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Analysis of phenolic acids by non-aqueous capillary electrophoresis after electrokinetic supercharging

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ABSTRACT

Electrokinetic supercharging (EKS), a new and powerful on-line preconcentration method for capillary electrophoresis, was utilized in non-aqueous capillary electrophoresis (NACE) to enhance the sensitivity of phenolic acids. The buffer acidity and concentration, leader and terminator length and electrokinetic injection time were optimised, with the optimum conditions being: a background electrolyte of 40 mM Tris–acetic acid (pH 7.9), hydrodynamic injection of 50 mM ammonium chloride (22 s, 0.5 psi) as leader, electrokinetic injection of the sample (180 s, −10 kV), hydrodynamic injection of 20 mM CHES (32 s, 0.5 psi) as terminator, before application of the separation voltage (−25 kV). Under these conditions the sensitivity was enhanced between 1333 and 3440 times when compared to a normal hydrodynamic injection with the sample volume <3% of the capillary volume. Detection limits for the seven phenolic acids were in the range of 0.22–0.51 ng/mL and EKS was found to be 3.6–7.9 times more sensitive than large-volume sample stacking and anion selective exhaustive injection for the same seven phenolic acids.

1. Introduction

Although capillary electrophoresis enjoys the advantages of speed and high efficiency when compared to traditional liquid chromatographic methods, it has the shortcomings of low sensitivity primarily due to combination of the short optical path length most commonly used for absorbance detection and the small sample volumes injected. To lower detection limits, a considerable number of methods for on-line concentration have been developed. Although on-line solid-phase extraction [1,2] and on-line liquid phase extraction [3,4] can be used to provide significant improvements in sensitivity, the modification of the instruments or capillaries are needed and these approaches are relatively complex. Most of the on-line preconcentration methods in capillary electrophoresis (CE) are based on changes in analyte migration due to conductivity difference, buffer pH difference or the association between the analytes and the surfactants, and the combination of these approaches. Thus methods such as field-amplified sample stacking (FASS) [5–7], field-amplified sample injection (FASI) [8], large-volume stacking using the EOF pump (LVSEP) [9–16], field-amplified sample injection with matrix removal via an EOF pump (FAEP) [17,18], dynamic pH junction (DypH) [19–21], transient isotachophoresis (tITP) [22,23], pseudo-transient isotachophoresis (Pseudo-tITP) [24–28], sweeping [29–32], micelle collapse (MC) [33,34] and various combinations including electrokinetic supercharging (EKS) [35–41], selective exhaustive injection [42] and selective exhaustive injection-sweeping (SEI-sweeping) [43–45], dynamic pH junction-sweeping [46], and large-volume stacking using the EOF pump-sweeping(LVSEP-sweeping) [47] have already been reported in the literature.

Electrokinetic supercharging (EKS) is the combination of FASI and tITP and was first developed by Hirokawa et al. to analyze trace rare earth ions [35,36]. It has been shown to be particularly powerful for on-line concentration with enhancements of 3–4 orders of magnitude readily achieved for peptide analysis [37], non-steroidal anti-inflammatory drugs in water samples [38,39] as well as inorganic cations such as Fe(II), Co(II) and Ni(II) analysis [40]. Previously we investigated EKS in non-aqueous conditions and obtained modest enhancements of 300–440 [41].

