	Methyl jasmonate	1	151,2	1	11,6	15	225,2	positive

GA4	Gibbrell in 4	-1	243	-1	10,4	15	331	negative
GA3	Gibbrell in 3	-1	142,9	-1	5,9	34	345,1	negative

Table 1. Mass spectrometric parameters used for analysis of nine target phytohormones and transition list used as input in the Skyline analysis.

- 4) Prepare serial dilutions of 0.1 ng/mL up to 400 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 phytohormones concentration (ng/mL) to generated linear curves (Figure 1).

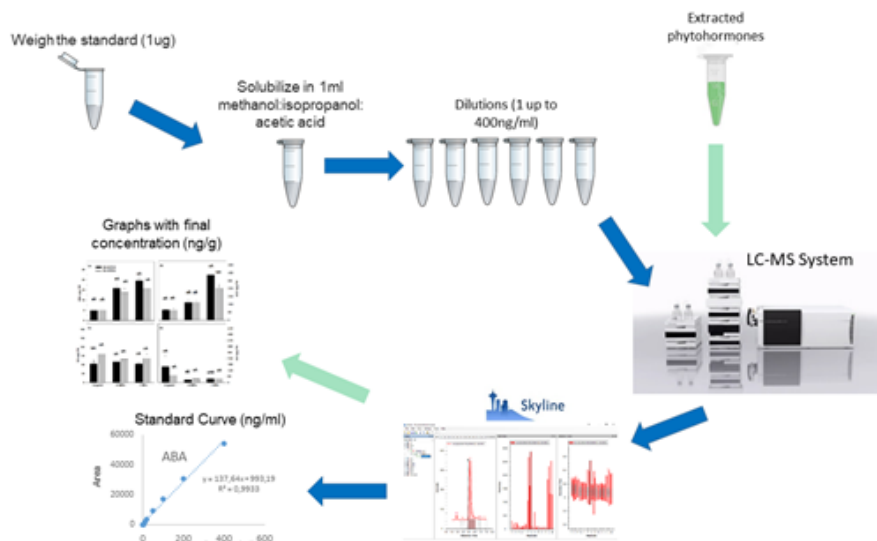


Figure 1. Schematic diagram showing the steps to prepare the standard curves and to final quantification of phytohormones

SAMPLE ANALYSIS

- 4 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 7).

Quantification of ABA for a Sample 1 with a XIC area of 6350:

ABA ; transition 263 >153; retention time 8.2 (Table 1).

Conversion to ng/mL:

Standard curve for ABA:

$y = 137.64x + 993.19$; where x is the sample concentration (ng/mL) and y is the XIC area (arbitrary units)

ABA concentration in the sample 1: 38.91ng/mL

Conversion of ng/mL to ng/g of fresh tissue:

38.91ng ---- 1000ul

x----- 800ul

Then: 31.13 ng of ABA from 110mg of fresh tissue, 282.98 ng/g fresh tissue.

ACKNOWLEDGMENTS

- 5 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).

REFERENCES

- 6 Coutinho, FS; Santos, DS; Lima, LL; Vital, CE; Santos, LA; Pimenta, MR; Silva, JC; Soares-Ramos, JRL; Mehta, A; Fontes, EPB; Ramos, HJO. Mechanism of the Drought Tolerance of a Transgenic Soybean Overexpressing the Molecular Chaperone BiP. Physiology and Molecular Biology of Plants 2019. DOI : 10.1007/s12298-019-00643-x
- Forcat, S., Bennett, M. H., Mansfield, J. W., & Grant, M. R. (2008). A rapid and robust method for simultaneously measuring changes in the phytohormones ABA, JA and SA in

plants following biotic and abiotic stress. Plant methods, 4, 16. doi:10.1186/1746-4811-4-16.

Müller M, Munné-Bosch S (2011) Rapid and sensitive hormonal profil-ing of complex plant samples by liquid chromatography coupled to electrospray ionization tandem mass spectrometry. Plant Methods 7(1):37. <https://doi.org/10.1186/1746-4811-7-37>

SUPPLEMENTARY MATERIAL: SKYLINE TUTORIAL

- 7 **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]

Install the Skyline Package (32 or 64 bits):

[https://skyline.ms/wiki/home/software/Skyline/page.view?](https://skyline.ms/wiki/home/software/Skyline/page.view?name=SkylineInstall_64_4-1&submit=false)

[name=SkylineInstall_64_4-1&submit=false](https://skyline.ms/wiki/home/software/Skyline/page.view?name=SkylineInstall_64_4-1&submit=false) or

