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Highlighting the tricarboxylic acid cycle: Liquid and gas chromatography–mass spectrometry analyses of ^{13}C-labeled organic acids

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ABSTRACT

The tricarboxylic acid (TCA) cycle is involved in the complete oxidation of organic acids to carbon dioxide in aerobic cells. It not only uses the acetyl-CoA derived from glycolysis but also uses breakdown products of proteins, fatty acids, and nucleic acids. Therefore, the TCA cycle involves numerous carbon fluxes through central metabolism to produce reductant power and transfer the generated electrons to the aerobic electron transport system where energy is formed by oxidative phosphorylation. Although the TCA cycle plays a crucial role in aerobic organisms and tissues, the lack of direct isotopic labeling information in its intermediates (organic acids) makes the quantification of its metabolic fluxes rather approximate. This is the major technical gap that this study intended to fill. In this work, we established and validated liquid and gas chromatography–mass spectrometry methods to determine ^{13}C labeling in organic acids involved in the TCA cycle using scheduled multiple reaction monitoring and single ion monitoring modes, respectively. Labeled samples were generated using maize embryos cultured with [^{13}C]glucose or [^{13}C]glutamine. Once steady-state labeling was reached, ^{13}C-labeled organic acids were extracted and purified. When applying our mass spectrometric methods to those extracts, mass isotopomer abundances of seven major organic acids were successfully determined.

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The tricarboxylic acid (TCA)[1] cycle, also known as the citrate or Krebs cycle, is the final catabolic pathway for the oxidation of fuel molecules such as proteins, fatty acids, and carbohydrates [1]. Four reactions of the TCA cycle transfer a pair of electrons from a substrate to an electron-accepting coenzyme (NAD or FADH; Fig. 1) [2]. The reducing power produced in this way can be further employed in respiratory processes to generate energy under the form of ATP [3]. The NADH and ATP synthesized here are crucial for biomass production and cellular maintenance. In addition, several precursor metabolites (amino acids) for protein biosynthesis are directly (glutamate and aspartate) or indirectly (proline, arginine, glutamine, threonine, and isoleucine) generated from the organic acids (OAs), intermediates of the TCA cycle. Thus, this cycle involves numerous carbon fluxes through central metabolism, and their regulation may lead to the identification of crucial steps for genetic engineering. Metabolic flux analysis (MFA) is the best-suited and commonly used tool to measure the distribution of carbon and its regulation.

MFA quantifies in vivo intracellular carbon fluxes through metabolic networks using ^{13}C isotopic substrates [4–10]. Labeled ^{13}C atoms are incorporated into intracellular metabolites and macromolecules [11] and then quantified by mass spectrometry (MS) and/or nuclear magnetic resonance (NMR) [12–14]. The resulting ^{13}C-labeling data are integrated into a mathematical model describing the metabolic network of a given cell or tissue [15]. Therefore, the quantification of the ^{13}C labeling in the major biosynthetic processes of a cell/tissue leads to a reliable flux map, which describes the pattern of carbon flow through a metabolic network.

Although the TCA cycle plays an important role in the central metabolism of aerobic cells and tissues, the quantification of ^{13}C labeling in its OAs for MFA has been poorly accessed. Indeed, labeling of these key metabolic intermediates often cannot be directly determined as a consequence of their instability and/or low concentration. To date, the information on labeling of OAs was indirectly accessed by analyzing the labeling in hydrolyzed molecules (i.e., proteinogenic amino acids) that are derived from the OAs of the TCA cycle [17–24]. Even though several analytical techniques have been applied to separate and quantify the levels of intracellular OAs [25–33], few of them were suitable to measure

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[1] *Abbreviations used:* TCA, tricarboxylic acid; OA, organic acid; MFA, metabolic flux analysis; MS, mass spectrometry; NMR, nuclear magnetic resonance; GC, gas chromatography; LC, liquid chromatography; MS/MS, tandem mass spectrometry; MRM, multiple reaction monitoring; SIM, single ion monitoring; KOH, potassium hydroxide; MTBSTFA, *N*-methyl-*N*-(*tert*-butyldimethylsilyl)-trifluoroacetamide; TBDMCS, *tert*-butyldimethylchlorosilane; EI, electron impact; TBDMS, *tert*-butyldimethylsilyl.

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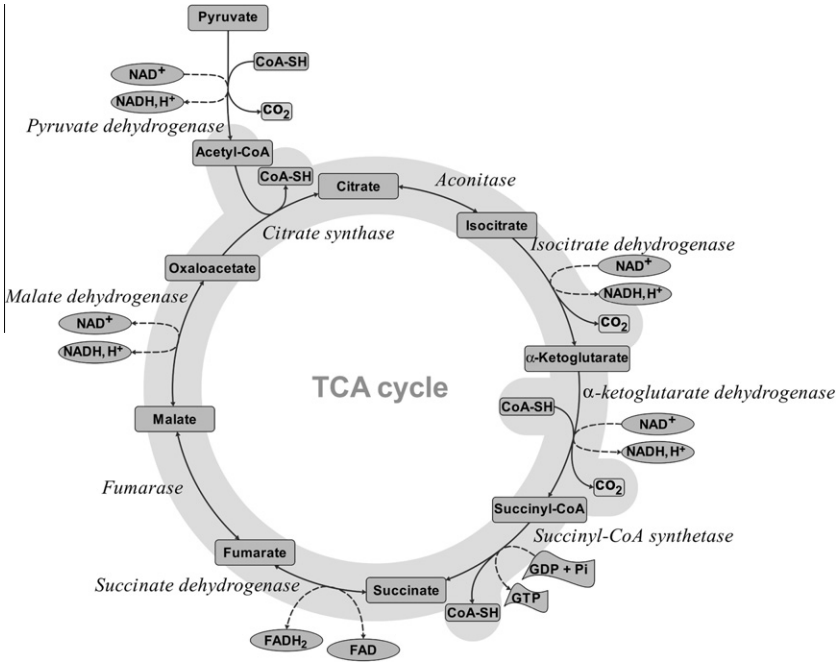

Fig.1. Overview of the TCA cycle in living cells. The metabolic map was drawn using Omix software [16] (http://www.13cflux.net/omix).

