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Two-step stacking in capillary zone electrophoresis featuring sweeping and micelle to solvent stacking: I. Organic cations

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ABSTRACT

Two-step stacking of organic cations by sweeping and micelle to solvent stacking (MSS) in capillary zone electrophoresis (CZE) is presented. The simple procedure involves hydrodynamic injection of a micellar sodium dodecyl sulfate solution before the sample that is prepared without the micelles. The micelles sweep and transport the cations to the boundary zone between the sample and CZE buffer. The presence of organic solvent in the CZE buffer induces the second stacking step of MSS. The LODs obtained for the four beta blocker and two tricyclic antidepressant test drugs were 20–50 times better compared to typical injection.

1. Introduction

On-line sample concentration or stacking is an important research area to improve the detection sensitivity in capillary electrophoresis (CE) [1–3]. There are several well known stacking techniques, e.g., field amplified or enhanced sample stacking [4,5], transient isotachophoresis (t-ITP) [6–8], sweeping [9,10], and dynamic pH junction [11,12] and the few new ones transient trapping [13], analyte focusing by micelle collapse (AFMC) [14,15] and micelle to solvent stacking (MSS) [16–18].

The sequential use of two stacking techniques, referred to here as two-step stacking had also became popular for small molecules during the last decade [1–3]. For charged analytes, the first step is usually stacking by field amplification followed by a sweeping or t-ITP step. Cation [19] and anion [20,21] selective exhaustive injection–sweeping are two-step stacking techniques where the sample is electrokinetically injected under field amplified conditions and then the concentrated zone is focused further by sweeping prior to separation by micellar electrokinetic chromatography (MEKC) [22]. Other two-step techniques reported in MEKC were the combination of dynamic pH junction and sweeping [23] and of sweeping via borate complexation [24] and sweeping with nonionic micelles [25,26]. In capillary zone electrophoresis (CZE) [27], there is the two-step technique called electrokinetic supercharging [28], where the first step is electrokinetic injection under field amplified conditions and the second step involves the injection of a terminator to induce t-ITP.

Here, initial studies on the two-step stacking of organic cations in CZE by sweeping and MSS, as the first and second stacking steps, respectively are presented. Sodium dodecyl sulfate was used as the anionic micellar phase for sweeping and MSS while methanol was used as the organic solvent additive in MSS and CZE. Beta blocker and tricyclic antidepressant drugs that contain a basic nitrogen group were used as the cationic model test analytes.

2. Experimental

2.1. Equipment

Electrophoresis and stacking experiments were performed on fused silica capillaries of 50 μm i.d. and 375 μm o.d. obtained from Polymicro Technologies (Phoenix, AZ). The total length was 50 cm and the length from the inlet to the detector was 41.5 cm. All electropherograms were obtained with Agilent 3D capillary electrophoresis systems (Waldbronn, Germany) with detection set at 200 nm using the diode array detector. The temperature of the capillary was controlled at 20 °C. Water was purified with a Milli-Q system (Millipore, Bedford, MA). The pH was measured using an Activon Model 210 pH meter (Pennant Hills, NSW, Australia).

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2.2. Reagents and solutions

All reagents (sodium dodecyl sulfate (SDS) for electrophoresis, ammonium acetate (≥98%), USP grade acetic acid, sodium hydroxide (≥0.97%), and HPLC grade methanol) were purchased from Sigma Aldrich (St. Louise, MA). Stock solutions of 100 mM sodium dodecyl sulfate and 250 mM ammonium acetate (pH 6.2) were prepared every two weeks in purified water. Background solutions (BGS) were prepared each day by dilution of the ammonium acetate stock solution with appropriate amounts of purified water and methanol. The measured pHs of the BGSs with methanol were around 7. Micellar solutions (MS) and sample matrices were prepared each day by dilution of the SDS and/or ammonium acetate stock solutions with purified water. The pH of the MS or sample matrices was 6.3. All solutions were filtered through 0.45 μm filters from MicroScience (Dassel, Germany) prior to use. All the samples of the highest purity available were obtained from Sigma Aldrich (St. Louise, MA). Stock solutions of the beta blocker (alprenolol, propranolol, nadolol, and labetalol) and tricyclics antidrepressant (nortriptyline and clomipramine) drugs were prepared in methanol at a concentration of 10 mg/mL each. Care should be taken when handling these chemicals. Sample solutions (S) were prepared by appropriate dilution of the sample stock aliquots with the different matrices. The composition of each matrix is described in the text or figures.