Non-aqueous capillary electrophoresis (NACE), is based on the use of electrolyte solutions prepared in pure organic solvents, and offers a number of attractive features such as improved selectivity by changing the solvent or solvent mixture, extended application scope with a better solubility for hydrophobic compounds, reduced electrophoretic currents and Joule heating and the easiness with which they can be coupled to a mass spectrometer. For these reasons, NACE has drawn much attention in recent years [48,49] including the implementation of on-line preconcentration methods

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such as FASS, LVSEP-ASEI, LVSEP and pseudo t-ITP [6,7,16,24,42]. Of all of these approaches, the most significant enhancements were obtained by Kim and Chung [42] who obtained 430-fold improvements in sensitivity using LVSEP-ASEI. In this paper, we build upon our previous work on the use of EKS in NACE to provide enhancements of at least 10^3. Introduction of a short injection of leading and terminating electrolytes and optimisation of the separation and injection conditions yielded enhancement factors ranging from 1333 to 3440 for a mixture of seven phenolic acids. This method has also been compared with a LVSEP-ASEI-NACE method and the sensitivity enhancement factor of this method is 3.6–7.9 times higher than the LVSEP-ASEI method.

2. Experimental

2.1. Instrumentation

CE analyses were carried out in a P/ACE MDQ capillary electrophoresis system with a photodiode array detector for absorbance measurements at 199 nm (Beckman Coulter, Fullerton, CA, USA). Uncoated fused-silica capillaries purchased from Polymicro Technologies (Phoenix, AZ, USA) were used. The dimensions of the capillary were 65.2 cm × 50 μm i.d. The effective length of the capillary was 55 cm. The temperature of the capillary was kept at 25 °C. The CE system was interfaced with a computer. 32 karat software (version 7.0) of Beckman was used for data acquisition.

2.2. Chemicals

2,5-Dihydroxybenzolic acid, 2,4-dihydroxybenzolic acid, p-toluic acid, 4-propylbenzoic acid, 3,5-dihydroxybenzolic acid and 4-heptylbenzolic acid were from Aldrich Chemical Company (Milwakee, USA). Tris and 2-(cyclohexylamino) ethanesulphonic acid (CHES) were from Sigma–Aldrich (St. Louis, MO, USA). Methanol (HPLC grade) and 4-hydroxybenzoic acid were from Merck-Schuchardt (Germany). Glacial acetic acid and ammonium chloride were of analytical reagent grade and were from BDH (Kilsyth, Australia). Ethyl acetate was of analytical reagent grade and was from Sinopharm Chemical Reagent Co. (China). Water of 18.2 mΩ was treated with a Millipore (North Ryde, Australia) water purification system.

A stock standard solution of 1 mg/mL of each analyte was prepared in methanol. A mixed standard solution of the seven analytes was prepared at a concentration of 0.1 mg/mL in methanol. The working standard solutions were prepared daily by diluting the stock standard solution with methanol. All solutions were stored in dark containers at 4 °C. The background electrolyte (BGE) was prepared in methanol and had a concentration of 40 mM of Tris–acetic acid (pH 7.9). The buffer solutions were prepared freshly each day, sonicated for 5 min and filtered through a 0.45 μm membrane filter before use.

2.3. Electrophoresis

Before use, the capillary was rinsed with 1 M sodium hydroxide, water, methanol, and separation medium for 10 min. Between analyses the capillary was washed with methanol for 2 min and then with the BGE for 4 min. Duplicate injections of the solutions were performed and average peak areas were used for quantification.

2.4. Stacking enhancement factor calculation

The enhancement factor was calculated by dividing limit of detection when a hydrodynamic sample injection (0.5 psi, 5 s) with that obtained when preconcentration was performed.

2.5. Large-volume sample stacking with EOF pumping-anion selective exhaustive injection (LVSEP-ASEI)

All analytes were dissolved in methanol and loaded with a pressure of 5 psi for 100 s. Thereafter, keeping the inlet of the capillary in the sample solution, a negative voltage of −10 kV was applied to load an additional amount of analytes electrokinetically for 500 s during the removal of the sample matrix. Then the inlet of the capillary was switched to the run buffer vial, and the subsequent separation was performed under a reverse voltage of −25 kV. For each run, a new sample solution was used.

