Application Note: 30075

irm-LC/MS: $\delta^{13}C$ Analysis of Organic Acids in Plants

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Key Words

- LC IsoLink™
- Isotope Ratio MS
- Organic Acids

Introduction

Organic acids play an important role in the metabolism of plants. They are involved in various anabolic and catabolic processes of metabolic pathways, such as citric acid cycle, synthesis of amino acids and fatty acids. They participate in the regulation of the stomata, control ion equilibrium in cells, and participate in the refixation of ammonium as well as to the adsorption of nutrients such as phosphorus, iron, copper, manganese, and zinc (Ryan et al., 2001). In addition, they ensure the tolerance of plants against aluminum and other heavy metals by forming organic heavy metal complexes (Srivastava et al., 1999). Compound-specific isotope analysis has been increasingly used to study metabolic pathways and their regulation. Thermodynamic and kinetic isotope effects discriminate isotopes in metabolic reactions and generate products with characteristic isotopic signatures (Schmidt, 2003). These isotopic signatures are unique for different metabolic pathways and are the "isotopic fingerprint" of metabolites (Schmidt, 2003).

Current research proves that stable isotope analysis offers a precise method to study metabolic pathways (Schmidt 2003, Hobbie and Werner, 2004), the effects of global sources and sinks on atmospheric CO_2 concentrations, and the influence of environmental processes and stress on the metabolism of plants (Gleixner et al., 1998, Ghashghaie et al., 2001, Damesin and Lelarge 2003).

So far no systematic investigations are present about changes in the isotopic signature of organic acids in the ontogenesis of plants. However, such investigations can be important to understand development of plant metabolism and to estimate the impact of environmental factors on plant metabolism. In first approaches, organic acids were separated by high performance liquid chromatography (HPLC). The different fractions are collected. The isotope ratio analysis was performed by an elemental analyzer which was linked with an isotope ratio mass spectrometer. This procedure showed reliable results but it is laborious and very time consuming.

The new Thermo Scientific LC IsoLink interface enables the on-line coupling of a liquid chromatograph to a stable isotope ratio mass spectrometer. It significantly reduces the preparation steps and the analysis time.

We present an analytical method for the isotope ratio analysis of organic acids in different plants extracts. First results are reported and discussed.

Taraxacum officinale

irm-LC/MS Technology

The LC IsoLink is the first high sensitivity interface connecting High Performance Liquid Chromatography (HPLC) with Isotope Ratio MS for the reproducible and accurate on-line determination of $^{13}C/^{12}C$ isotope ratios. All organic compounds eluting from an HPLC column are analyzed while maintaining the chromatographic resolution (Scheme 1).

In the LC IsoLink the sample is oxidized to CO_2 within the aqueous solvent eluting from the HPLC. The CO_2 is separated from the liquid phase and fed into the IRMS ion source. The oxidation reagent consists of two solutions, the oxidizing agent and phosphoric acid, both of which are pumped separately and added to the mobile LC phase. Silver nitrate can be added to the phosphoric acid as a catalyst, increasing the oxidation power. However silver nitrate cannot be used for samples containing halogenes, such as chlorides, because insoluble salts precipitate out. Alternatively, higher concentration of oxidizing agent can be used.

All organic compounds eluting from the HPLC column are oxidized quantitatively into CO_2 when passing through a heated reactor. Subsequently the CO_2 is removed from the liquid phase in a separation unit and transferred into a stream of He. The individual CO_2 peaks in He (which correspond one to one with the peaks of the individual compounds) are subsequently dried in an on-line unit (Nafion®) and then transferred to the Isotope Ratio MS via an open split interface.

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Figure 1: Scheme of the Thermo Scientific *irm*-LC/MS system with the LC IsoLink.

The LC IsoLink enables a second operation mode. The flow injection (µ-EA mode) offers the fast analysis of bulk samples, working standards and reference materials. Samples can be injected via a 6-port-valve of variable size which is located between the HPLC column and the interface.

Experimental Section

Sample Preparation

Plant material was sampled twice from the "Jena Experiment", the world´s largest biodiversity experiment (Rocher et al., 2004). The aboveground parts of five plants (*Plantago lanceolata, Plantago media, Medicago lupulina, Geranium pratense, Taraxacum officinale*) were collected on a sunny dry day in September 2003. A further nine plants (*Lolium perenne, Vicia cracca, Phleum pratense, Plantago lanceolata, Plantago media, Geranium pratense, Medicago lupulina, Trifolium hybridum, Poa pratensis*) were harvested in spring 2004 in dry, sunny weather. The plant material was directly frozen with dry ice after collecting, and stored frozen at - 80 °C before extraction.