Table 1
Organic acid-dependent MS parameters used for MRM.

Analyte	Parent ion formula	Product ion formula	Parent/product transition	Declustering potential (V)	Collision energy (V)	Collision exit potential (V)
Succinate	$C_4H_5O_4^-$	$C_4H_3O_3^-$	116.9/98.9	−40	−14	−1
Malate	$C_4H_5O_5^-$	$C_4H_3O_4^-$	132.9/114.9	−50	−14	−11
α-Ketoglutarate	$C_5H_5O_5^-$	$C_4H_5O_3^-$	145.0/101.0	−50	−10	−9
Fumarate	$C_4H_3O_4^-$	$C_3H_3O_2^-$	115.0/71.0	−50	−10	−13
Oxaloacetate	$C_4H_3O_5^-$	$C_3H_3O_3^-$	131.0/87.0	−40	−12	−9
Citrate and isocitrate	$C_6H_7O_7^-$	$C_6H_5O_6^-$	191.0/173.0	−75	−16	−21

^{13}C labeling [34,35]. Indeed, the ^{13}C enrichment data in a total of four OAs was determined by NMR [36,37]. Although this technique generates positional labeling information, its application is limited due to the lack of sensitivity; OA concentration needs to be in the millimolar (mM) range. Roessner-Tunali and coworkers [35] used gas chromatography (GC)–MS to follow the specific labeling in five OAs (succinate, fumarate, malate, citrate, and α-ketoglutarate) of potato tuber discs pulsed with [^{13}C]glucose. However, none of the approaches used to date has identified GC–MS fragments of OAs potentially useful for MFA. Such ion fragments will improve the sensitivity of the flux measurements through the TCA cycle and, consequently, the accuracy of in vivo network models.

In this work, we developed robust liquid chromatography–tandem mass spectrometry (LC–MS/MS) and GC–MS methods to quantify ^{13}C labeling in intermediates of the TCA cycle. Analyses were performed using scheduled multiple reaction monitoring (MRM) and single ion monitoring (SIM) modes, respectively. Methods were illustrated using maize embryos (*Zea mays* L.) incubated with ^{13}C-labeled substrates ([1,2-^{13}C]glucose, [U-^{13}C]glucose, and [U-^{13}C]glutamine). Our results showed a synergy between these two methods, which led to an accurate determination of the isotopic enrichment in the major OAs involved in the TCA cycle. The reliability of our results was further verified and validated by the equilibrium in the steady-state labeling maintained among succinic, fumaric, and malic acids as well as between citric and isocitric acids.

Materials and methods

Chemicals

All of the OA standards were purchased from Sigma (St. Louis, MO, USA). For LC–MS/MS analyses, to avoid carbonate contamination, the potassium hydroxide (KOH) was purchased in liquid form from ThermoFisher (Pittsburgh, PA, USA). All of the water used as eluent and reagent was deionized and degassed. The derivatization agent for GC–MS analyses was *N*-methyl-*N*-(*tert*-butyldimethylsilyl)-trifluoroacetamide (MTBSTFA) + 1% *tert*-butyldimethylchlorosilane (TBDMCS) obtained from ThermoFisher. [U-^{13}C]glucose, [1,2-^{13}C]glucose, and [U-^{13}C]glutamine were obtained from Isotec (Miamisburg, OH, USA).

In vitro embryo culture

Maize plants (*Zea mays* L.) were grown in a greenhouse as described previously [38]. Plants were hand pollinated, and the ears were detached at 15 DAP (days after pollination) and taken to a laminar flow bench for dissection. After removing the inner husks and silks, each maize ear was sterilized for 20 min in 50% (v/v) bleach, rinsed with 1 L of autoclaved water, and then washed for 20 min in autoclaved water. The developing embryos were dissected under aseptic conditions and transferred into the following culture medium: glucose (200 mM), fructose (200 mM), glutamine (5 mM), MS basal salts (4.3 g/L), 15% PEG (polyethylene glycol)

