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We use this protocol and it's working

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Liquid chromatography-mass spectrometry method for isomer separation and detection of sugars, phophorylated sugars and organic acids

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ABSTRACT

This standard operating procedure is used to achieve effective separation of a wide range of polar metabolites found in central carbon metabolism via a hybrid liquid chromatographic method (ion-exchange chromatography and hydrophilic interaction liquid chromatography (HILIC)) using an Intrada Organic Acid column (Imtakt) coupled with triple quadrupole mass spectrometry. This method gives improved resolution while showing enhanced sensitivity for the detection of low abundance phosphorylated sugars compared with standard HILIC methods.

MATERIALS

Reagents

- double distilled water (ddH2O) **Contributed by users**
- Formic acid (88%) **Sigma Aldrich Catalog #399388**
- Methanol (LCMS-grade) **Honeywell Catalog #14262**
- Acetonitrile (UV/HPLC-grade) **Honeywell Catalog #34888**
- Ammonium formate **Sigma Aldrich Catalog #70221-25G-F**
- Metabolite standards (Sigma-Aldrich)

BEFORE START INSTRUCTIONS

This protocol is part of the submitted paper "An efficient LC-MS method for isomer separation and detection of sugars, phosphorylated sugars, and organic acids".

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Keywords: Central metabolism, isomer separation, isotopic labeling, liquid chromatography-mass spectrometry, metabolite quantification, mixed-mode column chromatography

Abbreviations:

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- CE: collision energy
- CXP: collision cell exit potential
- ddH_2O: double-distilled water
- DP: declustering potential
- EP: entrance potential
- FW: fresh weight
- HILIC: hydrophilic interaction liquid chromatography
- HPLC: high-performance liquid chromatography
- ID: isotopologue distribution
- LC-MS: liquid chromatography-mass spectrometry
- MRM: multiple reaction monitoring
- PES: Polyethersulfone
- PIPES: Piperazine-N,N'-bis(2-ethanesulfonic acid)
- PVDF: Polyvinylidene fluoride

Apparatus

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- Vivaclear Mini →|← 0.8 µm PES clarifying filters (Sartorius #VK01P042)
- Durapore membrane filter, PVDF hydrophilic, →|← 0.22 µm, →|← 47 mm (EMD Millipore #GVWP04700)
- HPLC pump: Shimadzu Prominence-xR UFLC system
- HPLC column: Intrada Organic Acid (→|← 150 mm x →|← 2 mm, →|← 3 µm)
- MS detector: Sciex QTRAP 6500 triple quadrupole-linear ion trap MS with a Turbo VTM electrospray ionization source

Preparing metabolite mixes for an external standard curve

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3.1 A [M] 500 micromolar (µM) stock of alpha-ketoglutarate, 2-phosphoglycolate, 2-phosphoglyceric acid, 3-phosphoglyceric acid, 6-phosphogluconate, acetyl-CoA, adenosine diphosphate-glucose, aspartate, dihydroxyacetone phosphate, erythrose 4-phosphate, fructose, fructose 1,6-bisphosphate, glucose 1-

phosphate, glucose 6-phosphate, glyceraldehyde phosphate, glucose, glutamine, glutamate, glycerate, malate, phosphoenolpyruvate, piperazine-N,N'-bis(2-ethanesulfonic acid), raffinose, ribitol, ribulose-1,5-bisphosphate, ribulose 5-phosphate, sedoheptulose 7-phosphate, succinate, sucrose, and uridine diphosphate-glucose standards was prepared in ddH_2O containing [M] 0.2 % (v/v) formic acid.

3.2 Prepare a [M] 250 micromolar (µM) working stock in ddH_2O containing [M] 50 % (v/v) methanol and [M] 0.2 % (v/v) formic acid.

3.3 Filter the [M] 250 micromolar (µM) stock through a 0.8 µm PES clarifying filter at 2000 x g, 4°C 5m for 00:05:00.

3.4 Serially dilute the filtered [M] 250 micromolar (µM) stock in a filtered ddH_2O containing [M] 50 % (v/v) methanol and [M] 0.2 % (v/v) formic acid to create a standard curve containing 250, 125, 62.5, 31.25, 15.625, 7.813, 3.906, 1.953, 0.977, 0.488, 0.244, and 0.122 µM standards.

