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ARTICLE

Determination of Organic Acids in Tissues and Exudates of Maize, Lupin, and Chickpea by High-Performance Liquid Chromatography−Tandem Mass Spectrometry

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This article describes a fast and simple methodology for the extraction and determination of organic acids in tissues and root exudates of maize, lupin, and chickpea by LC/MS/MS. Its main advantage is that it does not require sample prepurification before HPLC analysis or sample derivatization to improve sensibility. The results obtained showed good precision and accuracy, a recovery close to 100%, and no significant matrix effect. Moreover, the sensibility of the method is in general better than that of previously described methodologies, with detection limits between 15 and 900 pg injected.

KEYWORDS: ESI-MS/MS; ion-exclusion chromatography; organic acids determination; maize; lupin; chickpea

INTRODUCTION

Different organic acids are considered to play important roles not only in the metabolism of plants but also in the acquisition of nutrients from the rhizosphere (*1*). Thus, a number of studies showed that under specific circumstances several plants increase the biosynthesis of some organic acids (*2*, *3*), which are released to the rhizosphere in order to mobilize, via complexation or other mechanisms, certain nutrients fixed by adsorption or precipitation, such as phosphorus and micronutrients with metallic character (*4*). Consequently, organic acid analysis is a powerful tool in the diagnosis of the efficiency of the plant to obtain nutrients that are not directly available for root uptake.

Several methods for organic acid analysis have been published, in plants and other matrices, such as coffee, fruit juices, root exudates, soil, honey, water, and urine (*5*−*11*). Capillary electrophoresis with ultraviolet (*12*, *13*) or mass spectrometry detection (*14*), gas chromatography (*6*, *15*−*20*), and liquid chromatography with ultraviolet (*6*−*9*, *11*), conductivity (*21*, *22*), refractive index (*23*), electrochemical (*24*, *25*), chemiluminescence (*26*), or mass spectrometry detectors (*5*, *10*, *18*, *27*−*32*) have been employed with this aim. Currently, the most sensitive methods involve GC/MS, LC with chemiluminescence, LC/MS, and LC/MS/MS. However, some require previous separation techniques such as anionic or/and cationic exchange resins (*5*, *15*−*17*, *21*, *27*). Moreover, GC/MS methodologies involve sample derivatization (*15*, *16*, *18*−*20*), and other methods based on liquid chromatography also use derivatization techniques to reach better sensibility (*18*, *26*). Recently, an ESI-MS method (*30*) that determines carboxylic acids in *Brassica juncea* root exudates by ion-exclusion chromatography has been published.

The aim of this work is to develop a fast and simple method to extract and analyze organic acids in shoots, roots, and root exudates of plants. The plants selected for the study were lupin, chickpea, and maize. Lupin is normally used as a model plant for root exudates as it releases high concentrations of organic acids. Chickpea is another frequently used plant for this type of assays, and maize was selected because of its agricultural importance. The method involves a simple water extraction and organic acid separation and quantitative determination using LC/MS/MS, a method that combines high sensitivity with mass selectivity and provides the advantage of avoiding both time-consuming preseparation techniques and sample derivatization without losing sensibility.

MATERIALS AND METHODS

Plant Growth. The experiment was carried out in hydroponics on maize (*Zea mays* L. cv. Sancia), chickpea (*Cicer arietinum* L.), and lupin (*Lupinus albus* L.) plants germinated for 10 days in a germination chamber at 24 °C and a relative humidity of 85% to be finally cultivated in a growth chamber with a photoperiod of 16 h (250 μmol m^{-2} s^{-1}), an average temperature of 24/18 °C day/night, and a relative humidity of 60%. Seeds were placed on paper towels, moistened with ultrapure water every two days, and maintained at 25 °C in dark conditions for 10 days during germination. Subsequently, the seedlings were transferred to half-strength nutrient solution with 0.25 mM P in 4 L of opaque plastic pots for the