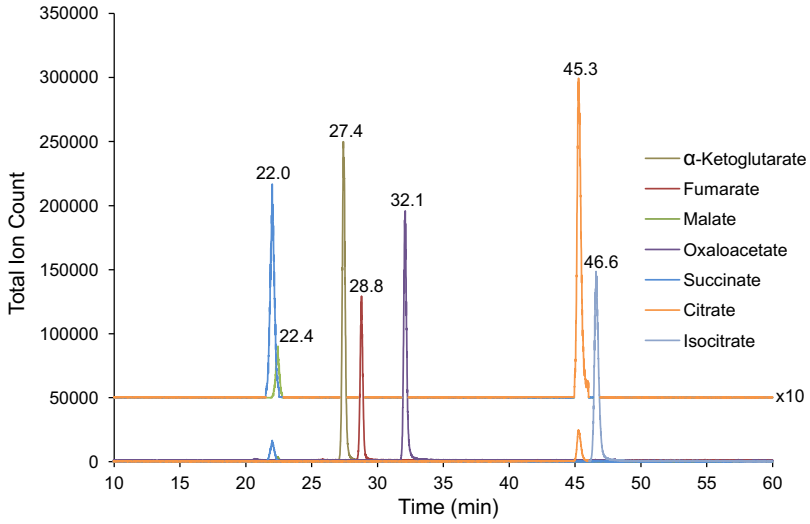

Fig.2. Analysis of a mixture of OA standards by LC–MS/MS. A mixture of OA standards, with 1 μM each except malate (0.5 μM) and oxaloacetate (5 μM), was monitored by LC–MS/MS using the separation and hardware described in Materials and methods. Different MRM transitions were followed: 116.9/98.9 for succinate, 132.9/114.9 for malate, 145.0/101.0 for α-ketoglutarate, 115.0/71.0 for fumarate, 131.0/71.0 for oxaloacetate, and 191.0/173.0 for citrate and isocitrate. A 10-fold vertical zoom shows the lower intensity peaks (succinate, malate, and citrate).

4000, and a mixture of vitamins containing nicotinic acid (5 μg/ml), pyridoxin hydrochloride (0.5 μg/ml), thiamine hydrochloride (0.5 μg/ml), and folic acid (0.5 μg/ml). MES (10 mM) was included as a buffer, and the pH was adjusted to 5.8 with 1 N of KOH. The embryos (four per plate) were placed on double-glass fiber filters soaked with 8 ml of the medium described above and cultured at 24 °C in the dark in a Petri dish sealed with surgical tape. For labeling experiments, substrates were replaced by (i) a mixture of 20% [U-^{13}C]glucose and 80% [1,2-^{13}C]glucose that has been shown to improve the identification and evaluation of the fluxes of interest [39] and (ii) 100% [U-^{13}C]glutamine. The embryos were cultured for 7 days, which is the time necessary to reach a metabolic and isotopic steady state [38].

Compound extraction

After incubation, maize embryos were collected and rinsed three times each with 10 ml of water to remove surface labeling. The embryos were then frozen with liquid nitrogen and lyophilized. One embryo (~10 mg dry weight) from each biological and technical replicate was used. Sequential extractions were carried out on a single embryo by first removing the oil following the protocol described in Refs. [38–40]. Water-soluble metabolites (soluble sugars, amino acids, and OAs) were extracted from the remaining pellet using 1 ml of boiling water and then placed for 10 min in a water bath set at 100 °C. The extracts were then transferred into ice and centrifuged at 14,000 × *g* for 5 min at 4 °C. The supernatants were recovered and filtered using 5-ml syringes and 0.22-μm filters. Remaining pellets were rinsed once with 1 ml of cold water (4 °C), vortexed, centrifuged, and then filtered as describe above. The syringes and filters were rinsed once with 1 ml of cold water each time. Finally, the water-soluble metabolite fractions were freeze-dried overnight.

OA purification

The lyophilized fractions of water-soluble metabolites (soluble sugars, amino acids, and OAs) were resuspended in 1 ml of sodium acetate buffer (50 mM, pH 5.5) and loaded through a cation exchange resin (Dowex 50X8) to remove the amino acids as

Table 2
Scheduled MRM transitions to monitor the abundance of ^{13}C-labeled OA isotopomers.

Analyte	Retention time (min)	Mass isotopomer	Parent/product transition
Succinate	22.0	m_0	116.9/98.9
		m_1	117.9/99.9
		m_2	118.9/100.9
		m_3	119.9/101.9
		m_4	120.9/102.9
Malate	22.4	m_0	132.9/114.9
		m_1	133.9/115.9
		m_2	134.9/116.9
		m_3	135.9/117.9
		m_4	136.9/118.9
α-Ketoglutarate	27.4	m_0	145.0/101.0
		m_1	146.0/101.0; 146.0/102.0
		m_2	147.0/102.0; 147.0/103.0
		m_3	148.0/103.0; 148.0/104.0
		m_4	149/104.0; 149.0/105.0
		m_5	150.0/105.0
Fumarate	28.8	m_0	115.0/71.0
		m_1	116.0/71.0; 116.0/72.0
		m_2	117.0/72.0; 117.0/73.0
		m_3	118.0/73.0; 118.0/74.0
		m_4	119.0/74.0
Oxaloacetate	32.1	m_0	131.0/87.0
		m_1	132.0/87.0; 132.0/88.0
		m_2	133.0/88.0; 133.0/89.0
		m_3	134.0/89.0; 134.0/90.0
		m_4	135.0/90.0
Citrate	45.3	m_0	191.0/173.0
		m_1	192.0/174.0
		m_2	193.0/175.0
		m_3	194.0/176.0
		m_4	195.0/177.0
		m_5	196.0/178.0
		m_6	197.0/179.0
Isocitrate	46.6	m_0	191.0/173.0
		m_1	192.0/174.0
		m_2	193.0/175.0
		m_3	194.0/176.0
		m_4	195.0/177.0
		m_5	196.0/178.0
		m_6	197.0/179.0

