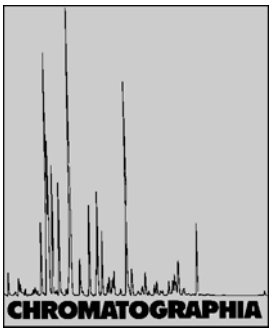
Application of Co-Electroosmotic Capillary Electrophoresis for the Determination of Inorganic Anions and Carboxylic Acids in Soil and Plant Extract with Direct UV Detection



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Key Words

Co-capillary zone electrophoresis
Direct UV detection
Organic acids and anions
Environmental samples
Soil and plant tissues

Summary

Indirect UV detection in capillary zone electrophoresis (CZE) is frequently used for the determination of inorganic anions and carboxylic acids. However, there are few reports on direct UV detection of these solutes in real samples. This paper describes the use of direct UV detection of inorganic anions and organic acids in environmental samples using co-electroosmotic capillary zone electrophoresis (co-CZE) at 185 nm. The best separation and detection of the solutes was achieved using a fused silica capillary with an electrolyte containing 25 mM phosphate, 0.5 mM tetradecyltrimethylammonium bromide (TTAB) and 15% acetonitrile *(v/v)* at pH 6.0. Four common inorganic anions (Cl^-, NO_2^-, NO_3^- and SO_4^{2-}) and 11 organic acids (oxalic, formic, fumaric, tartaric, malonic, malic, citric, succinic, maleic, acetic, and lactic acid), were determined simultaneously in 15 min. Linear calibration plots for the test solutes were obtained in the range 0.02 – 0.5 mM with detection limits ranging from 1 – 9 μM depending on the analyte. The proposed method was successfully used to determine inorganic anions and carboxylic acids in soil and plant tissue extracts with direct injection of the sample.

Introduction

Low molecular weight (LMW) carboxylic acids play an important role in soil chemical processes in the ecosystem [1]. These acids occur in soil solution and at the soil-root interface where their concentrations may exceed one millimolar due to microbial processes and root exudation [2]. Carboxylic acids in soils enhance anion availability to plants by participating in ligand exchange reactions on mineral surfaces [3–4]. Given the complex nature of chemical reactions that occur at the soil-root interface and its implications to plant uptake of nutrients and other toxic substances, analyses of the nature of LMW acids and the composition of soil solution have been the focus of much research during the past 10 years. However, the research has been limited by the lack of sensitive analytical tools for estimating low concentrations of LMW acids in soil solution. Rapid, sensitive and simultaneous analysis of inorganic anions and carboxylic acids is difficult because of the lack of chromophoric groups and poor separation selectivity. For these reasons, there is a need to develop an effective and reliable method for the analysis of inorganic anions and organic acids in real samples.

Gas chromatography (GC) and liquid chromatography (LC) are commonly used for the determination of anions and carboxylic acids in a wide variety of samples. Derivatization GC by trimethysilytation (TMS) provides excellent resolution and high detection sensitivity [5–6]. However, the procedure of derivatization is complex and time-consuming. Liquid Chromatographic methods based on ion-exclusion chromatography (IEC) have been widely employed for the analysis of carboxylic acids, where the separation selectivity for carboxylic acids depends on their first dissociation constants (pKa_1) and their hydrophobicity. Therefore, carboxylic acids with similar p*K*a's can not be separated well [7]. Furthermore, co-elution of anions such as Cl^-, NO_2^- and NO_3^-, which are importance in ligand exchange reaction on mineral surface [1], with oxalic, maleic and citric acids has been noted [8].

In recent years, capillary zone electrophoresis (CZE) has been recognised as a powerful separation technique for the analysis of ionic solutes in soils because of good resolution, high speed, simplicity and reduced sample preparation time of this technique [9]. Usually the CZE separation of organic anions is carried out in the co-electroosmotic mode, where the

