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Reversed phase LC-MS analysis of organic acids involved in the tricarboxylic acid cycle V.1

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Abstract

Organic acids of primary metabolism are essential for energy metabolism. They can range from formate (C1), glyoxylate (C2), to citrate (C6), or greater. While reversed phase (RP) C18 chromatography may not be the ideal separation technique for these acids, due to their polar nature, the use of mixed-mode polar RP stationary phases can provide retention through hydrogen bonding and other polar interactions. To this end, we have developed a RP-QTOF-MS method (via the Sulpeco Ascentis Express RP-Amide column) capable of separating, detecting, and quantifying organic acids involved in the tricarboxylic acid cycle (TCA).

Guidelines

Wear the appropriate PPE protection (i.e., gloves, safety goggles, and lab coat) and prepare solvents and LC-MS mobile phases in a chemical fume hood.
Store organic solvents in a flammable storage cabinet and peroxide-forming chemicals in the appropriate safety storage cabinets.

Materials

Solvents and chemicals used:
LC-MS grade methanol (part number LC230-4), LC-MS grade water (part number BJLC365-4), and LC-MS grade acetonitrile (part number LC015-4) were purchased from Honeywell Burdick & Jackson, Charlotte, NC, USA.

Reagents used:
Formic acid ([M] 98 % volume assay, part number 33015-500ML, from Sigma-Aldrich, St. Louis, MO, USA) and ammonium acetate (part number 17836, ACS reagent, from Sigma-Aldrich, St. Louis, MO, USA).

Analytical standards:
Chemical standards were purchased from Sigma-Aldrich. All analytical standard solutions were prepared (in 50:50, methanol:water, v/v) on ice and stored at Temperature-20 °C until LC-MS data acquisition.

MS calibrants:
Reference mass correction ([M] 100 millimolar (mM) trifluoroacetic acid ammonium salt (part number I8720243) and [M] 2.5 millimolar (mM) HP-0921 (part number 18720241)) and ESI-L-Low concentration tuning mix (part number G1969-85000) were purchased from Agilent Technologies (Santa Clara, CA, USA).

UHPLC system, LC column, and guard column:
The Agilent Technologies 1290 Infinity II UHPLC system was used throughout. An Ascentis Express RP-Amide column (150 mm length, 4.6 mm internal diameter, and 2.7 µm particle size; Supelco, Sigma-Aldrich, St. Louis, MO, USA), equipped with the appropriate guard column was connected to the column compartment of the UHPLC system.

UPLC conditions:
A sample injection volume of 2 µL was used throughout. The sample tray and column compartment were set to 4 °C and 50 °C , respectively. The mobile phases were composed of [M] 0.1 % volume formic acid/ [M] 84.9 % volume water/ [M] 15 % volume methanol (v/v/v) (A) and [M] 0.04 % volume formic acid and [M] 5 millimolar (mM) ammonium acetate in methanol (B).

QTOF-MS system:
The HPLC system was coupled to an Agilent Technologies 6545 Quadrupole Time-of-Flight Mass Spectrometer (QTOF-MS). An Agilent Technologies 6545 (part number G6545B) Quadrupole Time of Flight (QTOF) LC/MS system was used throughout. MS conditions were as follows: Drying and nebulizing gases were set to 10 L/min and 25 lb/in^2, respectively, and a drying-gas temperature of 300°C was used throughout. Sheath gas temperature and flow rate were 330 °C and 12 L/min, respectively. Electrospray ionization, via the Agilent Technologies Jet Stream Source, was conducted in the negative ion mode and a capillary voltage of 3500 V was utilized. The fragmentor, skimmer, and OCT 1 RF Vpp voltages were set to 100, 50, and 300 V,

respectively. The acquisition range was from 70-1,100 m/z, and the acquisition rate was 1 spectra/sec. Prior to data acquisition, the QTOF-MS system was tuned with the Agilent ESI-L Low concentration tuning mix (diluted 10-fold by adding 10 mL of ESI-L Low concentration tuning mix to 88.5 mL acetonitrile and 1.5 mL of water) in the range of 50-1700 m/z. Data acquisition and processing were conducted via the Agilent Technologies MassHunter Workstation software. Reference mass correction was performed with [M] 5 micromolar (µM) trifluoroacetic acid ammonium salt (part number I8720243) and 5 µM [M] 5 micromolar (µM) HP-0921 (part number 18720241) at a flow rate of 5 µL/min via a second ESI sprayer.