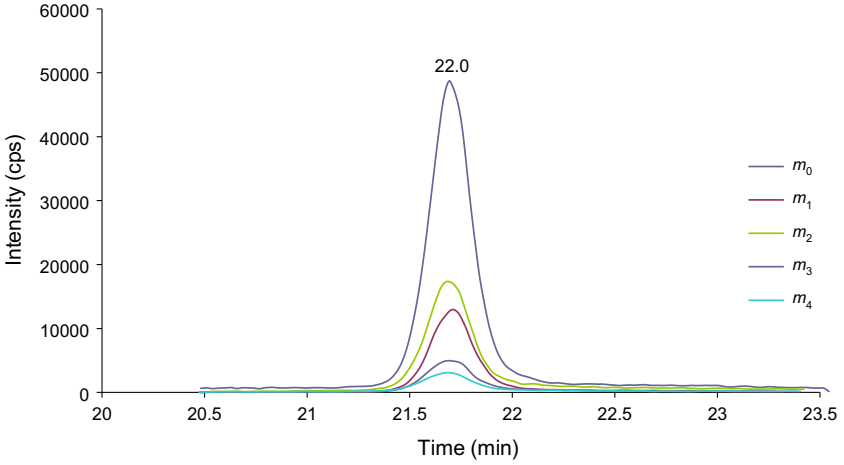

Fig.3. Scheduled MRM application to steady-state ^{13}C-labeling analysis of succinate. Maize embryos were incubated with a mixture of 20% [U-^{13}C]glucose and 80% [1,2-^{13}C]glucose until isotopic steady state was reached. OAs were extracted and purified as described previously and then were analyzed by LC–MS/MS. The collision parameters for succinate were optimized for a loss of water. Succinate has $n = 4$ carbons; therefore, $n + 1 = 5$ parent/product transitions were followed: 116.9/98.9 (m_0), 117.9/99.9 (m_1), 118.9/100.9 (m_2), 119.9/101.9 (m_3), and 120.9/102.9 (m_4). cps, counts per second.

Table 3
Steady-state mass isotopomer abundances of ^{13}C-labeled OAs.

Analyte	Mass isotopomer	[^{13}C]glutamine experiments		[^{13}C]glucose experiments	
		Average	Standard deviation	Average	Standard deviation
Succinate	m_0	0.885	0.011	0.566	0.007
	m_1	0.056	0.002	0.169	0.004
	m_2	0.015	0.002	0.167	0.003
	m_3	0.010	0.001	0.075	0.004
	m_4	0.034	0.006	0.023	0.001
Fumarate	m_0	0.870	0.015	0.555	0.008
	m_1	0.069	0.005	0.187	0.004
	m_2	0.013	0.004	0.173	0.009
	m_3	0.011	0.002	0.068	0.002
	m_4	0.037	0.006	0.017	0.001
Malate	m_0	0.892	0.010	0.579	0.004
	m_1	0.056	0.003	0.168	0.004
	m_2	0.013	0.003	0.162	0.004
	m_3	0.009	0.001	0.072	0.003
	m_4	0.031	0.004	0.020	0.001
α-Ketoglutarate	m_0	0.739	0.015	0.483	0.020
	m_1	0.071	0.003	0.157	0.009
	m_2	0.018	0.002	0.226	0.003
	m_3	0.037	0.002	0.077	0.003
	m_4	0.016	0.001	0.047	0.006
	m_5	0.121	0.009	0.011	0.001
Citrate	m_0	0.794	0.016	0.387	0.012
	m_1	0.095	0.006	0.184	0.001
	m_2	0.025	0.003	0.241	0.004
	m_3	0.026	0.003	0.111	0.002
	m_4	0.005	0.010	0.056	0.004
	m_5	0.055	0.005	0.019	0.001
	m_6	0.000	0.000	0.003	0.004
Isocitrate	m_0	0.789	0.013	0.418	0.015
	m_1	0.092	0.004	0.182	0.006
	m_2	0.023	0.005	0.230	0.007
	m_3	0.022	0.001	0.104	0.005
	m_4	0.023	0.004	0.048	0.002
	m_5	0.050	0.005	0.017	0.000
	m_6	0.001	0.001	0.001	0.000

Note: Maize embryos were incubated with either [U-^{13}C]glutamine or a mixture of 20% [U-^{13}C]glucose and 80% [1,2-^{13}C]glucose until isotopic steady state was reached. OAs were extracted and purified as described in Materials and methods and then were analyzed by LC–MS/MS using scheduled MRM mode. Mass isotopomer abundances were corrected for natural abundance as described previously [12]. The values reported are the averages of the relative abundances ± standard deviations from three biological replicates.

described previously [41,42]. The recovered fraction containing the sugars and OAs was applied to an anion exchange resin (Dowex 1X8 [formate form, 200–400, Supelco 13858]) to purify OAs as described previously [42]. The resin was washed with 5 ml of water to remove the remaining free sugars, and the retained OAs were eluted using 5 ml of formic acid (4 M). The collected fraction was dried under a nitrogen flow at 60 °C.

High-performance anion exchange chromatography mass spectrometry

The OA analyses were carried out using a modified version of Alonso and coworkers [43]. In brief, the LC was performed with a UHPLC (ultra high-pressure liquid chromatography) 1290 from Agilent Technologies (Santa Clara, CA, USA). An IonPac ATC-3 Anion Trap Column from Dionex (ThermoFisher), used to remove trace contaminants from KOH, was added before the injection valve. Samples in the autosampler were kept at 4 °C while the LC analysis was carried out at 23 °C. OAs were separated using an IonPac AS11 column (250 × 2 mm, Dionex) and a guard column AG11 (50 × 2 mm, Dionex) at a flow rate of 0.35 ml/min. A gradient of KOH was generated using 0.5 mM KOH (solvent A) and 75 mM KOH (solvent B). The gradient was defined as follows: A = 0 to 2 min 100% (0.5 mM KOH), 2 to 13 min 95.2% (4.1 mM KOH), 13 to 20 min 95.2% (4.1 mM KOH), 20 to 30 min 87.3% (10 mM KOH), 30 to 45 min 73.8% (20 mM KOH), 45 to 48 min 33.6% (50.3 mM KOH), and 48 to 50 min 0% (75 mM KOH). Cleaning of the column was achieved with 100% B (75 mM KOH) for 5 min. The initial conditions (100% A and 0.5 mM KOH) were restored in 0.1 min, and the column was equilibrated for 5 min. KOH was removed using a postcolumn ASRS 300 suppressor (Dionex) with a current and a reagent flow rate set at 50 mA and 2 ml/min, respectively.