2.6. Electrokinetic supercharging

Leader (50 mM ammonium chloride) was introduced into the capillary by hydrodynamic injection at 0.5 psi for 22 s, then the sample was injected electrokinetically by a negative voltage (−10 kV) for 180 s, followed by a small volume of the terminator (20 mM CHES) hydrodynamically injected at 0.5 psi for 32 s. A reverse voltage of −25 kV was applied for both the on-line focusing and the separation of the analytes.

2.7. Preparation of water samples

Water was collected from Zhangwei Nan River (Dezhou, China). Before analysis, the samples were filtered through a 0.45 μm membrane syringe filter in order to eliminate particulate matter. The samples were stored in the refrigerator at 4 °C.

2.8. Liquid–liquid extraction (LLE)

1 mL of water sample spiked with phenolic acids (2 ppb) was acidified with 0.1 mL of 1 mol/L hydrochloric acid, after shaking, then 0.5 mL of ethyl acetate was added. The sample was then centrifuged (TGL-16G centrifuger, ShangHai Anting Scientific Instrument Factory, Shanghai, China) at 3000 rpm for 3 min and the ethyl acetate layer was separated. The extraction process was repeated 3 times and the ethyl acetate layer was combined and evaporated to dryness under a N-EVAPTM 111 nitrogen evaporator (Organomation Associates, USA) and the dry residue was solved with 1 mL HPLC grade methanol.

3. Results and discussion

Kim and Chung [42] have analyzed ten weakly acidic organic compounds in NACE using methanol as the solvent and on-line concentration with LVSEP-ASEI. Detection limits of 0.5–4.0 nM were obtained, but this method is still insufficient for trace analysis especially when the detector was not very sensitive. In this work, we have developed an EKS method for the trace analysis of phenolic acids in NACE using methanol as the solvent and compare this to the previously developed LVSEP-ASEI method developed by Kim and Chung.

3.1. Optimisation of the separation

As no report has been published about the separation of the seven phenolic acids studied in this work with NACE, their separation had to be optimised. Methanol has been shown to be a suitable solvent for a number of phenolic acids. In addition to improving solubility it can also suppress EOF and thereby increase the electrophoretic mobilities of anionic solutes [42]. Originally, a methanol solution of ammonium acetate was used as the backgound electrolyte, however no acceptable separation could be achieved in this buffer system. A Tris–acetic acid buffer system with methanol as solvent was then tried and found to provide excellent separations.

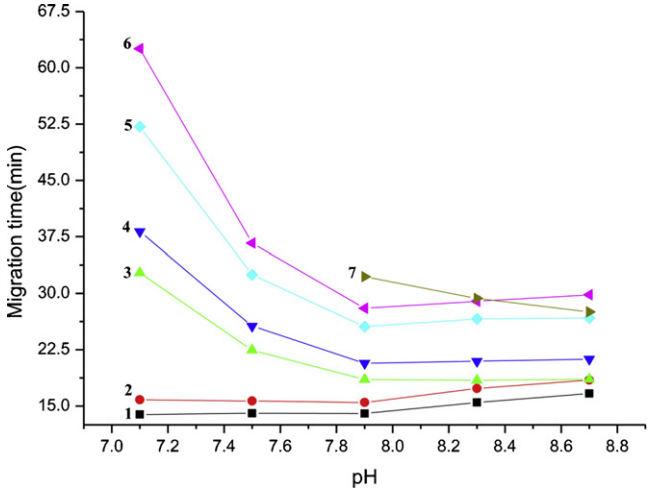

Fig. 1. Effects of pH on the migration time of the analytes:
1. 2,5-dihydroxybenzoic acid; 2. 2,4-dihydroxybenzoic acid; 3. p-Toluic acid; 4. 4-propylbenzoic acid; 5. 3,5-dihydroxybenzoic acid; 6. 4-hydroxybenzoic acid; 7. 4-heptylbenzoic acid. Conditions: 65.2 cm × 50 μm (55.2 cm to detector) fused silica capillary, BGE 40 mM Tris-Acetic acid (pH*7.9); Voltage, −25 kV; detection was at 199 nm. Sample: hydrodynamic injection of 100 μg/mL of each phenolic acid for 5 s at 0.5 psi.