2.3. General electrophoresis procedure

The capillary was conditioned (1 bar pressure) prior to use with 0.1 M NaOH (20 min), water (10 min), methanol (5 min), water (5 min), and finally BGS (10 min). The S and MS were injected into the capillary farthest from the detector end using pressure (50 mbar). Voltage (20 kV) was applied at positive polarity with the BGS at both sides of the capillary, until all peaks were detected. The capillary was conditioned, between consecutive analysis, with 0.1 M NaOH (1 min), purified water (1 min), and finally BGS (5 min). Other conditions are specified in the text or figures.

3. Results and discussion

3.1. Two-step stacking model

Fig. 1 shows the model for the two-step stacking of cationic analytes by sweeping followed by MSS using anionic SDS micelles in CZE. The movement of the cations (+), anionic SDS micelles (circles), and micelle to solvent stacking boundary (MSSB) in the presence of a homogenous electric field is illustrated. The electrokinetic velocities are positive and negative when movement is directed toward the cathode and anode, respectively. The velocity of the MSSB is the same as the electroosmotic flow (eof). The overall velocities of all zones are positive due to the strong eof. In the starting situation (Fig. 1A), the MS and the sample solution (S) both having similar conductivity as the background solution (BGS) are injected. Stacking and destacking effects due to differences in conductivity are absent. Upon application of voltage (Fig. 1B), the micelles from the anodic side of the MS zone sweep the cations [9]. This continues until all the cations are swept by the micelles. The direction of the electrophoretic mobility of the cations shift from positive to negative and the micelles transport the cations to the MSSB. The cations in the presence of SDS micelles depicted as + inside the circles in Fig. 1 have a negative effective electrophoretic mobility. When the micelles reach the MSSB (Fig. 1C), the second zone focusing due to MSS occurs. The effective electrophoretic mobility of the cations reverses from negative to positive due to the presence of organic solvent in the BGS. The analyte cations in the S bound to the micelles

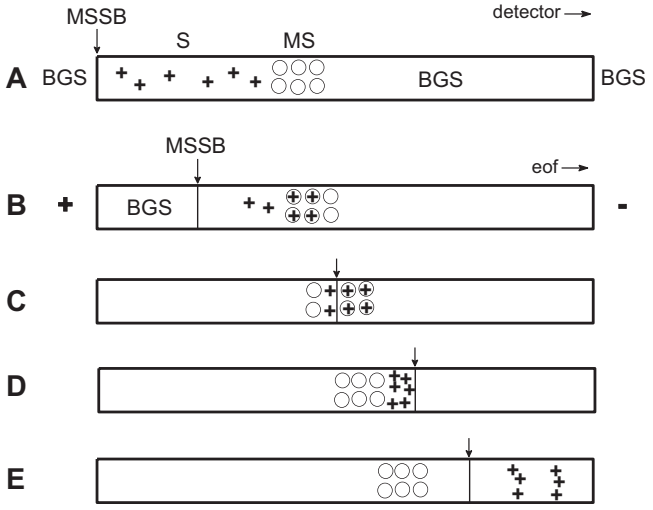

Fig. 1. Two-step stacking of cationic analytes by sweeping and MSS model. (A) The capillary is conditioned with the background solution (BGS) that contains an organic solvent. A MS is injected followed by the sample solution (S). The micelle to solvent stacking boundary (MSSB) is found at the inlet end of the S zone. (B) A voltage is applied with the anode and cathode at the inlet and outlet ends, respectively. A homogenous electric field across the capillary is assumed. The electroosmotic flow (eof) is directed toward the cathode. The MSSB moves with the velocity of the eof. The negatively charged SDS micelles (circles) sweep the cations (+). The effective electrophoretic velocity of the cations in the presence of SDS is directed to the anode. (C) The SDS micelles transport the cations to the MSSB. When the SDS micelles reach the MSSB, the second step of MSS occurs where the effective electrophoretic velocity of the cations reverses to the direction of the cathode. (D) The cations accumulate at the MSSB, and this stacking occurs until all the micelles from the injected MS zone traversed the boundary. (E) The two-stepped stacked cations separate by virtue of CZE and move toward the detector.

