JOURNAL OF
AGRICULTURAL AND FOOD CHEMISTRY

Determination of Organic Acids in Plants of *Silene paradoxa* L. by HPLC

MILUSCIA ARNETOLI,*,† GIORDANO MONTEGROSSI,§ ANTONELLA BUCCIANTI,# AND CRISTINA GONNELLI†

Section of Plant Ecology and Physiology, Department of Plant Biology, University of Florence, via Micheli 1, 50121 Firenze, Italy; CNR Institute Geosciences and Georesources, via G. La Pira 4, 50121 Firenze, Italy; and Department of Earth Science, University of Florence, via La Pira 4, 50121 Firenze, Italy

According to the general behavior that organic acids steadily bind metals, a specific and highly reproducible HPLC separation method with photodiode array detection has been improved for their determination and quantification in biological materials. The separation was carried out on an Alltima C-18 reverse phase column. The mobile phase was 125 mM KH_2PO_4, adjusted to pH 2.5 with concentrated H_3PO_4, and optimum separation efficiency was obtained by using a 2 mL min^{-1} flow rate. Detection wavelength for quantitative measurement was 210 nm. The run time of each sample was 20 min, with a spectra collection frequency of 5 spectra s^{-1}. Organic acids were identified by comparing the retention times of the samples against retention times of the standards and confirmed with spectral (190–700 nm) signature. Because organic acids could steadily bind metals in plant tissues and due to the strong matrix effect observed, the addition method was applied for quantitative analysis and its performance evaluated.

KEYWORDS: Organic acids; HPLC; plant; *Silene paradoxa*

INTRODUCTION

General Introduction. Environmental pollution by heavy metals occurs as a consequence of several anthropogenic activities. Serious problems of restricting heavy metal bioavailability in the soil−plant−animal pathway and of remediating contaminated soils and waterways are in need of solving. As plants show a remarkable ability to absorb and accumulate metals from the environment, in recent years there has been increasing interest in the development of cost-effective plant-based technologies to remove heavy metals from contaminated soils (*1–4*). The processes of heavy metal uptake, accumulation, distribution, and detoxification have been studied in a wide range of plant species (*5–7*). However, the physiological mechanisms involved are still largely unknown, as metal-imposed plant response is complex with considerable variation between species, specific effects for different metals, and metal concentration-dependent behaviors.

Low molecular mass organic acids, such as di- and tricarboxylic acids including oxalic, citric, malonic, malic, succinic, tartaric, fumaric, and glutaric acids, are almost ubiquitously found in the soil environment (*8, 9*). In soils, organic acids may be derived from plant, fungal, or microbial sources (*8, 9*) and are generally characterized as having metal-complexing properties. The effectiveness of carboxylates in metal binding depends on their number of carboxyl groups and molecular structure. Tricarboxylates (citrate) are generally more effective than dicarboxylates (e.g., malate, malonate) due to stronger ligand binding. Soil properties also have large effects on the effectivity of carboxylates; in fact, the stability of organic anion−metal complexes depends strongly on pH (*8*). Due to the several mechanisms by which organic acids may influence metal behavior in soils, the development of analytical methodology to quantify metal−organic acid complexes have a fundamental importance to improve our current understanding of many processes occurring in soils.

Following the uptake of heavy metals by plant roots, translocation to shoots, and detoxification within storage sites are two critical steps for tolerance and accumulation. This can be achieved by chelation, transport, trafficking, and sequestration by organoligands (*5, 10*), such as organic acids (e.g., carboxylates: malate, citrate, malonate, succinate, and oxalate) (*11*). Complexation of metals with these ligands results in decreased free ion activity, and thus reduced toxicity, and in plants the development of metal−organic acid complexes has been indicated using chromatography (*12–15*) and X-ray absorption spectroscopy (*16, 17*). Despite many indications that in plants organic acids are involved in tolerance, transport, and storage of heavy metals (*18–24*), so far accumulating evidence has shown that organic acids actually play an important role only in detoxifying aluminum (Al) both internally and externally,

* Corresponding author (telephone +39 055 2757392; fax +39 055 282358; e-mail miluscia@gmail.com).
† Department of Plant Biology, University of Florence.
§ CNR Institute Geosciences and Georesources.
# Department of Earth Science, University of Florence.

10.1021/jf072203d CCC: $40.75 © 2008 American Chemical Society
Published on Web 01/05/2008

forming stable complexes with this toxic ion and thereby preventing its binding to cellular components (*25*). In fact, Al-tolerant genotypes or species have been shown to be able to release more specific organic acids than sensitive ones (*26–34*). High concentrations of organic acids have been found in some Al-resistant species (*35*): oxalate and citrate in *Fagopyrum esculentum* (Polygonaceae) and citrate in *Cassia tora* (Caesalpinaceae) and *Hydrangea* spp. (Hydrangeaceae). The role of organic acids in the protection of the root apex from Al stress, and in its tissue detoxification and their importance in its xylem transport have been shown in *F. esculentum* and *Hydrangea* (*27, 28, 36*).

As plant vacuoles are the major repository for organic acids (*37*), the possible association between metals and organic acids suggests that metal detoxification occurs by vacuolar sequestration. In fact, for example, in hyperaccumulators, plants that have evolved not only the ability to survive in metal-rich soils but also to sequester and store exceptionally high levels of metals and metalloids in their aboveground tissues (*38*), levels of citric, malic, malonic, and oxalic acids have been shown to play a role in sequestering metal ions in the vacuoles (*39*). Furthermore, Salt et al. (*40*) suggested that metals, such as Ni and Zn, can be transported to shoots either as a metal−organic acid complex or as the hydrated cation. Once in the shoot, both Ni and Zn can accumulate in the vacuole, where co-ordination by organic acids is favored by the low pH (<6) value (*40*).

