MS2. Detailed protocol for the metabolomics analyses

By Georgia Ntatsi and Maira Lykogianni

Agricultural University of Athens

Required reagents for the analyses

For the derivatization of samples for GC/EI/MS analysis, the reagents ribitol, methoxylamine hydrochloride, N-methyl-N-(trimethyl-silyl)trifluoroacetamide (MSTFA) and pyridine are needed. Additionally, for the extraction of leaf tissues, the organic solvents methanol and ethyl acetate are required.

Plant material and sample preparation

Entire young, fully expanded, and healthy plant leaves from representative plants are collected and placed directly in liquid nitrogen for quenching, and then stored at -80 oC until further processing.

Metabolite extraction for gas chromatography-electron impact-mass spectrometry analysis (GC/EI/MS).

Plant leaves are pulverized using pestle and mortar in liquid nitrogen, and the pulverized tissues are placed in eppendorf tubes (2 mL) and stored in -80oC. For the metabolomics analysis, 50 mg are transferred in Eppendorf tubes (2 mL), and 1 mL of a solution of methanol:ethyl acetate (50:50 v/v) and 20 µL of ribitol (0,2 mg ml-1 methanol), serving as internal standard, are added.

The resulting suspensions are sonicated for 20 min in an ultrasonic bath and then agitated using an horizontal rotary shaker at 150 rpm for 2 h. The suspensions are then filtered using PSTFA filters (0,2 µm pore diameter) and are place again at new 2 mL eppendorf tubes (Fig. 1).

Figure 1. Sample suspensions placed in 2 mL eppendorf tubes.

Derivatization of samples for GC/EI/MS analysis is performed in a two-step process using methoxylamine hydrochloride in pyridine (20 mg mL-1) for methoxymation and MSTFA for silylation. The derivatized samples were finally transferred to microinserters (Fig. 2).

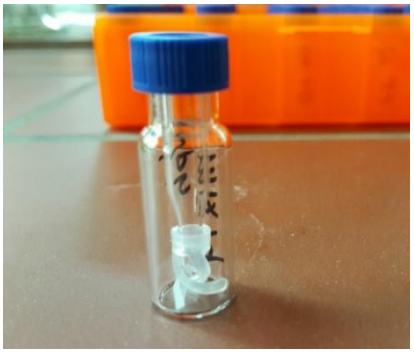

Figure 2. Samples placed at Microinserters after the derivatization method.

Gas chromatography–mass spectrometry analyses

The derivatized samples are analyzed by gas chromatography-electron impact-mass spectroscopy (GC/EI/MS) equipped with an inert mass selective detector 5793 (MSD) (Fig. 3). The electron ionization is set to 70 eV and full scan mass spectra is acquired at 50–800 Da in a rate of 4 scans per second with a 10-min solvent delay. The temperature for the ion source was set to 150 °C and for the transfer line to 230 °C. An HP-5MS capillary column (30 m, i.d. 0.25 mm, and film thickness 0.25 μm) is used, and helium (He 6.0) is the carrier gas. Samples are injected on column and the injector split ratio is set to 5:1. The initial temperature of the oven is 70 ^{o}C, stable for 5 min, followed by an increase of 5 ^{o}C per min to 295 ^{o}C.

Figure 3. GC/EI/MS Agilent equipped with an inert mass selective detector 5973 (MSD) and a 7683 autosampler.

Deconvolution and Data processing

For the metabolomics analyses, all experimental events are controlled using softwares like MSD Chemstation. The deconvolution of the acquired total ion chromatograms can also been perfomred by softwares like AMDIS (NIST-National Institute of Standards and Technology; Gaithersburg, MD, USA) and the MS database of the National Institute of Standards and Technology.

