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Journal of Pharmaceutical and Biomedical Analysis 41 (2006) 1022–1024
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Short communication

Development of a stacking-CZE method for the analysis of phenolic acids

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Received 17 October 2005; received in revised form 9 January 2006; accepted 10 January 2006
Available online 20 February 2006

Abstract

Eight phenolic acids were analyzed by capillary zone electrophoresis. On-line analyte preconcentration was carried out by hydrodynamic injection of large volume of sample followed by removal of the bulk of the low conductivity sample matrix by polarity switching. The optimal electrolyte system consisted of 50 mM sodium tetraborate of pH 9.0 (adjusted with 0.1 M phosphoric acid) containing 2% of α-cyclodextrin. The separations were carried out with a fused silica capillary (effective length 50 cm, i.d. 50 μm) and monitored at 200 nm. Under optimized preconcentration conditions (sample injection 99 s at 100 mbar and the polarity switching time 1.0 min) linear calibration ranges (0.1–2.0 μg/ml, $R = 0.9979$–0.9995), favourable limits of detection (0.01–0.025 μg/ml) and good repeatability of the peak areas (R.S.D.: 2.76–5.69%, $n = 6$) were achieved.

Keywords: Large-volume sample stacking; Stacking; Phenolic acids; Capillary electrophoresis

1. Introduction

The aim of the presented work was to develop CZE method for the determination of eight phenolic acids with antioxidant activity in *Herba epilobi*.

1.1. Large-volume sample stacking with polarity switching

Large-volume sample stacking technique with polarity switching mode was used to enhance the detection sensitivity in CZE. Initially a large plug of low conductivity sample was introduced hydrodynamically into the capillary and a negative voltage was applied. The large solvent plug was then electroosmotically pushed out of the capillary while the negative species stacked up at the boundary between the sample zone and the background electrolyte (BGE). Once the major part of the low conductivity zone has been pushed out of the capillary, the positive voltage was applied for the separation. The time of polarity switching was determined by monitoring the driving current value. As soon as the current reached the value close to the original one (about 95–99%), the polarity was switched [2–4].

1.2. Compounds under study

The studied antioxidants can be divided into two groups: derivatives of benzoic acid (vanillic, syringic and gentisic acids) and derivatives of cinnamic acid (cinnamic, *p*-coumaric, caffeic, ferulic and chlorogenic acids). These acids occur in *H. epilobi* and are responsible for the antioxidant effect of the plant.

The active compounds of *H. epilobi* were determined earlier by different techniques. Hiermann and Radl developed a CZE method using a fused silica capillary, phosphate buffer of pH 7.0 and UV detection for the analysis of aromatic acids occurring in plant extracts [1]. The polyphenolic metabolites were analyzed by RP-HPLC with UV and MS detection [5]. Gallotannins, ellagotannis and flavonoids were studied by HPLC-MS [6] and the HPLC with UV and MS detection was used for the analysis of flavonoid glycosides in various Epilobium species [7].

2. Experimental

2.1. Apparatus

Capillary electrophoretical system P/ACE^{TM} MDQ, Beckam Coulter equipped by PDA was used. Communication with PC and analysis control was supported by 32 Karat Software 4.01.

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doi:10.1016/j.jpba.2006.01.025

The separations were performed in fused silica capillary, i.d. 50 μm and total length 60 cm (effective length 50 cm).

2.2. Reagents

All chemicals were of analytical grade quality. Cinnamic, gentisic, coumaric, ferulic, vanillic, syringic and caffeic acids, α-cyclodextrin and methanol Chromasol® for HPLC were provided by Sigma–Aldrich. Sodium tetraborate and phosphoric acid were obtained from Lachema Brno.

Standard solutions of above mentioned aromatic acids (100 mg/ml) were prepared by dissolving these compounds in 50.00 ml of methanol and degassed in ultrasonic bath. This stock solution was diluted with water to contain 10% (v/v) of methanol. The electrolyte consisted of 50 mM sodium borate buffer with pH 9.0 containing 2% of α-cyclodextrin.

