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Separation and Identification of Organic Acids in Root Exudates of *Lupinus luteus* by Capillary Zone Electrophoresis

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The separation and identification of a mixture of organic acids by capillary electrophoresis, with direct detection by UV absorption at a wavelength of 200 nm is reported. A solution containing the following organic acids was studied: oxalic, formic, fumaric, malic, succinic, citric, acetic and lactic acid. Separation was achieved using a fused-silica capillary filled with phosphate buffer (200 mM; pH 6) containing *N*-acetyl-*N*,*N*,*N*-trimethylammonium bromide (0.5 mM) as cationic surfactant, necessary to modify the electrosmotic flow. The composition of organic acids in exudates of *Lupinus luteus* was determined by this method.

Keywords: Capillary electrophoresis; Organic acids; Exudates; *Lupinus*.

INTRODUCTION

The rhizosphere is a very active area, where a great number of chemical reactions and biological processes take place. The composition and amount of root exudates condition biological processes, since microorganisms will behave differently depending on the available substrate.

Organic acids are usually present in root exudates and they seem to be part of a nutrient mobilization strategy. Many studies report that organic acids are involved in manganese mobilization (Godo and Reisenauer, 1980; Uren, 1982). Ström *et al* (1994) showed oxalic acid to be the most efficient for phosphorus mobilization, while citric and malic acids are more efficient with iron. The same authors found different exudation profiles from calcifuge and acidifuge plants, and they proposed that the inability of calcifuge plants to exude citrate is related to their incapacity to mobilize iron and hence they are unable to live in soils poor in iron. Many studies on rice, corn and *Lupinus* report the existence of certain mechanisms by which the plant absorbs the mobilized metal–organic acid complex resulting in better nutrition for the plants (Dinkelaker *et al*, 1989; Mori *et al*, 1991; Jones and Darrah, 1992, 1995).

Certain organic acids act as the chemotactic signal necessary for *Azospirillum brasilensis* to approach roots (Reinhold *et al*, 1985). The release of organic acids in root exudates is also related to detoxification. Some organic acids such as malic, citric and oxalic (Mulette *et al*, 1974; Vaughan *et al*, 1993; Delhaize *et al*, 1993; Jones and Darrah, 1994; Ryan *et al*, 1995) are efficient chelators of aluminium, a very toxic metal in some soils.

The most frequently reported method for the determination of low molecular weight organic acids collected from exudates is ion exclusion high performance liquid chromatography (HPLC) (Hue *et al*, 1986; Fox and Comerford, 1990; Petersen and Bottger, 1991; Mench and Martin, 1991; Basu *et al*, 1994). Most of these methods lack specificity and are not able to separate a mixture of mono-, poly- and hydroxy-carboxylic acids in a single chromatographic run (Blake *et al*, 1987). Ion chromatography (IC), another approach, shows co-elution of both organic and inorganic species (Krzyszowska *et al*, 1996), and with both HPLC and IC, very little can be done to manipulate the selectivity since the analytical columns are used with simple buffers. Gas chromatography (GC) provides better resolution and specificity and has been widely used (Szmigielska *et al*, 1995), but requires sample extraction and derivatization and separate treatment of the samples for volatile and non-volatile acids. A comparative study of HPLC and GC (Paavilainen and Korpela, 1993) concludes that GC coupled with mass spectrometry (MS) provides the best resolution and permits the identification of peaks in the chromatograms, but HPLC gives a better quantitative reproducibility.

Capillary electrophoresis (CE), a modern and powerful separation technique, has proved to be a good choice for investigation of samples in aqueous media since usually no more than a simple dilution of samples is needed. Supporting this statement are the studies of Kenney (1991) who reported the separation of some organic acids of interest in a variety of food matrices with indirect detection employing a commercial package. Huang *et al* (1989) reported the separation of a six-component mixture of monocarboxylic acids from formate to hexanoate using conductivity detection. Devêvre *et al* (1994) presented the separation of 14 organic acids with

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indirect UV detection using a commercial modifier; however, fumaric acid was not detected under the working conditions used with this method. Turcat *et al* (1994) reported the determination of six organic acids in snow and rain water, and Shirao *et al* (1994) reported the determination of organic acids in urine.

