Sensitive analysis of poly-carboxylic acids in soil solution by capillary electrophoresis after excimer-forming fluorescence derivatization

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Abstract
A sensitive method for the determination of citrate and malate in sub-microliter samples of soil solution is described. The organic acids were derivatized with a pyrene reagent (1-pyrenebutanoic acid hydrazide) and analysed by micellar electrokinetic chromatography separation. The citrate and malate derivatives were detected within 10 min by using a fluorescence detector with a broad excitation wavelength of 240-400 nm and an emission wavelength of 400 nm. The detection limits (noise x 3) were about 0.24 μM for citrate and 0.72 μM for malate. By using internal standardization, this method was applicable to the determination of citrate and malate in soil solution. Furthermore, application of siphon injection with a commercial micropipette enabled the injection of a sub-microliter sample into the analytical system with acceptable reproducibility. When combined with a microsampling method, the method presented here will be useful for the sensitive and selective analysis of citrate and malate in soil solution with high spatial resolution.

Key Words
Organic acids, Soil solution, Capillary electrophoresis, Excimer-forming fluorescence derivatization

Introduction
Low-molecular-weight organic acids are involved in many processes of soil located in the vicinity of living or dead organisms, i.e. rhizosphere and detritusphere. These include nutrient acquisition, metal detoxification and mineral weathering. Since most of these roles rely to a large extent on the ability of the organic acids to complex metal cations, it is mainly the poly-carboxylic acids such as citrate, malate and oxalate that have been hypothesized to perform these processes (Jones *et al.* 2003). Analysis of organic acids in soil solution has often been made by high performance liquid chromatography (HPLC) and capillary electrophoresis (CE). The HPLC methods include reverse-phase chromatography and ion chromatography in ion-exchange and ion-exclusion modes (Tani *et al.* 2001), whereas the CE methods usually apply the indirect ultraviolet (UV) detection due to the low absorbing ability of organic acids in the UV and visible ranges (Dahlén *et al.* 2000). The smaller sample volume attained by on-capillary detection with a smaller path length is a suitable feature of CE for the analysis of soil solution with high spatial resolution. However, this feature also causes the lower detection ability than HPLC. Nohta *et al.* (2003) recently developed a very sensitive fluorescence assay for poly-carboxylic acids, which relies on intramolecular excimer-forming fluorescence derivatization with a pyrene reagent. They used an HPLC separation system. This fluorescent derivatization provides a promising sensitive and selective analysis of poly-carboxylic acids by CE. This is especially true of soil solutions whose concentrations of poly-carboxylic acids are sometimes too low to be detected by CE with the conventional indirect UV detection.

In this paper, we describe the application of this fluorescence derivatization method to CE with a special interest in the analysis of soil solution. In addition, we propose a novel and practical method for introducing sub-microliter samples into a CE capillary, which is useful for the analysis of soil solution at high spatial resolution.

Methods
Reagents and chemicals
1-pyrenebutanoic acid hydrazide (PBH) was purchased from Invitrogen. *N*-(3-Dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC-HCl) was purchased from Sigma-Aldrich. Other chemicals used were analytical grade. The solutions of PBH (5 mM in dimethylsulfoxide (DMSO)), EDC-HCl (0.2 M in water) and pyridine (40% v/v in DMSO) were prepared before use. The pyridine solution was mixed with the EDC-HCl solution at 1:1 (v/v), and this solution was further mixed with the PBH solution at 1:1 (v/v). This

reagent mixture (RM) was prepared immediately after preparation of each solution. The RM was stable for 2 days after preparation when it was stored in the dark at room temperature.

Derivatization procedure for standard solutions
Standard solutions of citrate and malate with a volume ranging from 500 nL to 100 µL were placed in a 0.5 mL microcentrifuge tube and the same volume of the RM was added to each. For manipulation of a 500 nL solution, a micropipette (Pipetman P2, Gilson) was used with care. Then the tube was sealed, heated at 40°C for 60 min in a block heater and cooled in ice for about 10 min (Nohta *et al.* 2003). The resulting solution was applied to the CE instrument without further dilution.