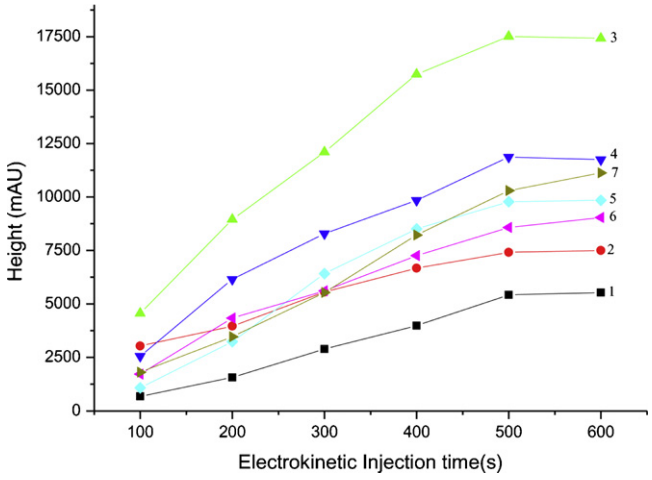

Fig. 2. Effects of electrokinetic injection time on the peak height in the LVSEP-ASEI system. Sample: hydrodynamic injection of 0.1 μg/mL of each phenolic acid for 100 s at 5 psi and EKI at −10 kV from 100 s to 600 s. All other conditions were the same as Fig. 1.

Keeping the buffer concentration constant at 40 mmol/L, the effect of buffer pH was studied in the pH 7.1–8.7 range. This range is highly relevant given that it is well known that the pK_a of weak acids in MeOH increases by approximately 5 pH units, meaning that the pK_a values for the weak acids used in this work are likely to fall near this range. A thorough search through the literature reveals pK_a values in MeOH for 2,4-dihydroxybenzoic acid of 8.48 (pK_a H_2O of 3.29, ΔpK_a 5.19) [50], 2,5-dihydroxybenzoic acid of 8.04 (pK_a H_2O of 2.97, ΔpK_a 5.07) [50] and 4-hydroxybenzoic acid of 9.99 (pK_a H_2O of 4.55, ΔpK_a 5.44) [51]. No values in MeOH have been reported for the remaining phenolic acids. Using the average ΔpK_a from the 3 known phenolic acids of 5.23, then it is possible to estimate the MeOH pK_a values for the others. They were estimated to be 9.59 for p-toluic acid (aqueous pK_a of 4.36 + ΔpK_a of 5.23), 9.60 for 4-propylbenzoic acid (aqueous pK_a of 4.37 + ΔpK_a of 5.23), 9.27 for 3,5-dihydroxybenzoic acid (aqueous pK_a 4.04 + ΔpK_a of 5.23), and 9.59 for 4-heptylbenzoic acid (aqueous pK_a 4.36 + ΔpK_a of 5.23). As shown in Fig. 1, as the pH of the buffer increased, the migration times of the analytes decreased rapidly in the pH 7.1–7.9 range, except for 2,5-dihydroxybenzoic acid and 2,4-dihydroxybenzoic acid, which remained almost constant. These two phenolic acids have the lowest pK_a values in MeOH (8.04 and 8.48), respectively, and will thus undergo less significant changes in ionization over this region when compared to the other 5 phenolic acids. Interestingly, when the pH was lower than 7.9, peak 7 did not appear in 50 min. This is a very interesting result given that it is estimated to have a similar pK_a value in MeOH similar to p-toluic and 4-propylbenoc acids (9.6), but will have a slower electrophoretic mobility due to its size. At pH values less than 7.9 its electrophoretic mobility is equal in magnitude but opposite in direction to the EOF, and when the pH is increased further, its mobility increases more. When the buffer pH was higher than 7.9, the migration times changed slowly presumably due to a more delicate balance of electrophoretic mobility and EOF. Consideration of both resolution and analysis time resulted in a pH of 7.9 being selected as the optimum buffer pH. At this pH, the effect of the buffer concentration was also studied over the range of 10–50 mmol/L. With the increase of buffer concentration, the migration time of the analytes decreased, and when the buffer concentration is 10 mmol/L, the peak of 4-heptylbenzoic acid again did not appear within 50 min. This is again because of changes in the EOF, with a higher EOF observed at lower ionic strength. Based on these results, 40 mmol/L Tris–acetic acid was selected as the optimum electrolyte concentration.