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Table 1. Compound Dependent Parameters of Mass Spectrometry Detection[a]

	transition		ionization and collision parameters				
compound	Q1 (uma)	Q3 (uma)	dwell time (ms)	DP (V)	EP (V)	CEP (V)	CE (V)
cis-aconitic	173	85	100	−13	−5	−14	−16
trans-aconitic	173	85	100	−13	−5	−14	−16
citric	191	87	100	−25	−4	−16	−22
iso-citric	191	73	100	−25	−4	−16	−26
fumaric	115	71	100	−15	−5.5	−10	−11
maleic	115	71	100	−15	−5.5	−10	−11
malic	133	115	100	−20	−4	−12	−13
malonic	103	59	100	−13	−4	−11	−13
oxalic	89	61	100	−17	−7	−10	−11
pyruvic	87	59	100	−15	−7	−9	−10
succinic	117	73	100	−18	−6	−12	−15
tartaric	149	87	100	−19	−7	−14	−16
d_4-succinic	121	77	100	−25	−5	−10	−18

[a] DP, declustering potential; EP, entrance potential; CEP, collision cell entrance potential; CE, collision energy.

first 3 days, and thereafter grown in full-strength nutrient solution with the P treatments applied. They were filled with 3.5 L of complete nutrient solution with continuum aeration with a pump (optimal membranpump 250 L/h). The nutrient solution, proposed according to plant nutrient requirements (*33*), consisted of 2 mM KNO_3, 4 mM $Ca(NO_3)_2$, 2.5 mM $MgSO_4$, 2 mM $CaCl_2$, 0.143 mM Fe (EDTA chelate), 0.054 mM Mn (EDTA chelate), 0.003 mM Cu (EDTA chelate), 0.045 mM Zn (EDTA chelate), 0.1 mM $Na_2B_4O_7$, 0.005 mM Mo (Na_2MoO_4), and 0.5 mM SiO_2 (to avoid etiolation problems).

The pH was adjusted to 6, and the conductivity was lower than 2 dS/m. The plants were placed on the top of the containers and supported by porexpan lids cut through the middle.

Chemicals. The organic acids analyzed were *cis*- and *trans*-aconitic, citric, iso-citric, fumaric, maleic, malic, malonic, oxalic, pyruvic, succinic, and tartaric acids. This selection was based on several studies focused on the role of plant-released organic acids in nutrient acquisition (*4*, *21*, *34*). d_4-Succinic acid, the internal standard, was purchased from Sigma-Aldrich (ref 293075). The standards *cis*- and *trans*-aconitic, citric, iso-citric, fumaric, maleic, malic, malonic, oxalic, pyruvic, succinic, and tartaric acids were obtained from Sigma-Aldrich-Fluka (ref A3412, 12.275-O, 27488, I1252, 47900, 63180, 02288, M129-6, 75688, 159740, S7501, and 251380, respectively). Acetic acid 100% employed to prepare the mobile phase was purchased from VWR (ref UN2789), and type I water was also employed to prepare the mobile phase and the standard solutions, as well as to extract the organic acids.

Sample Extraction. The extractants tested were 100% water, 90% water/10% methanol, and aqueous 0.1% acetic acid. The extraction methods assayed were 0.5 to 12 h shaking or Ultra-Turrax homogenization at 24000 rpm for 1 to 2 min. The finally selected procedure is described briefly: shoot and root fresh samples were harvested and immediately homogenized in a mill (IKA A11 basic) with liquid nitrogen, and stored at −80 °C in a Sanyo ultra low fridge. For the analysis, 0.1 g of the stored frozen tissue sample was weighed in 50 mL of polypropylene tubes (Sarstedt, ref 62.559). One hundred microliters of a 400 mg L^{-1} solution of the internal standard in water was added. Then, it was diluted with 40 mL of type I water and was shaken in a Heidolph Multi Reax 2 model vortex mixer at the maximum speed of 2025 rpm during 90 min for roots and 240 min for shoots. The extraction ratio selected (1:400) permitted both complete extraction and adequate concentration ranges. Subsequently, an aliquot was filtered through 0.45 μm polypropylene filters (Teknokroma, ref TR-200509) and analyzed directly by LC/MS/MS. The extractions were carried out at 22 °C. As far as root exudates are referred, roots were washed with type I water, and exudates were collected by immersion of root systems into 250 mL of aerated trap solutions of 0.5 mM $CaSO_4 \cdot 2H_2O$ for 2 h (*22*, *35*, *36*), and the solution was stored at −80 °C. Prior to analysis by LC/MS/MS, samples were thawed and filtered through 0.45 μm polypropylene filters. Four milliliters of each filtered sample were separated, and 10 μL of the internal standard was added to be finally transferred to chromatographic vials and analyzed.