3. Results and discussion

3.1. CZE with a standard hydrodynamic injection process (injection volume representing 1% of the overall capillary volume)

Alkaline BGE based on a borate buffer was employed to achieve ionization of the carboxylic and phenolic groups of the analytes and possibly to enhance separation of the analytes through complex formation of borate with vicinal –OH groups of dihydric phenols [8]. The effect of concentration of borate, α-cyclodextrin and of the content of methanol in the BGE was tested. The electropherogram of conventional CZE is shown in Fig. 1A.

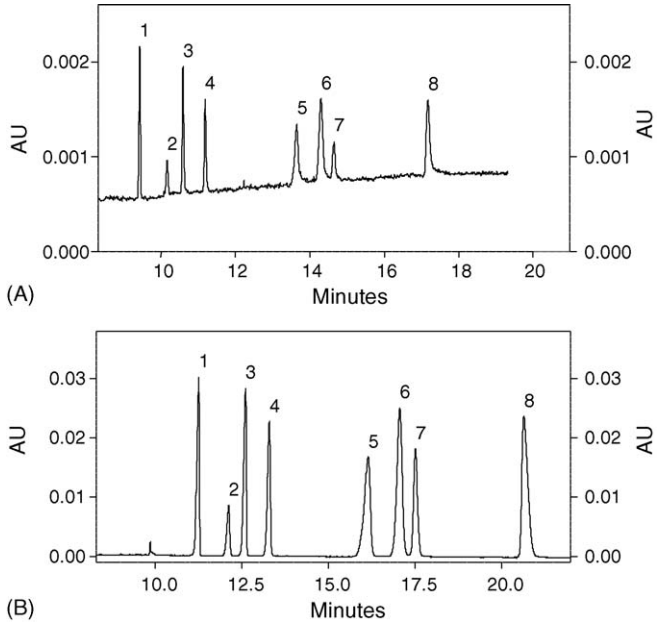

Fig. 1. Electropherogram of standard mixture ($c = 2$ μg/ml): (A) classical CZE and (B) CZE using large-volume sample stacking with polarity switching (for conditions see Section 3.1). (1) Cinnamic acid, (2) chlorogenic acid, (3) *p*-coumaric acid, (4) ferulic acid, (5) caffeic acid, (6) gentisic acid, (7) syringic acid and (8) vanillic acid.

3.1.1. Effect of borate concentration

Chlorogenic acid and caffeic acid form complexes with borate. It is caused by *ortho*-dihydroxyphenyl moieties in their structures. Effect of borate concentration was tested at pH 9 for 10, 30, 50 and 70 mM borate. Optimum peak symmetry and peak area were achieved with 50 mM borate.

3.1.2. Effect of pH

The pH of BGE is an important parameter influencing electroosmotic flow and ionization of analytes and thus it may act selectively to change the electrophoretic mobility of analytes. The effect of pH was examined with the borate-containing BGEs of pH 8.4, 8.6, 8.8, 9.0, 9.2 and 9.4. The pK_a values of the phenolic acids under study range between 4 and 5 and hence they are fully ionized in such BGEs [8,9]. Poor resolution of all analytes was observed below pH 9.0. Increasing pH above 9.0 resulted in longer analysis times. Therefore, pH 9.0 was selected as the optimum choice giving best results in terms of the resolution of the acids of interest.

3.1.3. Effect of addition of methanol

It is known that the content of methanol in BGE affects analysis time, peak symmetry and resolution. With the increasing methanol content in the BGE from 5% to 20% (v/v) the migration times of the analytes increased and the baseline quality and the resolution worsened. Therefore, in further experiments the BGE without the addition of methanol was used.

3.1.4. Effect of α-cyclodextrin

The separation quality was significantly influenced by the addition of 2–5% of α-cyclodextrin into the BGE of pH 9. The migration times and order of acids changed considerably and all the compounds were separated to baseline and the total analysis time was acceptable. Optimum content of α-cyclodextrin in the BGE was 2%.

3.2. CZE with a large-volume sample hydrodynamic injection, followed by the removal of the large plug of low conductivity sample matrix from the capillary using polarity switching (injected sample volume representing 70% of the capillary volume)

Cinnamic acid was chosen as a model standard to find suitable pressure, sample injection time and polarity switching time to increase the sensitivity of the method.