The MS/MS analysis was performed with a hybrid triple quadrupole/ion trap mass spectrometer, QTRAP 5500, from AB Sciex (Framingham, MA, USA). The mass spectra were acquired using TurboSpray ionization in negative ion mode and scheduled MRM with a detection window set to 180 s. The curtain gas was adjusted to 35 psi. The ion spray voltage, ion source gas 1, and ion source gas 2 were −4.5 kV, 60 psi, and 45 psi, respectively. The temperature of the source was fixed to 550 °C. The $[M–H]^-$ was fragmented by CAD (collision-activated dissociation) set to medium. The entrance potential was constant for each transition and kept at −10 V. LC–MS/MS data were acquired and processed using Analyst 1.6 software.

After lyophilization, OA fractions were resuspended in 1 ml of water, and then 50 µl was diluted five times and 20 µl was injected.

OA derivatization for GC–MS analysis

To remove residual moisture from purified and dried OAs, 200 µl of dichloromethane was added and then dried under a nitrogen flow. This procedure was repeated two more times. Then 300 µl of MTBSTFA + 1% TBDMCS/acetonitrile (1:1, v/v) was added to derivatize the organic acids. Tubes were flushed under a nitrogen flow and incubated for 1 h at 120 °C. After derivatization, samples were centrifuged for 2 min at 400 × *g*, and each supernatant was transferred into a GC–MS vial.

GC–MS conditions

OA derivatives were analyzed using a ThermoFisher GC–MS (DSQ II) instrument with a DB5 capillary column (5% phenyl/95% dimethylpolysiloxane, 30 m) from Agilent Technologies. The GC conditions were as follows. The initial temperature was set to 40 °C, increased to 100 °C at 50 °C/min, and held for 1 min. The oven temperature was then raised to 200 °C at 5 °C/min and maintained for 1 min before being increased to 320 °C at 10 °C/min and held for 5 min. The injection temperature was fixed to 280 °C, and

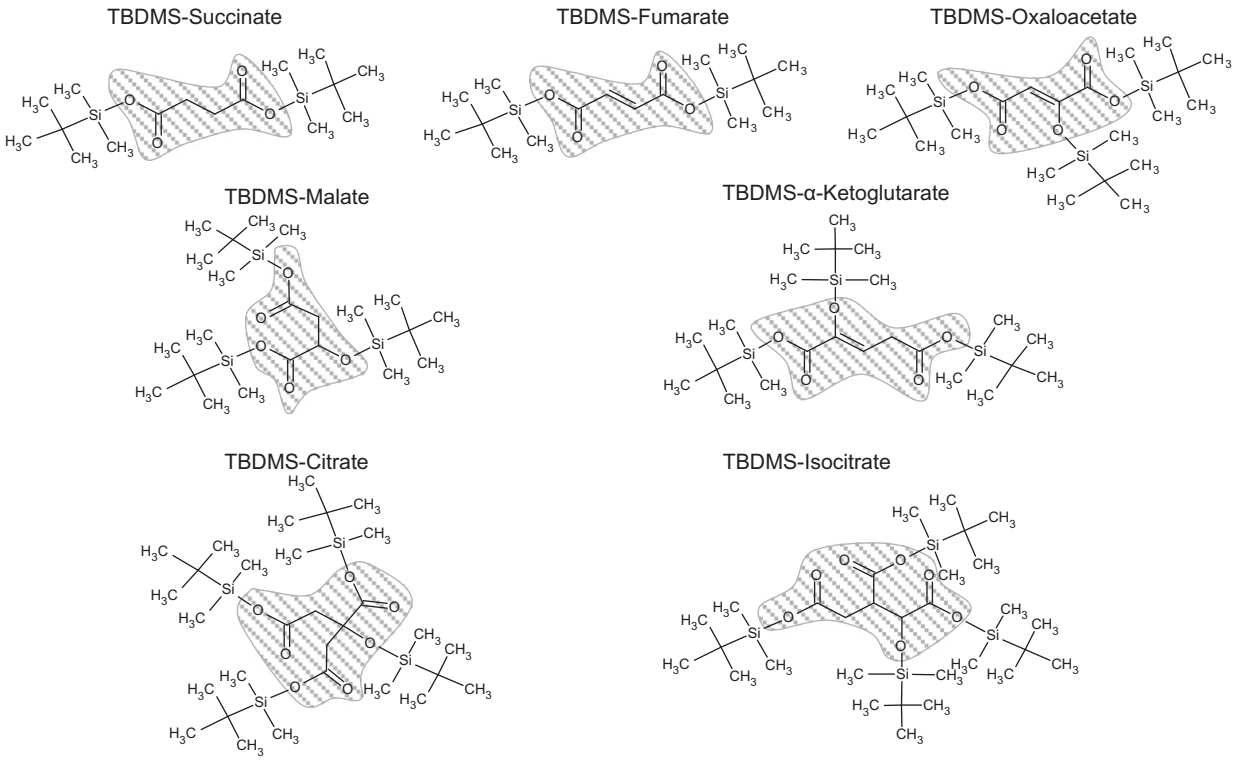

Fig.4. OA derivatives analyzed by GC–MS. The derivatization was performed using MTBSTFA + 1% TBDMCS reagent for 1 h at 120 °C. The shaded part corresponds to the atoms belonging to the OA. Molecules were drawn using ChemSketch software (http://www.acdlabs.com/resources/freeware/chemsketch).

