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Development of a New High-Performance Liquid Chromatography−Electrospray Ionization Time-of-Flight Mass Spectrometry Method for the Determination of Low Molecular Mass Organic Acids in Plant Tissue Extracts

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Supporting Information

ABSTRACT: A liquid chromatography−electrospray ionization time-of-flight mass spectrometry method has been developed for the direct and simultaneous determination of 10 low molecular mass organic acids in different plant tissue extracts. The method does not include a derivatization step. Quantification was accomplished using ^{13}C-labeled malic and succinic acids as internal standards. Good limits of detection (0.05−1.27 pmol) were obtained for malic, 2-oxoglutaric, succinic, quinic, shikimic, *cis*-aconitic, and citric acids, whereas for oxalic, ascorbic, and fumaric acids limits of detection were 255, 25, and 11 pmol, respectively. Repeatability values for the retention time and peak area were <5%, with the exception of ascorbic acid. Analyte recovery was between 92% and 110% in most cases, with the exception of oxalic (39−108%), 2-oxoglutaric (44−69%), and ascorbic (22−86%) acids, which may require specific extraction procedures and use of the corresponding ^{13}C-labeled organic acid as internal standards to improve accuracy. The method was applied to three types of plant materials: sugar beet leaf extracts, tomato xylem sap, and commercial orange juice.

KEYWORDS: carboxylates, mass spectrometry, organic acids, plant tissues

INTRODUCTION

Low molecular mass organic acids are involved in essential pathways in plant metabolism. For instance, citrate, malate, succinate, fumarate, and 2-oxoglutarate are carboxylate intermediates in the Krebs cycle, the central energy-producing pathway of the cell. This metabolic route not only provides reducing power to the cell, but also supplies important precursors for the synthesis of other biomolecules.[1] 2-Oxoglutarate is also a key metabolite for proper balancing of the carbon/nitrogen metabolism,[2] whereas shikimic acid is a precursor for aromatic amino acids, flavonoids, alkaloids, and lignin,[3] and ascorbate takes part in cellular redox control and antioxidative activities.[4] Oxalate, malate, and citrate are also involved in metal detoxification[5] and/or transport.[6] Furthermore, organic acids play important roles in the metabolic response and adaptation of plants to different micronutrient stresses such as Fe[7] and P[8] deficiencies and determine the organoleptic properties of fruit juices and other fresh produce.[9] The concentrations of carboxylates in plant tissues cover from the nanomole per kilogram to the millimole per kilogram fresh weight ranges, depending on the plant tissue and experimental conditions involved.

Many methods have been developed for the determination of low molecular mass carboxylates in a wide variety of complex matrixes, including plant tissue extracts.[10−14] Some of these methods tackle the direct determination of organic acids in sample extracts without any previous separation step. For instance, enzymatic assays using UV−vis detection have been used for the determination of some carboxylates; however, these techniques usually target a specific compound and cannot be used to determine the whole range of carboxylates occurring in the sample.[14−15] Most of the methods used nowadays combine a first separation step and a second detection one. Commonly used separation techniques are capillary zone electrophoresis, gas chromatography (GC), and high-performance liquid chromatography (HPLC), and the detection technique most often used is UV−vis spectrophotometry. However, UV−vis has a poor specificity due to the fact that many of the compounds present in the complex matrixes of sample extracts, which include carboxylates and others, can have very similar spectra.[16] Also, the relatively low UV−vis extinction coefficients of organic acids have led to the use of derivatization procedures.[17]

More recently, the analysis of carboxylates has been tackled using GC and HPLC coupled to mass spectrometry (MS) detection-based techniques. Mass spectrometry detection provides major advantages over UV−vis for the determination of carboxylates in complex matrixes that contain a large number of interfering compounds. First, selectivity is highly improved, especially when using high-resolution MS detection techniques such as time-of-flight (TOFMS); the good mass accuracy provided by high-resolution MS techniques allows for the unambiguous elucidation of molecular formulas in the case of small analytes such as carboxylates. This technique has been used for the determination of carboxylates in different plant tissues and plant exudates.[18,19] Furthermore, sensitivity is also frequently improved when using MS detection as compared to UV−vis, as shown for shikimic acid.[18] Derivatization has been used to further improve the

