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13C labeling analysis of sugars by high resolution-mass spectrometry for metabolic flux analysis

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ABSTRACT

Metabolic flux analysis is particularly complex in plant cells because of highly compartmented metabolism. Analysis of free sugars is interesting because it provides data to define fluxes around hexose, pentose, and triose phosphate pools in different compartment. In this work, we present a method to analyze the isotopomer distribution of free sugars labeled with carbon 13 using a liquid chromatography –high resolution mass spectrometry, without derivatized procedure, adapted for Metabolic flux analysis. Our results showed a good sensitivity, reproducibility and better accuracy to determine isotopic enrichments of free sugars compared to our previous methods [5, 6].

To better understand the metabolism of living cells, many omics tools have been developed and used in recent years. Fluxomics (metabolic flux analysis) is particularly interesting tool which facilitate a better understanding of physiological processes including enzyme prediction, identification of interactions between metabolites, mapping of the cellular functions, and analysis of the physiological changes resulting from genetic modification [1–3]. To obtain the metabolic flux, i) the metabolic steady state are required and ii) isotopic enrichments of metabolites are required either in isotopically stationary condition (MFA standard) or in isotopically nonstationary condition (INST-MFA) [1–4]. In plant cells, obtaining of the isotopic enrichments of free sugars is particularly interesting especially for glucose, fructose, sucrose and maltose because these occur in different compartments. These data are very useful to better determine reversible fluxes of the glycolysis, and transporters fluxes between compartments [5]. The isotopic enrichment can be quantitatively determined by mass spectrometry given mass isotopomer and/or nuclear magnetic resonance (NMR) given positional isotopomer [5–8]. Currently, the free sugar mass isotopomers are quantified in low resolution especially by GC-MS, based on the standard trimethylsilyl derivatives of saccharides [3–7]. However, these methods can be complex, time-consuming, and can lead to errors in determining the isotopic enrichments. In this case, it is necessary: i) to derivatize the sample, ii) to find the different fragments covering a portion or all of the carbons in the molecule which could be difficult, and iii) to possible overlap between the mass peaks of isotopomer fragments, especially by using low-resolution mass spectrometry. In previous methods developed [5,6], we have shown that, in most cases, chemical ionization is more suitable than the usual electron ionization for isotopomer quantification. Less fragmentation and larger fragments with low sensibility of mass-spectrometry are obtained [5,6]. Here, we propose a method for separation and quantification of mass isotopic enrichments of sugars by using liquid chromatography-high resolution mass spectrometry (LC-HRMS) for metabolic flux analysis. In this work we show: i) a better accuracy in mass isotopomer analysis by comparing the experimental mass isotopomer composition for the unlabeled molecules with the theoretically expected values, and our previous methods with a saving of time in the sample preparation [5,6], ii) the application of this method for the determination of the incubation time with the marked substrate (^{13}C) needed to reach the free sugar isotopic steady state in flaxseed embryos, necessary to obtain the metabolic flux.

Flax plant (*Linum usitatissimum* L.) were grown in a greenhouse in the presence of 60% moisture content, a photoperiod of 16 h day (400 μmol photon m^{-2} s^{-1}) and 8 h of darkness, at temperature

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20 °C day and 16 °C night. The plants were pollinated and capsules containing the seeds were harvested at 16 Days After Flowering (DAF). The sterilization of the capsules and the embryo culture medium was carried out using the protocol described by Refs. [7,9] with some modifications. The capsules were sterilized for 20 min in 50% (v/v) bleach and then washed for 20 min in autoclaved water. Seeds from sterile capsules were then recovered and dissected. 10 embryos were placed in a glass Petri box containing 16 ml of culture medium: 80 mM unlabeled glucose, 20 mM [U-^{13}C]glucose, 640 mg/L unlabeled glutamine, 160 mg/L [U-^{13}C] glutamine, 22% PEG (polyethylene glycol) 4000, 125 mg/L KNO_3, 370 mg/L $MgSO_4 \cdot 7H_2O$, 350 mg/L KCl, 125 mg/L, KH_2PO_4, 10 mg/L H_3BO_3, 10 mg/L $ZnSO_4 \cdot 7H_2O$, 0.25 mg/L Na_2MoO_4, 0.025 mg/L $CuSO_4$, 0.025 mg/L $CoCl_2$, 5 mg/L nicotinic acid, pH 5.8 (1 M KOH). The Petri box was then placed in a growth chamber at 18 °C under low-light intensity (20 μE m^{-2} s^{-1}), and with a humidity of 60%. After 24 h, 72 h, 120 h, 168 h of incubation, the embryos were harvested, washed 3 times with 10 mL water, frozen with liquid nitrogen and lyophilized. 10 mg of flax embryos were used and the water-soluble metabolites (free sugars, amino acids, organic acids) were extracted on oil free embryos as described by Koubaa et al. in 2013 [9]. The soluble fraction was frozen in liquid nitrogen and lyophilized. Purification of the free sugars was made using the methodology described by Koubaa et al. in 2013 and by Redgwell in 1980 [9,10]. Soluble compounds were resuspended in 1 mL of sodium acetate buffer (50 mM, pH 5.5), then loaded onto a Dowex 50 × 8 column to remove amino acids. Free sugars were purified using an anion exchange resin (Dowex1x8 [formate form, 200–400, Supelco 13858]) and washed with 5 mL of water. Free sugars were frozen with liquid nitrogen and lyophilized. The lyophilizate was resuspended in 500 μL of water, and 5 μL were injected into LC-HRMS. Free sugars were analyzed using ultra high-pressure liquid chromatography (UPLC 1290 Infinity) coupled with high resolution–mass spectrometry (HR-MS Q-TOF UHD 6538) from Agilent Technologies. In brief, 5 μL (sample or standard) was injected and the compounds were separated on a Thermo Hypersil Gold Hilic column (2.1 × 150 mm, 3 μm). The column temperature was set to 20 °C and the flow rate was set to 0.5 mL/min. The separation gradient was generated using 5 mM ammonium acetate in water (solvent A), and acetonitrile (solvent B). A gradient elution was performed initially with 3% A for 4 min, followed by a linear increase to 80% A until 30 min. Cleaning of the column was achieved with 90% B for 5min. The mass spectra were acquired using a dual electrospray ionization in negative-ion mode. The source temperature was set up at 200 °C. The nebulization gas, the ion spray voltage and the fragmentor were adjusted to 30psi, 3.5 kV and 140 V respectively. The range of mass detected on time of flight was 50 m/z and 1050 m/z, with a scan of two spectra per second. All data were acquired and processed using MassHunter B.07 software.