3.2. Analytical performance of LVSEP-ASEI

LVSEP-ASEI involves continuous electrokinetic injection of the sample during matrix removal after injecting a large volume of sample by hydrodynamic injection (5 psi, 100 s, full capillary length). The effect of the electrokinetic injection time was investigated in the 100–600 s range and governs the amount of analyte ions additionally injected into the capillary as well as the amount of sample matrix removed from the capillary. As shown in Fig. 2, the peak height increased as the electrokinetic injection time was increased from 100 to 500 s. After 500 s, no apparent increase for peak area and peak height was observed.

Under optimised conditions with a 500 s LVSS-ASEI injection (the molar amount of sampled analyte were in the 0.587–0.957 pmol range), the seven analytes can be separated in about 35 min and the electropherogram is shown in Fig. 3, with a total analysis time of approximately 51 min. As shown in Table 1,

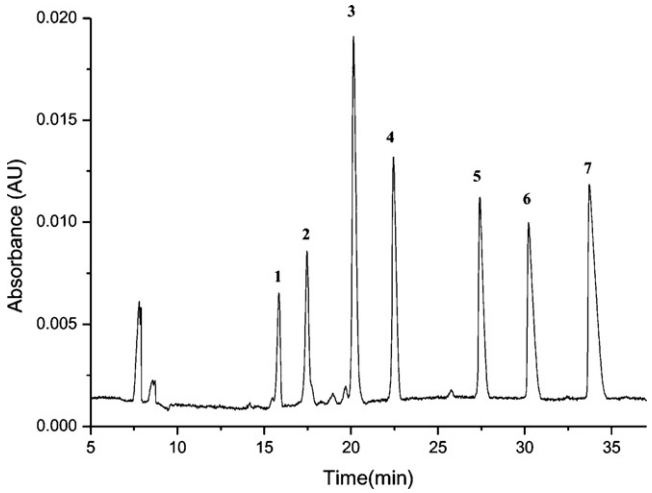

Fig. 3. Electropherogram of LVSEP-ASEI-NACE separation of seven phenolic acids with an injection time of 500 s. All other conditions are the same as those in Fig. 2.

Table 1
Limits of detection, enhancement factors and repeatability of LVSEP-ASEI and EKS.

Compounds	Normal	LVSEP-ASEI				EKS			
	LOD[a] (μg/mL)	LOD (ng/mL)	EF	RSD[b] (%)		LOD (ng/mL)	EF	RSD[c] (%)	
				Time	Area			Time	Area
2,5-Dihydroxybenzoic acid	0.60	3.56	168	0.81	3.95	0.45	1333	1.25	4.08
2,4-Dihydroxybenzoic acid	0.72	2.69	268	2.00	3.47	0.46	1565	0.97	5.23
p-Toluic acid	0.40	0.91	439	0.73	3.33	0.22	1818	1.56	3.92
4-Propylbenzoic acid	0.59	1.62	364	1.36	4.20	0.39'	1513	1.32	8.71
3,5-Dihydroxybenzoic acid	0.88	1.82	483	1.25	3.04	0.51	1725	1.58	9.26
4-Hydroxybenzoic acid	0.93	1.98	469	0.55	4.10	0.34	2735	0.89	7.09
4-Heptylbenzoic acid	0.86	1.61	534	1.11	3.32	0.25	3440	2.05	4.59