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chromatographic separation of carboxylates and ESI ionization efficiency, leading to selectivity and sensitivity increases.[19,20] Recently, derivatization with isotope-labeled tags has been used in metabolomic analysis, with a focus on profiling carboxylic acid-containing metabolites.[21] The occurrence of isobaric carboxylate compounds such as citric and quinic acids (both having an M_m of 192.1) can make necessary, if mass resolution is not sufficient, either a previous separation of these compounds or the use of molecular fragmentation MS techniques such as selected reaction monitoring (SRM). For instance, SRM has been used to determine carboxylates in different plant species and tissues by quadrupole ion trap MS[10] and in natural waters by triple-quadrupole MS.[22]

Although some recent electrospray ionization (ESI)-MS-based methods do not use internal standards (ISs),[18,23,24] such standards are needed to warrant the reproducibility and accuracy of any HPLC−ESI-MS analysis, especially when analyzing complex matrixes.[25,26] This is due to the nature of the ionization process required to couple the HPLC and MS devices, since analyte ionization efficiency not only is strongly affected by coeluting compounds, but also can change during day-to-day operation. Compounds used as ISs should have chemical characteristics as close as possible to those of the analytes, and for this reason stable isotope-labeled compounds are commonly used. For instance, D-labeled succinic acid[10] and ^{13}C-labeled oxalic and citric acids[19] have been recently used as ISs in the analysis of carboxylates in different plant materials by HPLC−ESI-MS.

The main aim of this study was to develop and validate a new HPLC−ESI-TOFMS method for the direct determination of low molecular mass organic acids in plant tissue extracts. The method does not include any derivatization step and is based on an isocratic HPLC separation and a high-resolution mass detection (TOFMS) using ^{13}C-labeled malic and succinic acids as ISs. The method has been applied to determine the concentrations of several carboxylates in different plant tissue extracts and fluids, including leaf extracts, xylem sap, and orange juice.

MATERIALS AND METHODS

Reagents. Oxalic, 2-oxoglutaric, *cis*-aconitic, malic, quinic, shikimic, fumaric, formic, and metaphosphoric (MPA) acids were purchased from Sigma (St. Louis, MO), succinic acid was obtained from Merck (Darmstadt, Germany), and citric and ascorbic acids were purchased form Riedel-de Häen (Seelze, Germany). Carbon-13-labeled malic ([$^{13}C_4$]malic acid, ^{13}C-malic acid) and succinic ([1,4-$^{13}C_2$]succinic acid, ^{13}C-succinic acid) acids were purchased from Cambridge Isotope Laboratories (Andover, MA). All extraction buffers, standard solutions, and mobile phases were prepared with analytical-grade type I water (Milli-Q Synthesis, Millipore, Bedford, MA).

Standard Solutions. Stock solutions of 10 mM oxalic (M_m = 90.0), *cis*-aconitic (M_m = 174.1), 2-oxoglutaric (M_m = 146.1), citric (M_m = 192.1), malic (M_m = 134.1), quinic (M_m = 192.2), ascorbic (M_m = 176.1), shikimic (M_m = 174.1), succinic (M_m = 118.1), and fumaric (M_m = 116.1) acids and the ISs ^{13}C-malic acid (M_m = 137.1) and ^{13}C-succinic acid (M_m = 120.1) were prepared in 0.1% (v/v) formic acid in water. Aliquots of each stock were conserved at −20 °C until analysis.

Plant Material. Leaves of sugar beet (*Beta vulgaris* L.), xylem of tomato (*Solanum lycopersicum* L.), and commercial orange juice were chosen to cover a wide range of analytes, analyte concentrations, and matrix compositions. Sugar beet and tomato plants were grown for 1 month in a controlled-environment growth chamber (16 h light/8 h dark photoperiod, 24 °C day/20 °C night). Tomato xylem sap was sampled by detopping shoots 3 cm above the cotyledons. The first xylem sap drops were discarded to avoid contamination, the cut surface was wiped dry, and xylem sap was collected for 20 min in eppendorf tubes kept on ice and immediately stored at −80 °C. Leaves of *B. vulgaris* were frozen in liquid N_2 and stored at −80 °C.