Separation and Detection of Organic Acids by LC/MS/MS. The chromatographic separation was carried out with an ion-exclusion column and the detection with an ESI-MS/MS method. Mass spectrometry detection parameters are shown in **Table 1**. **Figures 1** and **2** show the extracted chromatograms of a standard and lupin root, respectively.

Analytical Performance Characteristics. *Linearity*. The linearity was tested for standard solutions taking into account the low matrix effect as described below. Eight points were used for the calibration with four replicates for each concentration.

Limits of Detection (LOD) and Quantification (LOQ). The limits of detection and quantification in standards and plant extracts were calculated with a signal-to-noise ratio of 3 and 10, respectively, and were verified with standards of known concentrations on the order of these limits.

Precision. The precision of the method was evaluated by repeated analyses of quality control samples on different days. In this test, low, mid, and high concentrations of the calibration curves for each organic acid were analyzed through several replicates during 3 days. Low concentrations ranged from 0.01 to 0.1 mg L^{-1}, mid concentrations ranged from 0.1 to 0.25 mg L^{-1}, and the high concentration was 1 mg L^{-1}. The results were used to calculate intraday (repeatability) and day-to-day (reproducibility) precision.

Accuracy. The accuracy is calculated with the same concentrations analyzed in the repeatability study.

Recovery and Matrix Effect. In order to evaluate the recovery and matrix effect, the following study was carried out. To study the matrix effect, four samples of the plant tissue extract (root and shoot) and exudate were analyzed. The same samples were also analyzed spiking them after extraction with acid concentrations similar to those found in these samples. The matrix effect, expressed by the matrix factor, was calculated as the ratio of the difference between the spiked and nonspiked ones and the standards used for spiking. A value higher than unity means that the matrix causes an increase in the signal, and a result lower than unity indicates a decrease in the signal.

In order to study the recovery, four samples were analyzed without spiking and after having spiked before and after extraction with the same acid concentrations used for the matrix effect. The recovery was calculated as the ratio of the difference between the spiked before extraction and nonspiked ones, and