Thus, a number of analytical techniques have been reported for identifying and, but much less frequently, quantifying, metal−organic acid complexes in a range of environmental matrices (*41*). Moreover, many scientific disciplines, such as the study of metal tolerance physiology in plants, do need an accurate, sensitive, and fast determination methodology for light organic acids and their derivatives in various types of samples. Therefore, the aim of this study was to improve an HPLC method for the qualitative and quantitative determination of low molecular weight organic acids in plant tissues reducing as much as possible the matrix effect, representing an important obstacle in analytical method, as reported in the literature (*41*).

HPLC Analysis of Low Molecular Mass Organic Acids. In recent studies, organic acid contents have been analyzed to clarify their role in heavy-metal complexation and tolerance for many plant species (*6*). Many different methods have been used; phosphoric and sulfuric acids are mainly used as eluent, but there are no results on their performances in any condition, particularly when the matrix effect plays an important role (*41*). In many cases, phosphoric acid has been preferentially used because of its optimal organic acid separation (*42*). In some cases, sulfuric acid has been used, too, with the aim to separate organic acids, but its high acidity tends to drastically decrease pH values, with consequent metal precipitation and organic acid dissociations (*43*).

In HPLC methods, the determination of retention times is mainly based upon the distribution of a metal complex between a mobile and a stationary phase, but the influence of other parameters should be considered for an exhaustive evaluation of the procedure (*41*). In fact, retention is observed to be influenced by the effective charge of the analyte, the net charge of eluent, and the affinity of the stationary phase for analyte and eluent ions. The column stationary phase charge and the effective charge of the probe acids are pH-dependent and can significantly influence retention times. As observed by Lord and Stringham (*44*), an increase in the ionic strength resulted in a decrease of the retention times for all of the analytes at low and high pH values and the relative response of all acids increased as the ionic strength of the buffer decreased.

A further problem complicating the organic acid analysis is the analyte peak identification, due to the presence of vacant peaks that arise when eluent ions are displaced from the column by analyte ions and elute as a peak (*44*). The retention time of vacant peaks may be shorter or longer than or equal to that of the analyte of interest, thus interfering with it. Diluting the samples in the mobile phase could minimize the effect of vacant peaks, thus simplifying the interpretation of the chromatograms (*44*).

Chromatographic methods devoted to determining organic acids (*41*) can be affected by the presence of metals forming complexes with such molecules (*41*), and sometimes it is not possible to make any presumption about the identity of the metal complexes, because potentially more than one complex occurs at the same retention time. For example, the peaks attributed to the $(Al{-}oxalate)^+$, $(Al{-}citrate)^0$, and $(Al{-}H\ citrate)^+$ complexes have the same retention time (*45*), and this feature limits the application of the method to more complex natural solutions, because of the identification and quantification difficulty (*41*). Many separation methods have been investigated, but none has been usefully developed to separate these complexes (*41*). Furthermore, despite general methods based on UV–vis, in which standard external calibration of absorbance versus concentration is used, the determination of organic acid concentration in plant tissues needs an investigation of the influence of the matrix effect. The interference effect due to metal−acid complexes was investigated in greater detail for aluminum (*41, 45*), but, for example, oxalic acid could give strong complexes also with many other cations.

By taking into account all of the previous considerations, the approach presented here could be helpful in solving some problems affecting analytical measures. For instance, if oxalic acid has been portioned in several peaks, because of its chelation with any metal, the increase of its concentration can avoid the fractionation process of the metal−acid complexes that became thus negligible. The consequence is that the identification of oxalic acid could be possible and its quantification not underestimated. Moreover, if any superposition of metal−acid complex peaks with other acid peaks is present, by increasing the acid concentration while maintaining the weight of the interfering peak, a correct estimation of the acid concentration could be also possible.

MATERIALS AND METHODS

Apparatus. HPLC analyses were performed using a Perkin-Elmer series 200 HPLC system with TOTALCHROM v. 6.2 and TurboScan 200 v. 1.1 software, a pump (Perkin-Elmer series 200 pump), a filter and degasser system (Perkin-Elmer series 200 vacuum system), and a photodiode array detector (Perkin-Elmer series 200 DAD, 190–700 nm, 512 diode array). The separation was carried out on an Alltima C-18 reverse phase column (250×4.6 mm i.d., particle size $= 5\ \mu$m; Alltech, Deerfield, IL). The mobile phase was 125 mM KH_2PO_4, adjusted to pH 2.5 with concentrated H_3PO_4. After testing of 1, 1.5, 2, 2.5 mL min^{-1} flow rates, the optimum separation efficiency was obtained by using a 2 mL min^{-1} flow rate; in such conditions the resolution of the peak separation was unaffected, but overall run times were reduced. The detection wavelength for quantitative measurement was 210 nm (*18*). A few minutes of pressure equilibration (up to about 3300 psi) is required before injection. The injection volume was 3 mL, in order to clean the injection system, in a 6 μL injection loop. The run time of each sample was 20 min, with a spectrum collection frequency of 5 spectra s^{-1}. The column performance was carried out in a constant room temperature regimen. After three runs, a water (80%)/methanol (20%) mobile phase was employed to flush the column to avoid phosphate salt precipitation, which could damage the column and to clean the apparatus from low-polar organic compounds.