Organic Acid Extraction. Sugar beet leaves (100 mg of frozen powder) were supplemented with ^{13}C-malic and ^{13}C-succinic acids (200 nmol each), 1 mL of 4% cold MPA was added, and homogenization was carried out with an MM301 ball mill (Restch, Düsseldorf, Germany) for 3 min at 30 Hz. Homogenates were centrifuged at 15000*g* for 20 min at 4 °C, supernatants were collected, and the pellets were extracted again by vortexing with 4% MPA for 3 min. After centrifugation at 15000*g* for 5 min at 4 °C, both supernatants were pooled and then filtered through 0.22 μm poly(vinylidene fluoride) (PVDF) membranes. Leaf extracts were taken to a final volume of 2 mL with mobile phase (0.1% (v/v) formic acid) and analyzed immediately by HPLC−ESI-TOFMS. Five replications of each extract were made.

Tomato xylem sap and orange juice were diluted 5- and 20-fold, respectively, with 1 mL of 4% (w/v) cold MPA. At this time, the ISs ^{13}C-malic (200 nmol) and ^{13}C-succinic (200 nmol) acids were added, samples were extracted by vortexing for 3 min and centrifuged at 15000*g* for 5 min at 4 °C, and supernatants were collected and then filtered through 0.22 μm PVDF membranes. Extracts were taken to a final volume of 2 mL with 0.1% (v/v) formic acid and analyzed immediately. Five replications of each extract were made.

HPLC−ESI-TOFMS Analysis. Analyses were carried out with a micrOTOF II ESI-TOFMS apparatus (Bruker Daltonik, Bremen, Germany) coupled to an Alliance 2795 HPLC system (Waters, Milford, MA). To optimize the ESI-TOFMS signal, a direct injection of a combined standard solution (10 μM concentration of each carboxylate in 0.1% (v/v) formic acid) was carried out using a syringe pump (Cole-Parmer, Vernon Hills, IL) operated at 180 $\mu L \cdot min^{-1}$. All MS analyses were done in negative ionization mode. The nebulizer gas (N_2) pressure and drying gas (N_2) flow were kept at 1.6 psi and 8.0 $L \cdot min^{-1}$. The mass axis was calibrated, externally and internally, using Li−formate adducts (10 mM LiOH, 0.2% (v/v) formic acid, and 50% (v/v) 2-propanol). The internal mass axis calibration was carried out by introducing the solution containing Li−formate adducts with a divert valve at 3 and 17 min in the HPLC run. Spectra were acquired in the mass/charge range of 80−300 *m/z*. HPLC separation was carried out in a Supelcogel H 250 × 4.6 mm anion exchange column packed with a matrix of sulfonated polystyrene/divinylbenzene (Sigma). The autosampler and column temperatures were 6 and 30 °C, respectively. Samples (20 μL) were eluted isocratically at a flow rate of 200 $\mu L \cdot min^{-1}$ for 20 min using a mobile phase of 0.1% formic acid and 5% 2-propanol in water. The system was controlled with the software packages microTOF control 1.2 and HyStar 3.0 (Bruker Daltonik). Data were processed with Data Analysis 3.4 software (Bruker Daltonik). The Sigma Fit (Bruker Daltonik) algorithm was used for molecular formula determination.[27]

Validation was carried out using standard solutions of the different organic acids prepared in 2% MPA in 0.1% formic acid to obtain calibration curves corrected with the ISs ^{13}C-malic and ^{13}C-succinic acids to account for possible losses during sample extraction and also for possible variations in ESI efficiency. ^{13}C-malic acid (100 μM) was used for the quantification of oxalic, *cis*-aconitic, 2-oxoglutaric, citric, malic, quinic, and ascorbic acids, and ^{13}C-succinic acid (100 μM) was used for the quantification of shikimic, succinic, and fumaric acids. Limits of detection (LOD; defined as the amount of analyte that gives an S/N ratio of 3) and quantification (LOQ; defined as the amount of analyte that gives an S/N ratio of 10) were calculated using standards in extraction solution. Intra- and interday repeatabilities were assessed in 10 consecutive chromatographic runs for retention time and peak area using a 100 μM standard solution of each acid and also tomato xylem sap spiked with a combined standard solution with all carboxylates at a concentration of 80 μM. The interday repeatability was assessed by analyzing the same samples during