[a] Based on 3 times noise.
[b] Based on five determination of the standard mixture of 0.1 μg/mL.
[c] Based on five determination of the standard mixture of 0.01 μg/mL.

the enhancement factor of the seven analytes was in the 168–534 range. The repeatability was studied by 5 replicate injections of a 0.1 μg/mL standard mixture with the results shown in Table 1. The RSD values based on migration time and peak area were in the 0.81–1.36% and 3.04–4.20% range, respectively. Based on 3 times noise, the detection limits of the seven analytes were between 0.9 and 3.6 ng/mL.

3.3. Analytical performance of EKS

Electrokinetic supercharging (EKS) is the combination of t-ITP and FASI and enhancement factors more than 1000 have been reported in the literature [35–40]. EKS involves electrokinetically injecting the sample between hydrodynamically introduced leading and terminating ions. When the separation voltage is applied the diffuse band of analytes introduced during electrokinetic injection are restacked between the leader and terminator according to conventional ITP. When the ITP stage destacks, the analytes are separated by conventional CZE. The choice of the leader, terminator and the electrokinetic injection time is very important for achieving a good sensitivity. Previously, we examined the potential of EKS for the on-line concentration of 4 phenolic acids in NACE, with modest improvements in sensitivity of 300–440 [41]. In that work the EOF was reversed using a polyelectrolyte coating and acetate in the BGE was used as the leading electrolyte, with a short hydrodynamic injection of 20 mM CHES in MEOH used as the terminator. Due to the modest improvements in sensitivity obtained, in this work, here we tried to enhance both the FASI and tITP components of the EKS mechanism by using an unmodified fused-silica capillary with a low cathodic EOF (due to the use of MeOH) and also with the use of a leading ion (chloride) which has a much larger mobility difference to the phenolic acids. The simplest approach of using ammonium chloride as the BGE failed to provide acceptable separations of the phenolic acids. Thus the approach originally used by Hirokawa et al. [35] to demonstrate EKS using a small injection of leading electrolyte before injection of the sample was adopted. Because of its low electrophoretic mobility, and its use in previous reports, CHES was chosen as the terminator.

Based on previous work on optimisation of EKS by Dawod et al. [38] best results for EKS were obtained with relatively small amounts of leader and terminator, typically 2% of the capillary volume, even though volumes as large as 13% of the capillary were injected. We therefore examined the influence of leader and terminator over the range of 1–5% of the capillary volume with a constant electrokinetic injection time of 180 s. The results showed that when the leader injection time is shorter than 1.5% or the terminator injection time is shorter than 2.3% the analytes were not fully stacked and peak splitting occurred. As the amount of leader and terminator in the capillary became longer, resolution was decreased due to prolonged migration of the ITP zone through the capillary before destacking and the onset of separation. In order to maintain suitable resolution, the leader and terminator volumes were selected as 1.9% of 50 mM NH_4Cl (1.79 nmol) and 2.8% of 20 mM CHES (1.04 nmol), respectively. Having optimised the leader and terminator amounts, the injection time was varied to maximize the amount of injected analytes without compromising resolution. The electrokinetic injection time was varied from 60 to 270 s range, with the results shown in Fig. 4. As anticipated, as the injection time was increased, the peak height also increased. But for injection times higher than 180 s, the analytes were not stacked well and peak splitting occurred. Although this could be rectified by increasing the leader and terminator length and thereby achieve further improvements, this resulted in a reduction in resolution, so electrokinetic injection time was selected at 180 s (about 0.47–1.36 fmol for the phenolic acids) as a compromise.