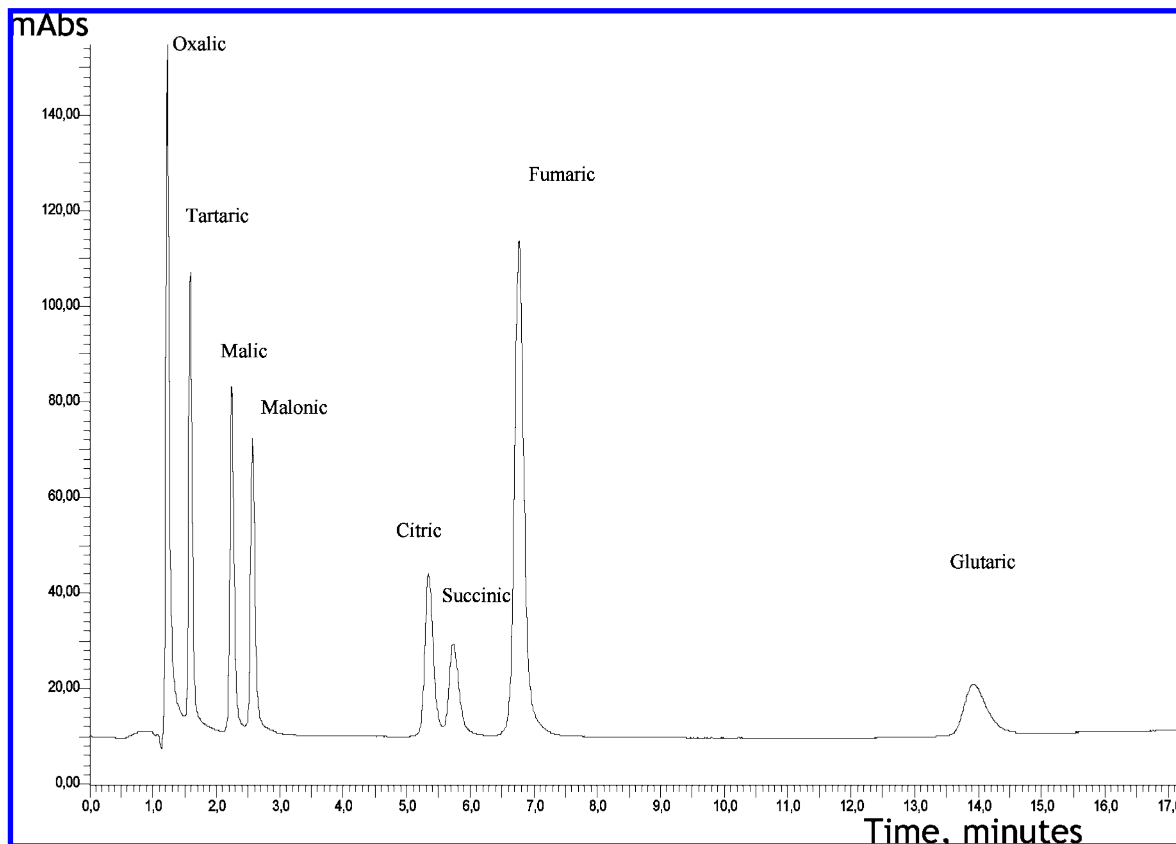

Figure 1. Mix of standards used to collect reference spectra. Chromatographic conditions are described in the text.

Organic acids were identified by comparing the retention times of the samples against retention times of the standards (**Figure 1**) and confirmed with spectral signature (UV/vis Spectra Database, 4th edition, 2005) (TurboScan 200 match index of standard collected spectra and sample peak spectra). The concentrations of organic anions in each sample were calculated from linear regression equations (slope and intercept of the calibration curve) obtained from the peak height of the sample (diluted sample with standard additions), with the exception of glutaric acid, for which we used peak area due to high retention time and nonhomogeneous broadening of the peak.

Root and shoot concentrations of carboxylates, in molar units per unit of organ dry mass, were calculated by relating the concentrations in the extract and the volume of the extract to the amount of organ mass used. For quantification of glutarate, peak area instead of peak height has been considered to have a higher sensitivity, because the glutarate peak was about 7 min farther than the fumarate peak, thus being far more broadened than the other peaks. Statistical analysis was performed according to standard statistical reference (*46*).

Standard and Reagent Preparation. The individual standards and a mix of all the standards, with the specific mix for the addition, were prepared by using malonic acid (Carlo Erba, purity = 99.5%), fumaric acid (Sigma, purity ≥ 98%), malic acid (Acros, purity = 99%), oxalic acid (Sigma, purity = 99%), L-(+)-tartaric acid (Carlo Erba, purity = 99.5%), citric acid (Carlo Erba, purity = 99.5%), succinic acid (Carlo Erba, purity = 99.5%), and glutaric acid (Sigma, purity ≥ 98%), with ultrahigh-grade quality (Milli-Q Reagent Grade Water System, Millipore) water. The standard solutions were injected to determine individual retention times, UV–vis (190–700 nm) spectra, and standard (210 nm) absorbance used for quantitative determination. Stock solutions of 10 mM oxalic acid, 25 mM tartaric acid, 50 mM malic acid, 50 mM malonic acid, 50 mM citric acid, 100 mM succinic acid, 25 mM fumaric acid, and 100 mM glutaric acid were prepared by dissolving pure standards into Milli-Q water for addition to the sample, whereas 1:4 v/v diluted solutions from stock solution were used for spectra and standard absorbance calibration. After 1 month, standard mix solution should not be used to avoid lower signal due to possible oxidations (*47*).