Capillary electrophoresis
Analysis was carried out in a custom-built capillary electrophoresis system (Bazzanella *et al.* 1998) equipped with a fluorescence detector (Argos 250 B, Flux Instruments, Basel, Switzerland) and a high-voltage power supply (HCN 6 M-30000, FuG Elektronik GmbH, Rosenheim, Germany). A fused-silica capillary of 75-µm I.D. and 363-µm O.D. (TSP075375, Polymicro Technologies, Arizona, USA) was used. The total length of the capillary was 80 cm, and the length from the inlet to the detector was 57 cm. On the injection side, about 2 mm of the polyimide coating was removed to reduce the outer diameter and facilitate the introduction of the sample using a micropipette as described below. Unlike the commercial system in which the inlet point of the capillary is sealed and inaccessible, the capillary in our system is flexible and accessible. Owing to this modification, it was possible to siphon sub-microliter solutions into the capillary by lifting the capillary and dipping it into a sample solution located in a micropipette tip (e.g., Diamond DL10, Gilson) as depicted in Figure 1. The difference in height between the inlet and the outlet electrode buffer (5 cm) was sufficient to set up a siphon. For sample introduction, this was run for 50 seconds. For sub-microliter samples, a volume of 500 nL was usually held in the pipette tip. The volume could be reduced to be about 200 nL by careful manipulation of a micropipette. For bigger samples (> 10 µL), injection was carried out by lifting the capillary and dipping it in a sample located in a horizontally inclined microcentrifuge tube rather than a pipette tip. For the separation of derivatized compounds, micellar electrokinetic chromatography was used. The buffer was composed of 50 mM sodium dodecyl sulfate (SDS), 20 mM sodium tetraborate and 5% (v/v) acetonitrile. It was used without adjustment of the pH (9.2). Separation was carried out at a constant voltage of 30 kV (negative at the detection side) with a current of between 80 and 100 µA. The instrument did not include temperature control of the capillary, which was run at ambient temperature. For detection, the fluorescence detector was operated at a broad excitation wavelength (240-400 nm) and an emission wavelength of 400 nm.

To compare the sensitivity of fluorescence detection with that of the conventional method, citrate and malate in water were also analysed by the indirect UV method according to Bazzanella *et al.* (1998).

Derivatization and analysis for soil solution samples
Two Cambisols were collected from a subsurface horizon (about 20-40 cm) at Henfaes Agricultural Research Station of Bangor University in Abergwyngregyn. One was located on a flat grassland used as a pasture (Cambisol-1), and the other was located on a hill slope predominated by bracken (Cambisol-2). Two Andosols were also collected from a surface 0-15 cm at experimental farms of National Institute of Agro-Environmental Sciences in Tsukuba (Andosol-1) and of Shimane University in Ohta (Andosol-2). Soil solution samples were collected from these four soils by adding deionized water at 50% (v/w) and vacuum suctioning the water out using a hollow fibre sampler after a few hours (Yanai *et al.* 1993). The pH and the concentrations of Ca and Mg in the soil solutions are given in Table 1. In order to evaluate the influence of solutes in soil solution on the derivatization reaction, the samples spiked with standards at the rate of 1:1 (v/v) were subjected to the above derivatization procedures. The influence of pure metal cations on derivatization was also evaluated.

Results
Performance of micropipette injection method
The sample volume introduced into the CE system is usually up to 10 nL, although this depends on the injection method. For commercial CE system, however, the volume required for analysis is usually more than 5 µL. This means that more than 99% of the sample prepared for injection is wasted. The micropipette injection method described here could decrease the required sample volume to a few hundred nanoliters. The reproducibility of our method was evaluated by introducing 500 nL of 1 µM Fluorescein sodium salt by

siphon under controlled conditions (50 sec, 5 cm height) and detecting it under the same conditions as those used for the analysis of poly-carboxylic acids. The relative standard deviations for retention time and the peak area were 2.99% and 8.02%, respectively, for 10 successive runs. These values indicate that this injection method can be influenced by some artefacts but still provides a simple and reliable way for treating sub-microliter samples with simple equipment. The reproducibility might be improved by the temperature control of the capillary.