were electrophoretically attracted to the anode. Upon reaching the MSSB containing the organic solvent, the affinity of the analytes to the micelles were significantly lowered. This causes reduction of the retention factor k to the extent that the cations migrate toward the cathode and experienced an electrophoretic reversal resulting to analyte accumulation at the MSSB [16,18]. A theoretical consideration of MSS was given in ref. 18. The stacking process is complete when all the micelles traversed the MSSB (Fig. 1D). The two-step focused cations then separate by virtue of CZE and migrate to the detector (Fig. 1E).

3.2. Experimental verification of the two-step stacking

Fig. 2 shows electropherograms obtained from typical (A), non-stacking (B), one-step stacking (C), and two-step stacking (D) injections of the test beta blocker drugs in CZE. The beta blockers were cationic at the pH of the BGSs (pH ~ 7). The pKa of alprenolol, propranolol, nadolol, and labetalol is 9.34, 9.25, 9.00, and 8.8, respectively [29]. The effects of stacking and destacking due to conductivity differences were negligible since the measured CE currents obtained for the MS and/or S were within 60–100% of the current obtained for the pertinent BGS. A homogenous electric field was provided to remove stacking or destacking effects, and thus only demonstrated the effects of sweeping and MSS.

Fig. 2A is from a typical injection (3 s at 50 mbar) of the beta blockers prepared in the BGS. Fig. 2B is obtained from a long injection (60 s at 50 mbar) of the samples in Fig. 2A that was diluted 20 times with the BGS. Broad peaks are observed under these non-stacking conditions. Fig. 2C is from a long injection similar to Fig. 2B but the samples were diluted in 25 mM ammonium acetate and the BGS contains only 30% methanol. A short plug (15 s at 50 mbar) of

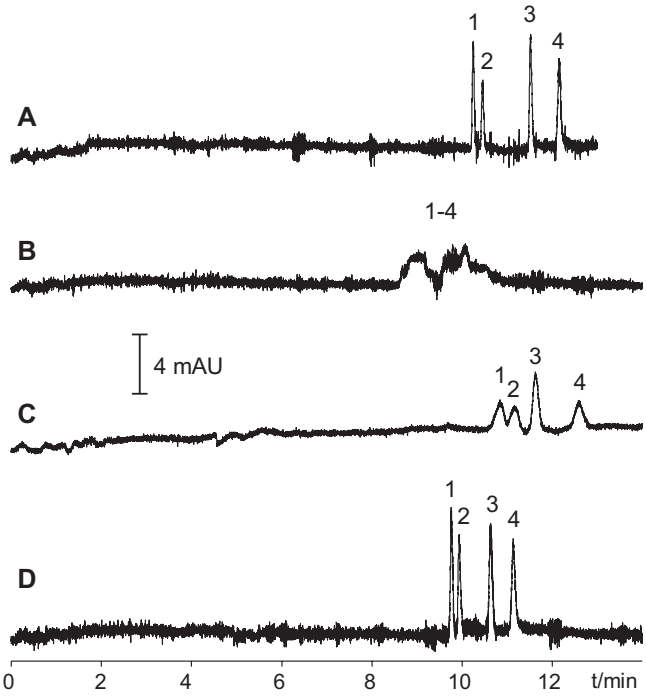

Fig. 2. Experimental verification of two-step stacking of organic cations (i.e., beta blocker drugs) in CZE by sweeping and MSS. Injection scheme: (A) 3 s at 50 mbar of 20 μg/mL beta blockers in BGS. (B) 60 s at 50 mbar of 1 μg/mL beta blockers in 25 mM ammonium acetate. (C and D) 15 s of 10 mM SDS with 12.5 mM ammonium acetate followed by 60 s of 1 μg/mL beta blockers in 25 mM ammonium acetate. BGS: 50 mM ammonium acetate in 50% MeOH (A, B, and D) and 50 mM ammonium acetate in 30% MeOH (C). Peak identity: alprenolol (peak 1), propranolol (peak 2), nadolol (peak 3), and labetalol (peak 4). Capillary: 50 μm i.d., 50 cm (total), 42.5 (effective length). Separation voltage: 20 kV. Detection wavelength: 200 nm.

