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Penicillin analyses by capillary electrochromatography-mass spectrometry with different charged poly(stearyl methacrylate–divinylbenzene) monoliths as stationary phases

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ABSTRACT

This study describes the ability of an on-line concentration capillary electrochromatography (CEC) coupled with mass spectrometry (MS) for the determination of eight common penicillin antibiotics. Poly(stearyl methacrylate–divinylbenzene) (poly(SMA–DVB)) based monolithic columns prepared under the same conditions but differing only in the charged monomer were used as separation columns. Vinylbenzyl trimethylammonium chloride (VBTA) and vinylbenzenesulfonate (VBSA) were employed as the positively charged monolith and negatively charged monolith, respectively. Results indicated that poly(SMA–DVB–VBTA) monolithic column provided reproducible performance for penicillin separation through ion-exchange interaction, while the negatively charged poly (SMA–DVB–VBSA) column produced unstable separation due to the electrostatic repulsion between the electrophilic analytes and the negatively charged stationary phase. On-line concentration steps of step-gradient elution combined with anion selective injection (ASEI) were used to enhance the detection sensitivity of the CEC-MS method and all penicillin detection sensitivities were further improved (reduction in the limits of detection from 1.9–31 μg/L (normal injection mode) to 0.05–0.2 μg/L (on-line concentration mode)). Finally, this optimal on-line concentration CEC-MS method was applied to trace penicillin analyses in milk samples.

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1. Introduction

Veterinary drugs are applied either to improve animal health or as growth promoters. β-lactam drugs, including penicillin, are widely used in veterinary medicine to treat bacterial infection as well as to increase feed efficiency [1]. However, residues of these drugs are retained in animal tissues which could possibly be transferred to humans through ingestion, and eventually pose potential harm [2,3]. Due to this reason, the European Union (EU), including Taiwan, set a maximum residue limit (MRL) of about 4–300 μg/L for these drugs in animal organ and milk [4]. So far, many methods such as liquid chromatography (LC) [5,6], ultra performance liquid chromatography (UPLC) [7], capillary zone electrophoresis [8], micellar electrokinetic chromatography [9], and microemulsion electrokinetic chromatography [10,11] were employed to detect these drugs. Due to the complexity of real samples, high sensitivity detectors are necessary to identify these compounds. Among them, LC coupled with mass spectrometry (MS) is commonly used to detect antibiotic residues, including penicillin, in food [12,13]; however, in this system, large amount of solvents are wasted.

Capillary electrochromatography (CEC) is a hybrid separation technique which combines the features of HPLC and CE; and has many advantages over LC including higher efficiency, lower waste production, lower sample amount, and faster separation [14–16]. But, when CE or CEC is coupled to MS system, due to an inadequate volume flow of mobile phase, a sheath liquid has to be used to provide a stable spray effect in a commercial CE-ESI interface. Similar to the observations of D'Orazio and Fanali [17], the sheath liquid (for example with a flow rate of about 220 μL/h) possibly caused a dilution effect to the chromatographic eluent as well as to the solutes leading to unavoidable decrease in the mass sensitivities. Our previous studies demonstrated that CEC-MS can provide comparable detection sensitivities with the best LC-MS methods reported so far, even if coupled with a commercial sheath liquid supported ESI interface [18,19]. The main reason could be due to the high column efficiency and no frits needed especially with polymeric monoliths as stationary phases; wherein their small-sized skeletons, large through pores and better compatibility with mass spectrometric detection have been documented [20,21]. Because of the many environmentally friendly features of CEC, this method was coupled to MS for

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penicillin residue detection, extending its potential in trace penicillin analyses in food samples. To the best of our knowledge, no penicillin analysis by the CEC-MS method has been previously reported in the literature.

In this study, we used CEC-MS to analyze penicillin antibiotics in poly(stearyl methacrylate–divinylbenzene) (poly(SMA–DVB)) based monolithic columns each prepared with different charged monomers to investigate the charged monomer effect on penicillin separation. This proposed CEC-MS method was employed for the determination of penicillin in milk samples with as low as 4 and 10 μg/L content by simple on-line concentration steps.