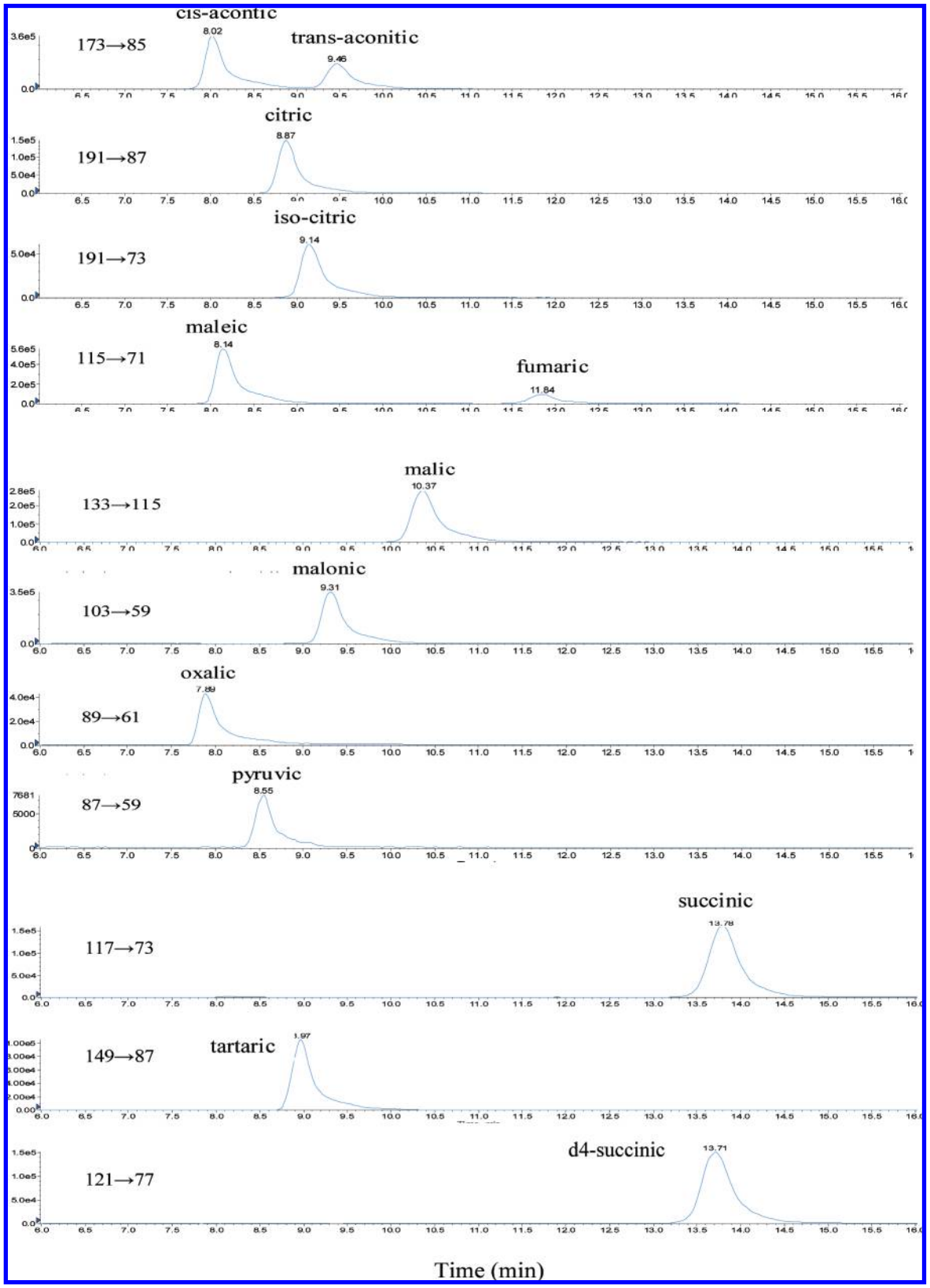

Figure 1. Extracted ion chromatograms of a standard (1 mg L^{-1}) of organic acids.

the difference between the spiked after extraction and nonspiked ones, and expressed as percentage.

Instrumentation. *Heidolph Multi Reax 2*. The Heidolph Multi Reax 2 vortex mixer was used as described.

HPLC-MS-MS. HPLC system: Alliance HT Waters 2795 (Waters, Mildford MA, USA). The separation column was a Rezex RHM-Monosaccharide H+ (8%) (300 × 7.8 mm) (ref OOH-0132-KO, Phenomenex, Torrance, CA, USA). The mobile phase consisted of aqueous acetic acid 0.1% (pH 3.20). The eluent flow rate was 0.5 mL/min, column oven temperature was maintained at 40 °C, and injection volume was 30 μL. Sample temperature was 20 °C.

Detection was carried out using a Q TRAP 3200 mass spectrometer (Applied Biosystems, MDS Sciex, Concord,

Ontario, Canada) equipped with a turboionspray (TIS) interface operating in the negative ion mode (NI).

Source parameters: temperature, 400 °C; CUR (curtain gas flow), 20.00 psi; IS (ion spray voltage), −4000.00 V; GS1 (nebulizer gas flow), 60.00 psi; GS2 (heater gas flow), 50.00 psi. Mass analyzer parameters: scan mode, multiple reaction monitoring (MRM); resolution Q1, unit; resolution Q3, unit; CAD gas (collision activated dissociation), medium; CXP (collision cell exit potential), −4.00 V.