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migration time of the anion can be significantly reduced by establishing a co-directional movement of electroosmotic flow (EOF) with the anionic solutes. A co-directional migration of the negatively charged solutes and the EOF can be achieved by coating the negatively charged inner surface of the fused silica capillary with cationic surfactant and reversing the polarity of the power supply [10]. This mode has been successfully used for the separation of many compounds, including inorganic anions [11], phenols [12] and organic acids [13]. Consequently, chromate [14], aromatic carboxylates such as pyromellitate, trimellitate, phthalate, benzoate [15], 2,6-naphtha-lenedicarboxylate [16], 2,6-pyridinedicarboxylic acid [17] and 5-sulfosalicylate [18], have been employed as background electrolytes (BGEs) for indirect UV detection. However, a serious problem encountered when using indirect UV detection is the presence of a concentration overloading effect. This effect causes broad triangular peaks when the effective mobility of a solute differs from that of co-ion in the buffer [19]. Recently, Volgger et al. [13, 20] separated carboxylic acids using co-CZE with direct UV detection at 185 nm with a borate-phosphate electrolyte using hexadimethrine bromide as an EOF modifier. However, some important carboxylic acids such as formic, fumaric, lactic, maleic, malic, malonic, oxalic and succinic acid that are commonly present in soil and plants were not identified.

In this paper, we describe the use of direct UV detection of organic acids separated by co-CZE in a phosphate electrolyte. The parameters affecting the separation selectivity for the organic anions of interest, including the type of cationic surfactants, the concentration of the running electrolyte and the organic modifier were systematically investigated. The problem of co-elution of some organic acids with inorganic anions was overcome and the proposed method was shown to be useful for the analysis of soil and plant extracts.

Experimental

All reagents obtained from Sigma and Aldrich (Sydney, Australia) were of analytical grade and used without further purification. Standards of the anions and organic acids tested were prepared daily from 10 mM stock solutions by dilution with Milli-Q water. Electrolytes required for CZE were prepared by dissolution of an appropriate amount of NaH_2PO_4 in Milli-Q water, which contained appropriate amounts of tetradecyltrimethylammonium bromide (TTAB) and organic solvents. All electrolytes were filtered through a Millipore 0.45 μm membrane filters and degassed in an ultrasonic bath prior to use. Electrolyte pH was adjusted with 0.1 M NaOH or 0.1 M H_3PO_4 solutions.

All CZE experiments were preformed using a Quanta 4000 (Waters, Milford, USA). The system was controlled by Millennium (Waters, Milford, USA) software. Separation was carried out on fused-silica capillaries with 50 μm I. D × 100 cm total length (95.5 cm effective length). The UV detector was set at 185 nm.

Prior to use, a new capillary was pretreated with the following cycles: 0.1 M NaOH for 30 min, 0.01 M NaOH for 30 min, deionized water for 30 min and then a 25 mM phosphate electrolyte for 30 min. The capillary was rinsed with phosphate electrolyte for 2 min between each run. Samples were injected in the hydrostatic mode for 30 s. The capillary was held at 25 °C, and the applied voltage was constant at −20 kV. 0.05% (*v/v*). Benzyl alcohol was used as a neutral marker for the determination of electroosmotic flow and electroosmotic mobilities were calculated from by the equation in described in reference [10]. Identification of each solute was verified by spiking with known standards.

Sandy soils with permanent pasture were shaken for 6 hrs at 20 °C on a mechanical shaker using deionsed water (soil: water = 1:5) as an extractant. The extracts were then centrifuged (4000 rpm for 3 min) and supernatant filtered through a 0.45 μm membrane filter. The soil extract was concentrated on anion-exchange membranes (Bio-Rad Laboratories) using bicarbonate as described by Szmigielska et al. [6]. Frozen leaf tissues (Spring wheat harvested at 4 weeks) were ground with liquid N in a mortar and pestle. Solute in powdered plant tissue were extracted twice with 5 mL water (0.1 g^{-1} 5 mL^{-1}) in water bath held at 50 °C for 1 h [15]. The extracts were then centrifuged (4000 rpm for 3 min) and the supernatants filtered through a 0.45 μm membrane filter before injection into CZE system.

Results and Discussion

Separation Conditions

Separation of anions is influenced by the ionic mobility of the solutes of interest and EOF. The mobility of an anion and the EOF are in turn controlled by the physical properties of the electrolyte, including pH, concentration of electrolyte and proportion of organic solvents present [10]. Hence, separation resolution also depends on these properties. Furthermore, to achieve a fast separation, it is required that the EOF moves in the same direction as anions and organic acids (co-EOF mode). To obtain high electrophoretic separation of the solutes, two common cationic surfactants, cetyltrimethylammonium bromide (CTAB) and teteradecyltrimethylammonium bromide (TTAB), were used.