The aim of the present study was to optimize the separation and identification of those organic acids most frequently exuded by plant roots, using *Lupinus* as a model, by capillary zone electrophoresis (CZE). A further objective was to establish patterns of organic acid exudates related to season, plant age or soils.

EXPERIMENTAL

Reagents and samples

Oxalic, formic, fumaric, malic, succinic, citric, acetic and lactic acids, and 2-morpholinoethanesulphonic acid (MES) were from Sigma (St Louis, MO, USA), *N*-cetyl-*N*,*N*,*N*,-trimethylammonium bromide (CTAB) was from Fluka (Buchs, Switzerland), potassium diphosphate, boric acid, sodium nitrate and potassium hydroxide were from Merck (Darmstadt, Germany), and methanol was from Scharlau (Madrid, Spain).

Exudates were collected from *Lupinus* according to Ström *et al* (1994). Sampling was carried out at the experimental plot of the Universidad San Pablo CEU (Madrid, Spain). Roots were gently washed (taking special care with the thinner roots) in order to remove the adhering soil. Plants with cleaned roots were placed in tubes (covered with aluminium foil to prevent algal growth) containing 60 mL of sterile distilled water, and placed in a culture chamber under a 14 h light: 10 h dark photoperiod and a corresponding 25°C: 18°C temperature regime. After 24 h the plants were transferred to new tubes and incubated under similar conditions for a further 24 h. The exudates collected after 24 and 48 h were immediately frozen for further analysis.

Prior to analysis, samples were defrosted, filtered through 0.22 μm filters (Millipore Corporation, Bedford, MA, USA) and then diluted 1:10 (v/v) with Milli-Q deionized water.

Instrumentation

The capillary electrophoresis system was a Beckman (Palo Alto, CA, USA) model P/ACE with the separation being carried out on an uncoated fused-silica capillary (57 cm × 75 μm i.d; Beckman). The injection mode used was hydrostatic for 10 s, and detection was performed at 200 nm. The capillary temperature was maintained at 25°C; the power supply and buffer conditions are discussed below.

RESULTS AND DISCUSSION

The conditions described for obtaining root extracts were the best available since this process is maximal when roots are stored in distilled water.

Detection of root exudate components

Normally, organic ionic species are detected by indirect UV spectroscopy, but when compounds are in low concentration or are present in mildly polluted media (such as is the case with the exudate samples considered here), this method is not sufficiently sensitive. Detection at 195 nm generally gave a greater absorbance than at 200 nm but the presence of an unassigned negative peak caused interference.

Optimization of separation parameters

Optimization of the parameters was carried out by considering the influence of electrolyte composition, pH, applied voltage and modifier concentration on the separation process. Based on literature data (Huang *et al*, 1989; Kenney, 1991; Devêvre *et al*, 1994; Turcat *et al*, 1994; Shirao *et al*, 1994), a negative potential difference was applied in order to make the species migrate towards the anode located near the detector, and a flow modifier was added to reverse electro-osmotic flow. Tests were carried out on a mixture of nitrate (which was always present in the samples) and the eight organic acids, oxalic and fumaric (at 2 ppm), formic, malic, succinic, citric and acetic (at 20 ppm), and lactic acid (at 50 ppm).

A variety of borate buffers ranging from 50 to 200 mM with increasing pH from 8.2 to 10.2 and containing 0.1–1.0 mM CTAB as modifier were used, but all gave poor separation. Initially, high pH values were chosen to ensure that all of the organic acids were in their anionic form, but since greater resolution was required, buffers with pH values closer to the pK_a values of the analytes used were assayed. Differences in the degree of ionization of the analytes resulted in different mobilities which are important for achieving separation in the CE system. When 100 mM MES buffers with pH values varying between 5.5 and 7.0 and containing between 0.1 and 1.0 mM CTAB were tested, noisy baselines and poor separation resulted. The best separations were obtained when phosphate buffers were employed and hence optimization was performed using these buffers.

As the interaction of phosphate with the capillary surface, and its binding with the silica surface is known and may be the cause of better separation (Li, 1993), an initial pre-conditioning of the capillary was made by passing sodium hydroxide for 10 min followed by water for 5 min, and then the tested phosphate buffer for 15 min.