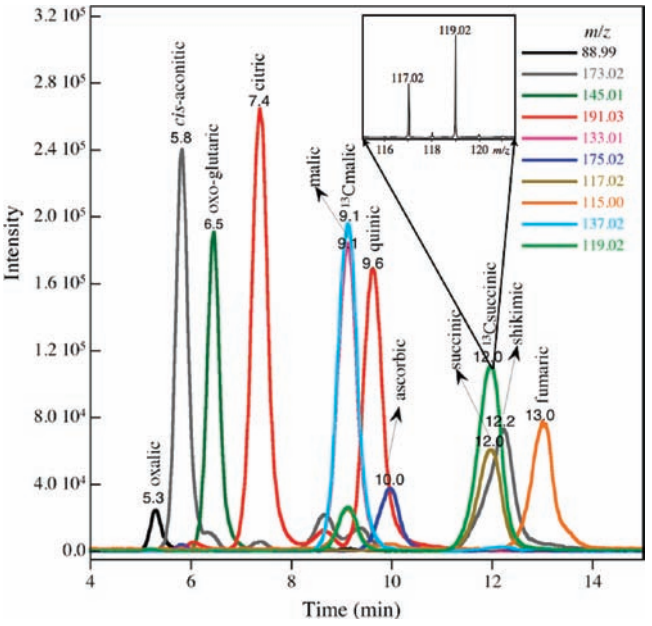

Figure 1. HPLC−ESI-TOFMS chromatogram of a mixture of organic acid standards. The concentration of all organic acids (including the internal standards) was 100 μM. Chromatogram traces correspond to the $[M - H]^-$ ± 0.03 m/z of each organic acid. The inset shows a zoomed mass spectrum, extracted at a 12 min retention time, with the $[M - H]^-$ peaks corresponding to unlabeled and ^{13}C-labeled succinic acid.

five consecutive days. For recovery assays, plant samples (sugar beet leaves, tomato xylem sap, and orange juice) were spiked with 200 nmol of each organic acid and extracted as described above.

RESULTS AND DISCUSSION

HPLC−ESI-TOFMS Analysis. Ionization parameters were optimized in negative mode using direct infusion of standard solutions containing all organic acids. Optimal values leading to a maximum signal intensity for most of the organic acids were (V) −500 for end plate offset, 3000 for the capillary, −82.1 for capillary exit, −41.4 for skimmer 1, and 80.0 for the hexapole radio frequency (rf). The ESI-TOFMS spectra of standard solutions gave major $[M - H]^-$ ions with m/z values of 89.98 for oxalic acid, 173.01 for *cis*-aconitic acid, 173.05 for shikimic acid, 145.01 for 2-oxoglutaric acid, 191.02 for citric acid, 191.06 for quinic acid, 133.01 for malic acid, 175.02 for ascorbic acid, 117.02 for succinic acid, 115.00 for fumaric acid, 137.01 for ^{13}C-malic acid, and 119.02 for ^{13}C-succinic acid.

Chromatographic separation of the different organic acids studied was carried out in approximately 14 min (Figure 1), in line with other recently published HPLC−MS methods. All peaks obtained were sharp, and most organic acids had a near-baseline separation, except for the pairs succinic/shikimic and quinic/ascorbic, which had a partial overlapping (Figure 1). The two ISs coeluted with the corresponding unlabeled compounds (Figure 1). On the other hand, the two isobaric couples, citric/quinic (with m/z 191.02 and 191.06, respectively) and *cis*-aconitic/shikimic (with m/z 173.01 and 173.05, respectively), were well separated (Figure 1). The high mass resolution of the TOFMS spectra is shown in the inset of Figure 1, which depicts the isotopic signatures of unlabeled and ^{13}C-labeled succinic acid.

Table 1. Molecular Formula of the Organic acids Analyzed by HPLC-ESI-TOFMS and the Theoretical and Experimental Mass-to-Charge (m/z) ratios of the corresponding $[M-H]^-$ Ions

organic acid	molecular formula	theoretical m/z	experimental m/z	error (mDa)
oxalic	$C_2H_2O_4$	88.9880	88.9849	3.17
cis-aconitic	$C_6H_6O_6$	173.0092	173.0083	0.90
2-oxoglutaric	$C_5H_6O_5$	145.0142	145.0131	1.18
citric	$C_6H_8O_7$	191.0184	191.0185	1.25
malic	$C_4H_6O_5$	133.0142	133.0136	0.69
quinic	$C_7H_{12}O_6$	191.0561	191.0559	0.24
ascorbic	$C_6H_8O_6$	175.0248	175.0248	0.06
shikimic	$C_7H_{10}O_5$	173.0455	173.0464	0.86
succinic	$C_4H_6O_4$	117.0193	117.0190	0.33
fumaric	$C_4H_4O_4$	115.0037	115.0029	0.75

The errors in m/z determination were always below 1.3 mDa, with the exception of the organic acid with the lowest m/z, oxalic acid (3.2 mDa) (Table 1).