Separation and detection of poly-carboxylic acid standards
An electropherogram obtained from a mixture of citrate and malate at 40 µM before derivatization is shown in Figure 2. Citrate and malate derivatives were separated from each other. The sensitivity was better for citrate than the first malate peak. When oxalate is present, the peak of the oxalate derivative overlapped with the second malate peak. This separation is therefore effective for citrate and malate but not for oxalate. A peak for derivatives of acetate and shikimate, which are mono-carboxylic acids, also appeared at the same retention time as the second malate peak only when they were present at mM concentrations. This shows that the detection is relatively specific to poly-carboxylic acids as described by Nohta *et al.* (2003). As for the retention time, citrate and malate peaks were found within 10 min. This was much shorter than the original HPLC method (50 min) (Nohta *et al.* 2003).

Limits of detection and quantification
From the data of Figure 2, the signal to noise ratios for citrate and malate were calculated to be 506 and 166, respectively. The detection limits (noise x 3) were about 0.24 and 0.72 µM for citrate and malate before derivatization. Using the indirect UV method with the same injection system, the signal to noise ratios for citrate and malate at 40 µM were calculated to be 6 and 8, respectively (data not shown). Compared with the indirect UV method, therefore, the sensitivity for citrate and malate was improved greatly, despite the additional dilution of the samples during derivatization. Calibration curves for citrate and malate standards showed a linear relationship between the signal and sample concentrations up to 20 µM (40 µM before derivatization) (data not shown). A signal of citrate at 0.5 µM (1 µM before derivatization) could be quantified.

Analysis of soil solution
Analysis of soil solution introduced additional problem, as both the pH of samples and cations present affect the derivatization of organic acids. As shown in Figure 3, peak areas of the citrate and malate derivatives were affected by soil solution matrices to various degrees. Thus, addition of target compounds to a sample prior to derivatization (internal standardization) is needed for the quantitative analysis of citrate or malate in soil solution. The cause for the reduced sensitivity for citrate may be due to cation interference, because polyvalent cations especially Fe^{2+} and Al^{3+} significantly inhibited the derivatization of citrate (Table 2). At the same time, using a phosphate buffer, it was found that the sensitivity for citrate decreased with an increase of the pH from 6 to 8 (data not shown). In terms of the sensitivity, it seems that this method can be applied to samples from a wide range of upland agricultural soils but that it may not be suitable for samples from submerged soils containing abundant Fe^{2+} or samples from extremely alkaline soils.

Conclusions
Excimer-forming fluorescence derivatization of poly-carboxylic organic acids followed by the analysis by CE provided rapid, specific and sensitive determination of citrate and malate in water and soil solutions. Furthermore, application of siphon injection with a commercial micropipette enabled to inject a sub-microliter sample into the analytical system with acceptable reproducibility.

Table 1. The pH and the concentrations of Ca and Mg in the soil solutions used in the experiment.

	pH	Ca (mM)	Mg (mM)
Cambisol 1	7.13	0.30	0.04
Cambisol 2	5.63	n.d.	n.d.
Andosol 1	6.25	0.62	0.42
Andosol 2	6.64	0.46	0.14

The pH was measured by a glass electrode.
Ca and Mg concentrations were measured by CE with the indirect UV detection (Bazzanella et al. 1998).

Table 2. (lower left). Influence of cations on peak area of citrate derivative. 20 µM citrate mixed with cation solutions (or deionized water as a control) at 1:1 (v/v) was subjected to derivatization. Values indicate the percentages of peak area relative to a control treatment (means ± S.D., n=3).

	Relative peak area (%)
1 mM Ca^{2+}	32.0 ± 2.7
10 mM Ca^{2+}	27.7 ± 2.2
1 mM Mg^{2+}	14.0 ± 1.1
10 mM Mg^{2+}	12.8 ± 1.3
100 µM Fe^{2+}	9.1 ± 4.1
1 mM Fe^{2+}	Undetectable
100 µM Al^{3+}	8.3 ± 4.6
1 mM Al^{3+}	Undetectable