LC-MS data acquisition and analysis software:
LC-MS data acquisition was performed via the Agilent MassHunter Workstation software (version 8). Data processing and analysis were performed via Agilent MassHunter Qualitative Analysis (version 6) and Profinder (version 8) software.

Troubleshooting

Sample preparation

1 This section describes the preparation of chemical standards for this work.

1.1 Dissolve analytical standards in 50:50 methanol:water (v/v) to make a stock solution of [M] 100 micromolar (µM) concentration.

1.2 Prepare a seven-point calibration curve was produced via a series of 2-fold serial dilutions (with 50:50, methanol:water, v/v), which were conducted from a concentration of [M] 100 micromolar (µM) to a concentration of [M] 0.39 micromolar (µM) .

Ultra high performance liquid chromatography (UHPLC) conditions

2 This section describes the set up and working operational parameters of the UHPLC method.

2.1 Turn on the UHPLC column compartment and set to 50 °C via the data acquisition software.

2.2 Connect the LC column (see Table 1) to the UHPLC column compartment (see Table 1) and flush with [M] 90 % volume acetonitrile at 0.2 mL/min for 10 minutes followed by 0.4 mL/min for 10 minutes to clean and equilibrate the LC column.

	A	B	C
	Component	Description	Part number
	1260 Infinity II Isocratic Pump	Isocratic pump for internal reference lock mass delivery	G7110B
	1290 Infinity II MCT	Column compartment	G7116B
	1290 Infinity II Multisampler	Autosampler for automatic sample injection	G7167B
	1290 Infinity II High Speed Pump	UHPLC pump	G7120A
	6545 Q-TOF LC/MS	Quarupole-time-of-flight mass spectormeter	G6545B

Table 1. The Agilent Technologies UHPLC-QTOF-MS system.

2.3 Flush the LC column with the starting mobile phase composition at 0.4 mL/min for 15 minutes.

2.4 Set up UHPLC gradient parameters (see Table 2).

	A	B	C	D
	Time (min)	Mobile phase B (%)	Flow rate (mL/min)	Maximum system back pressure (Bar)
	0	0	0.4	600
	5.5	30	0.4	600
	5.7	100	0.4	600
	7.7	100	0.4	600
	7.9	0	1	600
	10.4	0	1	600

Table 2. UHPLC gradient. The maximum allowable system backpressure for the LC column was 600 bar.

Please note, if the backpressure approaches 600 bar during the column conditioning step (i.e., from 7.9 minutes to 10.4 minutes), then reduce the flow rate during this step. An acceptable flow rate for the column conditioning step (i.e., from 7.9 minutes to 10.4 minutes) is 0.65 mL/min.

3 Set up the other UHPLC method parameters including sample injection volume, etc. (see Materials).

QTOF-MS method parameters

4 This section describes the set up and working operational parameters of the QTOF-MS method.

4.1 Set up the QTOF-MS method parameters in the data acquisition software (see Table 3).

	A	B
	QTOF-MS parameters	Values
	Acquisition range (m/z)	70-1100 m/z
	Acquisition rate (spectra/s)	1
	Nebulizer pressure (*Psi)	20
	Drying gas temperature (ºC)	300
	Drying gas flow rate (L/min)	10
	Sheath gas temperature (ºC)	330
	Sheath gas flow (L/min)	12
	Capillary voltage (V)	3500
	Fragmentor (V)	100
	Skimmer (V)	50
	OCT 1 RF Vpp (V)	300
	Nozzle voltage [Expt] (V)	2000

Table 3. QTOF-MS parameters. *Psi is lb/in^2.