The developed method allows the separation of the major free sugars (glucose, fructose, sucrose, maltose, raffinose) present in oilseed embryos for determining the status of isotopic enrichment in the stationary metabolic state. In our culture conditions, the stationary metabolic state is reached from 120 h of incubation (Data not shown). Two HILIC columns, Phenomenex Kinetex column (3.0 × 150 mm, 2.6 μm) and Thermo Hypersil Gold HILIC column (2.1 × 150 mm, 3 μm) were tested for the separation. Despite the optimization of the chromatographic parameters, sugars having the same mass (glucose/fructose), (sucrose/maltose) were separated only on a Thermo Hypersil Gold HILIC column under the conditions previously described (Fig. 1). The accuracy of mass isotopomer analysed using the method developed on a Thermo Hypersil Gold HILIC column was tested using standard (glucose, fructose, sucrose, maltose, raffinose) by comparing the experimental mass isotopomer composition for the unlabeled molecule with: i) the theoretically expected values, and ii) the experimental mass isotopomer composition obtained in our previous method [5,6]. Results show better sensitivity and accuracy for the isotopic mass

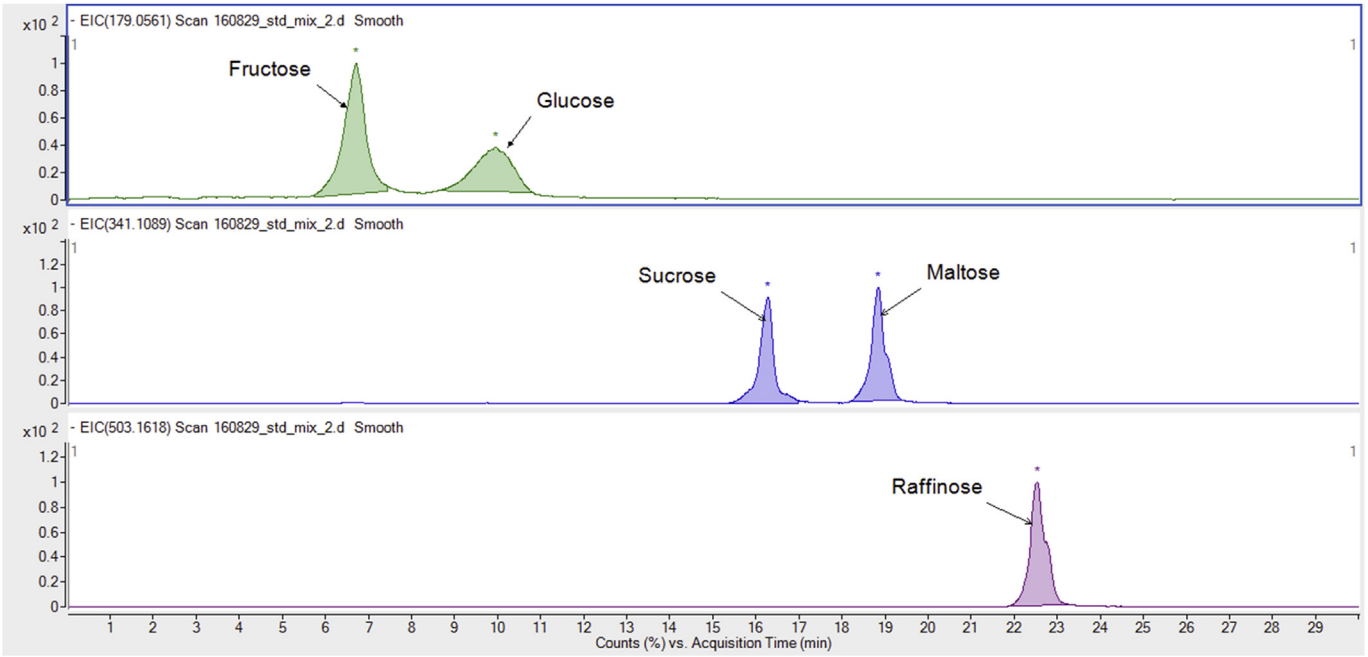

Fig. 1. HPLC-HRMS chromatogram of free sugars standards separated on a Thermo Hypersil Gold HILIC column (2.1 × 150 mm, 3 μm). Ten microliters was injected at 100 μg mL^{-1} in water solution.
A: HPLC-HRMS chromatogram of glucose and fructose.
B: HPLC-HRMS chromatogram of sucrose and maltose.
C: HPLC-HRMS chromatogram of raffinose.