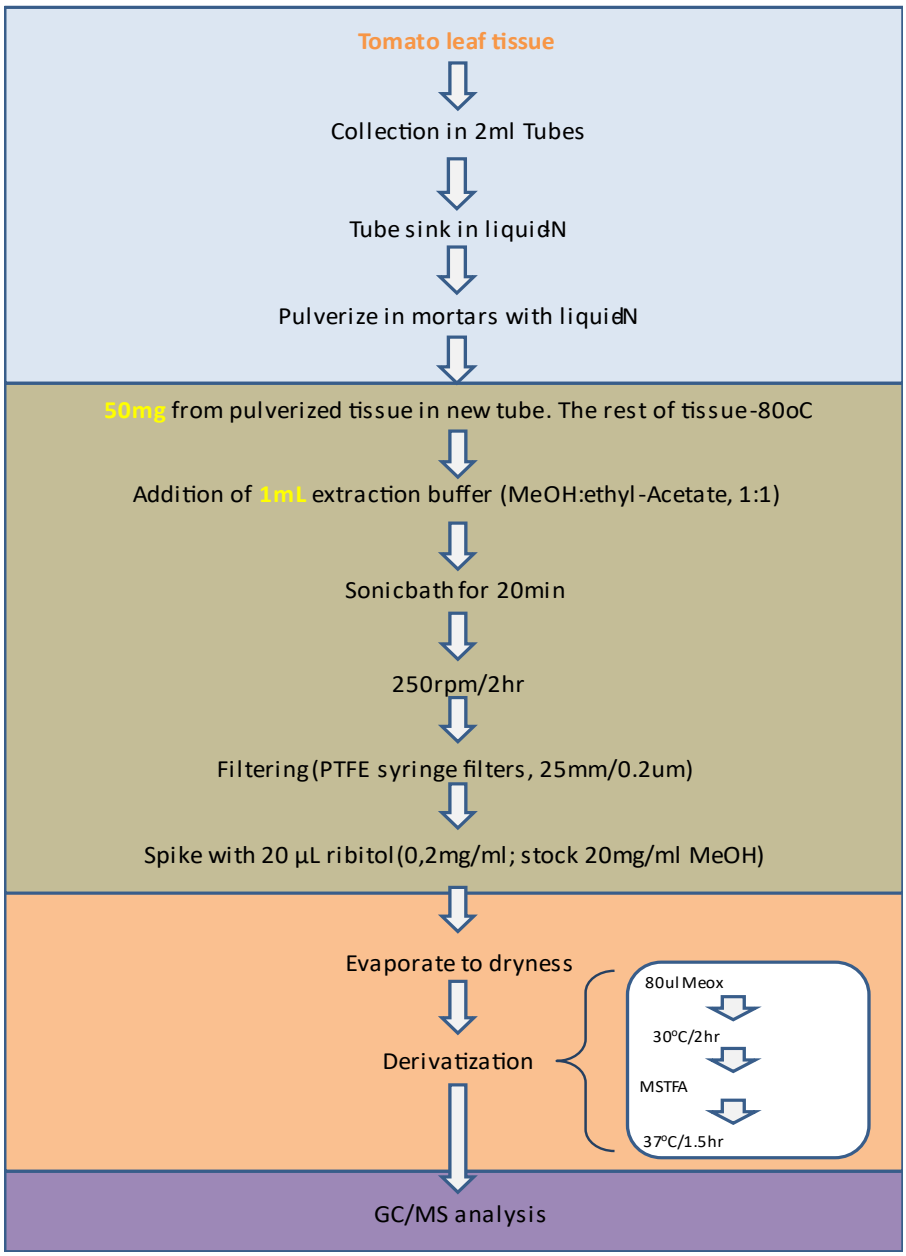

Figure 4. All steps of Metabolomics analysis procedure for analysis at tomato leaves

Notes:

A similar protocol is described by Kostopoulou et al. (2020), for metabolomics analysis at Lemna minor L., however, minor modifications might have been applied.

References

Kostopoulou, S., Ntatsi, G., Arapis, G., Aliferis, K.A. (2020) Assessment of the effects of metribuzin, glyphosate, and their mixtures on the metabolism of the model plant *Lemna minor* L. applying metabolomics. Chemosphere, 239, 124582.