Volgger et al. [13] reported that an electrolyte solution containing 5 mM tetraborate, 10 mM phosphate and 0.001% (*w/v*) polybrene at pH 3.9 could be used for the direct UV detection of carboxylic acids using co-CZE. A 25 mM phosphate containing 0.5 mM TTAB at pH 5.8 was used as the running electrolyte since pH at 5.8 exceeded the p*K*a of all acids studied in Table I and resulted in substantial dissociation. In addition, reports have demonstrated that the concentration of cationic surfactant greater than 0.5 mM resulted in a constant reversed EOF [21]. With the exception of fumaric and malonic acids, 15 inorganic anions and organic acids were separated by co-CZE using direct UV detection at 185 nm as shown in Figure.1. The poor resolution between fumaric and malonic acid resulted in the similar effective mobilities (fumaric: 5.02×10^{-4} and malonic: 4.99×10^{-4} $cm^2\ V^{-1}\ s^{-1}$). However, this initial test indicates that phosphate electrolyte could be used for the separation of the tested solutes [10]. The p*K*a and effective mobilities of the test solutes are given in Table I.

To investigate the influence of cationic surfactants on separation resolution, two EOF modifiers, CTAB and TTAB, were added to 25 mM phosphate electrolyte of pH 6.0. As shown in Figure 1(b), when CTAB was used as the EOF modifier, the migration time of test solutes and the separation window were dramatically reduced compared to that of TTAB (Figure 1(a)). This was probably due to the higher EOF in the presence of CTAB in the electrolyte (EOF: 4.58×10^{-4} $cm^2\ V^{-1}\ s^{-1}$). A

Table I. Mobility, molecular weight (Mt), Migration time, reproducibility, formula and pK_a of the test solutes.

	Mobility (10^{-4} cm^2/Vs)	Chemical formula	pK_a*	Detection limit (μM)	RSD% (n = 5)
Cl	7.10			5.0	3.78
NO_2	6.86			3.0	3.40
NO_3	6.63			1.0	2.92
SO_4^{2}	6.46			6.5	6.14
Oxalic	6.08	HOOC-COOH	1.23, 4.19.	2.0	1.71
Formic	5.11	HCOOH	3.75.	9.0	4.07
Fumaric	5.02	HOOCCH = CHCOOH	3.03,4.44	1.0	2.83
Malonic	4.99	$HCOOCH_2COOH$	2.83, 5.69.	1.0	3.46
Tartaric	4.89	HOOC-CH(OH)CH(OH)COOH	2.98, 4.34.	1.0	2.09
Malic	4.72	$HOOCCH_2CH(OH)COOH$	3.40, 5.11	1.0	3.01
Citric	4.66	$HOOC=CH_2)_2C(OH)COOH$	3.14, 4.77, 639.	2.0	4.35
Succinic	4.39	$HOOCCH_2CH_2COOH$	4.16, 5.61.	2.0	3.82
Maleic	4.28	HOOCCH=CHCOOH	1.83, 6.07	2.0	3.22
Acetic	3.58	H_3CCOOH	4.76	3.0	3.41
Lactic	3.21	$CH_3CH(OH)COOH$	3.08	2.0	5.51

* Data from Ref. [24]. Detection limts (S/N = 3). RSD%-repatability for the peak area (0.2 mM standard injected).

better resolution between solutes was therefore obtained using TTAB as the EOF modifier, due to lower EOF in the presence of TTAB in the electrolyte (EOF: 2×10^{-4} cm^2 v^{-1} s^{-1}), leading to a broader separation window. The mobility of solutes varies with the type of cationic surfactant electrolyte additive. Similar results were obtained using various cationic surfactants in electrolyte for the separation of carboxylic acids [13] and phenols [21]. The results indicate that the resolution of anionic solutes and the separation window in co-CZE can be manipulated by using surfactants of varying alkyl chain length.