2. Experimental

2.1. Chemicals and reagents

Oxacillin (Oxa) ($pK_{a_1}=2.61$), VBSA and DVB (80.1%, a mixture of *m*-DVB (55.5%) and *p*-DVB (24.6%)) were obtained from Fluka (Buchs, Switzerland). Penicillin V (PV) ($pK_{a_1}=2.62$) was purchased from ICN (Ohio, USA). Penicillin G (PG) ($pK_{a_1}=2.62$), ampicillin (Amp) ($pK_{a_1}=2.62$, $pK_{a_2}=7.4$), amoxicillin (Amo) ($pK_{a_1}=2.62$, $pK_{a_2}=7.2$, $pK_{a_3}=9.6$), cloxacilln (Clo) ($pK_{a_1}=2.44$) and dicloxacillin (Dic) ($pK_{a_1}=2.44$) were purchased from Sigma (St. Louis, MO, USA). Nafcillin (Naf) ($pK_{a_1}=2.61$) was purchased from MP (Eschwege, Germany). SMA was purchased from TCI (Tokyo, Japan). Azobisisobutyronitrile (AIBN) and ammonium formate were bought from Showa (Tokyo, Japan). *N*-Methyl-2-pyrrolidone (NMP) was purchased from Mallinckrodt Baker (Paris, KY, USA). VBTA was bought from ACROS (New Jesey, USA). Cyclohexanol was obtained from MERCK (Hohenbrunn, Germany). Polyimide coated fused-silica capillaries with 100-μm I.D. and 375-μm O.D. were purchased from Reafine Chromatography Ltd. (Hebei, China). DVB, which is a cross linker, was washed with 10% (w/v) aqueous sodium hydroxide to remove the inhibitors before use. All other chemicals were reagent-grade and were used as received. The above-mentioned eight penicillin standards used as test analytes in the study were individually dissolved in deionized water at a stock concentration of 1 mg/mL. Mobile phases were prepared by mixing acetonitrile and phosphate buffer (5 mM) (CEC-UV) or with ammonium formate buffer (5 mM) in different volume ratios, in which 1.0M HCl or NaOH (CEC-UV) or formic acid or 0.1M NH_4OH (CEC-MS) was then added to the mobile phase solution until the desired pH was achieved.

2.2. Apparatus

All CEC-UV experiments were performed in a Beckman Coulter MDQ CE system equipped with a photodiode array detector (Fullerton, CA, USA). Beckman Coulter MDQ 32 Karat software was used for CEC-UV instrumental control and data analysis. The CEC-ESI-MS experiments were performed in a configured in-house CE coupled to a Bruker Daltonics TOF mass spectrometer model microTOF II (Bremen, Germany) with an Agilent ESI source (model G1607-6001). The setup in this configured CE consisted of a platinum electrode in a vial containing a running buffer connected to CZE1000R high-voltage power supply (Spellman, Plainview, NY, USA). The microTOF control and Data AnalysisTM software were used for mass instrumental control and data analysis.

2.3. Preparation of the polymeric monolithic column

The inner wall of a 100-μm I.D. capillary column was treated according to the procedure described in our previous paper [22]. Then, the monolithic column was prepared as described in our previous report [18] with slight modification in the charged monomer used. Here, we prepared two columns each with different charged monomers, VBSA and VBTA.

2.4. Operation condition for CEC

The monolithic column was equilibrated according to the procedure described in our previous papers [18,19]. For normal injection, samples and standards were electrokinetically injected into the capillary for 3 s at a voltage of −10 kV (poly(SMA–DVB–VBTA)) or +10 kV (poly(SMA–DVB–VBSA)). Separations were carried out with an electrical voltage of −20 kV (poly(SMA–DVB–VBTA)) or +20 kV (poly(SMA–DVB–VBSA)). MS detection was performed in the selected ion mode. Since all penicillin had relatively strong molecular ion signals exhibited as $[M–H]^-$ form, these accurate mass molecular ion peaks (i.e. 413.116 m/z for Naf, 400.096 m/z for Oxa, 468.018 m/z for Dic and 433.057 m/z for Clo, 349.085 m/z for PV, 333.090 m/z for PG, 348.101 m/z for Amp and 364.096 m/z for Amo) were selected as monitored mass signals. Negative ions were generated through the application of 3.8 kV to the probe tip, and end plate off-set was fixed at −0.5 kV. Nitrogen gas was used as drying gas at 180 °C with a flow rate of 4 L/min. Nitrogen nebulization gas for electrospray was supplied at 0.4 psi. The sheath liquid (isopropyl alcohol (IPA)/water) (60/40, v/v) was delivered to the electrospray at 220 μL/h. The scanning mass range was from m/z 50 to 1000.