Table 4
SIM transitions to monitor the abundance of ^{13}C-labeled OA isotopomers using GC–MS.

Analyte	Retention time (min)	Fragment (s)	Mass isotopomers	*m/z* Range
Succinate	18.2	$[M-15]^+$ $[M-57]^+$	m_0–m_4	331–335 289–293
Fumarate	18.8	$[M-15]^+$ $[M-57]^+$	m_0–m_4	329–333 287–291
Malate	25.0	$[M-15]^+$ $[M-57]^+$	m_0–m_4	461–465 419–423
Oxaloacetate	25.6	$[M-15]^+$ $[M-57]^+$	m_0–m_4	459–463 417–421
α-Ketoglutarate	31.1	$[M-57]^+$	m_0–m_5	431–436
Citrate	31.3	$[M-15]^+$ $[M-57]^+$	m_0–m_6	633–639 591–597
Isocitrate	31.4	$[M-15]^+$ $[M-57]^+$	m_0–m_6	633–639 591–597

the injection mode was set to split with a split ratio of 5. For the MS, ion source and the interface temperatures were set to 280 °C with electron impact (EI) ionization. A SIM mode following the fragments $[M-15]^+$ and $[M-57]^+$ was used for the analyses. GC–MS data were acquired and processed using Xcalibur software.

Data correction for natural abundance

Mass distributions of chosen fragments were corrected for natural isotope abundance as described by Koubaa and coworkers [12].

Results and discussion

LC–MS/MS method development: Separation and identification of OAs

All of the intermediates of the TCA cycle are OAs (Fig. 1). Therefore, they should be separable by liquid anion exchange chromatography. For this purpose, we used an IonPac AG11 guard column (5 × 2 mm, Dionex) followed by an IonPac AS11 column (250 × 2 mm, Dionex). The triple quadrupole AB Sciex 5500 was used in negative ion mode to quantify the mass isotopomer distribution of OAs. It is important to note that we optimized the collision parameters for the loss of water or CO_2, which permitted us to follow each OA at a specific parent/product transition. Declustering potential, collision energy, and collision exit potential were optimized for each transition using AB Sciex Analyst 1.6 software (Table 1). MRM mode was used to track the different OAs simultaneously.

Optimized separation of a mixture of OA standards was achieved when applying the gradient described previously [43] with the following modifications: (i) KOH was used instead of sodium hydroxide and (ii) the chromatographic method was shortened (Fig. 2). Briefly, the gradient of eluent was started with 0.5 mM KOH for 2 min, linearly increased to 4.1 mM over 11 min, and maintained for 7 min. The KOH concentration was then linearly increased to 10 mM over 10 min, which allowed the elution of succinate, malate, α-ketoglutarate, and fumarate. A linear gradient from 10 to 20 mM KOH in 15 min was then applied to recover oxaloacetate and then applied from 20 to 50.3 mM KOH in 3 min to elute the two isomers citrate and isocitrate. The column was washed with 75 mM KOH for 5 min and finally allowed to equilibrate under initial conditions (0.5 mM KOH) for 5 min. Therefore, the separation and identification of seven OAs involved in the TCA cycle was achieved in 60 min by LC–MS/MS. It is important to note that this method is extremely sensitive, allowing the detection and quantification of OAs in the femtomole (fmol) range (see Supplementary File 1 in supplementary material).

LC–MS/MS method application: Analysis of ^{13}C-labeled OA isotopomers

Maize embryos were incubated with a mixture of 20% [U-^{13}C]glucose and 80% [1,2-^{13}C]glucose until isotopic steady state was reached [38]. OAs were extracted and purified as described in Materials and Methods and then were analyzed by LC–MS/MS. It is important to note that a scheduled MRM mode was applied to analyze ^{13}C labeling in OAs according to their respective retention