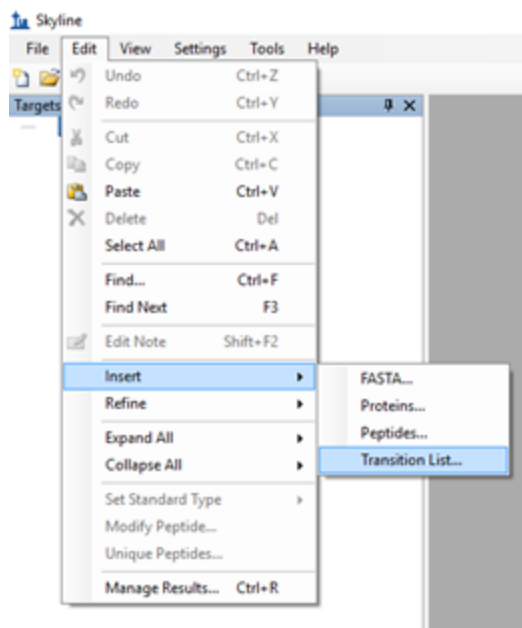
<https://skyline.ms/project/home/software/Skyline/begin.view>

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

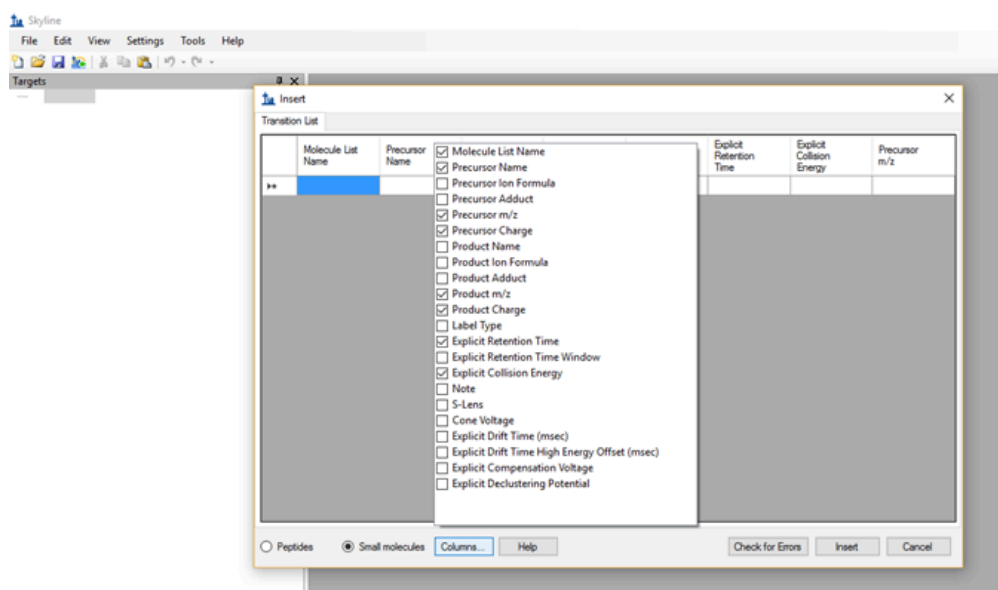
Open the *transition list* file in the OpenOffice software (Avoid language incompatibly). Select and Copy all lines, except the column heading.

Open Skyline package and click in *blank document*

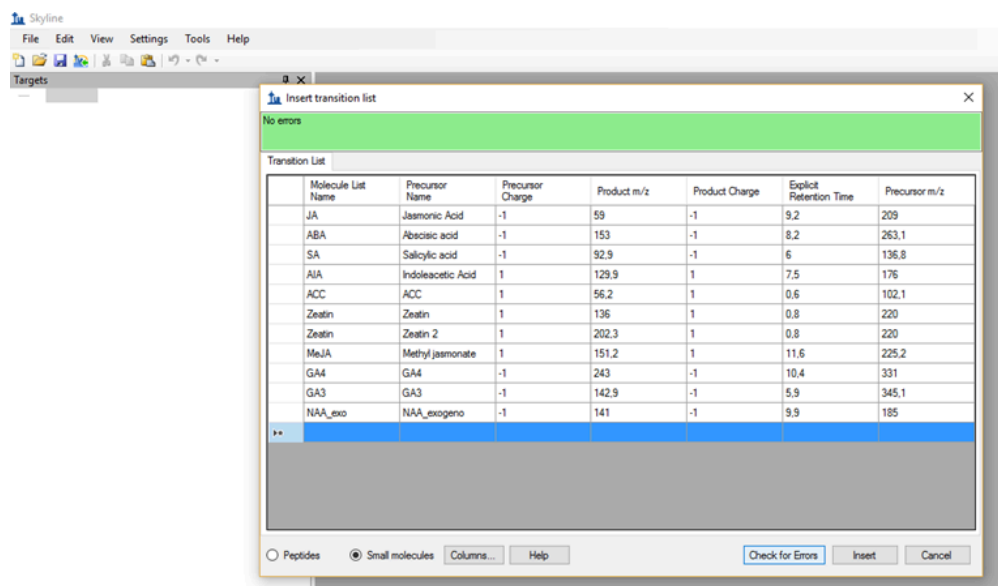
Proceed **edit>>>insert>>>transition list**