In co-CZE, ionic solutes are separated based on both their charge and size. Therefore, electrolyte pH has a significant effect on resolution. The solute mobility is pH dependent because the dissociation of solute is controlled by electrolyte pH [10]. Changes in the mobility of solutes were pronounced between pH 3 and 6 due to the dissociation of organic acids at their p*K*a. However, the use of electrolyte with pH values above the p*K*a of the organic acids lead to greater than 50% dissociation of organic acids and enhanced separation speed. Therefore, the separation of the 15 test solutes was examined over the pH range 5.0–6.5. Figure 2 shows the effect of electrolyte pH on the effective mobility of the test solutes. It is evident that the increased ionisation of organic acids at pH values above their p*K*a increases their effective mobilities. The effect of pH on mobility appears to be particularly pronounced for the di and triprotic acids (malic, fumaric, malonic, tartaric, malic, critic, succinic acid). In contrast, the mobilities of inorganic anions were nearly constant. Overlapping peaks between 2 acids (fumaric and malonic, malic and citric, malic and tartaric, succinic and maleic acid) were observed when the electrolyte pH was below 6.0 or above 6.5. Furthermore, as the electrolyte pH in-

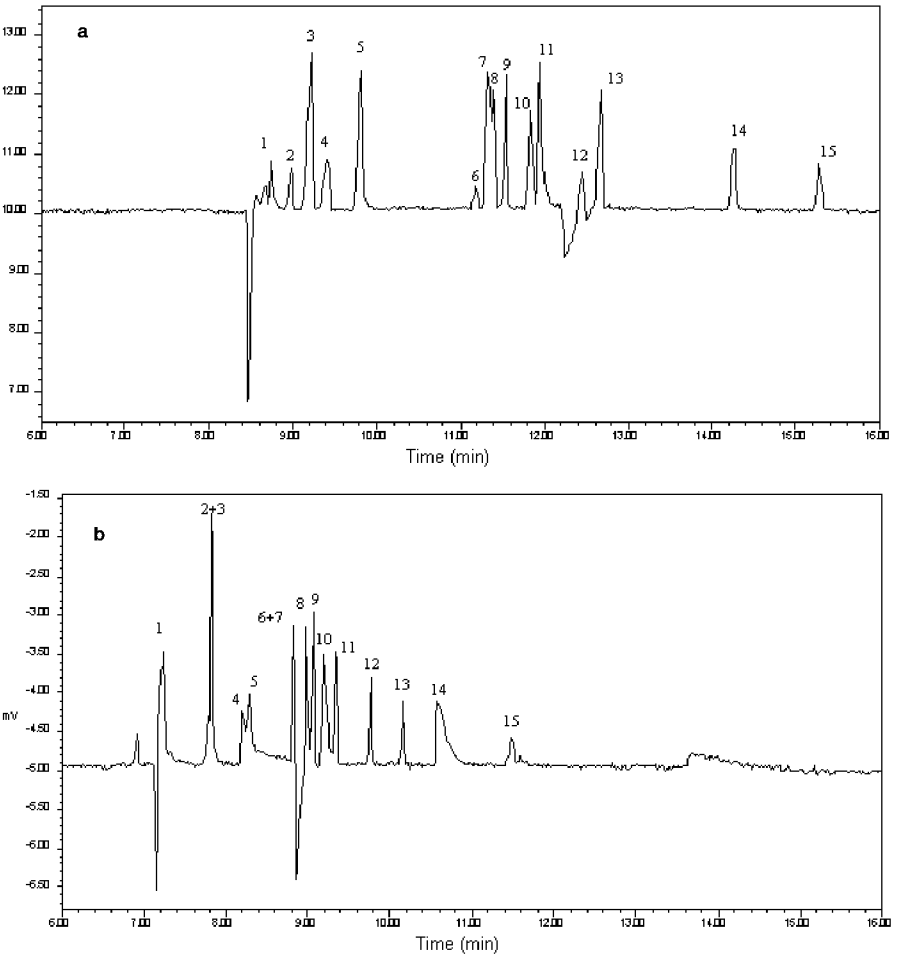

Figure 1. Co-electroosmotic capillary electrophoretic separation of anions and carboxylic acids with different cationic surfactants. (**a**) TTAB (0.50 mM) (**b**) CTAB (0.5 mM). Peaks: **1** = Cl ; **2** = NO_2 ; **3** = NO_3 ; **4** = $SO_4^{=}$; **5** = oxalic; **6** = formic; **7** = fumaric; **8** = malonic: **9** = tartaric; **10** = malic; **11** = citric; **12** = succinic; **13** = maleic, **14** = acetic; **15** = lactic acid. The concentration for each solute: 0.20 mM. Conditions: capillary, fused-silica capillary 50 μm × 100 cm (L: 95.5 cm); electrolyte, 25 mM sodium phosphate at pH of 5.8; applied potential, –20 kV; Hydrostatic injection: 30 s, UV detection at 185 nm. Capillary temperature, 25 °C.