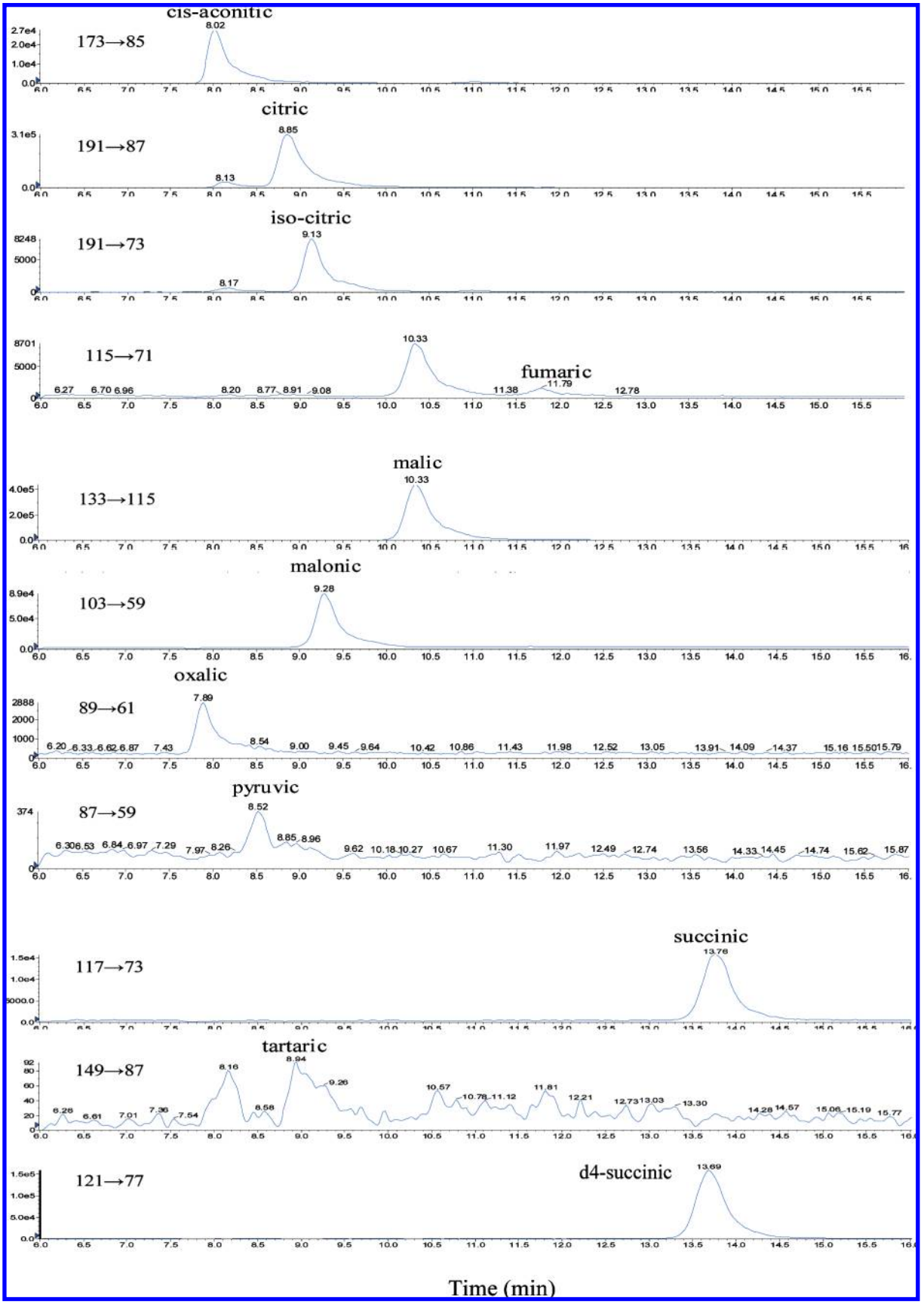

Figure 2. Extracted ion chromatograms of a water extracted sample of lupin root.