The mobile phase was prepared by dissolving the salt of 125 mM potassium phosphate monobasic anhydrous (KH_2PO_4) (Carlo Erba, purity 98%) in Milli-Q grade water adjusted to pH 2.5 with concentrated phosphoric acid (H_3PO_4) (Carlo Erba, 85%, ca. 17 cm^3). The mobile phase solvents were filtered through a 0.20 μm filter (Sarstedt), refiltered, and degassed before HPLC analysis.

Biological Materials. Adult plants of two Tuscan populations of *Silene paradoxa*L. were harvested from the polymetallic sulfide deposit of Fenice Capanne (FC), characterized by significant contents of polluting elements such as As, Cu, Pb, and Zn (*48*), and from the uncontaminated soil of Colle Val D'Elsa (CVD), characterized by low metal (Cd, Cu, Fe, Ni, Pb) and As concentrations (*49*). Roots were separated from shoots; plant material was carefully rinsed with Milli-Q water to remove excess soil and impurities, blotted with paper tissue, weighed, frozen in liquid nitrogen, and stored at −20 °C. Before freezing, the root system was transferred to a beaker of suitable size, in which the rhizosphere soil was washed off the roots by gently shaking the beaker after the addition of a measured amount of 10 mM calcium chloride ($CaCl_2$) solution at 4 °C for 10 min (*50*), to remove metals adhering to the root cell walls. Organic acids were extracted by homogenizing 1 g of fresh weight of frozen material in 10 mL of distilled water, using a mortar and pestle and liquid nitrogen; then homogenates were centrifuged for 20 min at 10000 rpm at 4 °C (*18*). The supernatant was stored at −20 °C and filtered through a 0.20 μm filter (Sarstedt) before analysis.

RESULTS AND DISCUSSION

According to the general behavior that organic acids could steadily bind metals in plant tissues, the presence of different peaks suggested the presence of different organic acid−metal complexes. This result indicates that some determinations can be affected by under- or overestimation, and a devoted procedure is here developed to overcome this problem.

The acid−metal complexes were broken by diluting the sample 1:4 with Milli-Q water, and a decreasing of pH was

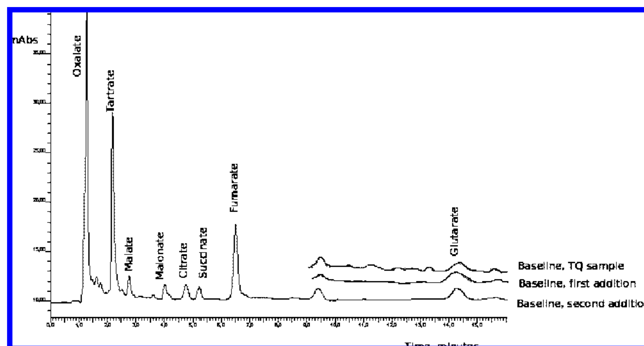

Figure 2. Differences between chromatograms before and after addition of standard acid organic solutions in root and shoot of *S. paradoxa*. The multipeak of oxalic acid converged in a single peak. Chromatographic conditions are described in the text, and the scales are equal in both chromatograms.

Table 1. Significance of Calibration Curves, Pearson Squared Coefficient (R^2), Relative Error, and MDL

organic acid	concn added to sample 1 mM	2 mM	0 mM	R^2 (min)	error (%) (max)	MDL (av, mmol L^{-1})	MDL (max mmol L^{-1})
oxalate	1	2	0	0.95	5.30	0.0106	0.0148
tartrate	2.5	5	0	0.99	1.19	0.0202	0.0210
malate	5	10	0	0.97	2.71	0.0596	0.0704
malonate	5	10	0	0.99	0.66	0.0702	0.0751
citrate	5	10	0	1.00	0.48	0.0602	0.0689
succinate	10	20	0	1.00	0.01	0.2240	0.2288
fumarate	2.5	5	0	0.99	1.28	0.0013	0.0014
glutarate	10	20	0	0.98	1.66	0.1836	0.2612

obtained through a strong addition of each organic acid; in this condition, dilution with an eluent could favor the described process. Metal−acid complexes have been broken prior to the analysis, as shown in **Figure 2**. Thus, the strong expected matrix effect was eliminated by using the addition method, so allowing a quantitative analysis. To investigate the modifications in the shape of the chromatograms attributable to the additions, graphical comparison of the highest addition with respect to undiluted solution is shown in **Figure 2**. The absence of a side peak (e.g., after oxalic acid), at the same absorbance scale, is observed, thus indicating the breakdown of acid−metal complexes.

To perform the analysis by addition method three points were enough to check for proportionality between concentration and peak height (mAbs) response. The three points were obtained for 1.25 mL of the sample by the addition of 0, 0.5, and 1 mL of the standard mix and then adjusted to 5 mL with Milli-Q water. To check the linearity of the concentration−peak height (mAbs) response up to high concentrations (i.e., the concentration of acid in the stock solution used for the addition), a calibration curve for each organic acid was determined and the linearity evaluated by using the Pearson squared coefficient (R^2), which were found to be >0.95 (**Tables 1** and **2**). These results confirmed the linearity of the signal/concentration ratio within the considered working range and represented the base to evaluate the accuracy and precision of the measurements (*51*).