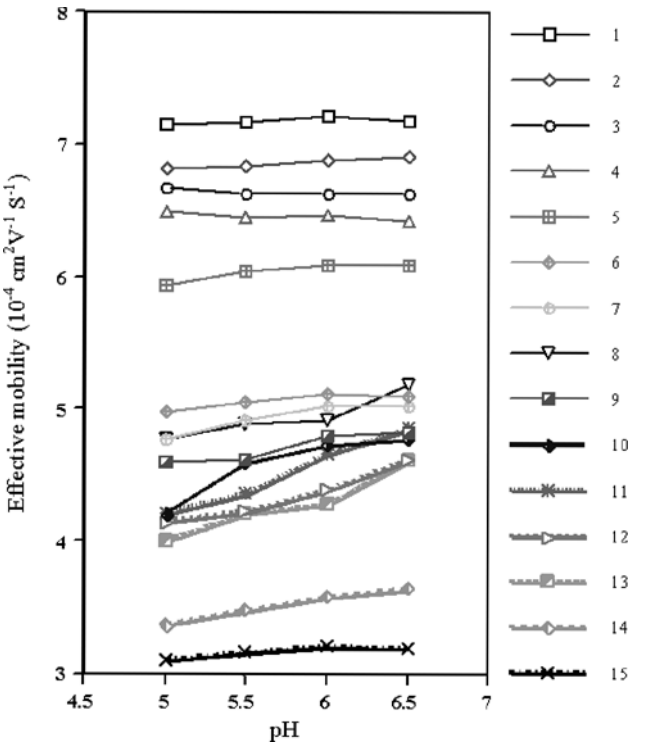

Figure 2. Effect of electrolyte pH on the effective mobility of test solutes. Condition: electrolyte, electrolyte, 25 mM sodium phosphate + 0.5 mM TTAB + 15% acetonitrile (*v*/*v*). Other conditions as in Figure 1.

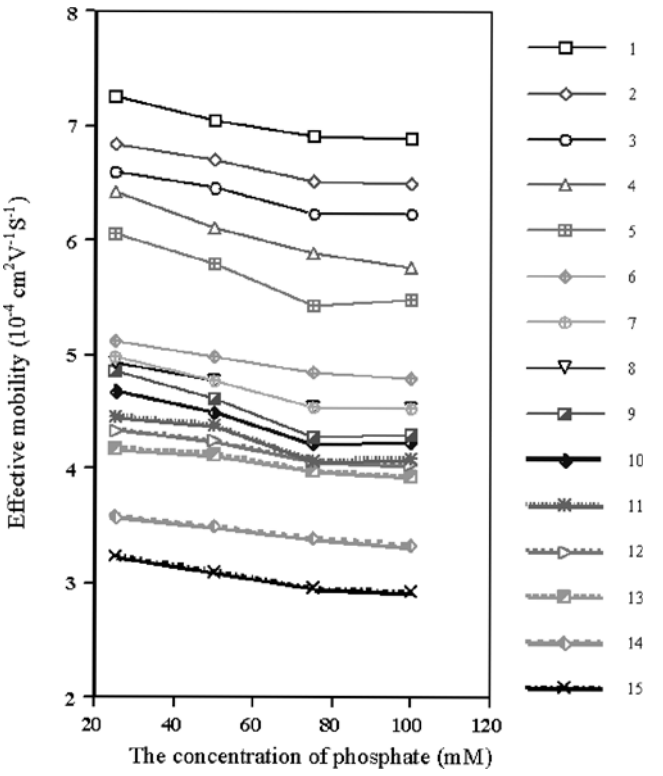

Figure 3. Effect of the concentration of electrolyte on the effective mobility of test solutes. Other conditions as in Figure 2.