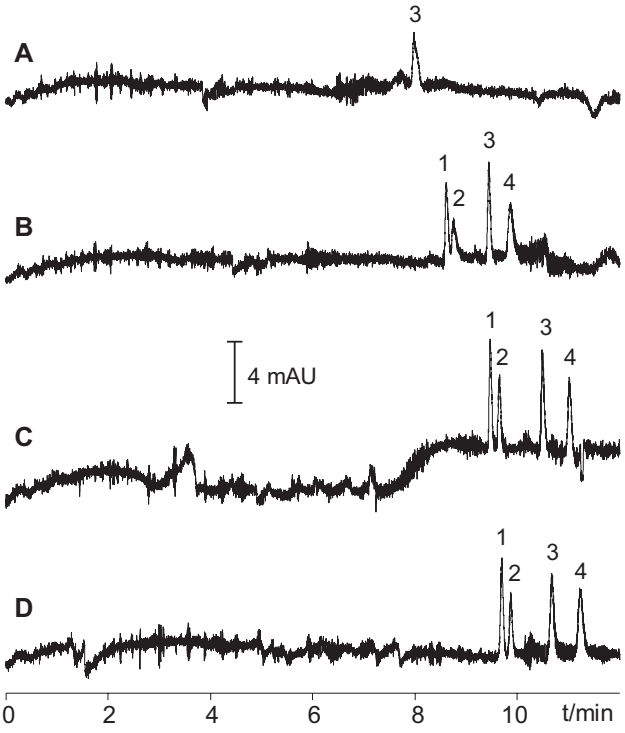

Fig. 3. MSS optimisation for beta blocker drugs. BGS: 50 mM ammonium acetate in 30 (A), 40 (B), 50 (C), and 60% (D) MeOH. Sample matrix: 10 mM SDS with 12.5 mM ammonium acetate. Samples: 2 μg/mL of each beta blocker. Injection: 30 s at 50 mbar. Other conditions and peak identity: see Fig. 2.

MS (10 mM SDS with 12.5 mM ammonium acetate) was injected before the sample to induce sweeping. Note that the peaks found in Fig. 2C are sharper compared to those in Fig. 2B.

The swept zones in Fig. 2C can be focused by a second stacking step which was accomplished by using a higher concentration of methanol in the BGS to induce MSS. This is shown in Fig. 2D where the concentration of methanol in the BGS was 50%. Note that the injection scheme of the MS and S was the same in Fig. 2C and D. The peaks in Fig. 2D are much sharper compared to those in Fig. 2C. The sharpness of the peaks is also comparable to those obtained from typical injection (see Fig. 2A). The enhancement in sensitivity can be obtained by comparing the peak heights and concentrations of the samples in Fig 2A and D. Note that the drugs in Fig. 2D were 20 times less concentrated compared to the samples in Fig. 2A. The enhancements in sensitivity obtained from two-step stacking compared to typical injection were 24, 30, 20, and 20 for alprenolol (peak 1), propranolol (peak 2), nadolol (peak 3), and labetalol (peak 4), respectively.

3.3. Two-step stacking method development

3.3.1. MSS conditions

In the development of the two-step stacking method, the MSS conditions must first be identified. Fig. 3 shows the effect of methanol content (30% (A), 40% (B), 50% (C), and 60% (D)) in the BGS on the MSS of the beta blocker drugs. Other typical organic solvents such as acetonitrile in CE may be utilized to induce MSS [16]. The concentration of ammonium acetate in the BGS was kept at 50 mM. The S in all conditions contained 2 μg/mL of each drug and was injected for 30 s at 50 mbar. The sample matrix (i.e., 10 mM SDS with 12.5 mM ammonium acetate) was chosen because 10 mM SDS was previously found effective for the MSS of beta blocker drugs [16]. The neutral pH of the BGSs (~7) and sample matrix (6.3) produced a positive charge on the drugs (see pKa values above).