Identification of fragments for each organic acid and optimization of compound-dependent parameters were accomplished by direct infusion of 10 mg L^{-1} solutions of the acids with a syringe pump into turboionspray interface. The parameters are described in **Table 1**.

Detector parameters: CEM (channel electron multiplier), 2400.0 V. Software version, Analyst 1.4.2.

RESULTS AND DISCUSSION

Separation and Detection of Organic Acids by LC/MS/MS. In a preliminary study, different columns and mobile phases were tested.

A C18 column (150 × 2.00 mm, 4 μm) with good resolution of highly polar compounds under 100% aqueous mobile phase conditions was assayed with mobile phases of acetic or formic acids between 0.1 and 0.5%, and methanol between 0 and 10%. The results obtained showed low chromatographic retentions, which could increase the importance of matrix effect, and poor resolutions.

A column with hydrophilic interaction liquid chromatography (HILIC, 150 × 2.00 mm, 3 μm) separation mode was proved with different mobile phases recommended by the manufacturer of the column. Whereas good results were achieved for certain acids, such as fumaric, maleic, malonic, succinic, and *cis*- and *trans*-aconitic, bad results were obtained for the rest.

A C8 column (250 × 4 mm, 5 μm) was tested with mobile phases of acetic or formic acid between 0.1 and 0.5%, and methanol between 5 and 20%. The best results corresponded to a gradient of formic acid 0.5% and methanol. The resolution of the majority of the acids was good except for pyruvic, oxalic, and tartaric acids, its determination being impossible at concentrations lower than 0.25, 10, and 0.5 mg L^{-1}, respectively.

A column for carbohydrate and organic acid analysis (Rezex RHM-Monosaccharide), similar to that used by several authors (*10*, *30*, *31*) was assayed with mobile phases of acetic or formic acids between 0.1 and 0.5%, and methanol between 0 and 5%. Higher signal intensity was obtained with acetic acid, especially at 0.1%. This is in line with previous studies (*30*). Inclusion of methanol in the mobile phase did not improve nor did chromatographic signal intensity and peak resolution. A flow rate of 0.5 mL/min was used according to maximum pressure of the column. This column proportioned good resolution for the studied acids with the conditions previously described (**Figures 1** and **2**). Citric and iso-citric peaks were not totally resolved with this column using the main transition for both acids (191 → 111), but they can be perfectly determined using the transitions 191 → 87 for citric acid and 191 → 73 for iso-citric acid, although it involves a decrease in sensibility.

Different mass modes were assayed: MS scan technique with Q1 scan mode or Q1 multiple ion scan mode and MS/MS scan technique with multiple reaction monitoring (MRM) and LIT scan mode with enhance mass scan (EMS). MRM was selected due to its better selectivity and sensibility.

Sample Extraction. As regards the extractants, the best results were obtained with 100% water, especially for *cis*-aconitic, iso-citric, and fumaric acids. As for the extraction procedure, the results were similar, but the stability of the extract was better for shaking extraction. A certain degradation of citric acid was observed at extraction times above 1.5−2 h for roots, without increasing the extractions of the rest of the acids. However, in shoots, there was an increase of the extractions of some organic acids during the first 4 h, without degradation problems. Therefore, different extraction times were used for each tissue. Filtered extracts were stable at 20 °C at least during 24 h.

Analytical Performance Characteristics. *Linearity*. The equations and correlation coefficients of calibration curves are presented in **Table 2**. As can be observed, the correlation coefficients (R values) obtained were all over 0.998. The regression equations are of a polynomial form.