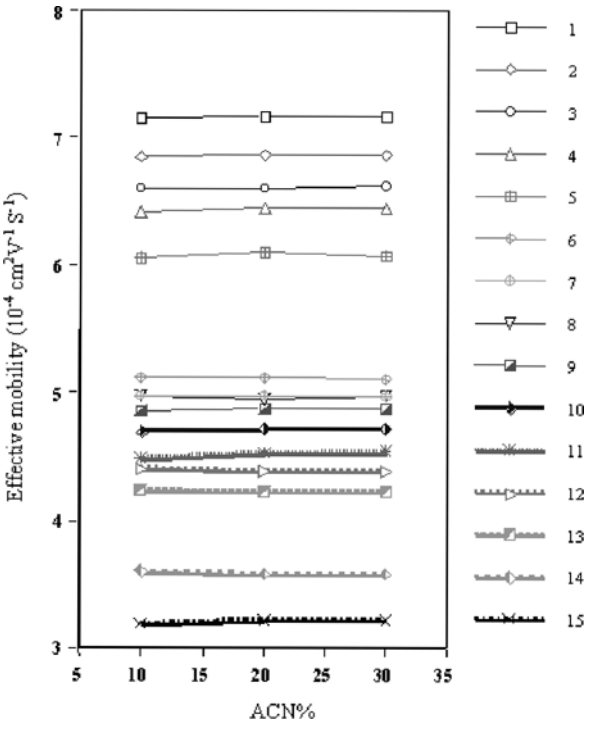

Figure 4. Effect of the content of acetonitrile in the electrolyte on the mobility of test solutes. Other conditions as in Figure 2.

creased, the magnitude of the reversed EOF remained nearly constant. This can be attributed to the ionization of surface sianol group within the capillary (p*Ka* 5.3). The number of the ionized silanol group at the surface of the wall is constant when the electrolyte pH ranged between 5.0–6.0 [22]. Further adsorption of the surfactant on surface of the wall was saturated, leading to a constant of EOF [21].

Previous work on the effect of electrolyte concentration on solute mobility has shown that the mobility of solutes decrease with increasing electrolyte concentration [10]. Therefore, by varying electrolyte concentration peak resolution may be improved. Hence, various concentrations of the electrolyte containing 0.5 mM TTAB at pH of 6.0 were examined to improve solute peak resolutions. Figure 3 shows the relationship between the mobility of the solutes and the concentration of phosphate electrolyte. It can be seen that the mobility for all test solutes decreased as the concentration of phosphate increased. In addition, a decrease in the magnitude of the reversed EOF with increased in electrolyte concentration was observed. The increase in ionic strength results in a decrease in the thickness of the double layer and the zeta potential of the capillary wall and shrinking of the double layer dominating the surfactant adsorption [22]. As a consequence, the mobility for all test solutes and the EOF was reduced. In addition, an electrolyte concentration in the range of 20–50 mM phosphate yielded sharper peak and consequently gave better resolution. However,

co-migration of fumaric and malonic acids in this case caused significant problem for the separation of these organic acids. Furthermore, a decrease in detection sensitivity with increasing electrolyte concentration was observed. Therefore, considering resolution and detection sensitivity, a 25 mM phosphate electrolyte was considered for all subsequent studies.

The influence of acetonitrile on the mobility of solute and the EOF was studied by adding acetonitrile to an electrolyte containing 25 mM phosphate at pH 6.0. Figure 4 shows that the effective mobility of the test solute is almost constant with increasing acetonitrile in the electrolyte. However, it was found that EOF decreased significantly as the content of acetonitrile was increased, whilst the observed mobility of the solute decreased with increasing content of acetonitrile in the running electrolyte. Studies on the influence of organic modifiers on the EOF indicate the decrease of EOF with increasing content results mainly from a decreased electrolyte dielectric constant in electrolyte, which leads to a low value for the zeta potential of the capillary wall [22]. In addition, a decrease in observed mobility of the test solutes with increasing concentration was observed. This can be attributed both to a dynamic equilibrium in which organic modifier and TTAB are adsorbed onto the capillary surface and to structural changes of the hemimicelle itself [23]. Therefore, considering both separation time and resolution, 15% acetonitrile (*v*/*v*) was added to the electrolyte.