3.5 Transfer 50 µL of each standard concentration into separate 300 µL LC vials with inserts.

Preparation of 100 µg/mL PIPES-Ribitol-Norvaline internal standards fo

4 These internal standards are used for calculation of extraction efficiencies (PIPES for organic acids, ribitol for sugars and phosphorylated sugars, and norvaline for amino acids).

4.1 Prepare separate [M] 1 mg/mL stocks for each internal standard in 1 mL chilled ddH_2O.

4.2 For 1 mL of [M] 100 µg/mL standard mix, mix 100 µL of each [M] 1 mg/mL stock with 700 µL chilled ddH_2O.

4.3 Add 15 µL [M] 100 µg/mL PIPES-Ribitol-Norvaline as internal standards during sample extraction. 5m Polar metabolites are extracted using the protocol adapted from Ma et al., 2017, with only a single water

extraction step performed and samples being resuspended in 50 µL ddH_2O containing [M] 50 % (v/v) methanol and [M] 0.2 % (v/v) formic acid and subsequently filtered through 0.8 µm PES clarifying filters at 2000 x g, 4°C for 00:05:00.

CITATION

Ma F, Jazmin LJ, Young JD, Allen DK (2017). Isotopically Nonstationary Metabolic Flux Analysis (INST-MFA) of Photosynthesis and Photorespiration in Plants.. Methods in molecular biology (Clifton, N.J.).

LINK

https://doi.org/10.1007/978-1-4939-7225-8_12

HPLC conditions

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- Solvent A: ddH_2O containing [M] 100 millimolar (mM) ammonium formate and [M] 10 % (v/v) acetonitrile
- Solvent B: ddH_2O containing [M] 1 % (v/v) formic acid and [M] 75 % (v/v) acetonitrile
- Seal wash: ddH_2O containing [M] 20 % (v/v) methanol and [M] 0.5 % (v/v) formic acid
- Autosampler wash 1: ddH_2O containing [M] 25 % (v/v) methanol
- Autosampler wash 2: ddH_2O containing [M] 75 % (v/v) methanol
- Vacuum filter buffers using 0.22 µm PVDF hydrophilic membrane filters into clean bottles.

Table 1. HPLC mobile phase gradient

Time (min)	Flow (mL/min)	%A	%B
0	0.225	0	100
1	0.225	0	100
5	0.225	12	88
7	0.225	12	88
8	0.225	16	84
10	0.225	16	84
13	0.225	25	75
15	0.225	100	0
19.5	0.25	100	0
20	0.25	0	100
25	0.25	0	100

- All samples are diluted in ddH_2O containing [M] 50 % (v/v) methanol with [M] 0.2 % (v/v) formic acid.
- A 3 µL injection volume is used, the sample tray in autosampler is held at 4 °C, and the column temperature is held at 40 °C.

LC-MS conditions (specific for QTRAP 6500 triple quadrupole MS)

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- Source temperature: 450 °C
- Ion spray voltage: -4500 V
- Curtain gas: 30 psi
- Ion source gas 1: 30 psi
- Ion source gas 2: 35 psi

Table 2. LC-MS parameters for each compound

Q1	Q3	Compound ID	DP (v)	CE (v)	CXP (v)	EP (v)
145	101	Alpha-ketoglutarate	-5	-12	-11	-10
155	79	2-phosphoglycolate	-70	-20	-12	-10
275	79	6-phosphogluconate	-65	-22	-13	-10
808	408	Acetyl-CoA	-35	-46	-23	-10
588	346.1	Adenosine diphosphate-glucose	-30	-32	-21	-10
132	88	Aspartate	-20	-16	-43	-10
199	97	Erythrose 4-phosphate	-5	-32	-11	-10
339	97	Fructose 1,6-bisphosphate	-60	-24	-11	-10
145.1	108.9	Glutamine	-30	-18	-5	-10
146.05	102	Glutamate	-16	-15	-8	-10
179	89	Hexoses	-25	-10	-11	-10
259	79	Hexose phosphates	-10	-28	-7	-10
133	115	Malate	-40	-20	-3	-10
229	79	Pentose 5-phosphates	-25	-60	-15	-10
167	79	Phosphoenolpyruvate	-5	-14	-9	-10
185	79	Phosphoglyceric acids	-30	-20	-11	-10
301	193	PIPES	-35	-34	-31	-10
87	43	Pyruvate	-10	-15	-3	-10
151	89	Ribitol	-50	-16	-9	-10
309	79	Ribulose 1,5-bisphosphate	-55	-70	-9	-10
289	79	Sedoheptulose 7-phosphate	-60	-58	-9	-10
104.001	74	Serine	-16	-15	-8	-10
117	73	Succinate	-5	-16	-7	-10
341	179	Sucrose	-110	-18	-13	-10
169	97	Triose phosphates	-10	-12	-11	-10