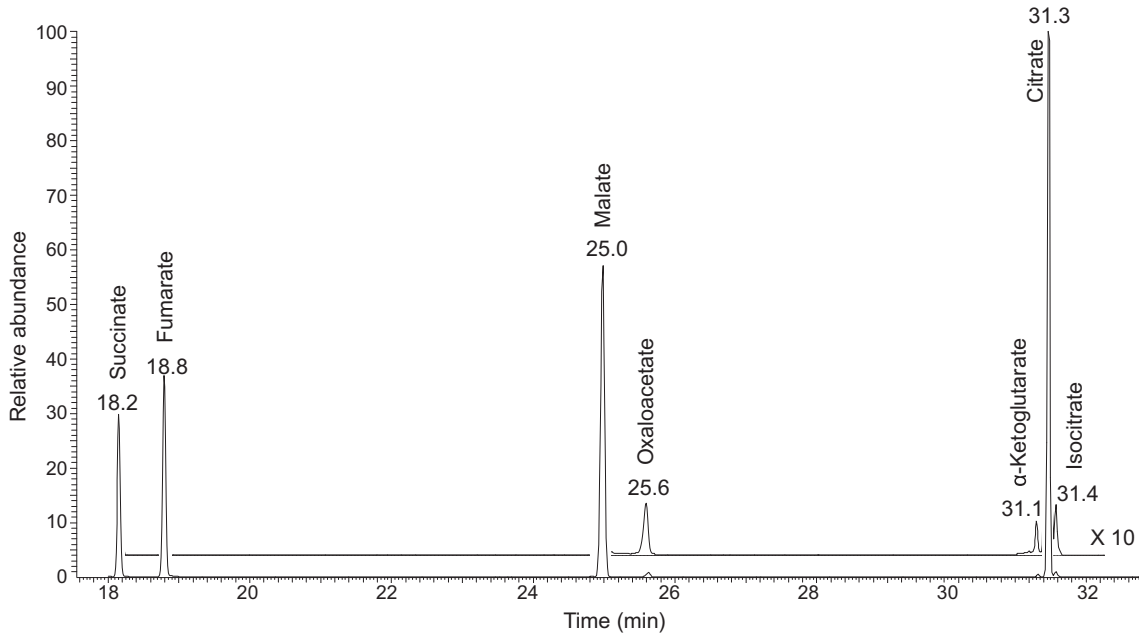

Fig.5. Chromatogram representing the OAs extracted from maize embryo incubated for 7 days with [U-^{13}C]glutamine. OAs were TBDMS derivatized and analyzed by GC–MS using EI and SIM mode at 70 eV. A 10-fold vertical zoom shows the oxaloacetate, α-ketoglutarate, and isocitrate peaks.

time previously determined using standards (Fig. 2 and Table 2). For an OA containing n carbon atoms, $n + 1$ or $2n$ transitions were followed when it lost water or CO_2, respectively (Table 2).

Six OAs were identified in the maize extracts: succinate, malate, α-ketoglutarate, fumarate, citrate, and isocitrate (data not shown). Oxaloacetate has been shown to be unstable in aqueous solution [32]. Extracts from samples were resuspended in water and run through a resin to purify the OAs. For LC–MS/MS, OA extracts were constantly kept in water where oxaloacetate is unstable and, therefore, is not detected. ^{13}C labeling in each carbon of the molecular ion can then be assessed directly from the intensity of the selected parent/product transitions (Fig. 3 and Table 3).

Mass isotopomer distribution was determined for OAs extracted from ^{13}C-labeled maize embryos. As we followed the carbon labeling, data were then corrected for natural abundance as described previously [12] and reported in Table 3. The LC–MS/MS method developed in the current study led to accurate and sensitive determination of the isotopomer abundance for six OAs involved in the TCA cycle.

GC–MS method development: Separation and identification of OAs

Because of the low volatility of the TCA cycle intermediates and the difficulty in separating them by GC, a derivatization step was performed in order to make them volatile. Similar to amino acid

Table 5
Mass isotopomer abundances of OAs after incubation with [^{13}C]glutamine and [^{13}C]glucose.

Analyte	Fragment(s)	Mass isotopomers	m/z	[^{13}C]Glutamine experiments		[^{13}C]Glucose experiments	
				Average	Standard deviation	Average	Standard deviation
Succinate	$[M-15]^+$	m_0	331	0.873	0.002	0.585	0.004
		m_1	332	0.062	0.003	0.163	0.003
		m_2	333	0.022	0.002	0.180	0.004
		m_3	334	0.008	0.002	0.062	0.004
		m_4	335	0.046	0.002	0.024	0.001
	$[M-57]^+$	m_0	289	0.867	0.005	0.572	0.012
		m_1	290	0.065	0.002	0.167	0.006
		m_2	291	0.021	0.002	0.185	0.004
		m_3	292	0.009	0.002	0.063	0.003
		m_4	293	0.049	0.001	0.024	0.003
Fumarate	$[M-57]^+$	m_0	287	0.872	0.014	0.552	0.010
		m_1	288	0.074	0.003	0.195	0.004
		m_2	289	0.015	0.003	0.167	0.006
		m_3	290	0.012	0.003	0.078	0.003
		m_4	291	0.036	0.008	0.020	0.001
Malate	$[M-15]^+$	m_0	461	0.876	0.014	0.577	0.007
		m_1	462	0.072	0.002	0.198	0.007
		m_2	463	0.013	0.001	0.159	0.004
		m_3	464	0.015	0.007	0.072	0.003
		m_4	465	0.040	0.011	0.015	0.001
	$[M-57]^+$	m_0	419	0.874	0.018	0.564	0.007
		m_1	420	0.073	0.005	0.203	0.003
		m_2	421	0.014	0.004	0.164	0.006
		m_3	422	0.013	0.003	0.072	0.001
		m_4	423	0.041	0.011	0.016	0.001
Oxaloacetate	$[M-57]^+$	m_0	417	0.835	0.006	0.528	0.005
		m_1	418	0.074	0.003	0.201	0.004
		m_2	419	0.049	0.013	0.192	0.005
		m_3	420	0.019	0.001	0.079	0.002
		m_4	421	0.040	0.004	0.023	0.002
Citrate	$[M-15]^+$	m_0	591	0.792	0.029	0.403	0.002
		m_1	592	0.095	0.009	0.194	0.001
		m_2	593	0.015	0.004	0.232	0.001
		m_3	594	0.021	0.007	0.106	0.002
		m_4	595	0.034	0.008	0.052	0.002
		m_5	596	0.052	0.018	0.018	0.002
		m_6	597	0.002	0.001	0.003	0.001
	$[M-57]^+$	m_0	633	0.800	0.018	0.398	0.006
		m_1	634	0.083	0.004	0.188	0.002
		m_2	635	0.019	0.002	0.237	0.002
		m_3	636	0.023	0.004	0.111	0.004
		m_4	637	0.032	0.008	0.052	0.001
		m_5	638	0.050	0.014	0.019	0.001
		m_6	639	0.002	0.000	0.004	0.000
Isocitrate	$[M-57]^+$	m_0	591	0.785	0.017	0.402	0.019
		m_1	592	0.079	0.006	0.195	0.006
		m_2	593	0.028	0.007	0.233	0.010
		m_3	594	0.031	0.005	0.108	0.006
		m_4	595	0.030	0.010	0.050	0.003
		m_5	596	0.052	0.021	0.016	0.004
		m_6	597	0.005	0.007	0.003	0.006