2.5. On-line concentration step

On-line sample concentration steps, which combined step gradient elution with ASEI, were used to enhance the detection sensitivity of penicillin. For Amp and Amo to achieve the best sensitivity in CEC-MS, the CEC column was filled with a pH 3 mobile phase (60% ammonium formate solution (5 mM), 40% ACN), and standards or samples which were first mixed with ammonium formate solution (0% ACN, pH 7, 5 mM) in a volume ratio of 1:1 (i.e. the volume ratio of buffer solution, D.I water and ACN was 50%:50%:0%), were then electrokinetically injected into the capillary for 90 s at a voltage of −10 kV. For the other analytes to achieve the best sensitivity in CEC-MS, the CEC column was filled with a pH 2 mobile phase (60% ammonium formate solution (5 mM), 40% ACN), and standards or samples which were first mixed with ammonium formate solution (20% ACN, pH 7, 5 mM) in a volume ratio of 1:9 (i.e. the volume ratio of buffer solution, D.I water and ACN was 72%:10%:18%), were then electrokinetically injected into the capillary for 105 s at a voltage of −10 kV. After sample injection, a voltage of −20 kV was applied with the original mobile phase in the inlet vial and then the CEC separation proceeded.

Further experimental details such as real sample pretreatment are described in the Supporting Information.

3. Results and discussion

3.1. Effect of different charged monomers on penicillin separation

Unlike in the LC system, wherein the mobile phase is driven by high pressure, in the CEC system, the electroosmotic flow (EOF) created by the charged monomer is responsible for mobile phase flow through the column. Studies in most CEC reports were focused on the effect of charged monomers on the EOF magnitude and migration direction, but few reported the effect of their polarity on analytes chromatographic behavior. Our previous reports used the same type of poly (SMA–DVB) column but with different charged monomers (cationic VBTA or anionic VBSA) to separate neutral compounds (i.e. NSAIDs on poly(SMA–DVB–VBSA) or sulfonamides on poly(SMA–DVB–VBTA)) [18,19]; however, no comparison on analytes' separation performance as well as sensitivity enhancement with different polar charged monomers were carried out.

In this study, we used anionic VBSA and cationic VBTA as charged monomers to prepare the negatively charged poly(SMA–DVB) and the positively charged poly(SMA–DVB) monoliths, respectively and then evaluated their effect on penicillin separation.

3.1.1. Anionic VBSA as charged monomer

As shown in Fig. 1(a), all penicillin analytes were baseline separated on the poly(SMA–DVB–VBSA) but with poor signal reproducibility observed between run-to-run This is possibly due to the negative charges present in the stationary phase brought by the anionic VBSA charged monomer thus acting like a nucleophile. Except for positively charged Amp and Amo, the rest of the penicillin analytes (pK_a around 2.4) were neutral or partially anionic under pH 2 mobile phase. In addition, the penicillin structure carried many lone pair electrons coming from oxygen, nitrogen and hydroxyl groups which would likely act as nucleophiles causing electrostatic repulsion with the negatively charged stationary phase. As a result, the retention of penicillin compounds on the negatively charged poly(SMA–DVB–VBSA) was inadequate, causing poor separation reproducibility.

3.1.2. Cationic VBTA as charged monomer

VBTA was chosen as the cationic charged monomer in the subsequent experiments. Results indicated that poly(SMA–DVB–VBTA) column exhibited relatively good separation for the tested analytes (resolution $(R) > 2$), but mobile phases of different pHs had to be used for Amp/Amo and the rest of the penicillin (pH 3 and pH 2, respectively) (Fig. 1(b)) as described in Section 3.2.2. As mentioned in Section 3.1.1, penicillin antibiotics have many lone pair electrons in the structure which behaved like nucleophiles, while poly(SMA–DVB–VBTA) carried positive charges and acted like electrophiles. As a result, an apparent interaction between the electrophile poly(SMA–DVB–VBTA) and the analytes caused strong retention on the stationary phase therefore, good baseline separation for penicillin was obtained.

On the other hand, morphologies of the poly(SMA–DVB) monoliths carrying different charged monomers were evaluated by SEM to clarify if the significant variation in the retention ability and analyte elution order observed even if all other polymerization conditions were the same and all the test penicillin compounds were neutral (except for Amp and Amo) (Fig. 1(a) and (b)) were influenced by the charged monomers or to the morphology changes brought by these charged monomers.

SEM micrographs showed very similar morphology between poly(SMA–DVB–VBSA) and poly(SMA–DVB–VBTA) (Fig. S1), which implied that the poly(SMA–DVB) polymer granules and their interconnected pores were not altered by the type of charged monomers. This result suggested that the chromatographic behaviors of penicillin analytes shown in Fig. 1(a) and (b) were influenced solely by the polarity of the charged monomers used. Consequently, this positively charged poly(SMA–DVB–VBTA) monolith, which provided a better and reproducible separation (Table 1), was employed as the optimal separation column for penicillin analyses.