LC-MS data acquisition

5

5.1 Set up the sample worklist in the data acquisition software.

5.2 Perform a system check tune using the calibrant mix (see Materials).

5.3 Run worklist sequence via the data acquisition software.

LC-MS data analysis

6

6.1 Use MassHunter Qualitative Analysis to determine the retention times of organic acids via extracted ion chromatograms.

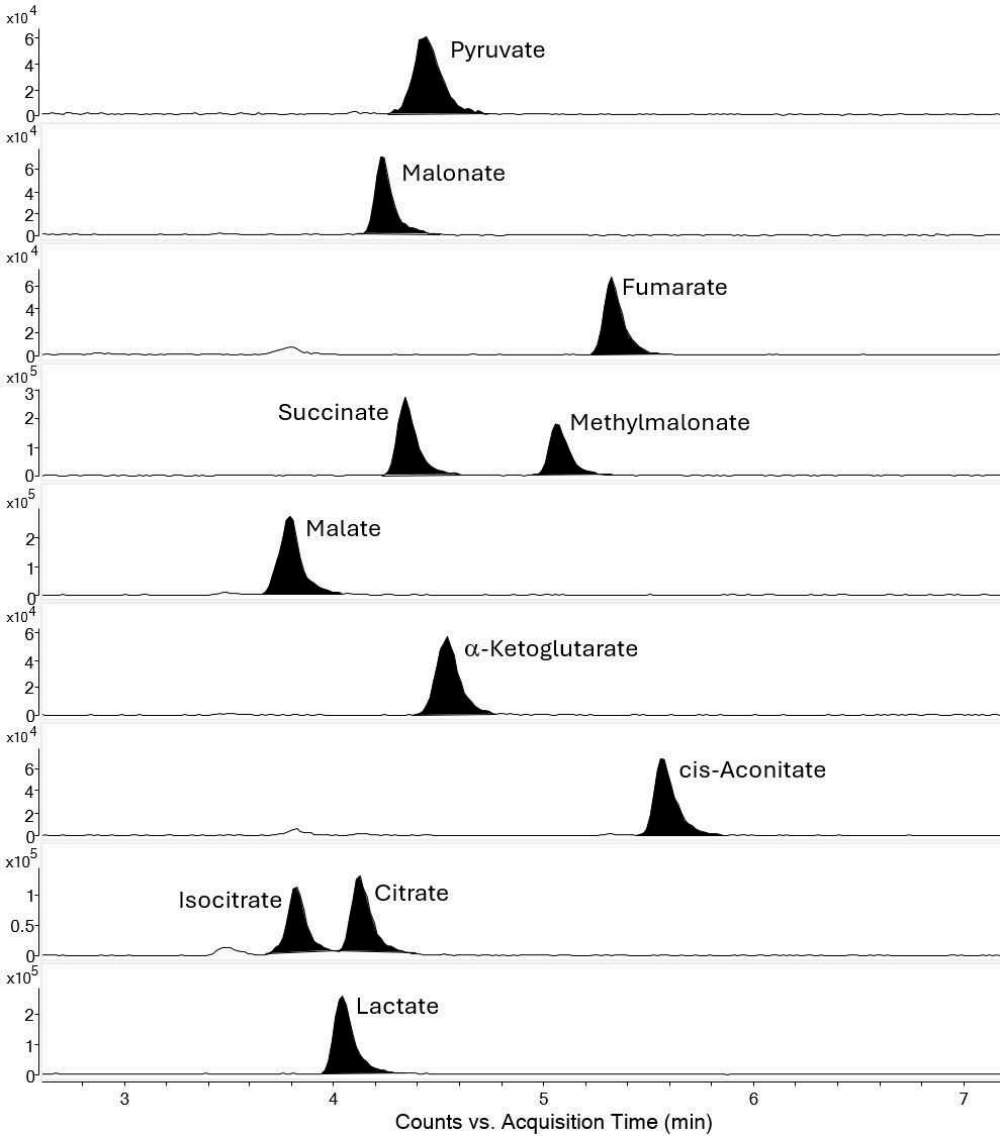

Figure 1. Extracted ion chromatograms of organic acids. Chemical standards at concentrations of 3.125 µM each were used.