Validation of the HPLC−ESI-TOFMS Method. For validation of the method, standard solutions of the different carboxylates were prepared in 2% MPA in 0.1% formic acid, and calibration curves in the 0.1−1000 μM range were obtained after correction with the corresponding IS. Different ranges of linearity were observed for the organic acids analyzed (all correlation coefficients (R^2) obtained were higher than 0.992), and the best results were obtained for *cis*-aconitic, 2-oxoglutaric, malic, and succinic acids, which had linearity ranges from 0.1−0.4 to 1000 μM (Table 2). In the cases of oxalic, citric, ascorbic, and fumaric acids, linearity ranges were from 0.3−10.0 to 500 μM. Linearity ranges for quinic and shikimic acids covered only from 0.2 to 70 μM and from 0.1 to 100 μM, respectively (Table 2). Linear ranges obtained cover the carboxylate concentrations usually found in plant tissues.[17,28]

LODs were between 0.05 pmol (for malic acid) and 255 pmol (for oxalic acid), whereas LOQs ranged between 2.80 pmol (for citric acid) and 337 pmol (for oxalic acid) (Table 2). For most of the organic acids measured, LODs and LOQs were below 2 and 5 pmol, respectively (Table 2). For 2-oxoglutaric, malic, succinic, and shikimic acids, sensitivity was better than those of previous HPLC−MS methods[10,19,22,24] (see Supplementary Table 1 in the Supporting Information). For some of the acids analyzed (e.g., citric and malic acids) the sensitivity obtained with this method was 3 orders of magnitude better than those of other methods. For citric, malic, shikimic, and ascorbic acids, the sensitivity was even better than those of methods targeted to the determination of only one or two carboxylates,[18,23,25] whereas for quinic acid the LOD (0.85 pmol) was of the same order of magnitude as that of another HPLC−MS method.[30] The high LOD of oxalic acid when compared with those of the other organic acids is due to poor ionization efficiency, as indicated by the differences in the calibration curve slopes (Table 2).

The intraday repeatability was assessed for retention time and peak area using a combined standard solution containing all carboxylates (at 100 μM each) in 10 consecutive chromatographic runs (Table 3). The interday repeatability was assessed by analyzing the same standard during five consecutive days. The relative standard deviation (RSD) for peak retention time was always lower than 0.2% and 0.4% in the intra- and interday tests,

Table 2. Calibration Data, LODs, and LOQs Obtained for the Different Organic Acids[a]

	calibration data			LOD		LOQ	
organic acid	linearity range	equation	R^2	μM in solution	pmol injected	μM in solution	pmol injected
oxalic	10.0−500.0	$y = 0.0006x - 0.0069$	0.995	12.8	255	16.9	337
cis-aconitic	0.2−1000.0	$y = 0.0093x + 0.2121$	0.996	0.058	1.16	0.245	4.91
2-oxoglutaric	0.2−1000.0	$y = 0.0067x - 0.0065$	0.997	0.007	0.13	0.208	4.15
citric	0.3−500.0	$y = 0.0093x + 0.0169$	0.992	0.063	1.27	0.140	2.80
malic	0.1−1000.0	$y = 0.0085x + 0.0416$	0.996	0.003	0.05	0.162	3.24
quinic	0.2−70.0	$y = 0.0115x + 0.0269$	0.991	0.043	0.85	0.184	3.68
ascorbic	1.0−500.0	$y = 0.0035x - 0.0236$	0.995	1.25	24.9	2.417	48.3
shikimic	0.1−100.0	$y = 0.0085x + 0.0213$	0.994	0.050	1.00	0.367	7.35
succinic	0.4−1000.0	$y = 0.0088x + 0.0652$	0.998	0.021	0.42	0.158	3.15
fumaric	1.0−1000.0	$y = 0.0069x + 0.0567$	0.997	0.540	10.8	1.87	37.5

[a] All data were obtained by analyzing standard mixtures prepared in 2% metaphosphoric acid.