Since peak width depends on the ratio of concentration in the original sample solution to that of the buffer in the column (an effect known as sample stacking) an initial buffer concentration of 200 mM phosphate was chosen with pH values between 5.5 and 7.0, adjusted with potassium hydroxide, in the presence of 0.5 mM CTAB. Satisfactory results were obtained with a phosphate buffer at pH 6.0. However, ionic strength and buffer concentration also have significant effects on solute mobilities, and so three phosphate buffer concentrations (100, 200 and 300 mM) each at pH 6.0 and each containing 0.5 mM CTAB were studied (Fig. 1). Resolution was improved by moving from 100 to 200 mM buffer, whilst at 300 mM buffer the operating time increased and the electric current was elevated without further improvement in resolution.

The capillary used was 57 cm in length; the use of a

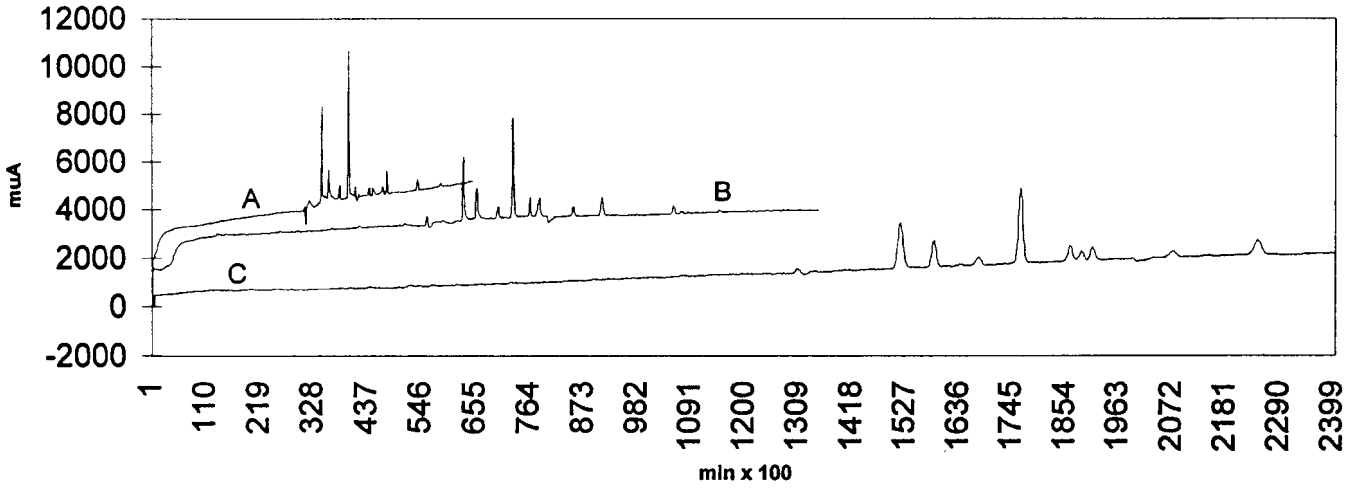

Figure 1. Electropherograms of the eight organic acids together with nitrate carried out at pH 6.0 in phosphate buffers of concentration: (**A**) 100 mM; (**B**) 200 mM; and (**C**) 300 mM.

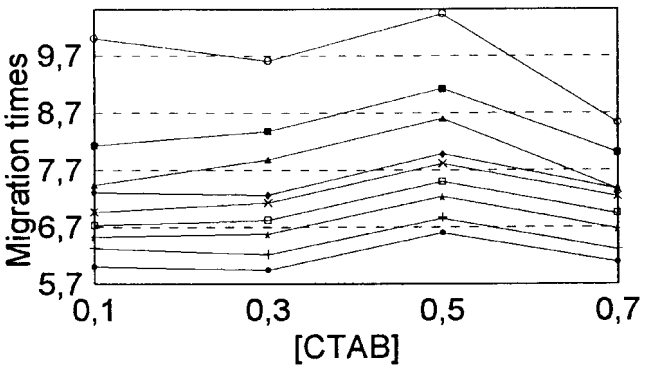

Figure 2. The effects of the concentration of CTAB (mM) on the migration times of: (●) nitrate; (+) oxalic acid; (★) formic acid; (□) fumaric acid; (×) malic acid; (◆) succinic acid; (▲) citric acid; (■) acetic acid; and (○) lactic acid (for assay conditions see Experimental section).

shorter capillary gave poor resolution of some peaks, whilst a longer capillary gave unacceptably long retention times.