Note: OAs were extracted and purified as described previously and then analyzed by GC–MS using SIM mode. Correction for natural abundance was performed as described previously [12]. The average for each isotopomer abundance was calculated based on three biological replicates for each labeling experiment.

analysis by GC–MS [44–46], OA standards (succinate, fumarate, malate, oxaloacetate, citrate, isocitrate, and α-ketoglutarate) were derivatized for 1 h at 120 °C to their *tert*-butyldimethylsilyl (TBDMS) forms (Fig. 4). OA derivatives (or TBDMS–OAs) were separated using a DB5 capillary column (5% phenyl/95% dimethylpolysiloxane, 30 m) plugged to a single quadrupole ThermoFisher GC–MS instrument (DSQ II).

The mixture of TBDMS–OA standards (fumarate, malate, and succinate: 200 μM; oxaloacetate: 500 μM; α-ketoglutarate: 2 mM; citrate and isocitrate: 150 μM) was injected with a split ratio of 10. The retention time for each TBDMS–OA was determined using a full scan mode between m/z 50 and 650 (see Supplementary File 2 in supplementary material). The separation and identification of seven OAs (in the nanomole [nmol] range) involved in the TCA cycle was achieved in 40 min by GC–MS. The analysis of the corresponding mass spectrum for each TBDMS–OA shows that the most prominent analyzed fragments were $[M-57]^+$ and $[M-15]^+$, molecules that lost a $-C(CH_3)_3$ and a $-CH_3$, respectively (data not shown). These fragments were determined based on previous TBDMS–amino acid analysis by GC–MS [44]. Once the retention time and the most prominent fragments for each OA derivative were determined (Supplementary File 2), the GC–MS method was adapted to SIM mode, which included all isotopomers corresponding to the $[M-57]^+$ and $[M-15]^+$ fragments. For each fragment, $n+1$ transitions were monitored (Table 4).

GC–MS method application: Analysis of ^{13}C-labeled OA isotopomers

OAs extracted from ^{13}C-labeled maize embryos were purified using anion exchange resin (Dowex 1X8), dried under nitrogen (60 °C), and derivatized using MTBSTFA + 1% TBDMCS reagent. OA derivatives were then analyzed by GC–MS using SIM mode (Fig. 5). The detected OA derivatives from maize embryos were succinate (18.2 min), fumarate (18.8 min), malate (25 min), oxaloacetate (25.6 min), α-ketoglutarate (31.1 min), citrate (31.3 min), and isocitrate (31.4 min).

It is important to note that both fragments $[M-57]^+$ and $[M-15]^+$ gave the same isotopic information. In our samples from maize embryos, we were unable to use the fragment $[M-15]^+$ for fumarate due to its superposition with another peak at m/z 331 as well as for oxaloacetate and isocitrate due to their low abundance in maize extracts. After correcting unlabeled α-ketoglutarate for natural abundance, the mass distribution was 0.97 for m_0 and 0.03 for m_2, showing that this fragment is not suitable for MFA (see Supplementary File 4 in supplementary material). The distribution of mass isotopomers for each OA extracted from ^{13}C-labeled maize embryos was determined and then corrected for natural abundance as described in the LC–MS/MS result section and reported in Table 5. The GC–MS method developed here complemented the LC–MS/MS method, permitting the relative quantification of ^{13}C labeling in seven OAs involved in the TCA cycle.

Validation of LC–MS/MS and GC–MS methods

First, the suitability of the LC–MS/MS and GC–MS methods for isotopomer analysis of the different fragments was verified using the OAs extracted from maize embryos incubated for 7 days with unlabeled substrates (Supplementary Files 3 and 4). After correction for natural abundance, including all atoms of the fragment, the corrected mass distributions were not significantly different from 1 and 0 for m_0 and all of the other masses (±1% of total), respectively. Second, the LC–MS/MS and GC–MS methods were validated according to the ^{13}C labeling of OAs after incubating maize embryos with ^{13}C substrates until isotopic steady state (Tables 3 and 5). Isotopomer abundances were not significantly different between the two chromatographic methods. Moreover, they were also found to be similar in intermediates known to be in close equilibrium such as between succinic, fumaric, and malic acids as well as between citric and isocitric acids.

Conclusion

In this work, we developed and validated robust LC–MS/MS and GC–MS methods to monitor ^{13}C isotopomer abundances in the major OAs involved in the TCA cycle. This is likely to further improve the definition of the carbon fluxes through this cycle. It is important to note that (i) although the methods were validated in plant samples, they are fully applicable to other aerobic systems, and (ii) the ability to directly measure mass isotopomer abundances in intermediates of the TCA cycle opens the possibility to monitor pulse (i.e., dynamic) labeling experiments. This technical advance is important to study the metabolism of the TCA cycle and understand its regulation in any aerobic organisms and tissues.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ab.2013.01.027.

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