Liquid chromatography tandem mass spectrometry for measuring ^{13}C-labeling in intermediates of the glycolysis and pentose-phosphate pathway

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Running Head: ^{13}C-analysis of phosphorylated metabolites by LC-MS/MS

i. Abstract

This chapter describes a procedure to analyze ^{13}C-labeled phosphorylated compounds by liquid chromatography tandem mass spectrometry. Phosphorylated compounds, intermediaries of the glycolysis and pentose phosphate pathway, are separated by anion exchange chromatography and their isotopic labeling is determined by mass spectrometry. A sensitivity in the fmole range is achieved using scheduled multiple reaction monitoring mode.

ii. Key Words:

Anion exchange chromatography, LC-MS/MS, phosphorylated compounds, multiple reaction monitoring, ^{13}C-labeling, metabolic flux analysis.

ABBREVIATIONS:

hexose-phosphates, hexose-Ps; sedoheptulose 7-phosphate, S7P; glycerol 3-phosphate , glycerol 3-P; phosphoenolpyruvate, PEP; pentose 5-phosphates, P5Ps; ribulose 1,5-bisphosphate, rib 1,5-bisP; 2/3-phosphoglycerate, 2/3-PGA; fructose 1,6-bisphosphate, fruc 1,6-bisP; 6-phosphogluconic acid, 6PG; disaccharide-phosphate, disac-P; galactose 1-phosphate, gal 1-P; glucose 1-phosphate, glc 1-P; mannose 1-phosphate, man 1-P; glucose 6-phosphate, glc 6-P; fructose 6-phosphate, fruc 6-P; mannose 6-phosphate, man 6-phosphate; sucrose 6-phosphate, S6P; trehalose 6-phosphate, T6P; liquid chromatography, LC; mass spectrometry, MS; dry weight, DW; multiple reaction monitoring, MRM; potassium hydroxide, KOH.

1. Introduction

Intracellular metabolic rates (fluxes) provide critical insights into the metabolic state of an organ or cell. ^{13}C-Metabolic Flux Analysis (^{13}C-MFA) is a powerful tool to analyze the flow of carbon through metabolic networks. For this purpose, a plant organ is incubated with a combination of ^{13}C-labeled substrates (that mimics its growth *in planta*) until the isotopic steady state is reached. Labeled substrates are metabolized by cells, resulting in the incorporation of ^{13}C-atoms into intracellular metabolites and biomass products. ^{13}C-labeling is usually measured in hydrolyzed macromolecules (proteins, starch, and triacylglycerols) as they are more abundant than intermediary metabolites. These ^{13}C-measurements, directly reflecting the labeling of macromolecules precursors (amino acids, plastidic glucose 6-phosphate, acetyl CoA) are used within the context of a retrobiosynthetic approach to map carbon fluxes through central metabolism. Indeed, metabolic fluxes are calculated from these labeling measurements using a model-based approach that maximizes the best fit between the measured and model-predicted labeling distributions(*1-5*). This approach has been successfully applied to characterize the *in vivo* metabolic fluxes in plants for: i) understanding carbon partitioning in maize root tips(*6-8*), tomato cells(*9*) , tobacco cells(*10*) and Arabidopsis cells(*11*); ii) unraveling new pathways/reactions in plants, such as the "RuBisCo bypass" in Brassica napus embryos(*12*) and a new "futile" cycle(*13*); iii) understanding the storage metabolism in seeds, such as maize kernels(*14, 15*), and soybean(*16, 17*), Brassica napus(*18, 19*), sunflower(*20*), and Arabidopsis embryos(*21*); iv) determining the impact of a mutation on carbon partitioning in maize root tips(*6*) and Arabidopsis embryos(*21*). However, ^{13}C-MFA and retrobiosynthetic approach are limited to plant organs/cells that can reach metabolic and isotopic steady and pathways resulting in

carbon rearrangement. For systems that do not meet those requirements, such as leaves or plant cell wall biosynthetic pathway, the direct labeling in intermediary metabolites is required.