The minimum detection limit (MDL) obtained by the calibration curves was significantly low, and sensitivity was quite high for the proposed analysis, whereas the maximum percentage error, equal to 5.30, was obtained for oxalate (**Tables 1** and **2**). Average and maximum MDL were determined, too, for each organic acid analysis, as shown in **Table 1**. It is noteworthy to report that the average MDL

Table 2. Analytical Results with Respective Relative Errors (Samples Have Been Diluted 4 Times To Perform the Method of Addition

sample	organic acid	intercept (micro-absorbance)	slope (micro-absorbance)	concn of extract (mmol)	error	concn of sample (mmol g^{-1} of dw)
CVD shoot	oxalate	24749.07	69004.6	1.43	0.0075	13.43
	tartrate	−135.28	19029.61	d.l[a]	<d.l	<d.l
	malate	15631.6	5678.98	11.01	0.0271	103.09
	malonate	1174.29	5913.5	0.79	0.0023	7.44
	citrate	2436.91	5804.44	1.68	0.0048	15.72
	succinate	−61.92	1747.92	<d.l	<d.l	<d.l
	fumarate	48156.5	290519.44	0.66	0.0094	6.21
	glutarate	32143.5	2397.8	53.62	0.0166	502.10
FC shoot	oxalate	81401	50422.25	6.46	0.0530	42.74
	tartrate	−3092.94	20457.6	<d.l	<d.l	<d.l
	malate	2372.83	7100.3	1.34	0.0171	8.85
	malonate	58.33	5324.6	0.04	3.11E−05	0.29
	citrate	−1446.57	6932.2	<d.l	<d.l	<d.l
	succinate	−549.04	1775.76	<d.l	<d.l	<d.l
	fumarate	6193.67	305917.6	0.08	0.0002	0.54
	glutarate	−2134.67	1531.3	<d.l	<d.l	<d.l
CVD root	oxalate	163571.56	27047.75	24.19	0.0337	212.94
	tartrate	−434.15	19662.4	<d.l	<d.l	<d.l
	malate	1096.62	7126.77	0.62	0.0006	5.42
	malonate	445.9	6071.76	0.29	0.0008	2.59
	citrate	868.18	7010.27	0.5	0.0003	4.36
	succinate	76.43	1821.9	0.17	5.28E−05	1.48
	fumarate	46913.07	293448.1	0.64	0.0107	5.63
	glutarate	−689.59	3532.11	<d.l	<d.l	<d.l
FC root	oxalate	174022	29216.6	23.83	0.0016	173.65
	tartrate	−81.07	19931.28	<d.l	<d.l	<d.l
	malate	458.77	7205.38	0.25	0.0002	1.86
	malonate	5595.67	5537.96	4.04	0.0066	29.46
	citrate	77.97	7002.26	0.04	0.0002	0.32
	succinate	173.84	1799.91	0.39	0.0001	2.82
	fumarate	49914.36	297812.95	0.67	0.0128	4.89
	glutarate	984.28	2071.74	1.9	0.0006	13.85

[a] d.l, detection limit.

could be increased by using a larger loop because the injected sample volume could be increased to any value that does not affect the peak separation.

The possible interference between inorganic ions and oxalic acid as a function of concentration was investigated, and in **Figure 3** the effect of high concentration is reported. From a general point of view, results indicated that the retention time of inorganic ions was about 1.15 min and, consequently, because that of oxalic acid was about 1.30, no significant overlapping between peaks was obtained for low concentrations. This interfering peak was generally well resolved, and only in case of strong inorganic ion concentrations (100 mM chloride, 12.5 mM sulfate, or 10 mM nitrate) was an overlap with the oxalate peak observed (**Figure 3**).

The repeatability was tested by analyzing three times each sample, and because the differences are less than the overall analytical error, results are not reported in **Table 1**.

A statistical evaluation of the differences between the standard and the improved addition method can be performed by investigating frequency distribution of the difference values between the two analytical methods, independently from the sample and the organic acid species, as reported in **Figure 4**. The Kernel density estimation (a smooth estimator of the histogram) is also

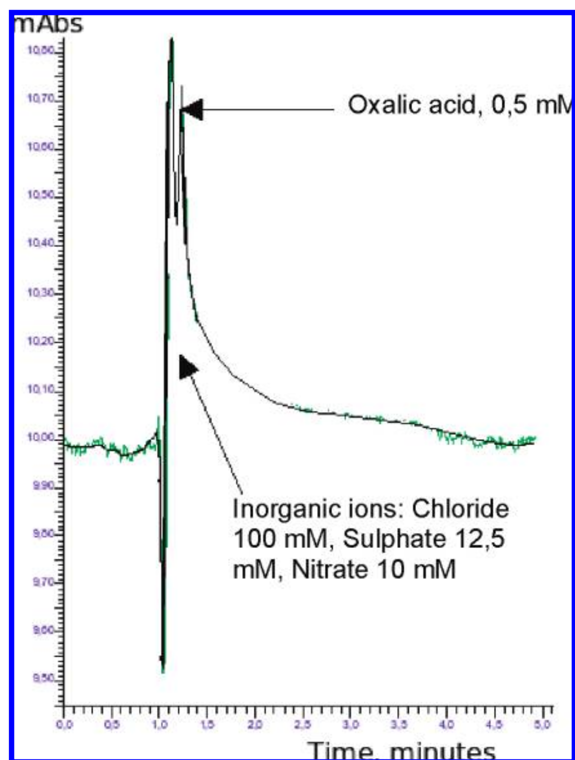

Figure 3. High-saline water; separation between inorganic anion (100 mM chloride, 12.5 mM sulfate, 10 mM nitrate) peak and oxalic acid (0.25 mM). Chromatographic conditions are described in the text.