Limits of Detection (LOD) and Quantification (LOQ). The results obtained ranged from 0.5−30 μg L^{-1} for LOD and 2−100 μg L^{-1} for LOQ in standards and exudates, and 0.4−12 mg kg^{-1} for LOD and 1.2−40 mg kg^{-1} for LOQ in tissues depending on the acid (**Table 3**). In general, these

Table 2. Equations of Calibration Curves and Linearity

organic acids	calibration curves	R
cis-aconitic	$y = -0.831x^2 + 2.25x + 0.0036$	>0.998
trans-aconitic	$y = -0.0952x^2 + 0.827x + 0.00008$	>0.999
citric	$y = -0.0306x^2 + 0.644x + 0.0032$	>0.999
iso-citric	$y = -0.0422x^2 + 0.438x + 0.00135$	>0.999
fumaric	$y = 0.0718x^2 + 0.406x - 0.000003$	>0.999
maleic	$y = -0.831x^2 + 2.25x + 0.0040$	>0.999
malic	$y = -0.398x^2 + 1.81x + 0.0036$	>0.999
malonic	$y = -0.232x^2 + 1.56x + 0.00238$	>0.999
oxalic	$y = 0.0302x^2 + 0.125x - 0.00039$	>0.999
pyruvic	$y = 0.00134x^2 + 0.0214x + 0.00003$	>0.998
succinic	$y = 0.0274x^2 + 0.933x - 0.0004$	>0.999
tartaric	$y = -0.246x^2 + 0.67x + 0.0009$	>0.998

Table 3. Detection (LOD) and Quantification (LOQ) Limits

	standards		shoots and roots		exudates	
organic acids	LOD (μg mL^{-1})	LOQ (μg mL^{-1})	LOD (μg g^{-1}FW)	LOQ (μg g^{-1}FW)	LOD (μg mL^{-1})	LOQ (μg mL^{-1})
cis-aconitic	0.5	2.0	0.4	1.2	1.0	3.0
trans-aconitic	2.0	5.0	0.8	2.0	2.0	5.0
citric	2.0	6.0	1.2	4.0	3.0	8.0
iso-citric	5.0	15.0	2.0	6.0	5.0	15.0
fumaric	5.0	15.0	2.0	6.0	5.0	15.0
maleic	0.5	2.0	0.4	1.2	1.0	3.0
malic	1.0	4.0	0.4	1.6	1.0	4.0
malonic	3.0	8.0	1.2	3.2	3.0	8.0
oxalic	15.0	50.0	6.0	20.0	15.0	50.0
pyruvic	30.0	100.0	12.0	40.0	30.0	100.0
succinic	3.0	10.0	1.2	4.0	3.0	10.0
tartaric	1.0	4.0	0.4	1.6	1.0	4.0

Table 4. Precision and Accuracy (%) of the Method

	low concentration			mid concentration			high concentration		
	repeatability	reproducibility	accuracy	repeatability	reproducibility	accuracy	repeatability	reproducibility	accuracy
cis-aconitic	12.6	7.7	−7.8	1.0	0.9	8.5	1.2	2.2	8.6
trans-aconitic	6.3	5.7	−1.7	0.9	4.8	−8.7	1.1	2.7	−12.8
citric	6.4	6.7	1.9	1.7	3.4	1.2	2.1	3.9	3.7
iso-citric	6.3	4.1	4.6	2.1	3.5	4.2	2.2	2.4	7.2
fumaric	2.8	5.4	−2.3	1.8	4.0	−2.0	1.5	1.5	2.0
maleic	3.7	5.4	−12.0	0.5	1.3	2.5	1.4	1.5	8.6
malic	4.4	4.3	−2.0	1.1	0.9	2.0	1.2	1.7	7.2
malonic	3.8	5.9	−13.8	3.0	2.7	−4.0	0.9	1.6	−4.8
oxalic	4.0	11.5	−10.0	3.6	3.5	−12.8	1.1	1.7	−6.0
pyruvic	5.0	3.9	−5.8	5.5	5.0	−12.2	2.5	2.2	−6.3
succinic	5.0	3.4	7.0	0.9	1.5	3.4	0.6	0.5	8.6
tartaric	4.6	5.2	7.5	2.0	1.4	7.7	1.8	1.9	11.8