Several metabolic pathways, such as glycolysis and oxidative pentose-phosphate pathway (OPPP), exclusively involve phosphorylated compounds. Determining the levels and labeling distribution in these metabolites is therefore important to access carbon fluxes in central metabolism. Several studies have reported the separation of phosphorylated metabolites using liquid chromatography coupled with tandem mass spectrometry(*22*-*24*). Anion exchange chromatography was shown to be effective at separating various intermediates involved in central metabolism(*25*-*29*), such as but not limited to glycolysis, Calvin cycle, plant cell wall biosynthesis, and tricarboxylic acid cycle, especially when coupled with the high specificity and sensitivity of a triple quadrupole mass spectrometer. Moreover, this technique can be used to determine the mass isotopomer distribution of labeled compounds(*30*), specifically in phosphorylated metabolites.

This protocol describes the use of LC-MS/MS for measuring ^{13}C-labeling in intermediaries of the glycolysis and pentose-phosphate pathway. Metabolites are extracted from ^{13}C-labeled plant organs using boiling water. Phosphorylated compounds are separated by an anion exchange column, and then detected by triple quadrupole mass spectrometer using multiple reaction monitoring (MRM) mode: i) the first quadrupole selects for particular m/z ratios of the ionized precursor compounds; ii) the second one fragments the precursor into product ions; and iii) the third quadrupole selects for a specific product ion which is here the phosphate group. For a phosphorylated metabolite containing *n* carbon atoms, *n+1* transitions are followed. Mass isotopomer distribution are determined for each

phosphorylated compound extracted from ^{13}C-labeled plant organ; those labeling data can then be used for MFA.

2. Materials

2.1. Liquid chromatography supplies

Chromatography supplies are purchased from Dionex:

1. IonPac® ATC-3 Anion Trap Column (4 x 35 mm).
2. Guard column AG11 (50 x 2 mm).
3. IonPac AS11 column (250 x 2 mm).
4. ASRS 300 suppressor.
5. SRS controller.

2.2 Liquid chromatography solutions

1. Degas fresh ultra pure water (18.2 megaOhm) in a 2 L flask containing a stir bar for 30 minutes using a pump system.
2. Make a 2 M KOH solution in degassed ultrapure water. Pre-weigh a 100 mL graduated cylinder and add KOH from a 45 % KOH liquid solution (cat # SP236500, ThermoFisher) to have 2 M final. Complete to 100 mL and flush with helium. This KOH solution is used to condition the IonPac® ATC-3 Anion Trap Column (*see* **Note 1**).
3. Prepare 0.5 mM (solvent A) and 75 mM (solvent B) potassium hydroxide solutions from a 45 % KOH liquid solution (cat # SP236500, ThermoFisher). First, weigh KOH in a weighing boat to have a final concentration of 75 mM in a desired volume. Transfer KOH into a graduate cylinder and add degassed ultra pure water to have a final concentration of 75 mM

KOH (solvent B). Cover top of graduate cylinder with parafilm and invert 2-3 times. Add your solution into a 2 L plastic bottle (cat # 45903, Dionex). Do not use borosilicate bottle to avoid leaching of borate that will interfere with the separation of your phosphorylated compounds. Then, take a desired volume from the 75 mM KOH solution to make the 0.5 mM (solvent A) KOH solution. Finally, flush both solutions of KOH with helium as well as the 45% KOH stock solution.

4. Solvent for needle wash: 50% (v/v) LC-MS grade methanol in ultrapure water.

2.3 Sample preparation

1. Phoshorylated compounds were purchased from Sigma and stored accordingly to the manufacturer instructions.

2. Plant tissue labeled with ^{13}C substrates were weighed (~ 10 mg dry weight) using an analytical scale.

3. Method

3.1. Extraction of phosphorylated compounds

1. Place a 50 mL conical tube of ultrapure water in a water bath set at 100ºC.

2. Add one tungsten bead (5 mm) per 2 mL screw cap tube containing your plant tissue.

3. Shake in a mill mixer for 3 minutes at a constant frequency of 30 Hz. Remove tubes and insure that you have a powder.

4. Add 1 mL of boiling ultrapure water to the 2 mL screw cap tubes and incubate at 100°C for 5 minutes in a water bath **(***see* **Note 2).**

5. Take out, vortex the samples and put them back in the boiling water bath for 5 more

minutes.