Table 3. Intraday (n = 10) and Interday (n = 5) Repeatabilities of the HPLC−ESI-TOFMS Method[a]

		intraday		interday	
organic acid		mean	RSD (%)	mean	RSD (%)
oxalic	RT	5.36	0.15	5.35	0.22
	A_S/A_{IS}	0.06	1.56	0.05	3.70
cis-aconitic	RT	5.87	0.08	5.87	0.06
	A_S/A_{IS}	1.15	1.47	1.04	3.19
2-oxoglutaric	RT	6.48	0.04	6.47	0.07
	A_S/A_{IS}	0.61	2.28	0.66	2.27
citric	RT	7.42	0.04	7.41	0.13
	A_S/A_{IS}	1.74	1.62	1.73	0.99
malic	RT	9.18	0.04	9.17	0.11
	A_S/A_{IS}	0.82	2.32	0.85	2.91
quinic	RT	9.64	0.05	9.64	0.08
	A_S/A_{IS}	1.06	0.84	0.88	4.14
ascorbic	RT	9.99	0.06	9.97	0.35
	A_S/A_{IS}	0.22	2.74	0.25	19.9
shikimic	RT	12.2	0.03	12.2	0.10
	A_S/A_{IS}	1.08	1.92	1.08	4.41
succinic	RT	12.1	0.05	12.0	0.10
	A_S/A_{IS}	0.53	0.52	0.51	1.36
fumaric	RT	13.2	0.06	13.2	0.16
	A_S/A_{IS}	0.730	1.11	0.72	1.52

[a] A 100 μM standard solution containing all ten organic acids and the two internal standards was used. RT = retention time, A_S = area of the standard peak, and A_{IS} = area of the internal standard peak.

Table 4. Recoveries (%) Obtained for the Different Organic Acids in Sugar Beet Leaf Extracts, Tomato Xylem Sap, and Commercial Orange Juice[a]

organic acid	sugar beet leaves	tomato xylem sap	orange juice
oxalic	107.8 ± 3.6	46.5 ± 1.0	38.9 ± 1.0
cis-aconitic	99.2 ± 1.7	102.8 ± 2.3	95.8 ± 2.0
2-oxoglutaric	66.2 ± 0.9	68.8 ± 1.8	43.9 ± 0.7
citric	100.0 ± 1.8	109.6 ± 2.2	96.7 ± 1.5
malic	104.0 ± 1.4	100.6 ± 1.0	101.0 ± 0.5
quinic	98.0 ± 0.4	96.7 ± 2.4	98.2 ± 1.4
ascorbic	21.5 ± 0.4	85.5 ± 1.9	76.4 ± 0.5
shikimic	103.2 ± 0.8	97.4 ± 2.5	91.9 ± 1.9
succinic	100.4 ± 0.6	96.8 ± 2.5	96.4 ± 2.5
fumaric	93.2 ± 0.5	94.8 ± 3.1	93.5 ± 2.7

[a] Results are means ± SE (n = 5).

respectively. It should be taken into account that in the case of slight shifts in the retention time, the identity of any given peak can be confirmed using the molecular formula determination derived from the isotopic pattern information, in combination with the Sigma Fit (Bruker Daltonik) algorithm.[27] The RSD for peak area was in the range of 0.5−3% in the intraday test; in the interday test, the RSD was in the range of 1−4% except for ascorbic acid (RSD = 20%), a compound that is known to be very labile and easily degraded with time. Using a tomato xylem sap sample, the peak area repeatability values were similar to those obtained with a standard mixture, in the ranges of 0.8−4.5% in the intraday test and 3.0−5.6% in the interday test. Again, the ascorbic acid showed the highest RSD values (12.2% in the interday test).

Recovery assays were carried out for all 10 organic acids in sugar beet leaf extracts, tomato xylem, and orange juice by spiking samples with known amounts of analyte standards and isotope-labeled ISs. Recovery was calculated by dividing the amount of analyte found in the spiked sample by the sum of the amount originally found in the sample plus the amount spiked. Good analyte recoveries were found (in the range 92−110%; 99% average value) for *cis*-aconitic, citric, malic, quinic, succinic, shikimic, and fumaric acids. However, the recovery values for oxalic, oxoglutaric, and ascorbic acids were adequate only in some specific plant materials (Table 4), whereas in other cases the recoveries were as low as 22% (e.g., ascorbic acid in sugar beet leaves). Ascorbic acid measurements can be affected significantly by the presence of other compounds in plant tissues and xylem sap, including metals, flavonoids, amino acids, and sugars.[29] Ascorbate is oxidized by light and air, and it is only detectable by ESI-MS when in the reduced state, so that recovery may be increased by protecting samples during extraction by using a low temperature and a green safe light.[25] For this particular compound, the method described elsewhere[25] could be recommended, although sensitivity was not as good as that of the method described here. Regarding the low recovery of oxalic acid in tomato xylem and orange juice, some studies suggest that pH