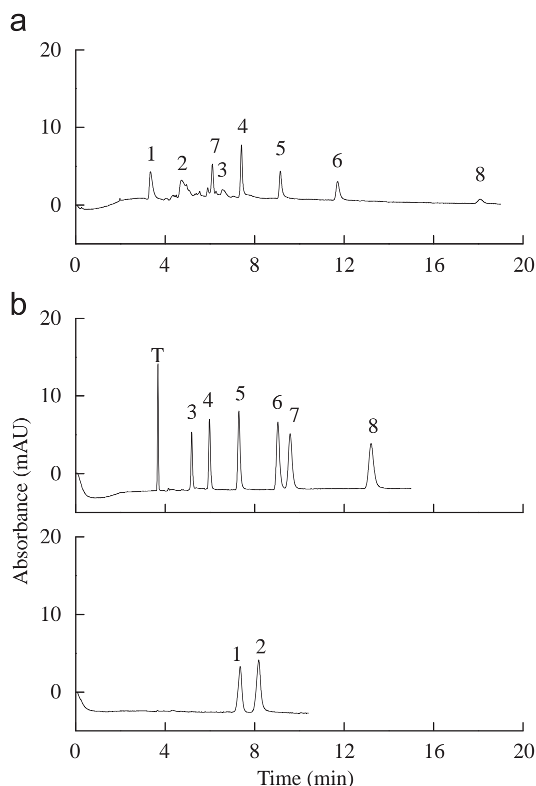

Fig. 1. Electrochromatograms of 8 penicillin separated in (a) poly(SMA–DVB–VBSA) VBSA charged monomer and (b) poly(SMA–DVB–VBTA) monolithic columns. VBTA charged monomer, Separation conditions: mobile phase (phosphate buffer/ acetonitrile, v/v), (a) 65:35, pH 2; (b) 60:40, pH 2; 50:50, pH 3. Separation voltage, (a) +20 kV, (b) −20 kV; sample matrix, mobile phase: standards = 1:3; sample injection, (a) +10 kV, (b) −10 kV for 3 s was applied to a 30 cm capillary tube (20 cm active length filled with monolithic stationary phase). Wavelength: 214 nm. 250 μg/ml each penicillin; (T) thiourea (1) amoxicillin, (2) ampicillin, (3) penicillin G, (4) penicillin V, (5) oxacillin, (6) cloxacillin, (7) nafcillin, (8) dicloxacillin.

3.2. Separation mechanism of penicillin on poly(SMA–DVB–VBTA)

Various interactions such as π–π interaction [23], size exclusion [24,25], hydrophilic interaction [26,27], hydrophobic interaction [28,29] and ionic interaction [30,31] dominate the retention behaviors of the analytes on the stationary phase in order to achieve good baseline separation in chromatography. In order to clarify the retention mechanism of the test analytes on the poly(SMA–DVB–VBTA) column, different mobile phase composition (solution pH, electrolyte concentration and organic solvent percentage) were examined, and results are discussed in the succeeding sections.

Table 1
Separation performance of penicillin standards in CEC-UV method with poly (SMA–DVB–VBTA) stationary phase[a].

Penicillin compounds	Column repeatability [b] Run-to-run (n=3)		Column reproducibility [c] Day-to-day (n=3)	
	Retention time (min) (RSD%)	Peak area (RSD%)	Retention time (min) (RSD%)	Peak area (RSD%)
Amoxicillin	5.96 (0.67)	3.88	6.04 (1.75)	3.02
Ampicillin	6.55 (0.64)	1.94	6.68 (2.59)	2.04
Penicillin G	5.13 (0.42)	5.92	5.14 (0.29)	3.68
Penicillin V	5.89 (0.02)	1.45	5.90 (0.23)	3.94
Oxacillin	7.11 (0.09)	2.46	7.18 (1.17)	3.57
Cloxacillin	8.79 (0.17)	3.49	8.84 (0.48)	4.81
Nafcillin	9.32 (0.11)	5.56	9.37 (0.54)	2.21
Dicloxacillin	12.9 (0.42)	3.04	13.0 (1.24)	2.69

[a] Separation conditions: mobile phase (40/60, v/v) acetonitrile and 5 mM phosphate buffer (pH 2 and pH 3). Standards were electrokinetically injected into the capillary at a voltage of −10 kV for 180 s.

[b] Values of column repeatability were means of three intra-day replicates on the same column. The value in parenthesis indicates the RSD of migration time in percentage.

[c] Data of column reproducibility were means nine of inter-day replicates on the same column.

3.2.1. Effect of organic solvent percentage in the mobile phase

Several solutions of pH 2, composed of ACN and phosphate solution in different volume ratios (35:65 to 50:50), were used as mobile phases. Baseline separations were obtained in three out of four volume fractions of ACN tested (35%, 40% and 45%), while the mobile phase composed of 50% ACN could not separate well the Clo and Naf analytes (R < 0.9). In addition, the plots of log k′ values of the analytes versus ACN content of the mobile phase did not approach exact linearity (R^2=0.9981–0.9985 for test analytes), which implied partial deviation of the reversed-phase mechanism of the penicillin retention on the poly(SMA–DVB–VBTA) column in the tested mobile phase (pH 2).