6. Place quickly the tubes on ice. Wait 3-5 minutes until samples cool down.

7. Centrifuge the tubes at 20,000 x g at 4°C for 5 minutes.

8. Collect and filter each supernatant through a 5 mL syringe with a 0.22um filter into a 15 mL tube kept on ice. Syringes and filters are conserved.

9. Wash tubes containing disrupted plant tissue with 1 mL of 4°C ultrapure water, vortex to resuspend material and centrifuge at 20,000 x g at 4°C for 5 minutes.

10. Collect and filter each supernatant as explained above, using previous syringes (so syringes and filters get rinsed too).

11. Rinse syringes and filters with 1 mL of cold ultrapure water.

12. Spin down 15 mL tubes, poke holes on each cap.

13. Freeze-dry extracts using lyophilizer.

14. Dried samples are resuspended in 500 µL of cold ultrapure water and applied on a 0.5 mL Amicon ultra filter (Millipore) with a 3 kDa cut off.

15. Tubes are centrifuged at 20,000 x g at 4°C for 30 minutes.

16. The flow through, containing 13C labeled phosphorylated metabolites, is ready for LC-MS/MS analyses. Samples are diluted (5:1) in water.

3.2 Optimization of MS parameters for each compound

Tandem mass spectrometry analysis is performed with a hybrid Triple Quadrupole/Ion trap mass spectrometer QTRAP 5500 (AB Sciex). The mass spectra are acquired with Analyst 1.6 software using Turbo Spray ionization in negative ion mode.

Automatic optimization of MS parameters (*see* **Table 1**) associated to the different compounds will give the best sensitivity for MS/MS analysis.

1. Prepare each metabolite individually in water at a final concentration of 10 µM.

2. Load metabolite in a 1 ml Hamilton syringe and inject by direct infusion at a flow rate of 7 µL/min.

3. Insure that compound is detected (*see* **Note 3**).

4. Proceed to automatic compound optimization (*see* **Note 4**) and save file. This file contains optimized declustering potential (DP), entrance potential (EP), collision energy (CE) and collision exit potential (CXP) for each metabolite. Note that automatic compound optimization can generate 5 product ions by metabolite that will be optimized for the parameters cited above.

3.3 Flow Injection Analysis (FIA)

To optimize Gas/Source parameters to have the best sensitivity for MS/MS analysis.

1. Prepare a vial with a mixture of phosphorylated compounds (avoid isomers) in water at a final concentration of 10 µM.

2. Select compound optimization, then FIA experiment for MS/MS analysis. Two µL injection for FIA experiments is suitable to test different value for curtain gas, collision gas (CAD), ionspray voltage (IS), heater temperature (TEM), nebulizer gas (GS1) and heater gas (GS2) (*see* **Note 5**). Water is used as mobile phase at a 350 µL/min flow rate.

3. Optimized curtain gas, CAD, IS, TEM, GS1 and GS2 are saved for the mixture of phosphorylated compounds (*see* **Table 2**).

3.4 Chromatographic conditions

1. LC is performed with an Agilent Ultra High Pressure Liquid Chromatography (UHPLC) 1290.

2. Column: Anion exchange IonPac AS11 column (250 x 2 mm). Column is kept at 23ºC. Guard column AG11 (50 x 2 mm). IonPac® ATC-3 Anion Trap Column (4 x 35 mm) is used to remove contaminant ions from KOH solvents.

3. Gradient for LC-MS/MS analysis is shown in **Table 3**.

4. Sample injection: 5 µL of standard or biological sample are injected with a 30 s needle wash.

5. Salt suppression (*see* **Note 6**): KOH is removed using a post-column anion self-regenerating suppressor ASRS 300 (Dionex). It is operated in the Autosuppression External Water Mode for high sensitivity analysis. The current and a reagent flow rate are set respectively at 50 mA and 2 $mL.min^{-1}$.

3.5 Determination of retention time (see Ana's paper)

1. Specific metabolite-dependent MS parameters (*see* **Table 1**) are used to determine retention time of phosphorylated standards on a full Multiple Reaction Monitoring (MRM) run.

2. Retention time of phosphorylated compounds is shown in **Figure 1**. Each metabolite is associated to a specific color representing a parent/daughter ion pair.