6.2 Generate a csv. script containing formula, retention time, theoretical mass, and compound name for targeted chromatographic peak identification and integration via Masshunter Profinder.

This is a compound formula database file used by Agilent MassHunter applications.
Copy to a different file if you wish to make changes to database contents
You can set the version (below) to any string value.
We recommend changing it when you update the database contents.
#
#
Version: the most recent-one
#
#
#
WARNING: User should not change the format of this file.
#
Each record in this file contins 5 columns described in the last comment in this comment block.
Retention time and Description are optional.
Each record should be kept on an individual line.
Fields are separated by comma. Use quotes around a field that contains a comma.
#
The first two lines have to be comments which start with the '#' character.
First line is 'Agilent TOF Formula data store'
Second line is 'Version:' followed by a version number
#
Addition comments (such as these) may be inserted on individual
lines by specifying a '#' character at the beginning of the line
#
#

### Formu	Retention	Mass	Compour	Description
# Formula	RT	Mass	Cpd	Comments
C3H4O3	4.438	88.01604	Pyruvate	87.00877
C6H8O7	4.14	192.027	Citrate	191.0197
C6H6O6	5.571	174.0164	cis-Aconit	173.0092
C6H8O7	3.822	192.027	Isocitrate	191.0197
C5H6O5	4.538	146.0215	Ketoglutar	145.0142
C4H6O4	4.339	118.0266	Succinate	117.0193
C4H4O4	5.321	116.011	Fumarate	115.0037
C4H6O5	3.8	134.0215	Malate	133.0142
C3H6O3	4.04	90.03169	Lactate	89.02442

Figure 2. Script for data targeted integration.

6.3 Integrate peaks via Masshunter Profinder.

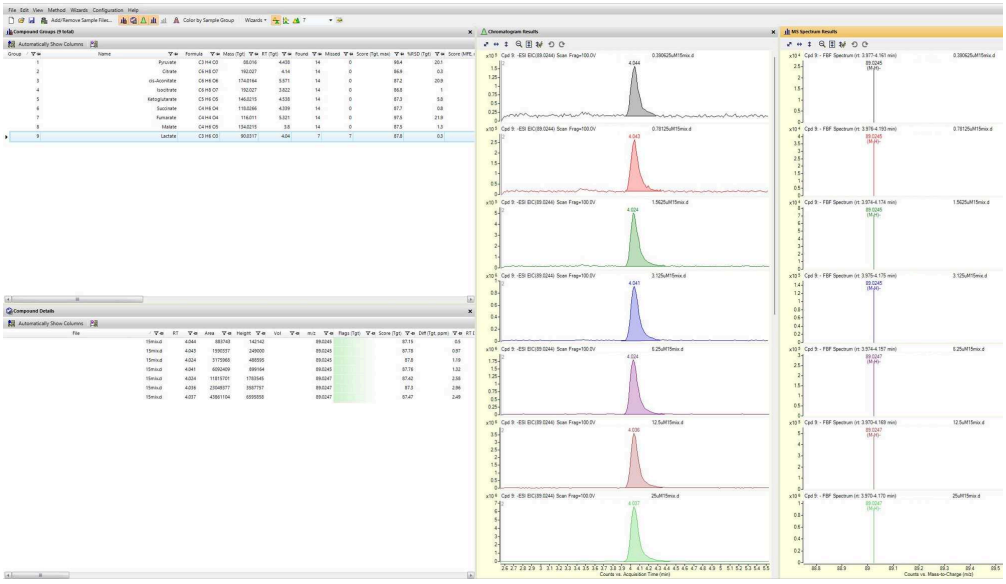

Figure 3. Masshunter Profinder extracted ion chromatograms.