Table 5. Matrix Factors of Organic Acids in Exudates, Shoot, and Root Samples of Maize, Chickpea, and Lupin

	maize			chickpea			lupin		
	exudates	roots	shoots	exudates	roots	shoots	exudates	roots	shoots
cis-aconitic	1.02	1.11	1.15	0.98	0.88	0.91	0.43	0.93	0.98
trans-aconitic	0.93	0.95	0.89	1.01	0.89	0.85	0.98	0.92	0.86
citric	0.93	0.83	0.85	0.93	0.98	0.93	0.85	0.94	0.86
iso-citric	0.97	0.93	0.95	0.99	0.89	0.89	1.00	1.01	0.97
fumaric	1.02	0.86	0.89	1.01	0.89	0.82	1.01	0.84	0.74
maleic	0.84	1.15	1.08	0.87	1.01	1.12	0.75	1.02	1.08
malic	1.05	1.12	0.89	0.87	1.01	0.98	0.96	0.96	0.79
malonic	1.04	1.02	0.97	0.93	1.11	0.98	1.00	1.03	0.95
oxalic	0.76	1.01	0.96	1.11	0.92	0.89	0.93	1.09	1.25
pyruvic	0.98	0.99	1.00	1.14	0.91	1.01	0.96	0.98	1.02
succinic	1.03	0.96	0.97	0.99	0.91	1.00	1.00	0.98	0.98
tartaric	0.91	0.93	0.83	0.58	0.90	0.79	0.91	0.90	0.89
d_4-succinic	0.96	0.96	0.96	0.96	0.96	0.93	1.01	1.02	1.02

Table 6. Recovery (%) of Organic Acids in Shoot and Root Samples of Maize, Chickpea, and Lupin

	maize		chickpea		lupin	
	roots	shoots	roots	shoots	roots	shoots
cis-aconitic	100.3	96.5	99.3	75.9	101.9	79.6
trans-aconitic	103.6	84.8	98.7	94.1	102.3	97.2
citric	105.5	97.5	103.9	92.7	103.9	98.9
iso-citric	97.7	95.5	100.9	71.5	103.9	93.8
fumaric	102.7	100.4	99.0	96.1	104.8	92.9
maleic	99.4	99.6	99.3	103.6	103.9	101.6
malic	105.2	92.4	96.2	95.7	108.0	103.6
malonic	89.7	98.8	94.0	96.8	98.3	93.4
oxalic	114.8	111.1	100.6	97.8	109.4	102.2
pyruvic	94.4	92.5	96.7	92.7	93.6	86.2
succinic	100.9	96.7	96.3	99.4	100.0	101.7
tartaric	100.9	99.3	98.4	100.7	101.3	105.5
d_4-succinic	98.5	96.9	98.7	98.3	99.6	94.4

values are better than others described in the literature (*10*, *28*−*32*).

Precision. All RSD values for repeatability and reproducibility were below the maximum accepted value of 15% (*37*) as can be observed in **Table 4**.

Accuracy. In all cases, the results obtained, shown in **Table 4**, were below the maximum accepted value of 15% (*37*), thus showing the good accuracy of the analytical method.

Recovery and Matrix Effect. The results, shown in **Table 5**, indicate that the matrix effect is rather low. Only in some cases, a small matrix effect that can be considered irrelevant can be appreciated. Regarding the recovery of the different acids, in general the results obtained showed a complete recovery (**Table 6**).

Maximum values of organic acids measured in roots, shoots, and exudates were 1800, 4800, and 12 μg g^{-1} FW, respectively, for maize, 5300, 5600, and 120 μg g^{-1} FW, respectively, for chickpea, and 3700, 11400, and 73 μg g^{-1} FW, respectively, for lupin.

In conclusion, the method provides a good extraction and separation of the organic acids studied. Moreover, the sensibility is very high, achieving better detection limits than other published techniques. The principal advantage of the method is that it does not require pretreatment to purify the samples before HPLC analysis, nor does it require sample derivatization to improve sensitivity.

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