565	323.1	Uridine diphosphate-glucose	-125	-32	-2	-10

All analytes are measured in negative ionization mode, with ions being detected using a targeted MRM approach.

Determining sample metabolite concentrations from an external standa

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7.1 Run a full set of all 12 concentrations of the external standard mix on the LC-MS three times total, at the start, middle, and end of sample runs.

7.2 Integrate peak areas for each standard peak using the quantitation wizard tool in the Analyst instrument control and data processing software (v.1.6.2) and export the data to Excel.

7.3 In Excel, convert peak area units from µM to µmols of compound by multiplying by sample volume (50 µL).

7.4 Plot the peak areas vs the standard concentrations and fit a linear regression to the data.

7.5 For sample runs, compare the measured amounts of PIPES, ribitol, and norvaline internal standards with the known amounts added during sample preparation to calculate the percent metabolite recovery, using PIPES for organic acids, norvaline for amino acids, and ribitol for sugars, nucleotide sugars, and phosphorylated sugars.

7.6 For each metabolite, correct for metabolite loss during extraction using the relevant factor.

7.7 For each metabolite, calculate the concentration from the sample by solving for x using the linear regression equation of that metabolite's standard curve.

Calculation of limits of detection (LOD) and limits of quantitation (LOQ)

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8.1 For each metabolite, determine the lowest concentration on the standard curve that still showed a change in peak area. Calculate the standard deviation of this concentration (SD_{low}) for all three injections.

8.2 Calculate the limits of detection and quantitation for each metabolite by multiplying this standard deviation by 3 or 10 and dividing by the slope of the standard curve.

$$LOD = \frac{SD_{low} \times 3}{slope\ of\ standard\ curve}$$

$$LOQ = \frac{SD_{low} \times 10}{slope\ of\ standard\ curve}$$

Interpreting 13C-labeling in metabolites

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9.1 For sample runs investigating ^{13}C-tracer incorporation, the MRM list for compounds of interest was expanded with the set of possible labeled carbon isotopologues in the Q1 ion and the Q3 fragment (if applicable). Since the Q3 fragment for a compound like 3-PGA does not contain any carbons, an increase in label is only possible in the Q1 ion.

Table 3. Monitored isotopologue distribution (ID) for select metabolites

Q1	Q3	Compound ID (13C in Q1/13C in Q3)
133	115	MAL-0/0
134	116	MAL-1/1
135	117	MAL-2/2
136	118	MAL-3/3
137	119	MAL-4/4
185	79	PGA-0/0
186	79	PGA-1/0
187	79	PGA-2/0
188	79	PGA-3/0

9.2 Once runs are completed, extract the peak intensities (or areas) of the set of isotopologues for each compound.

9.3 To determine isotopologue distribution (ID) of a particular isotopologue (M_i), divide the abundance (A) of that isotopologue by the sum of all isotopologue abundances for that compound.

$$ID = \frac{A}{\sum_{i=0}^{n}(M_i)}$$

with *n* being the number of carbons in that compound

9.4 Once all of the isotopologue distributions have been determined, the average ^{13}C-enrichment for each compound can be calculated by determining the sum of each isotopologue multiplied by the number of labeled carbons present and dividing by total number of carbons in that compound.

$$Average\ 13C\ enrichment = \frac{\{ID_{(M0)} \times 0\} + \{ID_{(M1)} \times 1\} + \cdots + \{ID_{(Mn)} \times n\}}{n}$$