Immediately before the extraction plant material was fixed three times 10 s in the microwave (Popp et al., 1996). The extraction was performed in the ASE™ 200 (Dionex Corporation) with the mixture ethanol/water (90:10) with p = 1450 psi twice at 80 °C and once with 100 °C. The resulting extracts were centrifuged (10 min, 2000 d/min). The clear supernatant was acidified with the 0.1 M HCl to a pH value of 2.5.

The solution was applied to a column (10 mm, inner diameter) containing 20 mL of a cation exchange resin (bio wheel AG 50W-X8, 50-100 mesh, acid form) preequilibrated with 12 mL HCl solution, pH 1.7. The fractions containing organic acids and sugars were eluted from the column by 0.1 M HCl and water-soluble amino acids remained on the column. The resulting eluate was neutralized with 1 N NaOH to pH 7. This was applied to a column (10 mm, inner diameter) containing 13.5 mL of an anion exchange resin (bio wheel AG1-X8, 200-400 mesh, chloride form) preequilibrated with 12 mL 1 N NaCl. The column was washed with 10 mL (5 x 2 mL) of distilled water in order to separate the sugar fraction from the organic acid fraction. The acid fraction was eluated from the anion exchanger column with 10 mL (5 x 2 mL) of 0.5 N HCl. The resulting extracts were analyzed by the LC IsoLink Interface connected with a Thermo Scientific DELTA^{plus} XP.

irm-LC/MS Analysis

The interface was coupled with the Thermo Scientific Surveyor HPLC system using an Allure Organic Acid column (300 mm x 4.6 mm, particle size 5 µM; Restek Corporation) with 0.1 M potassium dihydrogen phosphate buffer at pH 3.0 (Table 1). All organic acids were separated in 34 min at 25 °C with a flow rate of 0.5 mL min^{-1}.

The oxidation reagents (sodium peroxodisulfate, and phosphoric acid) were added to the mobile phase at a constant flow of 70 µL min^{-1} (Table 1).

HPLC Parameters	
Pump:	Thermo Scientific Surveyor MS Pump
Mobile Phase:	100 mM KH_2PO_4, pH 3 (adjusted with phosphoric acid), degassed with Helium
HPLC Column:	300 mm x 4.6 mm, non-endcapped, particle size: 5 µm spherical, pore size: 60 Å.
Temperature:	ambient
Flow:	500 µL/min

Interface Parameters	
Reagent Pump 1:	1.05 M $Na_2S_2O_8$
	Flow: 70 µL/min
Reagent Pump 2:	1.3 M H_3PO_4 (density: 1.88 g/cm3)
	Flow: 70 µL/min
Reactor Temperature:	99.9 °C
Sample Loop 2:	5 µL

Table 1: HPLC and LC IsoLink parameters.

HPLC-grade water was used to prepare the oxidation reagents and mobile phase. The solutions were degassed 5 min under vacuum using ultrasound. The reaction is carried out in a reactor maintained at 99.9 °C.

Results and Discussion

The HPLC column satisfactorily resolved a standard mixture of tartaric, malic, lactic, citric and succinic acid, which frequently occur in plants (Figure 2).

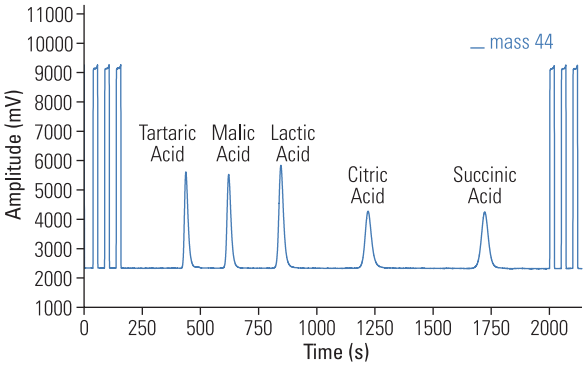

Figure 2: *irm*-LC/MS chromatogram (m/z 44 trace) of an organic acid mixture.

The most dominant organic acids studied in C3 plants were malic and citric acid (Figure 3-5). The reproducibility of these $\delta^{13}C$ values by *irm*-LC/MS was always below 0.3 ‰ with a mean of 0.08 ‰ (Table 2).