Under the optimised EKS injection conditions, the seven phenolic acids were separated in 25 min, with the electropherogram shown in Fig. 5. As shown in Table 1, the enhancement factor of the seven analytes based on peak height were in the 1333–3440 range, which is 3.57–7.91 times higher than those obtained by LVSEP-ASEI method, while the analysis time is 16 min shorter. Detection limits, based on 3 times noise, ranged from 0.22 to 0.51 ng/mL. The repeatability (%RSD) based on migration time and peak area ranged from 0.89% to 2.05% and 3.92% to 9.26%, respectively. The %RSDs are poorer than those obtained with LVSEP-ASEI method,

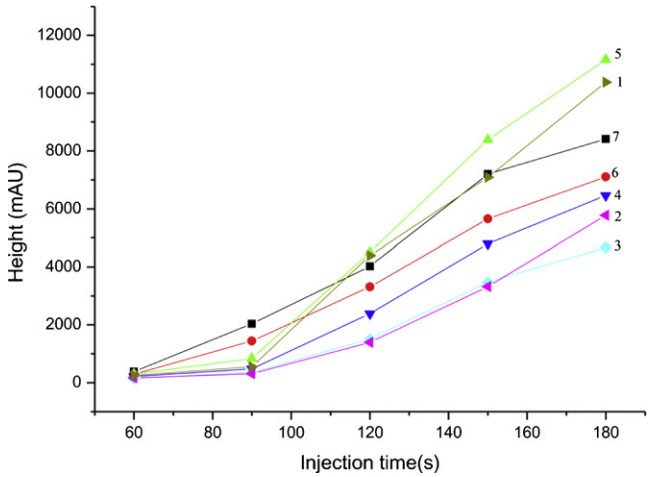

Fig. 4. Effects of electrokinetic injection time on peak height in the EKS system. Sample: hydrodynamic injection of 50 mM NH_4Cl at 0.5 psi for 22 s, EKI of a mixture of 0.01 μg/mL of each phenolic acid at −10 kV from 60 to 180 s hydrodynamic injection of 20 mM CHES at 0.5 psi for 32 s. All other conditions were the same as Fig. 1.

Table 2
The regression equations in EKS.

Compounds	Regression equation[a]	Correlation coefficient	Linear range (ng/mL)
2,5-Dihydroxybenzoic acid	$Y = 3774.42X + 4068.38$	0.9989	2–50
2,4-Dihydroxybenzoic acid	$Y = 4486.63X - 975.20$	0.9980	2–50
p-Toluic acid	$Y = 10553.84X - 312.99$	0.9952	1–20
4-Propylbenzoic acid	$Y = 6160.41X - 3612.16$	0.9978	2–50
3,5-Dihydroxybenzoic acid	$Y = 5156.55X - 3778.78$	0.9970	2–50
4-Hydroxybenzoic acid	$Y = 7736.66X + 660.95$	0.9988	1–20
4-Heptylbenzoic acid	$Y = 7382.53X + 5041.34$	0.9991	1–20

[a] In the regression equation, the X value is the concentration of analytes (ng/mL), the Y value is the peak area.

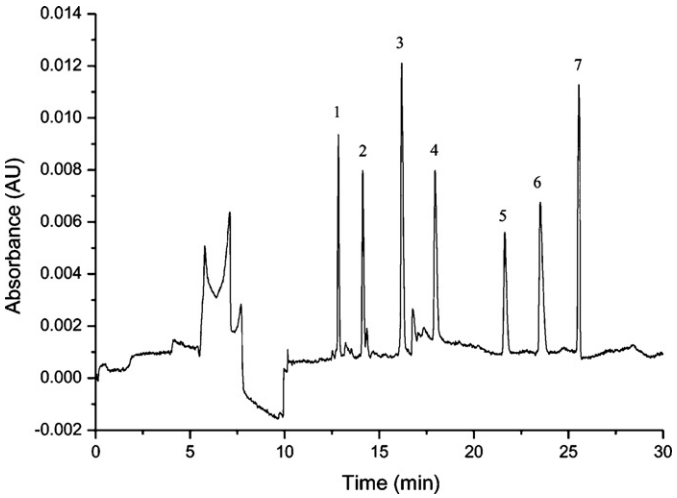

Fig. 5. Electropherogram of EKS-NACE separation of seven phenolic acids with an injection time of 180 s. All other conditions are the same as for Fig. 4.

but considering the improved detection limits and the reduced sample-to-sample time, it is still acceptable for many applications. The linearity was also studied, with these results shown in Table 2.