Huang *et al* (1989) showed that, to detect the kind of anion considered here, it is necessary to reverse the polarity of the electric field and also to control the electro-osmotic flow by adding a cationic surfactant. Figure 2 shows the effect on migration time of adding CTAB in the concentration range 0.1–0.7 mM. From 0.2 to 0.5 mM only slight differences are apparent, although the best separation is at 0.5 mM. At 0.7 mM the migration times decrease, owing to the reversion of electro-osmotic flow, but the resolution is worse. Under these conditions all of the analytes migrate to the detector more rapidly than the electro-osmotic flow, as was shown by the injection of the neutral marker acetone which had a migration time of 15.3 min, longer than all of the components in the mixture.

Since higher voltages give shorter analysis times and narrower peaks (provided that the equipment has an effective heat dissipation system), all of the studies reported here were made at the maximum working voltage which, following Ohm's law determined from an Ohm's law plot, was found to be −10 kV.

Repeatability and reproducibility of migration times

One of the criteria for peak assignment or confirmation of the presence of a compound in a mixture is based on migration time, and hence the accuracy of assignment depends on the reproducibility of the measured migration time.

Repeatability was determined by carrying out six successive injections of a mixture of standard acids under the selected working conditions (phosphate buffer (200 mM) containing CTAB (0.5 mM) and working at an applied voltage of −10 kV). Between the injections, the capillary was washed with sodium hydroxide for 5 min, with water for 5 min, and with running buffer for 15 min in order to restore capillary conditions. Initially,

Table 1. Relative Standard Deviation (RSD) values for the migration times of analytes

	Migration time (min)	RSD (%) Single buffer stock[a]	RSD (%) Two buffer stock[b] Day 1	RSD (%) Two buffer stock[b] Day 2	RSD (%) Two buffer stock[b] Days 1 + 2
Nitrate	5.60	2.33	1.42	1.94	1.63
Oxalate	5.83	2.27	1.36	1.90	1.60
Formate	6.19	2.24	1.30	1.90	1.57
Fumarate	6.44	2.17	1.29	1.88	1.55
Malate	6.73	2.13	1.27	1.84	1.51
Succinate	6.90	2.12	1.21	1.80	1.47
Citrate	7.48	2.04	1.17	1.76	1.43
Acetate	7.98	1.96	1.14	1.73	1.40
Lactate	9.23	1.86	1.02	1.69	1.33

[a] Same buffer stock used for washing the capillary and for the separations.

[b] Different buffer stocks used for washing the capillary and for the separations.