3.6 Scheduled MRM for ^{13}C-labeling determination

1. Retention time of phosphorylated standards, previously determined, is used to define the different windows for scheduled MRM experiment (*see* **Table 4**). A 180 s window is applied for each ^{13}C-labeled phosphorylated compounds.

2. Scheduled MRM for ^{13}C-S7P are shown in **Figure 2**.

4 Notes

1. The IonPac® ATC-3 Anion Trap Column needs to be conditioned following Document No. 031835-01 (Dionex) to remove trace anions from KOH gradient. The use of the ATC-3 column permits to decrease the background and/or eliminate contaminant peaks. Therefore, the signal to noise of each metabolite is increased as well as the sensitivity.

2. Add 1 mL of boiling ultrapure water to one tube at a time and quickly transfer the tube in the water bath sets at 100ºC. Proceed to the next tube and continue until you are done with your samples. 6-8 samples per extraction are recommended.

3. A Q1 scan survey is performed to see whether or not the metabolite injected through direct infusion is detected. The mass range, EP, DP are set respectively from 100 to 500 amu, -10 V and -50 V. In the case that the compound is not detected, the DP is optimized from -250 to 0 V. The DP value giving the best signal is entered in the new Q1 scan survey which allows the detection of the metabolite.

4. Select compound optimization as well as MS/MS experiment. Enter parameters in the following order: negative polarity, unit resolution, product ion of at least 14 amu, compound name, monoisotopic mass, charge of 1. Start compound optimization and save file corresponding to each individual metabolite.

5. For FIA experiments a set of values is entered for each parameter (*see* **Table 5**)

6. Before connecting the liquid chromatography system to the mass spectrometer, a conductometer is used to check whether or not salt are removed according to Dionex instructions. Indeed, the conductivity should not exceed 4 to 7 µS.

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Figure Captions

Fig. 1. Analyses of a mixture of phosphorylated metabolites by LC-MS/MS using MRM. Phosphorylated metabolite standards, approximately 10 µM each, are monitored by LC-MS/MS at the transition $[M-H]^-/79$ using Table I MS parameters and Table IV gradient. Total ion count is reported for each phosphorylated metabolite: PEP, phosphoenolpyruvate; Glycerol-Ps, glycerol-phosphates; 2/3-PGA, 2/3-phosphoglyceric acids; E4P, erythrose 4-phosphate; P5Ps, pentose 5-phosphates; Rib 1,5-bisP, ribulose 1,5-bisphosphate; Gal 1-P, galactose 1-phosphate; Glc 1-P/Man -1P, glucose 1-phosphate/mannose 1-phosphate; Glc 6-P, glucose 1-phosphate; Fruc 6-P, fructose 6-phosphate; Man 6-P, mannose 6-phosphate; 6PG, 6-phosphogluconic acid; S7P, sedoheptulose 7-phosphate; T6P, trehalose 6-phosphate; S6P, sucrose 6-phosphate.

Fig. 2. Scheduled MRM application for unlabeled and ^{13}C-labeled S7P.

Corn embryos were grown with unlabeled or ^{13}C-labeled substrate sources following the procedure described previously(*25*). Phosphorylated metabolites were extracted as previously described and analyzed by LC-MS/MS. The selected daughter ion was $[PO_3]^-$ (m/z=79) for S7P. For the S7P containing 7 atoms of carbon we follow the transitions 291/79 through 298/79 (*see* **Table 3**). Mass isotopomer distribution of S7P after 7 days of corn embryo culturing with respectively unlabeled (**A**) or 13C labeled (**B**) substrates.

Table Captions

Table 1. Specific metabolite-dependent MS parameters used for LC-MS/MS.

* Declustering potential; ¶ Entrance potential; # Collision energy; § Collision exit potential

Table 2. Optimal gas/source parameters for scheduled MRM.

Table 3. Transitions and retention time used in scheduled MRM.

*: The transition of pentose phosphates (P5Ps) is used to follow the ribulose 1,5-bisphosphate which looses a phosphate in the source.

#: 2 peaks for the glycerol-phosphate standard meaning that 2 glycerol-phosphate isomers are present. The 2 peaks are usually integrated into one.

Table 4. Liquid chromatography gradient used for phosphorylated compounds.

Table 5. Parameter for FIA experiments.