Species	$\delta^{13}C$ [‰]				
	Malic acid	Std. dev.	Citric acid	Std. dev.	Δ*
			May 2004		
Poa pratensis	-25.98	0.05	-27.46	0.10	1.48
Lolium perenne g	-27.13	0.00	-29.05	0.02	1.92
Lolium perenne k	-27.16	0.01	-29.50	0.10	2.34
Vicia cracca	-24.84	0.02	-27.09	0.30	2.25
Phleum pratense	-25.25	0.04	-27.17	0.03	1.92
Trifolium hybridum	-23.88	0.00	-27.25	0.04	3.37
Geranium pratense	-23.96	0.07	-26.02	0.07	2.06
Medicago lupulina	-26.25	0.06	-28.08	0.16	1.83
Plantago media	-26.24	0.21	-27.35	0.06	1.11
Plantago lanceolata	-25.34	0.08	-28.17	0.24	2.83
			September 2003		
Geranium pratense	-21.20	0.04	-24.27	0.02	3.07
Medicago lupulina	-23.74	0.01	-25.33	0.03	1.59
Plantago media	-28.65	0.07	-27.66	0.18	-0.99
Plantago lanceolata	-25.34	0.01	-27.19	0.07	1.85
Taraxacum officinale	-23.47	0.09	-25.93	0.04	2.46
Mean					2.15
Standard deviation					0.63
*Malic acid - Citric acid					

Table 2: Precision of $\delta^{13}C$ values of organic acids in plant extracts analyzed by *irm*-LC/MS. Plants were isolated from aboveground parts of meadow plants.

The $\delta^{13}C$-values of the malic and citric acid were up to 4 ‰ more positive, than bulk values of plants (data not shown). For malic acid $\delta^{13}C$ values between - 21.2 and - 28.7 ‰ are found in September 2003 (Figure 6a), and between - 23.9 and - 27.2 ‰ in May 2004 (Figure 6b). The $\delta^{13}C$ values of the citric acid were in the range of - 27.7 and - 24.3 ‰ in September 2003 and - 26.0 and - 29.5 ‰ in May 2004.

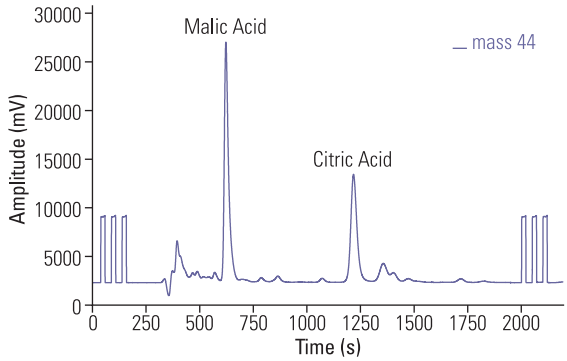

Figure 3: *irm*-LC/MS chromatogram (m/z 44 trace) of a *Poa pratensis* extract, harvest May 2004.

Poa pratensis

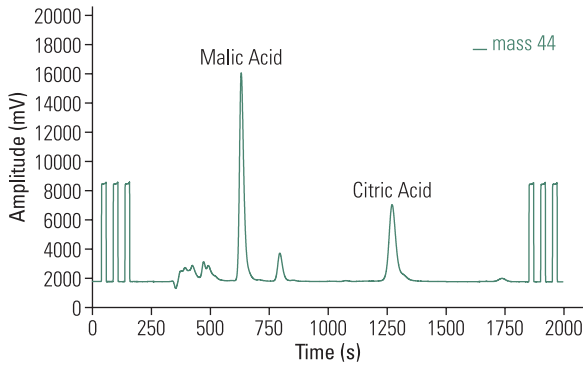

Figure 4a: *irm*-LC/MS chromatogram (m/z 44 trace) of a *Geranium pratense* extract, harvest September 2003.

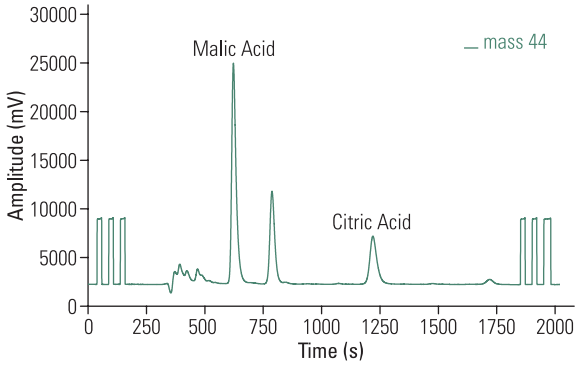

Figure 4b: *irm*-LC/MS chromatogram (m/z 44 trace) of a *Geranium pratense* extract, harvest May 2004.