3.2.2. Effect of mobile phase pH

Separation behaviors of penicillins were compared in mobile phases with pH 2–7. When pH 2 mobile phase was used, penicillin separation, except for Amp and Amo, attained better resolutions, more symmetric signals, higher peak intensities and shorter retention time as shown in Fig. 2. Although most penicillin analytes were predominantly neutral, both Amp and Amo were converted into cations under pH 2 (the pK_{a_1} is around 2.44 and pK_{a_2} is about 6.81–7.14 for Amp and Amo). Thus, they were stocked at the inlet electrode (i.e. negative polarity) at an injection or separation voltage of −10 or −20 kV, while the other neutral penicillin analytes were then introduced by EOF. As a result, no Amp and Amo were found in the electrochromatogram. When the mobile phase pH was higher than 2, all analytes except for Amp and Amo were converted into anions and their peak shapes and signal intensities worsen, which was likely due to the very strong electrostatic attraction that occurred between penicillin anions and positively charged stationary phase, as mobile phase pH was raised further. In contrast to the six penicillin, Amp and Amo analytes which became neutral or anion at pH 3–7 mobile phases, were then introduced into the column successfully, and baseline separations as well as good symmetric signals were observed in all mobile phases except for pH 4–5 (Fig. 2). However, difficulty in the sample introduction for Amp/Amo was encountered with pH 2 mobile phase, whereas peak tailing and reduced signal intensities were observed with higher pH. It was therefore decided that pH 3 mobile phase would be used for Amp/Amo and pH 2 for the rest of the analytes as depicted in Fig. 2 because best separation and sensitivity were achieved. Compared to the rest of the mobile phase pHs employed, pH 3 allowed the separation of Amp and PV, though resolution (R~0) was still inadequate. To solve this, variations in column length and ACN level in the pH 3 mobile phase were tried in the subsequent CEC-MS experiments (Section 3.4).

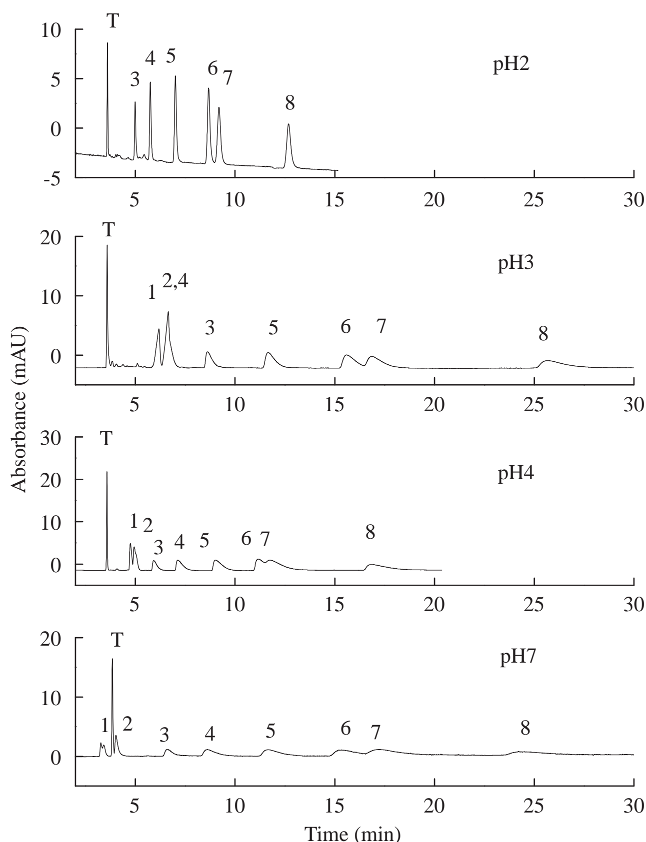

Fig. 2. Electrochromatograms of 8 penicillin separated on positively charged poly(SMA–DVB–VBTA) columns in different mobile phase pHs. Separation conditions: mobile phase: phosphate buffer/ acetonitrile=60:40 (pH 2–3) or 50:50 (pH 4–7); sample matrix, mobile phase: standards=1:4; penicillin standard, 200 μg/ml each. All other conditions are the same as in Fig. 1.