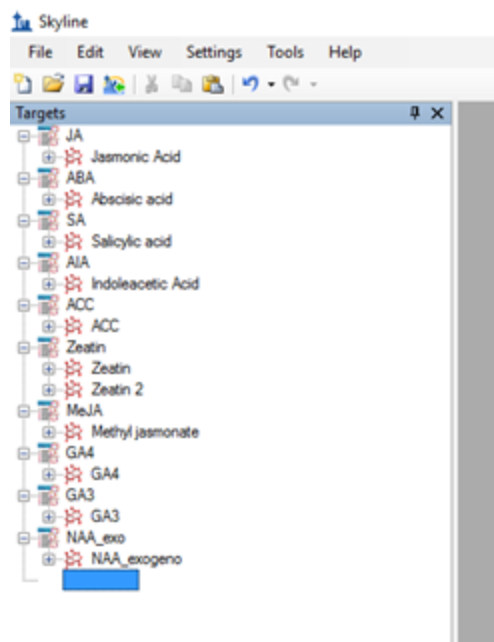
Click in "**columns**" to **edit**. Select in accordance with the transition list create before. Use the mouse to dragging and to change the column order.



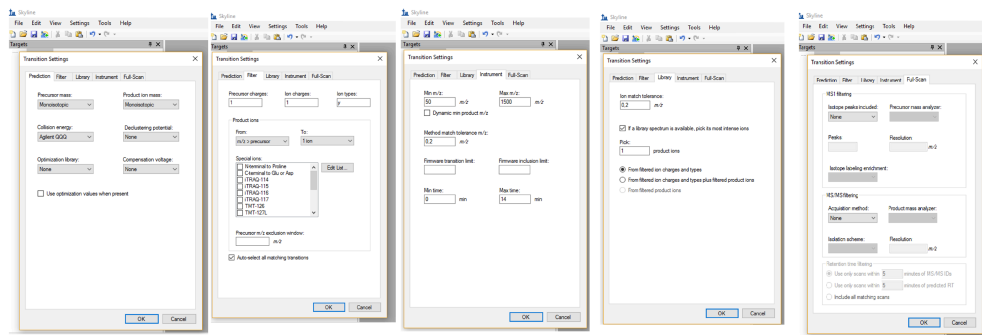
Paste the transition list information. Click in **check for errors**



Click in **"insert"**.

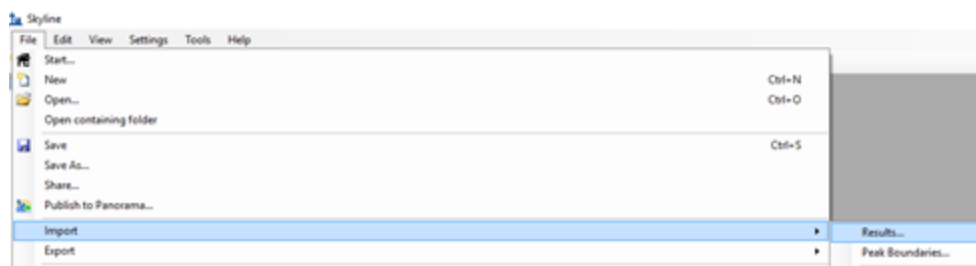


Open **Setting >>> transitions setting** and **configure** to processing the raw data from the QqQ mass spectrometry



Open **Setting>>> Save Current** .

Open File>> import >> Results

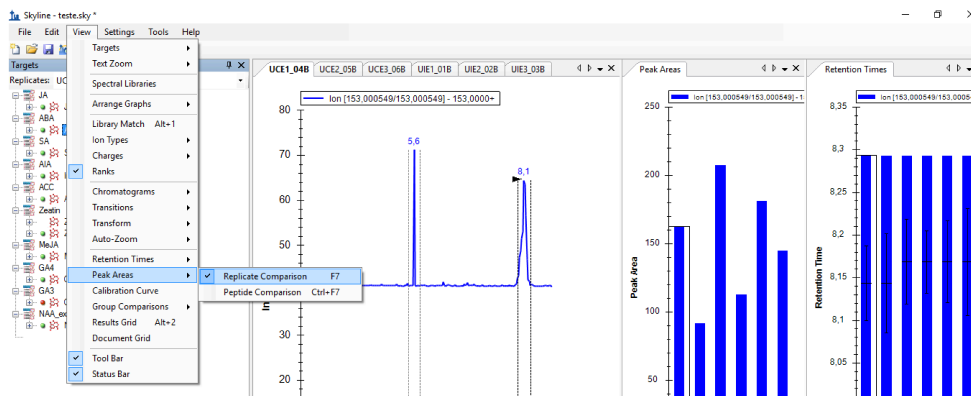


Click in **Ok** to import and **save the Project as myprojectname. Sky**

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

After the data **uploading**, open **View >>>> retention times >>>** and select **Replicate Comparison**

Open **View>>>> Peak Areas>>>** and select **replicate comparison**.



Proceed a **double-click** in the tabs "**Peak Areas**" and "**Retention Times**".

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!!!

Save the project.

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.**