Geranium pratense

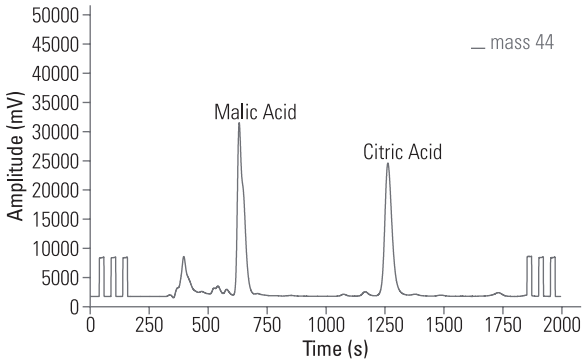

Figure 5a: *irm*-LC/MS chromatogram (m/z 44 trace) of a *Medicago lupulina* extract, harvest September 2003.

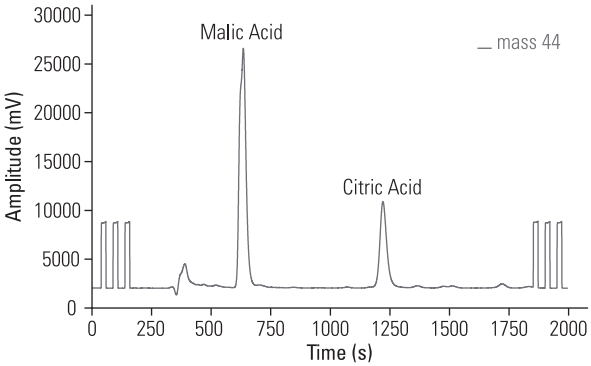

Figure 5b: *irm*-LC/MS chromatogram (m/z 44 trace) of a *Medicago lupulina* extract, harvest May 2004.

Medicago lupulina

The isotopic composition of the organic acids is characteristic for these species and the maximum difference between different kinds was up to 3.3 ‰ for plants collected in May 2004 and 7.5 ‰ for plants, collected in September 2003. These differences may result from variations in the RUBISCO and PEP carboxylase activity within different plant types or the impact of photo respiration (Ivlev, 2004).

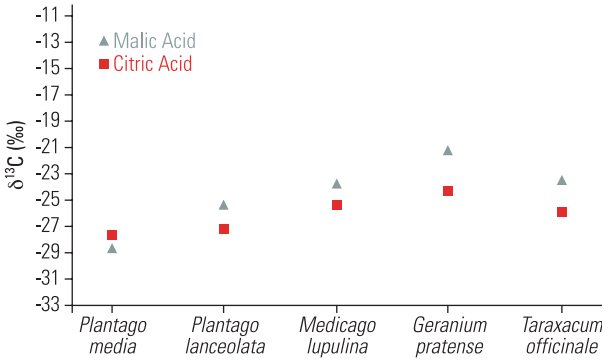

Figure 6a: $\delta^{13}C$ values of malic and citric acids in plant extracts analyzed by *irm*-LC/MS, harvest September 2003.

Vicia cracca

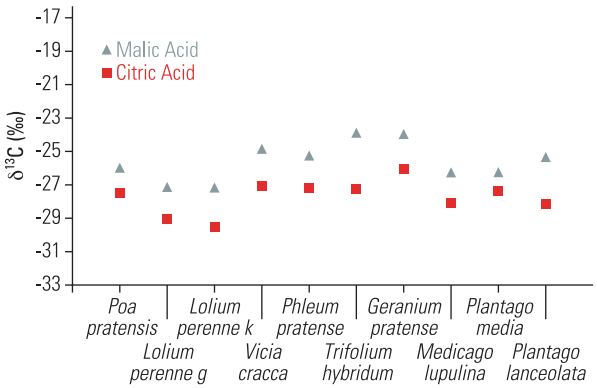

Figure 6b: $\delta^{13}C$ values of malic and citric acids in plant extracts analyzed by *irm*-LC/MS, harvest May 2004.