3.2.3. Effect of electrolyte concentration

The profiles in Fig. 2 showed that peak tailing became worse when the mobile phase of pH higher than 2 was used because in these conditions, the analytes were converted into anions; therefore, an ionic attraction between the anionic analytes and the positively charged stationary phase possibly predominated their retention behaviors. To verify this assumption, the electrolyte concentration in the pH 3 mobile phase was varied from 2.5 to 10 mM. In order to emphasize ionic attraction, a mobile phase of pH 3 was used to convert most penicillin antibiotics into anion forms; and both Amp and Amo, that were neutral at pH 3, were not tested. Results indicated that the EOF velocity was almost the same in each electrolyte concentration; but with an increase in the electrolyte concentration, the retention time of penicillin compounds was reduced (Fig. S2). Obviously, with higher electrolyte concentration, more electrolyte anions such as PO_4^{3-} competed with penicillin anions to adsorb on the positively charged surface of the stationary phase, and thus causing a reduced interaction between the analytes and the stationary phase; as a result, these analytes had weaker retention at higher electrolyte concentration. Considering the results in the variation of the pH value and electrolyte concentration in the mobile phase (Fig. 2 and Fig. S2), it could be concluded that an ion exchange interaction also contributed to the penicillin retention on this positively charged monolith.

3.3. Optimization of on-line concentration step for penicillin analysis

3.3.1. Step gradient elution

Fig. S3 shows the effect of on-line concentration step (step gradient elution) on penicillin intensities, in which the ACN content in the sample matrix was varied from 9% to 27% (pH 2, Fig. S3a–c) or 0% to 5% (pH 3, Fig. S3d–f). The penicillin standards (1 μg/mL each) were first introduced electrokinetically at −10 kV for 180 s and the elution step was then carried out with a mobile phase composed of 40% (pH 2, Fig. S3a–c) or 50% (pH 3, Fig. S3d–f) acetonitrile(v/v) at −20 kV separation voltage. Results indicated that the larger difference in the eluent strength between the mobile phase and the injection solvent created higher signal intensities for all analytes (for example, SER_{height}=(dilution factor × (peak height obtained with on-line concentration step/ peak height obtained with normal injection)) was around 52–61, 52–57 and 23–52 at the sample matrix of 9%, 18% and 27% ACN, respectively; while SER_{height} of Amo and Amp was around 69–85 and 37–68 at 0% and 5% ACN level, respectively). Obviously,

decreasing the ACN amount in the sample solution (0% (pH3 mobile phase for Amp and Amo) or 18% (pH 2 mobile phase for the rest of the analytes)) improved the solute retention on the poly(SMA–DVB–VBTA) material, thus enabled the penicillin to accumulate at the column entrance in higher concentrations which led to larger signal enhancement.

3.3.2. Combining step gradient elution and anion-selective injection

Since all penicillin compounds existed as anions at pH higher than 2.5 or 7, thus the injected amount was possibly further enhanced by anion selective injection through changing the sample matrix pH. Fig. S4 showed the electrochromatograms of penicillin compounds by combining on-line concentration modes of step gradient elution and ASEI, in which analyte standards were prepared in different pH buffers (pH 2–7). All analytes were successfully injected and stacked in all pHs, except for Amo and Amp, which were not found at pH 2 (the reason is mentioned in Section 3.2.2). Comparison of the profiles in Fig. S4 indicated that higher stacking effect was achieved at pH 7 sample matrix for most analytes, for example, SER_{height} was increased from 52–57 (pH 2, i.e. only step gradient elution) to 102–130 (pH 7), while Amp and Amo had higher intensities at pH 7 sample matrix, for example, SER_{height} was increased from 69–85 (pH 3, i.e. only step gradient elution) to 130–244 (pH 7). The highest SER_{height} was attained when the sample matrix was increased to pH 7, therefore this condition was chosen as the optimized sample matrix in this on-line concentration mode.

3.4. On-line CEC coupled with MS detection for penicillin analysis

An attempt was made to develop a CEC coupled to mass spectrometric detection with poly(SMA–DVB–VBTA) monolith as stationary phase for penicillin separation.

3.4.1. Penicillin analysis by normal injection and on-line concentration CEC-MS

The CEC-MS electrochromatograms of penicillin standards (1 ppm each) using the previously optimized conditions obtained from CEC-UV system are shown in Fig. 3(a) and Fig. S5(a), wherein phosphate electrolyte in the CEC-UV mobile phase was replaced with ammonium formate (5 mM). The baseline separation for the six penicillin and both Amp/Amo standards was still acquired within 11 and 8 min at pH 2 mobile phase containing 40% ACN (Fig. 3(a)) and pH 3 mobile phase containing 50% ACN (Fig. S5(a)), respectively; even if a longer monolithic column (30-cm column length filled with stationary phase) was used in the CEC-MS. As mentioned earlier (Section 3.2.2, CEC-UV), at pH 3 mobile phase, all eight analytes including Amo and Amp were successfully detected but with poorer resolution and signal intensities (Fig. 2). In contrast to CEC-UV, when a longer monolithic capillary (30-cm total length filled with poly(SMA–DVB–VBTA) and a decrease of the ACN level in the pH 3 mobile phase (40% ACN)) was used in this CEC-MS, all eight penicillin antibiotics were baseline separated and with highly symmetric signals, even though the whole separation took almost 58 min (Fig.3(b)). This implied that better separation was achieved in pH 3 mobile phase with longer column which allowed sufficient ionic interaction between the penicillin anions and the positively charged poly(SMA–DVB–VBTA) stationary phase. Consequently, these two mobile phases (pH2 and pH3, 40% ACN) were simultaneously employed in the following on-line concentration CEC-MS.