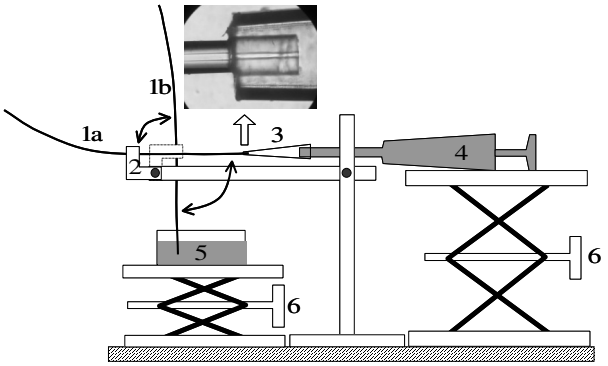

Figure 1 (upper right). Set up for micropipette injection system. 1, capillary at the positions for injection (a) and separation (b); 2, flexible arm; 3, pipette tip; 4, micropipette (Pipetman P2); 5, inlet buffer with an electrode; 6, lift.

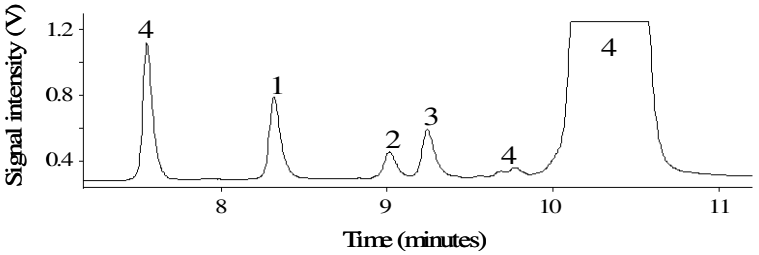

Figure 2. An electropherogram of fluorescent derivatives of citrate and malate, each of which was 40 µM before derivatization. Peaks: 1, citrate; 2, malate; 3, malate (oxalate, acetate or shikimate if present); 4, reagent peaks.

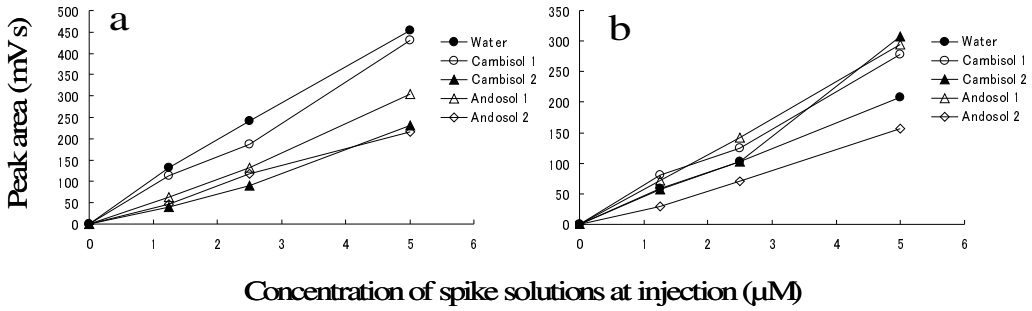

Figure 3. Influence of different soil solution matrices on peak areas of derivatives of citrate (a) and malate (b).

References

Bazzanella A, Lochmann H, Tomos AD, Bächmann K (1998) Determination of inorganic cations and anions in single plant cells by capillary zone electrophoresis. *J. Chromatogr. A*. **809,** 231-239.

Dahlén J, Hagberg J, Karlsson S (2000) Analysis of low molecular weight organic acids in water with capillary zone electrophoresis employing indirect photometric detection. *Fresenius J. Anal. Chem*. **366**, 488-493.

Jones DL, Dennis PG, Owen AG, van Hees PAW (2003) Organic acid behavior in soils – misconceptions and knowledge gaps. *Plant Soil* **248**, 31-41.

Nohta H, Sonoda J, Yoshida H, Satozono H, Ishida J, Yamaguchi M (2003) Liquid chromatographic determination of dicarboxylic acids based on intramolecular excimer-forming fluorescence derivatization. *J. Chromatogr. A*. **1010**, 37-44.

Tani M, Shida KS, Tsutsuki K, Kondo R (2001) Determination of water-soluble low-molecular-weight organic acids in soils by ion chromatography. *Soil Sci. Plant Nutr*. **47**, 387-397.

Yanai J, Araki S, Kyuma K (1993) Use of a looped hollow fiber sampler as a device for non-destructive soil solution sampling from the heterogeneous root zone. *Soil Sci. Plant Nutr*. **39**, 737-743.