Hydrodynamic injections were preformed under pressures ranging from 50 to 200 mbar for 99 s. The best results were achieved at 100 mbar, which corresponds to an injected volume representing 70% of the capillary volume.

The polarity was switched as soon as the experimental current reached 95% of its standard value observed during conventional CZE separation performed with the same BGE. The electropherogram of standards mixture obtained using large-volume sample stacking is shown in Fig. 1B.

Table 1
Calibration parameters and relevant analytical data of CZE method using large-volume sample stacking with polarity switching

Acid	Calibration curve, $y = kx + q$	s_k	s_q	r	$s_{residual}$	Time (min) ± R.S.D. (%)	Area R.S.D. (%)
Cinnamic	$y = 77585x - 2439$	1685	2065	0.9993	2560	10.77 ± 0.50	2.76
Chlorogenic	$y = 26418x - 2734$	957	1173	0.9980	1454	11.55 ± 0.58	3.53
Coumaric	$y = 77029x - 3346$	3856	4725	0.9983	5857	12.01 ± 0.86	3.50
Ferulic	$y = 63113x - 2475$	2165	2654	0.9982	3290	12.64 ± 0.94	2.93
Caffeic	$y = 107467x - 6599$	1880	2304	0.9995	2856	15.08 ± 0.85	4.11
Gentisic	$y = 140207x - 6994$	2553	3129	0.9995	3879	15.89 ± 1.07	3.37
Syringic	$y = 76853x - 7742$	2949	3614	0.9979	4479	16.37 ± 1.34	5.69
Vanillic	$y = 141081x - 10104$	3688	4519	0.9990	5602	19.08 ± 1.62	3.74

y, Corrected peak area; x, concentration in μg/ml; s, standard deviation.

3.3. Comparison of conventional CZE and CZE with large volume sample stacking

According to our results the corrected peak areas (peak area/migration time) increased by the factor of 40 compared to the peak areas obtained by conventional CZE. No drifting of the baseline was observed during the CZE separations with large-volume sample stacking.

4. Method validation

4.1. Calibration parameters

The calibration was carried out with five standard solutions containing 0.1, 0.5, 1.0, 1.5 and 2.0 μg/ml of each analyte and relevant pieces of information are given in Table 1.

4.2. LOD and LOQ

LOD (S/N = 3) and LOQ (S/N = 10) were found to be 0.01 and 0.033 μg/ml for cinnamic, *p*-coumaric, ferulic, gentisic and syringic acids. The same values for caffeic and vanillic acid were 0.02 and 0.066 μg/ml, respectively. The highest values were obtained for chlorogenic acid (0.025 and 0.083 μg/ml).

4.3. Repeatability

Solution of standard acids containing 1 mg/ml of analytes was repeatedly ($n = 6$) injected. Repeatability data are included in Table 1.

The repeatability of the results, expressed by the relative standard deviation of six CZE measurements is satisfactory. The R.S.D. values of migration times do not exceed 2% whereas R.S.D.s of peak areas are lower than 5% except of syringic acid (R.S.D. = 5.69%).

4.4. Resolution

Resolutions of neighbouring peaks were calculated using 32 Karat Software 4.01. The resolution factor (R_s) values determined at optimal conditions were 3.1 for the cinnamic–chlorogenic acid peaks, 2.0 for chlorogenic–coumaric acid peaks, 2.3 for coumaric–ferulic acid peaks, 5.9 for ferulic–caffeic acid peaks, 1.7 for caffeic–gentisic acid peaks, 1.4 for gentisic–syringic acid peaks and 6.9 for syringic–vanillic acid peaks.

5. Conclusions

Large-volume sample stacking with polarity switching has proved to be a powerful tool for sensitivity enhancement in CZE analysis of selected aromatic acids. A 40-fold sensitivity enhancement was attained for the quantitative analysis of extremely diluted analytes in low conductivity matrices. The proposed method is intended for the identification and assay of the listed phenolic acids in the methanolic extract of *H. epilobi*.

Acknowledgments

This work was supported by the Grant Agency of Charles University No. 306/2005/BCH, Internal grant Agency of the Ministry of Health of the Czech Republic No. NL/7689-3 and by the MSM Project 0021620822 of the Czech Ministry of Education.

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