The retention factor k affects the focusing effect of sweeping and MSS. The k is related to the octanol–water distribution or partition coefficient. The log experimental partition coefficient ($\log P_{app}$) at pH 7 reported for alprenolol, propranolol, nadolol, and labetalol were 0.81, 1.16, −1.00, and 0.99, respectively [28]. This means that the k of propranolol and nadolol is the highest and lowest, respectively. The low k of nadolol explains the behaviour of this drug in Fig. 3A that shows the MSS sharpening of nadolol using the lowest concentration of methanol (30%) in the BGS. The reversal of the effective electrophoretic mobility induced by organic solvent content is easier to achieve for lower k than higher k analytes. Note that the other drugs that all have k values higher than nadolol were not focused. The effect of MSS improved for all the drugs when the concentration of methanol in the BGS was increased from 30% to 50% (see Fig. 3A–C). The effect was quite similar using 50% or 60% methanol (see Fig. 3C and D), thus the 50% methanol content in the BGS was chosen as optimum for MSS.

Higher concentration of SDS (up to 50 mM) in the MS was studied in order to increase the focusing effect of sweeping using 50% methanol in the BGS. However, the focusing effect of MSS was lowered with the increase in the SDS concentration and the results obtained with the 10 mM were found optimum.

3.3.2. Injection scheme optimisation in two-step stacking: injection time for the MS and S

3.3.2.1. Beta blocker drugs. Fig. 4 shows the effect of varying the injection time (5 s (A), 10 s (B), 15 s (C), 30 s (D), and 60 s (E) at 50 mbar) of the MS in the two-step stacking of beta blockers. The MS is the sample matrix in the MSS study described in section 3.3.1. The injection of the S that contained 2 μg/mL of each drug was

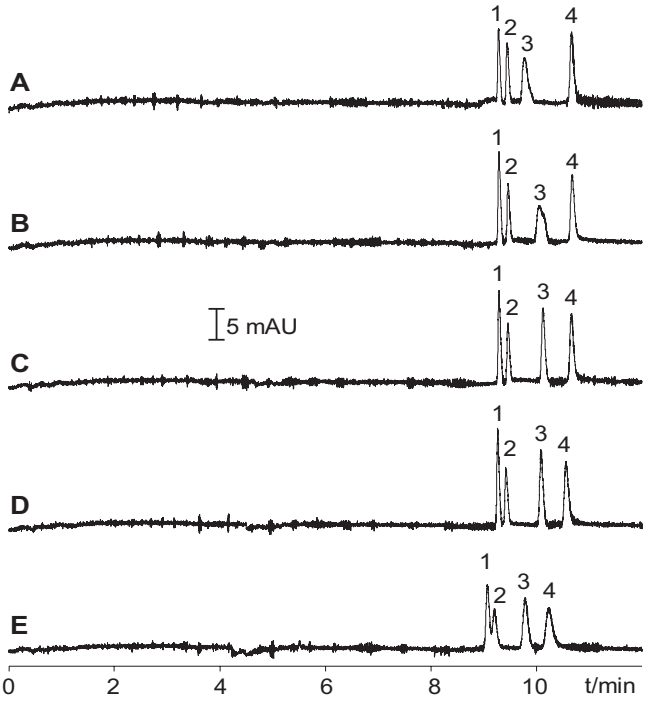

Fig. 4. Optimisation of the injection time of MS and sample solution (S). Injection scheme at 50 mbar: 5 s (A), 10 s (B), 15 s (C), 30 s (D), and 60 s (E) of MS followed by 60 s injection of S. MS: 10 mM SDS with 12.5 mM ammonium acetate. S: 2 μg/mL beta blockers in 25 mM ammonium acetate. Other conditions and peak identity: see Fig. 2.