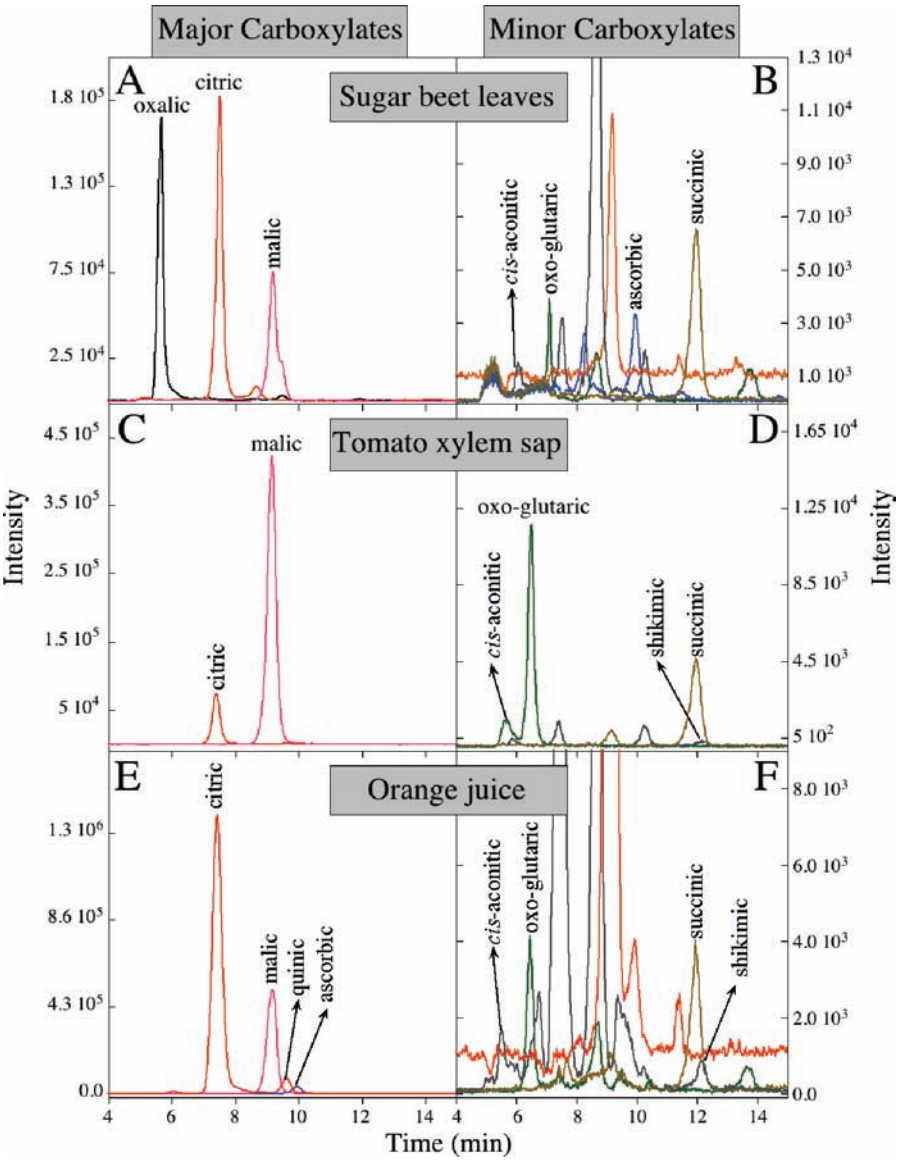

Figure 2. HPLC−ESI-TOFMS chromatograms of the major (A, C, E) and minor (B, D, F) carboxylates found in sugar beet leaves (A, B), tomato xylem sap (C, D), and orange juice (E, F) samples. Chromatogram traces correspond to the $[M - H]^-$ ± 0.03 m/z of each organic acid.

and Ca^{2+} have an impact on oxalic solubility, leading to the formation of insoluble precipitates;[15] however, oxalic acid recovery in sugar beet leaves was good. The low recoveries of ascorbic or oxalic acid in some plant matrixes (Table 4) indicate that although the method developed is suitable for a large number of organic acids and plant matrixes, some organic acid/plant matrix combinations may require specific extraction procedures and/or the use of ^{13}C-labeled ISs.