Also, the optimized on-line concentration step in CEC-UV (section 3.3) was used to enhance the penicillin sensitivity in the CEC-MS system. Fig. 3(c and d) shows the electrochromatogram of the on-line concentration CEC-MS, in which the penicillin standards (5 μg/L) were first prepared in pH 7 sample matrix and then electrokinetically injected for 105 s (Fig. 3(c), pH 2) or 90 s (Fig. 3(d), pH 3) at a voltage of −10 kV. These injection time were the maximum allowable length in the CEC-MS system according to our testing. Comparison of the CEC-MS profiles in Fig. 3 obviously indicated that the on-line concentration steps of step

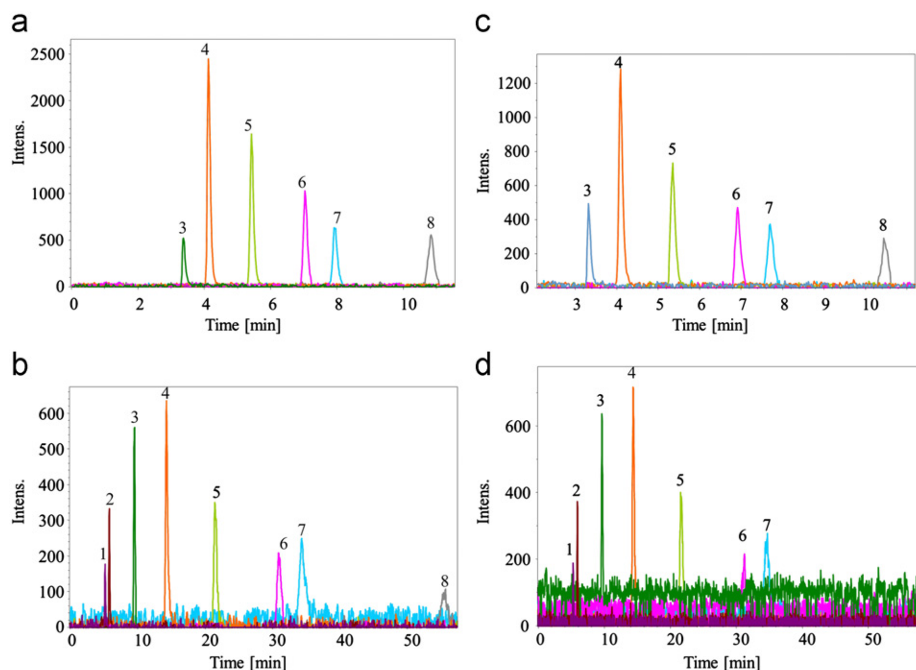

Fig. 3. Electrochromatograms of 8 penicillin separated using poly(SMA–DVB–VBTA) monolithic column in CEC-MS by (a and b) normal (−10 kV for 3 s), (c) on-line concentration (−10 kV for 105 s) and (d) on-line concentration (−10 kV for 90 s) injection modes. Mobile phase, 5 mM ammonium formate/ACN (60:40, v/v): (a and c) pH 2, and (b and d) pH 3 Penicillin standards, 1 μg/mL each, sample matrix, mobile phase: standards = 1:3 (a, b). −20 kV was applied to a capillary tube of 30 cm filled with monolithic stationary phase. Penicillin standards, 10 μg/L per penicillin; sample matrix: pH 7, 20% ACN buffer solution: standards = 1:9 (c), pH 7, 0% ACN buffer solution: standards = 1:1 (d). Sheath liquid: 220 mL/h, IPA/water (60/40, v/v); 4 L/min dry gas flow rate; 180 °C dry gas temperature.

gradient combined with ASEI developed in CEC-UV system, also improved the mass sensitivities of all tested analytes without loss in separation velocity and resolution.