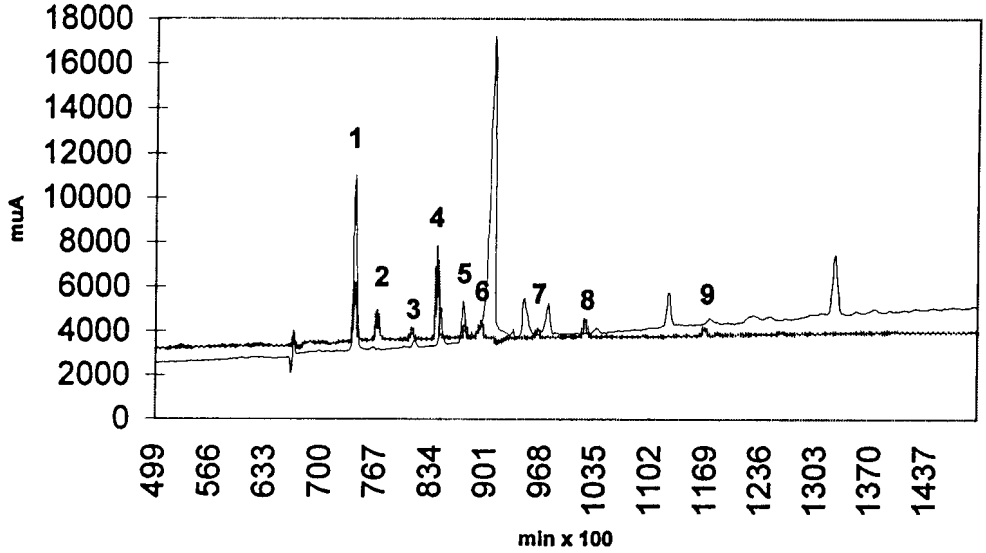

Figure 3. Electropherograms of an exudate from *Lupinus* (solid line) and a mixture of standards (dotted line). Key to peak identity: (**1**) nitrate; (**2**) oxalic acid; (**3**) formic acid; (**4**) fumaric acid; (**5**) malic acid; (**6**) succinic acid; (**7**) citric acid; (**8**) acetic acid; (**9**) lactic acid (for assay conditions see Experimental section).

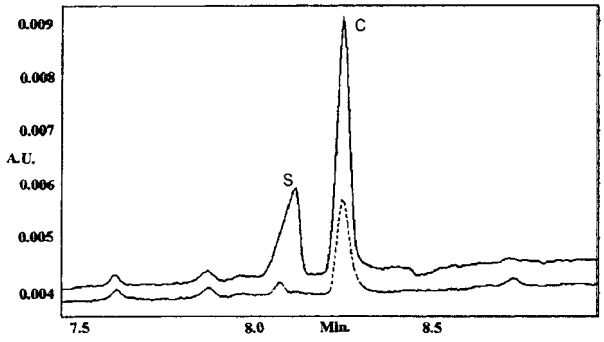

Figure 4. Electropherograms showing succinic and citric acids in an exudate from *Lupinus* (diluted 1:25): the separation buffer employed was 200 mM phosphate (pH 6.0) containing 10% methanol. The dotted line shows the exudate extract and the solid line shows the same extract spiked with citric (c) and succinic (s) acids.

the same buffer was used for washing and for separations, giving a relative standard deviation (RSD) for migration times of around 2.12% (Table 1). Further experiments used two different stocks of phosphate buffer, one for conditioning and filling the capillary and another for the separations, thus ensuring that the filling buffer was always fresh and had not been degraded by the operating voltage. A smaller RSD, of around 1.24%, was achieved and hence this technique was employed for the remainder of the work.

Reproducibility was evaluated by repeating the same experiment with six injections on different days and pooling the results (Table 1). The results were in the range of values reported for CE (Baker, 1995).

Qualitative analysis of samples

In order to identify which peaks in the sample corresponded to each of the organic acids studied, sample and standard electropherograms were overlaid. As the migration times might differ depending on the other components in the mixture, 2 ppm of nitrate were injected together with each standard and sample, and the nitrate peaks were superimposed. The results shown in Fig. 3 indicate that all eight organic acids appear to be present, although it cannot be excluded that other compounds present in the sample might have migration times identical to an organic acid component. Therefore, the sample was spiked with each of the corresponding standards to see if the appropriate peak became more intense without deformation. The previous peak assignment was confirmed using this technique; however, in the sample, succinic and citric acid could not be separated and so methanol was added to the sample as a way to increase the viscosity of the running buffer and to change its dielectric constant. Figure 4 shows the resolution of succinic acid (migration time 8.11 min) and citric acid (migration time 8.28 min) following the addition of 10% methanol to the running buffer. The lower trace (dotted) corresponds to the sample diluted 1:25 with water, whilst the upper trace shows the same sample spiked with the respective standards. Peaks for both organic acids are clearly separated and increase symmetrically in size when spiked.

CONCLUSION

For organic acids in root exudates, the proposed CZE method provides good separation in a relatively short time, without time-consuming preparations, and using a very limited amount of sample and reagents. The developed methodology will be useful in further studies aimed at determining the effects of different parameters on rhizosphere processes, and will become a facile manner by which to establish the patterns of organic acids in different species.

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