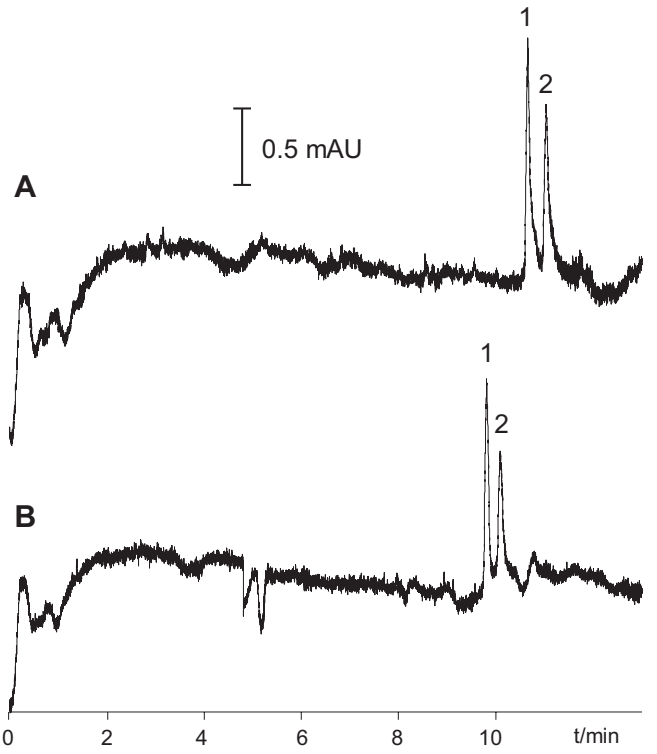

Fig. 5. Typical injection (A) and two-step stacking (B) of two tricyclic antidepressant (TCA) drugs. BGS: 50 mM ammonium acetate in 50% methanol. Injection scheme at 50 mbar: 3 s of S (A) and 10 s of MS followed by 120 s of S (B). Sample matrix: BGS (A) and 25 mM ammonium acetate (B). MS: 10 mM SDS with 12.5 mM ammonium acetate. Concentration of each TCA drug: 4 μg/mL (A) and 0.08 μg/mL (B). Peak identity: nortriptyline (peak 1) and clomipramine (peak 2). Other conditions are the same as in Fig. 2.

fixed at 60 s at 50 mbar. For sweeping purposes, the sample matrix (i.e., 25 mM ammonium acetate, pH 6.3) used to make the S was SDS free and had a conductivity value similar to the BGS. As seen in Fig. 4A–C, the lowest *k* nadolol (peak 3) was the only analyte significantly affected by the injection length of the MS. A minimum 15 s injection of the MS (see Fig. 4C) was needed to sweep this analyte prior to MSS focusing. The other 3 analytes were nicely focused using a 10, 15, or 30 s injection of the MS (see Fig. 4B–D). A MS injection of 60 s (see Fig. 4E) produced lower peak heights and this is due to the longer flux of micelles that broadened the focused zones at the MSSB. The injection scheme of 15 s MS followed by 60 s of S (injection ratio = 1:4) described in Fig. 4C was then chosen for further optimisation.

Supplementary information Fig. 1 shows the effect of increasing the injection time of the MS and S where the injection ratio = 1:4. The injection of the MS/S was varied from 15 s/60 s, 22.5 s/90 s, 30 s/120 s, 37.5 s/150 s, and 45 s/180 s. No significant increases in peak heights were observed and the 15 s/60 s injection scheme was found optimum. In addition, the resolution of the peaks decreased as the injection times were increased.

3.3.2.2. Tricyclic antidepressant drugs. For the two test tricyclic antidepressant drugs (i.e., nortriptyline and clomipramine), the BGS, MS, and sample matrix employed for the beta blockers were also employed. However, the optimum injection ratio (i.e., 1:12) determined was different. The *k* values of these drugs were higher than the beta blockers, thus a shorter plug of MS sufficed. The optimum injection scheme was 10 s of MS and 120 s of S (see Fig. 5B). Compared to typical injection (see Fig. 5A), the improvement in peak heights was 46 and 40 times for nortriptyline (peak 1) and clomipramine (peak 2), respectively. In addition, the peaks are sharper in the two-step stacking method (Fig. 5B) compared to typical injection (Fig. 5A).