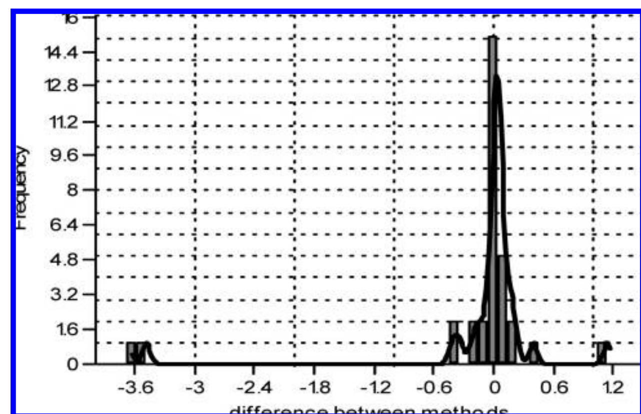

Figure 4. Histograms of the values difference between the two methods and Kernel density estimation curve.

plotted and may be useful to visualize how the data tend to group around the zero value (*52*). If the data included in the interval ± 0.6 are considered, the Kolmogor−Smirnow test allows us to accept the null hypothesis about normality ($p > 0.05$), indicating that the two methods are comparable in the whole range of values and conditions here investigated.

From the comparative reports of results obtained by using standard and addition methods (**Table 3**), differences that are located out of the ±0.6 interval are reported in CVD shoot, for which glutaric acid was 1.54 and 0.41 mmol L^{-1} respectively; in CVD root, for which oxalic acid was 2.37 and 6.05 mmol L^{-1} respectively; and in FC root, for which oxalic acid was 2.47 and 5.96 mmol L^{-1} respectively. Higher values are reported by using the addition method for oxalic acid and, in our opinion, this result is generally predictable because of a lower influence of the matrix effect. Thus, analytical data showed that without the adoption of the addition method this organic acid could be undervalued. The particular behavior of the glutaric acid in CVD shoot could be explained by taking into account the possible unpredictable contribution of a vacant peak in the standard methodology.

Table 3. Comparison of the Analytical Results Obtained by External Calibration and by Using the Addition Method

sample	organic acid	molar absorption coefficient (mAbs/mmol)	concn using external calibration (mmol L^{-1})	concn using addition method (mmol L^{-1})
CVD shoot	oxalate	70.25	0.40	0.36
	tartrate	18.48	0.05	0.00
	malate	7.10	2.59	2.75
	malonate	5.99	0.33	0.20
	citrate	6.34	0.20	0.42
	succinate	1.73	0.05	0.00
	fumarate	20.56	0.36	0.17
	glutarate	19.72	1.54	0.41
FC shoot	oxalate	70.25	1.26	1.61
	tartrate	18.48	0.01	0.00
	malate	7.10	0.72	0.33
	malonate	5.99	0.02	0.01
	citrate	6.34	0.01	0.00
	succinate	1.73	0.04	0.00
	fumarate	20.56	0.02	0.02
	glutarate	19.72	0.03	0.00
CVD root	oxalate	70.25	2.37	6.05
	tartrate	18.48	0.00	0.00
	malate	7.10	0.22	0.15
	malonate	5.99	0.16	0.07
	citrate	6.34	0.20	0.12
	succinate	1.73	0.00	0.04
	fumarate	20.56	0.13	0.16
	glutarate	19.72	0.20	0.00
FC root	oxalate	70.25	2.47	5.96
	tartrate	18.48	0.00	0.00
	malate	7.10	0.10	0.06
	malonate	5.99	1.15	1.01
	citrate	6.34	0.05	0.01
	succinate	1.73	0.04	0.10
	fumarate	20.56	0.05	0.17
	glutarate	19.72	0.06	0.48

When the discussed results and the statistical comparison between the two methodologies are taken into acount, our approach seems to be appropriate if organic acids have to be determined in the presence of a high matrix effect as occurs in biological samples, thus avoiding underestimation.

LITERATURE CITED

(1) Chaney, R. L.; Malik, M.; Li, Y. M.; Brown, S. L.; Brewer, E. P.; Angle, J. S.; Baker, A. J. M. Phytoremediation of soil metals. Curr. Opin. Biotechnol. **1997**, *8*, 279–284.

(2) Garbisu, C.; Alkorta, I. Phytoextraction: a cost-effective plant-based technology for the removal of metals from the environment. Bioresour. Technol. **2001**, *77*, 229–236.

(3) Salt, D. E.; Smith, R. D.; Raskin, I. Phytoremediation. Plant Mol. Biol. **1998**, *49*, 643–668.

(4) Terry, N.; Bañuelos, G. *Phytoremediation of Contaminated Soil and Water*; Lewis Publishers: Boca Raton, FL, 2000.

(5) Clemens, S. Molecular mechanisms of plant metal tolerance and homeostasis. Planta **2001**, *212*, 475–486.