CZE Separation and Sample Analysis

Figure 5 shows the separation of 4 anions and 11 carboxylic acids, which play important roles in soil chemistry. The running electrolyte contained 25 mM phosphate, 0.5mM TTAB and 15% acetonitrile at pH 6.0. Clearly, the anions and organic acids were separated well with reasonable resolution between solutes and detected by UV at 185 nm as sharp symmetrical peaks with the exceptions of furmaric and malonic acids. However, no relation between migration order and pKa_1 was found between mono, di and tricarboxylic acids. The pKa_1 [24] and the effective mobility for each tested solute are listed in Table I. The solutes migrated in the order Cl^-, NO_2^-, NO_3^-, SO_4^{2-}, oxalic, formic, fumaric, tartaric, malonic, malic, citric, succinic, maleic, acetic, and lactic acid. This migration order could be attributed to the difference in charge and size.

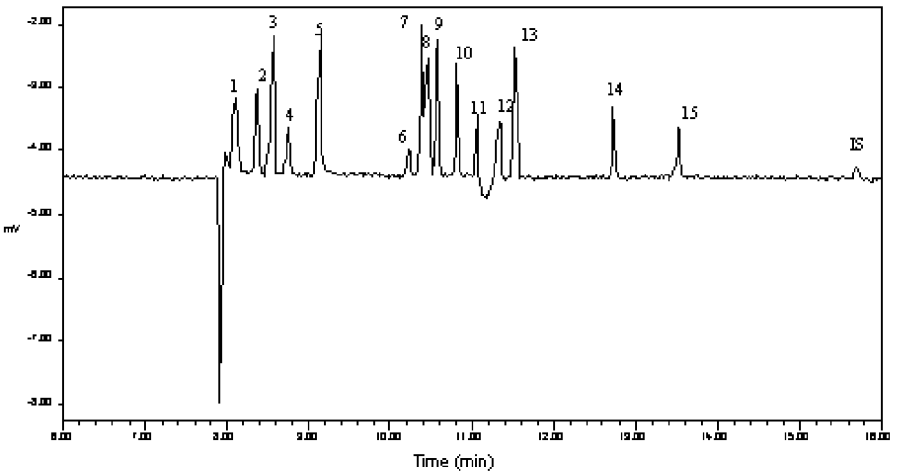

Figure 5. A typical electropherogram obtained using optimised conditions. Conditions: capillary, fused-silica capillary 50 μm × 100 cm (L: 95.5 cm); electrolyte, 25 mM sodium phosphate + 0.5 mM TTAB + 15% acetonitrile (v/v) at pH 6.0; applied potential, –20 kV; injection pressure; 30 s; capillary temperature, 25 °C. Other conditions as in Figure 1 the concentration for each solute: 0.20 mM.