6.4 Generate csv export integration file and analyze data via the appropriate software.

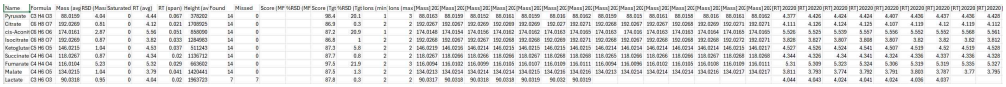

Figure 4. Masshunter Profinder export csv file.

6.5 Analyze data via appropriate software (e.g., Microsoft excel, Jupiter Notebook, etc.).

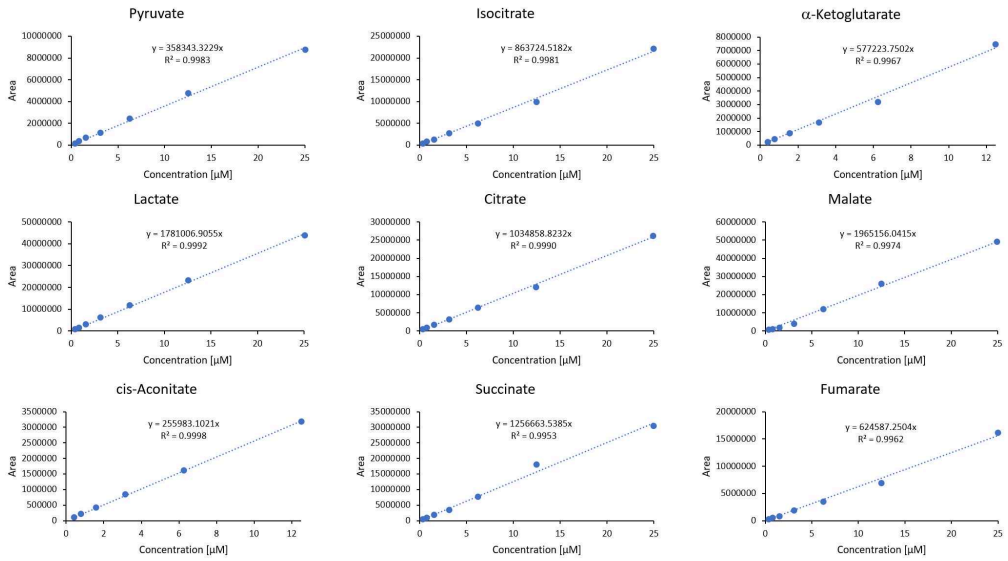

Figure 5. Organic acid calibration curves.

Compound	Theoretical mass-to-charge ratio (m/z)	Average retention time (min, n = 7)	%RSD of retention time (n = 7)	Limit of detection (s/n = 3) [nM]	Limit of quantitation (s/n = 10) [nM]	Linearity (R^2 coefficient)
Pyruvate	87.008768	4.44	0.11	148.34	494.46	0.9983
Citrate	191.019726	4.12	0.21	87.78	292.60	0.999
cis-Aconitate	173.00916	5.57	0.15	67.74	225.79	0.9998
Isocitrate	191.019726	3.82	0.25	96.45	321.50	0.9981
Ketoglutarate	145.014247	4.53	0.21	96.06	320.18	0.9885
Succinate	117.019332	4.34	0.14	84.61	282.04	0.9953
Fumarate	115.003682	5.33	0.09	122.71	409.03	0.9962
Malate	133.014247	3.79	0.30	78.65	262.16	0.9974
Lactate	89.024418	4.04	0.21	96.85	322.83	0.9992

Figure 6. Method validation table. All other compounds were detected via [M - H]- ions. %RSD denotes percent relative standard deviations.

Conclusions

7 The RP-Amide-QTOF-MS method was able to successfully separate and detect all of the organic acids tested. The method was able to separate isomers such as citrate and isocitrate as well as succinate and methylmalonate. TCA cycle intermediates and lactate showed LODs and LOQs in the nM range and good linearity, with R^2 coefficients of >0.99. The method also showed good repeatability with %RSD of ≤0.3. These measurements suggest the method may be applicable to the detection and quantification of TCA cycle intermediates, lactate, and other organic acids in biological matrices.

Acknowledgements

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