In the cases of succinic and malic acids, where isotope-labeled compounds were used as ISs, recoveries in all tissues tested were 96−104%. Therefore, it seems likely that results could be improved by using the corresponding isotope-labeled IS for each analyte, although a compromise between the number of expensive ISs and total analysis costs is needed. The rationale is that a given IS may provide coverage for carboxylates eluting within a range of retention times. The use of a third ^{13}C-labeled organic acid eluting close to oxalic and 2-oxoglutaric acids could lead to improved recoveries for these carboxylates. We used ^{13}C-labeled ISs instead of D-labeled ones[10] because proton exchange reactions with the extraction media and/or mobile phase can occur easily when using the latter compounds.[31]

Analysis of Plant Tissue Extracts. Typical chromatograms obtained with sugar beet leaf extracts, tomato xylem sap, and orange juice are shown in Figure 2, and the corresponding carboxylate concentrations are shown in Table 5. The major carboxylates in sugar beet leaf extracts were oxalic, citric, and malic acids, whereas *cis*-aconitic, 2-oxoglutaric, ascorbic, and succinic acids were also detectable but in lower amounts (Figure 2 A,B, Table 5). In tomato xylem sap, major carboxylates were citric and malic acids, whereas *cis*-aconitic, 2-oxoglutaric, shikimic, and succinic acids were minor components (Figure 2C,D, Table 5). In orange juice, major carboxylates were citric, malic, quinic, and ascorbic acids, whereas *cis*-aconitic, 2-oxoglutaric, shikimic, and succinic acids were found in lower concentrations (Figure 2 E,F, Table 5). The method has also been applied elsewhere for the determination of organic acids in peach roots[32] and roots and xylem sap of sugar beet.[33]

Table 5. Organic Acid Contents Found in the Different Plant Tissues and Fluids Analyzed Using the HPLC−ESI-TOFMS Method[a]

organic acid	sugar beet leaves	tomato xylem sap	orange juice
oxalic	29992 ± 832	nd	nd
cis-aconitic	9.4 ± 0.3	20.1 ± 0.0	10.5 ± 0.9
2-oxoglutaric	7.6 ± 0.0	19.4 ± 0.2	26.7 ± 0.5
citric	2076 ± 62	268 ± 2	12427 ± 257
malic	1260 ± 38	433 ± 3	3088 ± 25
quinic	nd	nd	253 ± 2
ascorbic	297 ± 10	nd	914 ± 8
shikimic	nd	11.9 ± 0.0	47.5 ± 0.0
succinic	159 ± 5	16.2 ± 0.2	59.2 ± 7.1
fumaric	nd	nd	nd

[a] Results are means (nmol g^{-1} FW for sugar beet leaves, μM for tomato xylem sap and orange juice) ± SE of five independent sample extracts. Quantification was carried out as described in the text. nd = not detected.

In summary, an HPLC−ESI-TOFMS method has been developed and validated to determine simultaneously 10 different low molecular mass carboxylates in plant tissue extracts. In seven cases (*cis*-aconitic, citric, malic, quinic, succinic, shikimic, and fumaric acids), the results were satisfactory, whereas oxalic, 2-oxoglutaric, and ascorbic acids may require specific extraction procedures and/or use of the corresponding ^{13}C-labeled organic acid as the IS to improve accuracy. Carboxylate analysis is carried out following a rapid and easy extraction procedure, and time-consuming steps such as sample prepurification, preconcentration, and derivatization are not necessary. The method is based on a separation with anion exchange HPLC, followed by ionization of the sample by electrospray and analyte detection with a TOFMS device. The method is highly selective, with the identification of carboxylates being unequivocal, based on both the retention time and exact molecular mass. The newly developed method integrates a number of features that, taken together, improve the existing methodologies: the determination of a considerable number of organic acids, in a large range of concentration levels, with good sensitivities, without any derivatization step, and using two ^{13}C-isotope-labeled ISs. Furthermore, the recoveries for the different organic acids have been measured in three different plant matrixes.

■ ASSOCIATED CONTENT

Supporting Information. Supplementary Table 1 showing a comparison of LODs obtained with those of other recently published HPLC−ESI-MS methods. This material is available free of charge via the Internet at http://pubs.acs.org.

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