Table 1

Analyte	Parent ion formula	Product ion formula	Parent/product transition	DP* (V)	EP¶ (V)	CE# (V)	CXP§ (V)
Disac-P	$C_{12}H_{22}O_{14}P^-$	$[PO_3]^-$	421/79	-95	-10	-130	-15
E4P	$C_4H_8O_{14}P^-$	$[PO_3]^-$	199/79	-70	-10	-84	-11
Fruc 1,6-bisP	$C_6H_{13}O_{12}P_2^-$	$[PO_3]^-$	339/79	-50	-10	-88	-13
Glycerol -Ps	$C_3H_8O_6P^-$	$[PO_3]^-$	171/79	-60	-10	-42	-9
Hexose-Ps	$C_6H_{12}O_9P^-$	$[PO_3]^-$	259/79	-40	-10	-90	-11
P5Ps/Rib 1,5-bisP	$C_5H_8O_8P^-$	$[PO_3]^-$	229/79	-45	-10	-74	-37
PEP	$C_3H_4O_6P^-$	$[PO_3]^-$	167/79	-40	-10	-52	-9
6PG	$C_6H_{12}O_{10}P^-$	$[PO_3]^-$	275/79	-45	-10	-108	-15
2/3-PGA	$C_3H_6O_7P^-$	$[PO_3]^-$	185/79	-35	-10	-63	-12
S7P	$C_7H_{14}O_{10}P^-$	$[PO_3]^-$	291/79	-80	-10	-100	-9

Table 2

Source/Gas parameters	Value
Curtain Gas (CUR)	35
Collision Gas (CAD)	Medium
IonSpray Voltage (IS)	-4500
Temperature (TEM)	550
Ion Source Gas 1 (GS1)	60
Ion Source Gas 2 (GS2)	40

Table 3

Time (min)	Flow rate (µL/min)	A (%)	B (%)
0.0	350	100.0	0.0
2.0	350	100.0	0.0
13.0	350	95.2	4.8
20.0	350	95.2	4.8
30.0	350	87.3	12.7
45.0	350	73.8	26.2
65.0	350	33.6	66.4
70.0	350	0.0	100.0
75.0	350	0.0	100.0
75.1	350	100.0	0.0
80.0	350	100.0	0.0

Table 4

Analyte	Retention time (min)	Mass isotopomers	Parent/product transition
PEP	46.16	m_0 to m_{+3}	167/79 to 170/79
Glycerol-Ps	16.23/16.66[#]	m_0 to m_{+3}	171/79 to 174/79
2/3PGA	41.75	m_0 to m_{+3}	185/79 to 188/79
E4P	59.30	m_0 to m_{+4}	199/79 to 203/79
P5Ps	30.63	m_0 to m_{+5}	229/79 to 234/79
Rib 1,5-bisP*	39.82	m_0 to m_{+5}	229/79 to 234/79
Gal 1-P	15.80	m_0 to m_{+6}	259/79 to 265/79
Glc 1-P/Man 1-P	16.45	m_0 to m_{+6}	259/79 to 265/79
Glc 6-P	27.56	m_0 to m_{+6}	259/79 to 265/79
Fruc 6-P	29.03	m_0 to m_{+6}	259/79 to 265/79
Man 6-P	30.17	m_0 to m_{+6}	259/79 to 265/79
6PG	39.94	m_0 to m_{+6}	275/79 to 281/79
S7P	32.49	m_0 to m_{+7}	291/79 to 298/79
Fruc 1,6-bisP	56.91	m_0 to m_{+6}	339/79 to 345/79
S6P	19.71	m_0 to m_{+12}	421/79 to 433/79
T6P	16.16	m_0 to m_{+12}	421/79 to 433/79

Table 5

Source/Gas parameters	Value
Curtain Gas (CUR)	30; 35; 40
Collision Gas (CAD)	Low; Medium; High
IonSpray Voltage (IS)	-4500; -4000; -3500; -3000; -2500; -2000
Temperature (TEM)	300; 350; 400; 450; 500; 550; 600; 650
Ion Source Gas 1 (GS1)	35; 40; 45; 50; 55; 60; 65; 70
Ion Source Gas 2 (GS2)	35; 40; 45; 50; 55; 60; 65; 70