Generally, the $\delta^{13}C$-values of malic acid were more positive than citric acid (approximately 2.2 ‰). This was also found for leafs from potato (Gleixner et al., 1998) and tobacco plants (Jamin et al., 1997). Uptake and release of carbon in the citric acid cycle might be reasonable. On the one hand heavy carbon can to be taken up by anaplerotic reactions, catalyzed by the malic enzyme or PEP carboxylase (Melzer and O´Leary 1987). On the other hand light carbon molecules can be split off during the rearrangement of citrate followed by the release of two CO_2 molecules (Figure 7).

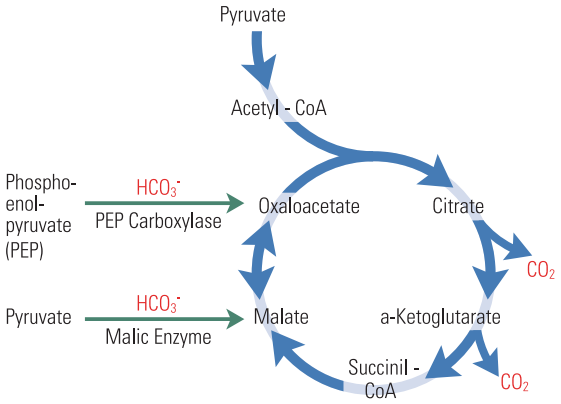

Figure 7: Anaplerotic reactions and Krebs cycle.

The use of chemical degradations of the organic acids in combination with HPLC separations of the breakdown products in the Thermo Scientific LC IsoLink will give position specific isotope ratios to differentiate between both possibilities.

Species	$\Delta = \delta^{13}C_{September\ 2003} - \delta^{13}C_{May\ 2004}$ **[‰]**	
	Malic acid	Citric acid
Geranium pratense	2.76	1.75
Medicago lupulina	2.51	2.75
Plantago media	-2.41	-0.31
Plantago lanceolata	0.00	0.98

Table 3: The change of $\delta^{13}C$ values of organic acids in plant extracts, collected in September 2003 and May 2004.

Four plant species were harvested in both seasons (*Plantago media, Plantago lanceolata, Medicago lupulina, Geranium pratense*). Table 3 reveals different seasonal alterations of the $\delta^{13}C$ values of organic acids depending on the species.

Medicago lupulina and *Geranium pratense* exhibit more negative $\delta^{13}C$ values of malic and citric acid in spring than in autumn.

Plantago lanceolata only shows a small difference of $\delta^{13}C$ values of citric acid, whereas the organic acids of *Plantago media* are more enriched in May 2004. The data support that the biochemical production of organic acids is differently influenced by plant species, environmental conditions and plant growth.

Conclusions

$\delta^{13}C$ values of organic acids can easily be analyzed by *irm*-LC/MS. The ^{13}C enrichment of malic acid in comparison with citric acid is in agreement with the hypothesis of isotope enrichment by anaplerotic reactions of the citric acid cycle. The metabolism of malic and citric acid is affected by environmental conditions, plant growth and age. The seasonal change of $\delta^{13}C$ values is characteristic for the plant species investigated.

irm-LC/MS analysis of organic acids will be applied in ongoing projects. Further investigation of several plant species of different ages under controlled environmental conditions will help to identify factors for alteration of the isotope composition of organic acids.

The new LC IsoLink interface opens a wide range of research in the cellular metabolism and this will induce a new research field within the metabolomics.

Plantago media

Plantago lanceolata

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Elena Hettmann is a biologist, interested in the use of stable isotopes of Carbon to investigate the plant metabolism and to trace the carbon flow from plants to soil and ground water. She studied Biology at the Ural State University (Yekaterinburg, Russia) and obtained diploma in Plant Physiology. At this time she is a PhD-Student at the Max Planck Institute for Biogeochemistry.

Gerd Gleixner is a biogeochemist interested in the use of stable isotopes of Hydrogen, Carbon and Nitrogen to explore the flow of elements in natural systems. Current research interests deal with metabolite fluxes in plants, plant-water relations, palaeoclimate reconstructions, and Carbon and Nitrogen cycling in soil organic matter. He obtained a PhD in agricultural science at the Technical University of Munich in the lab of H.-L. Schmidt focussing on inter- and intramolecular isotope distribution in natural compounds. At the Max Planck Institute for Biogeochemistry in the lab of E.-D. Schulze and at the Friedrich Schiller University in Jena, Germany, he was tenured for organic geochemistry. Currently he is Research Associated Professor at the Max Planck Institute for Biogeochemistry.

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