In chemical industry production, some phenolic acids can be discharged into waste water. If this waste water flows into the river unprocessed then there is the potential for environmental damage and it is therefore important to be able to detect these compounds in natural waters [52]. To demonstrate the applicability of the developed method for this application, river water was collected from Zhangwei Nan River (Dezhou, China) and spiked with 2 ng/mL of each of the phenolic acids. No significant peaks were observed in the sample, most likely due to the presence of other salts which increased the conductivity of the sample and may also be preferentially injected due to a higher electrophoretic mobility. Detection of the spiked phenolic acids could only be detected after LLE with ethyl acetate. Fig. 6 shows separations of LLE extracts from the river water sample and the same sampled spiked with 2 ng/mL of the phenolic acids. As can be seen, the separation looks similar to that obtained with the standards. Recovery values of the phenolic acids were 75.24%, 78.16%, 48.97%, 61.84%, 44.93%, 42.25% and 58.556% for 2,5-dihydroxybenzoic acid, 2,4-dihydroxybenzoic acid, p-toluic acid, 4-propylbenzoic acid, 3,5-dihydroxybenzoic acid, 4-hydroxybenzoic acid and 4-heptylbenzoic acid, respectively.

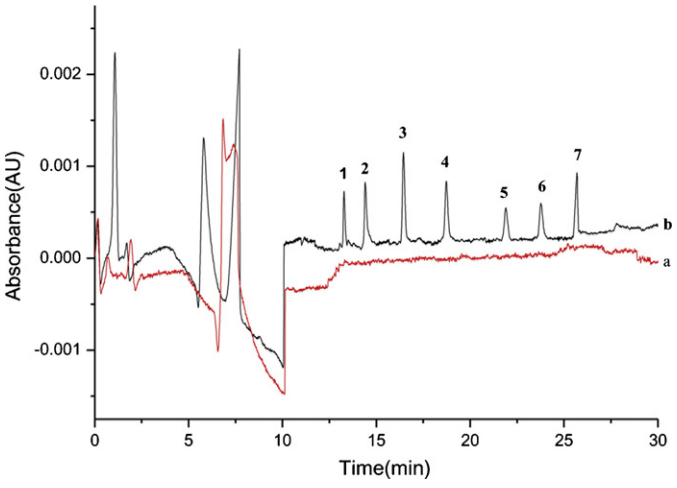

Fig. 6. Electropherogram obtained from EKS-NACE for samples after liquid–liquid extraction (a) blank water sample after liquid-liquid extraction and (b) water sample spiked with 2 ppb of the phenolic acids after liquid–liquid extraction. CE conditions is the same as in Fig. 5.

4. Conclusion

A non-aqueous capillary electrophoresis method with EKS on-line preconcentration was developed for the separation of seven phenolic acids. Using this method, the sensitivity enhancement factors ranged from 1333 to 3440, which are 3.57–7.91 times higher than those reported for the previous best NACE system of LVSEP-ASEI. The sample-to-sample time of the EKS method is also about 16 min shorter than the LVSEP-ASEI method primarily due to the absence of the matrix removal step. The repeatability of the EKS method is slightly worse, but is still acceptable for most analytical applications. The applicability of the method for the detection of trace environmental pollutants was demonstrated with the injection of LLE extracts of spiked river water. To the best of our knowledge, this is the highest improvement in sensitivity obtained in NACE without the use of on-line liquid–liquid or solid-phase extraction.

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