(6) Hall, J. L. Cellular mechanisms for heavy metal detoxification and tolerance. J. Exp. Bot. **2002**, *53*, 1–11.

(7) Williams, L. E.; Pittman, J. K.; Hall, J. L. Emerging mechanisms for heavy metal transport in plants. *Biochim. Biophys. Acta* **2000**, *1465*, 104–126.
(8) Jones, D. L. Organic acids in the rhizosphere - a critical review. *Plant Soil* **1998**, *205*, 25.
(9) Strobel, B. W. Influence of vegetation on low-molecular-weight carboxylic acids in soil solution: a review. *Geoderma* **2001**, *99*, 169–198.
(10) Clemens, S.; Palmgren, M. G.; Krämer, U. A long way ahead: understanding and engineering plant metal accumulation. *Trends Plant Sci.* **2002**, *7*, 309–315.
(11) Baker A. J. M.; McGrath S. P.; Reeves R. D.; Smith J. A. C. Metal hyperaccumulator plants: a review of the ecology and physiology of a biological resource for phytoremediation of metal-polluted soils. In *Phytoremediation of Contaminated Soil and Water*; Terry N., Bañuelos G., Eds.; Lewis Publishers: Boca Raton, FL, 2000; pp 85–107.
(12) Brooks, R. R.; Shaw, S.; Asensi Marfil, A. The chemical form and physiological function of nickel in some Iberian *Alyssum* species. *Physiol. Plant.* **1981**, *51*, 167–170.
(13) Kersten, W. J.; Brooks, R. R.; Reeves, R. D.; Jaffré, T. Nature of nickel complexes in *Psychotria douarrei* and other nickel accumulating plants. *Phytochemistry* **1980**, *19*, 1963–1965.
(14) Pelosi, P.; Fiorentini, R.; Galoppini, C. On the nature of nickel compounds in *Alyssum bertolonii* Desv-II. *Agric. Biol. Chem.* **1976**, *40*, 1641–1642.
(15) Sagner, S.; Kneer, R.; Wanner, G.; Cosson, J.-P.; Deus-Neumann, B.; Zenk, M. H. Hyperaccumulation, complexation and distribution of nickel in *Sebertia acuminata*. *Phytochemistry* **1998**, *47*, 339–347.
(16) Krämer, U.; Pickering, I. J.; Prince, R. C.; Raskin, I.; Salt, D. E. Subcellular localization and speciation of nickel in hyperaccumulator and non-accumulator *Thlaspi* species. *Plant Physiol.* **2000**, *122*, 1343–1353.
(17) Salt, D. E.; Prince, R. C.; Baker, A. J. M.; Raskin, I.; Pickering, I. J. Zinc ligands in the metal hyperaccumulator *Thlaspi caerulescens* as determined using X-ray absorption spectroscopy. *Environ. Sci. Technol.* **1999**, *33*, 713–717.
(18) Godbold, D. L.; Horst, W. J.; Collins, J. C.; Thurman, D. A.; Marschner, H. J. Accumulation of zinc and organic acids in roots of zinc tolerant and non-tolerant ecotypes of *Deschampsia caespitosa*. *Plant Physiol.* **1984**, *116*, 59–69.
(19) Krotz, R. M.; Evangelou, B. P.; Wagner, G. J. Relationships between cadmium, zinc, Cd-peptide, and organic acid in tobacco suspension cells. *Plant Physiol.* **1989**, *91*, 780–787.
(20) Ma, J. F.; Ryan, P. R.; Delhaize, E. Aluminium tolerance in plants and the complexing role of organic acids. *Trends Plant Sci.* **2001**, *6*, 273–278.
(21) Mathys, W. The role of malate, oxalate, and mustard oil glucosides in the evolution of zinc-resistance in herbage plants. *Physiol. Plant.* **1977**, *40*, 130–136.
(22) Nigam, R.; Srivastava, S.; Prakash, S.; Srivastava, M. M. Cadmium mobilisation and plant availability: the impact of organic acids commonly exuded from roots. *Plant Soil* **2001**, *230*, 107–113.
(23) Tiffin, L. O. Translocation of iron citrate and phosphorus in xylem exudate of soybean. *Plant Physiol.* **1970**, *45*, 280–283.
(24) Yang, X. E.; Baligar, V. C.; Foster, J. C.; Martens, D. C. Accumulation and transport of nickel in relation to organic acids in ryegrass and maize grown with different nickel levels. *Plant Soil* **1997**, *196*, 271–276.
(25) Ma, J. F.; Furukawa, J. Recent progress in the research of external Al detoxification in higher plants: a minireview. *J. Inorg. Biochem.* **2003**, *97*, 46–51.
(26) Delhaize, E.; Ryan, P. R.; Randall, P. J. Aluminum tolerance in wheat (*Triticum aestivum* L.). II. Aluminum-stimulated excretion of malic acid from root apices. *Plant Physiol.* **1993**, *103*, 695–702.
(27) Ma, J. F.; Hiradate, S.; Nomoto, K.; Iwashita, T.; Matsumoto, H. Internal detoxification mechanism of Al in hydrangea: identification of Al form in leaves. *Plant Physiol.* **1997a**, *113*, 1033–1039.
(28) Ma, J. F.; Zheng, S. J.; Matsumoto, H. Specific secretion of citric acid induced by Al stress in *Cassia tora* L. *Plant Cell Physiol.* **1997**, *38* (9), 1019–1025.