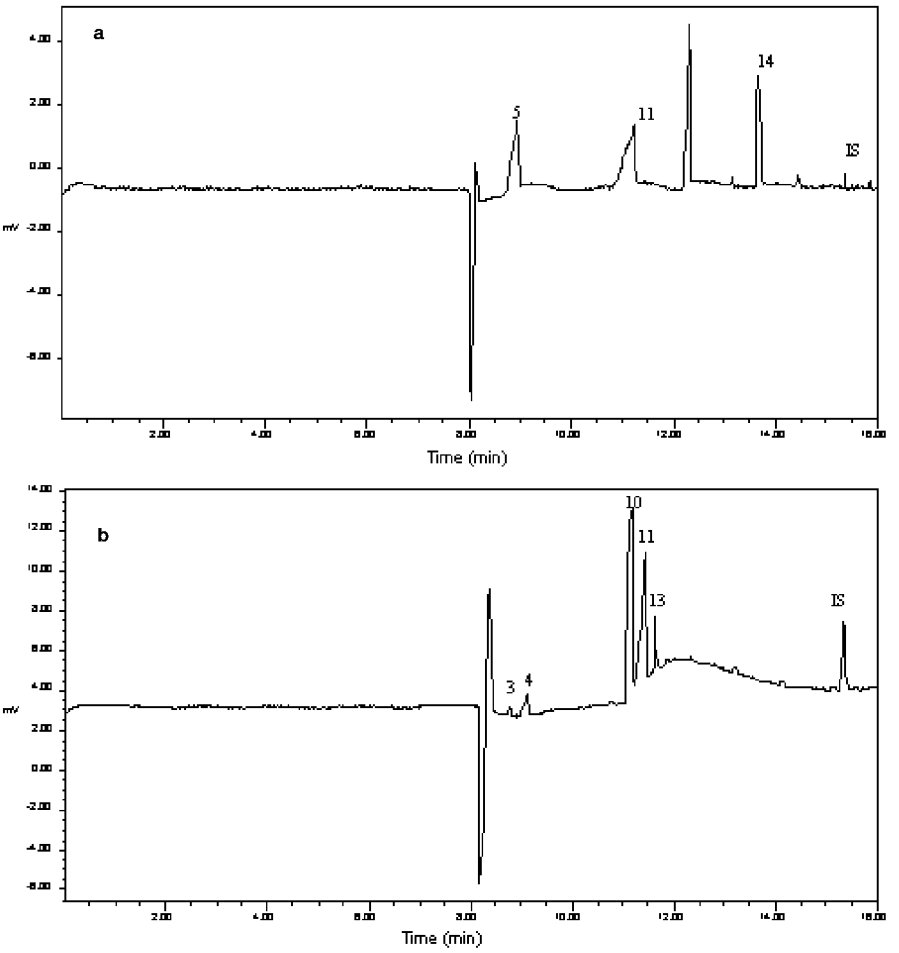

Figure 6. Soil and plant tissue samples analysed by the proposed method. (**a**) soil, (**b**) plant tissue. Conditions as for Figure 5.

Calibration plots were obtained by plotting peak area versus concentration of the test solutes. The relationship was linear in the concentration range of 0.02–0.5 mM. Correlation coefficients were in the range of 0.9992 to 0.9998. The detection limits (S/N = 3) ranged from 1–9 μM and

Table II. Concentration of anions and carboxylic acids in soil and plant extracts determined by the proposed method.

Solute	Plant extract				Soil extract			
	Concentration (mM)	Spiked	Found	Recovery (%)	Concentration (mM)	Spiked	Found	Recovery (%)
NO_3	0.03	0.20	0.21	91.3	–	–	–	–
SO_4^{2}	0.45	0.20	0.63	96.9	–	–	–	–
Oxalic	–	–	–	–	0.49	0.20	0.63	91.3
Formic	–	–	–	–	–	–	–	–
Malic	1.74	0.20	1.90	97.9	–	–	–	–
Citric	1.96	0.20	2.06	95.4	1.20	0.20	1.37	94.2
Maleic	0.28	0.20	0.45	93.7	–	–	–	–
Acetic	–	–	–	–	1.36	0.20	1.45	93.5

– not detected.

the reproducibility for the migration time (RSD% n = 5) from injecting a 0.2 mM standard mixture range from 0.15–0.43%, and the reproducibility for the peak area ranged between 1.71–6.14%.

The proposed method was used to determine the concentration of inorganic anions and organic acids in soil and plant extracts. Typical electropherograms are presented in Figure 6 a–b. A reasonable resolution for the solutes was obtained with direct injection of the sample. It seems that the proposed co-CZE method exhibits less interference from sample matrices than ion-exclusion chromatography [7] since using co-CZE has higher selectivity for the test solutes. Therefore, solutes such as Cl-, NO_2^- and NO_3^- or oxalic, citric and malic acids were well resolved, although co-elution occurred in ion-exclusion chromatography[8]. Concentrations of solutes in the samples and their recoveries determined by spiking with known standards are listed in Table II. The recoveries for the tested solute are satisfied.

Conclusion

Simultaneous analysis of inorganic anions and organic acids can be performed by co-EOF capillary electrophoresis with direct UV detection at 185 nm. The electrolyte pH and concentration, the type of the cationic surfactant and the content of organic modifiers significantly affect separation resolution. The proposed method used a 25 mM phosphate electrolyte containing 0.5 mM TTAB, 15% acetonitrile (*v/v*) at a pH of 6.0 to analyse anions and organic acids in real samples. It offered low detection limits, good reproducibility of migration time and simple sample preparation. Compared with ion-exclusion chromatographic separation of organic acids, the CZE method used in this study provides higher selectivity and eliminates co-elution of organic acids with inorganic anions using direct sample injection.

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