3.4. Linearity, reproducibility, and LOD

The analytical figures of merit for the two-step stacking method were studied using standard solutions. For the beta blocker drugs, the optimum conditions are given in Fig. 2D. The linearity, intraday, and interday reproducibility were all acceptable. The R^2 from injections of 0.5, 1, 2, and 5 μg/mL of each drug using peak heights and corrected peak areas were all >0.99. The corrected peak areas were calculated by dividing the peak area by the retention time. The reproducibility was studied using the 1 μg/mL standard. The %RSD ($n = 3$) for retention time, peak height, and corrected peak area were in the range from 0.7% to 0.8%, 4.2% to 10.2%, and 4.2% to 9.1%, respectively. For intraday reproducibility, the %RSD ($n = 6$) were in the range from 0.3% to 0.5%, 4.1% to 9.1%, 4.8% to 8.1%, correspondingly. For interday reproducibility, the %RSD ($n = 6$) were in the range from 1.8% to 2.1%, 3.5% to 5.0%, and 3.6% to 8.5%, correspondingly. The linearity and %RSD values are similar to that obtained in the one-step stacking by MSS in CZE using ESI-MS detection of pindolol and metoprolol [16]. The LOD for each drug was 0.2 μg/mL, which is 30 times better compared to the LOD obtained from typical injection (6 μg/mL).

For the tricyclic antidepressant drugs, the optimum conditions are given in Fig. 5B. The linearity, intraday, and interday reproducibility were all acceptable. The R^2 from injections of 0.08, 0.3, 0.5, 1, and 2 μg/mL of each drug using peak heights and corrected peak areas were all >0.98 and >0.99, respectively. The reproducibility was studied using the 1 μg/mL standard. The %RSD ($n = 3$) for retention time, peak height, and corrected peak area for nortriptyline was 0.3%, 1.9%, and 6.1%, respectively. The values for clomipramine were 0.3%, 3.6%, and 9.4%, correspondingly. For intraday reproducibility, %RSD ($n = 6$) for retention time, peak height,

and corrected peak area for nortriptyline was 0.3%, 5.3%, and 4.9%, respectively. The values for clomipramine were 0.3%, 7.0%, and 7.7%, correspondingly. For interday reproducibility, %RSD ($n = 6$) for retention time, peak height, and corrected peak area for nortriptyline was 0.3%, 3.3%, and 4.8%, respectively. The values for clomipramine were 0.3%, 4.1%, and 7.5%, correspondingly. The LOD for each drug was 0.02 μg/mL, which is 50 times better compared to the LOD obtained from typical injection (1 μg/mL).

The following were results obtained using typical injection. Linearity study of the beta blocker (7–100 μg/mL) and tricyclic antidepressant (3–40 μg/mL) drugs gave R^2 of ≥0.97 and ≥0.98 for peak heights and corrected peak area, respectively. Intraday reproducibility for the beta blocker (30 μg/mL) and antidepressant (7 μg/mL) drugs gave %RSDs ($n = 3$) of 1.0–1.5% for retention time, 3.7–5.9% for peak height, and 7.8–15.1% for corrected peak area. Interday reproducibility gave %RSDs ($n = 6$) of 1.7–2.3% for retention time, 7.0–9.9% for peak height, and 6.5–18.5% for corrected peak area. The analytical figures of merit for the two-step stacking method were similar or better compared to typical injection.

4. Conclusion

A new two-step stacking strategy in CZE that combines two online concentration techniques, namely sweeping and MSS, afforded 20–46 fold improvements in peak height sensitivity for the test organic cations (i.e., beta blocker and tricyclics antidepressant drugs). The LODs were more than a magnitude better compared to typical injection. As a stacking strategy in CZE, the improvements in sensitivity were 2–5 times better compared to one-step stacking by MSS [16]. It is noted that stacking by field amplification or hydrodynamic injection of the sample prepared in water can only afford up to 10 times enhancement in sensitivity. Cationic analytes can be focused more effectively by sweeping in MEKC. The advantage over sweeping MEKC is that the current stacking strategy is compatible with ESI-MS detection since the focused zones were detected in the absence of surfactants that interfere with the detection.

This two-step stacking procedure using 10 mM SDS micelles was not useful for anionic or neutral analytes. The specific focusing of target cationic analytes can be regarded as an advantage over other stacking techniques such as field amplification where both anionic and cationic analytes can be focused. A similar two-step stacking procedure but using cationic micelles was developed for anionic analytes, this will be reported elsewhere. For neutrals, a more feasible second stacking step is AFMC where a lower concentration of SDS must be used in the MS for easy dilution of SDS to below its CMC.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2010.10.020.

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