(29) Ma, J. F. Role of organic acids in detoxification of aluminium in higher plants. *Plant Cell Physiol.* **2000**, *41*, 383–390.
(30) Ryan, P. R.; Delhaize, E.; Randall, P. J.; Aust, J. Malate efflux from root apices and tolerance to aluminium are highly correlated in wheat. *Plant Physiol.* **1995a**, *22*, 531–536.
(31) Ryan, P. R.; Delhaize, E.; Randall, P. J. Characterisation of Al-stimulated efflux of malate from the apices of Al-tolerant wheat roots. *Planta* **1995b**, *196*, 103–110.
(32) Miyasaka, S.; Buta, J.; Howell, R.; Foy, C. Mechanisms of aluminium tolerance in snap beans. Root exudation of citric acid. *Plant Physiol.* **1991**, *96*, 737–743.
(33) Pellet, D. M.; Grunes, D. L.; Koehian, L. V. Organic acid exudation as an aluminium-tolerance mechanism in maize (*Zea mays* L.). *Planta* **1995**, *196*, 788–795.
(34) Yang, Z. M.; Sivaguru, M.; Horst, W. J.; Matsumoto, H. Aluminium tolerance is achieved by exudation of citric acid from roots of soybean (*Glycine max*). *Physiol. Planta.* **2000**, *110*, 72–77.
(35) Barceló, J.; Poschenrieder, C. Fast root growth responses, root exudates, and internal detoxification as clues to the mechanisms of aluminium toxicity and resistance: a review. *Environ. Exp. Bot.* **2002**, *48*, 75–92.
(36) Ma, J.; Hiradate, S.; Matsumoto, H. High aluminium resistance in buckwheat. *Plant Physiol.* **1998**, *117*, 753–759.
(37) Matile, P.; Wiemken, A. Interactions between cytoplasm and vacuole . In *Transport in Plants III: Intracellular Interactions and Transport Processes*; Encyclopedia of Plant Physiology; Stocking, C. R., Heber, U., Eds.; Springer-Verlag: Berlin, Germany, 1976; Vol. 3, pp 255–287.
(38) Brooks, R. R. *Plants that Hyperaccumulate Heavy Metals*: CAB International: Wallingford, U.K., 1998.
(39) Callahan, D. L.; Baker, A. J. M.; Kolev, S. D.; Wedd, A. G. J. Metal ion ligands in hyperaccumulating plants. *Biol. Inorg. Chem.* **2006**, *11*, 2–12.
(40) Salt, D. E.; Prince, R. C.; Pickering, I. J. Chemical speciation of accumulated metals in plants: evidence from X-ray absorption spectroscopy. *Microchem. J.* **2002**, *71*, 255–259.
(41) Collins, R. N. Separation of low-molecular mass organic acid-metal complexes by high-performance liquid chromatography, review. *J. Chromatogr., A* **2004**, *1059*, 1–12.
(42) Veneklaas, E. J.; Stevens, J.; Cawthray, G. R.; Turner, S.; Grigg, A. M.; Lambers, H. Chickpea and white lupin rhizosphere carboxylates vary with soil properties and enhance phosphorus uptake. *Plant Soil* **2003**, *248*, 187–197.
(43) Shui, G.; Leong, L. P. Separation and determination of organic acid and phenolic compounds in fruit juices and drinks by high-performance liquid chromatography. *J. Chromatogr., A* **2002**, *977*, 89–96.
(44) Lord, B. S.; Stringham, R. W. Liquid chromatographic determination of organic acids used as pharmaceutical counterions. *Anal. Chem.* **1996**, *68*, 1067–1070.
(45) Bertsch, P. M.; Anderson, M. A. Speciation of aluminium in aqueous solutions using ion chromatography. *Anal. Chem.* **1989**, *61*, 535–539.
(46) Snedecor, G. W., Cochran, W. G., Eds. *Statistical Methods*, 8th ed.; University Press: Ames, IA, 1989.
(47) *Standard Methods for the Examination of Water and Wastewater*, 16th ed.; American Public Health Association, American Water Works Association, Water Pollution Control Federation: Washington, DC, 1985.
(48) Mascaro, I.; Benvenuti, M.; Corsini, F.; Costagliola, P.; Lattanzi, P.; Parrini, P.; Tanelli, G. Mine wastes at the polymetallic deposit of Fenice Capanne (southern Tuscany, Italy). Mineralogy, geochemistry and environmental impact. *Environ. Geol.* **2001**, *41*, 417–429.

(49) Arnetoli, M. Tossicità e tolleranza all'arsenico in due popolazioni di *Silene paradoxa* L. Bachelor's thesis, University of Florence, Firenze, Italy, 2004.
(50) Gonnelli, C.; Galardi, F.; Gabbrielli, R. Nickel and copper tolerance and toxicity in three Tuscan populations of *Silene paradoxa*. *Physiol. Planta.* **2001**, *113*, 507–514.
(51) Green, J. M. A practical guide to analytical method validation. *Anal. Chem.* **1996**, *68*, 305A–309A.
(52) Bowman, A. W.; Azzalini, A. *Applied Smoothing Techniques for Data Analysis. The Kernel Approach with S-Plus Illustrations*; Clarendon Press: Oxford, U.K., 1997.

Received for review July 23, 2007. Revised manuscript received November 19, 2007. Accepted November 26, 2007.

JF072203D