3.4.2. Comparison of the optimal on-line concentration CEC-MS method with previous literatures for penicillin analyses

The qualitative and quantitative performances of the proposed on-line concentration conditions are shown in Table 2 and Table S1. The LODs for penicillin were in the range of 3.7–31.8 μg/L for normal injection mode (−10 kV for 3 s) and of 0.05–0.2 μg/L for on-line concentration mode (−10 kV for 105 s or 90 s) (S/N=3). Previous studies on the analyses of penicillin compounds reported that the best detection limits were around 1 to 10 μg/L in CE-MS system [32], 0.1 to 10 μg/L in LC-MS system[13] and 2.5 to 5 μg/L in UP LC-MS system [7]. Comparison of these methods for penicillin analyses revealed that the sensitivity of on-line concentration CEC-MS is the best. In addition, separation ability is better than in LC system because baseline separation of eight penicillins was achieved. In this study, two mobile phase conditions were chosen for penicillin residues detection using CEC-MS methods, one was pH 3 mobile phase, which separated Amp and Amo within 10 min, and the other was pH 2 mobile phase, which separated the rest of the penicillin compounds within 11 min. Although the proposed CEC-MS methods needed two separate runs, this still provided the best LOD compared to the other techniques; in addition to the advantages of lower solvent consumption and/or lower instrumental and maintenance costs. Therefore, this first CEC-MS report for the trace penicillin analyses has relatively comparable ability with other CE and LC methods with MS as detector.

3.5. Real sample analyses

Finally, the proposed on-line concentration CEC methods were used to analyze penicillin in milk samples. Results indicated that no penicillin residues were found in the samples. In order to examine the separation and detection ability of the proposed CEC methods, the milk sample (penicillin-free) spiked with eight penicillin compounds (4 μg/L), except for Amp and Amo (10 μg/L), (with spiked amounts less than or equal to the maximum residue limits (MRLs) set for penicillin in foodstuffs (4–300 μg/L) in Taiwan and in the European Union) was also analyzed by the optimal CEC conditions. Consequently, these trace-level penicillin compounds in the milk sample were successfully determined by the CEC-MS method (Fig. 4). The recoveries of these spiked analytes were in the range of 57–103%. Some of analyte recoveries in this CEC-MS method were low likely due to matrix effect and ion suppression. Sample extraction condition for milk sample could be optimized in future experiments. With our results compared to those published in literatures (Table S2), our proposed method is still applicable to milk sample analysis at present. The above results demonstrated that the proposed on-line concentration CEC-MS method has high potential to analyze trace penicillin residues in milk samples after a simple sample pretreatment.

4. Conclusion

In this paper, a highly sensitive CEC method using poly(SMA–DVB) monolithic column was developed to analyze trace penicillin related drugs present in milk samples. The type of charged monomer on poly(SMA–DVB)-based column were found to have strong

Table 2
Limit of detection, repeatability of retention time, calibration curve and coefficients of determination of penicillin standards in the optimal on-line concentration CEC-MS method[a].

Penicillin compounds	LOD (μg/L) (S/N=3)	Retention time (min) (%RSD)	Peak area (%RSD)	Calibration curves	Coefficient of determination for calibration curves (R^2)
(1) Amoxicillin	0.10	5.3 (1.89)	6.60	$Y=725.80x-6754.47$	0.9967
(2) Ampicillin	0.05	6.0 (0.97)	3.94	$Y=1309.79x-11042.6$	0.9951
(3) Penicillin G	0.11	3.3 (1.77)	15.8	$Y=897.00x-4150.33$	0.9997
(4) Penicillin V	0.08	4.0 (1.43)	4.09	$Y=1989.48x+1402.47$	0.9994
(5) Oxacillin	0.09	5.3 (0.00)	5.95	$Y=1891.22x-6577.62$	0.9999
(6) Cloxacillin	0.05	6.9 (0.84)	5.56	$Y=1571.58x-10736.29$	0.9981
(7) Nafcillin	0.05	7.7 (0.75)	14.1	$Y=1165.66x-6772.51$	0.9995
(8) Dicloxacillin	0.22	10.4 (0.55)	4.17	$Y=954.58x-9845.63$	0.9966

Calibration curves were constructed from three replicate measurements at each concentration in the range of 3–600 μg/L (3, 30, 300, 600 μg/L).

[a] Separation conditions: mobile phase (40/60, v/v) acetonitrile and 5 mM ammonium formate (pH 2 and pH 3).Standards were electrokinetically injected into the capillary at a voltage of −10 kV for 90 s (pH3) or 105 s (pH2). 5 ppb of penicillin standards was selected to calculate the RSDs of peak area. This is based on the maximum residue limit (MRL) of about 4–300 μg/L for these drugs in animal organ and milk set by the European Union (EU), including Taiwan. The data for LOD and calibration curves did not include sample pretreatment. Values are means of three intra-day replicates on the same column. Values in parenthesis indicates % RSDs of retention time.

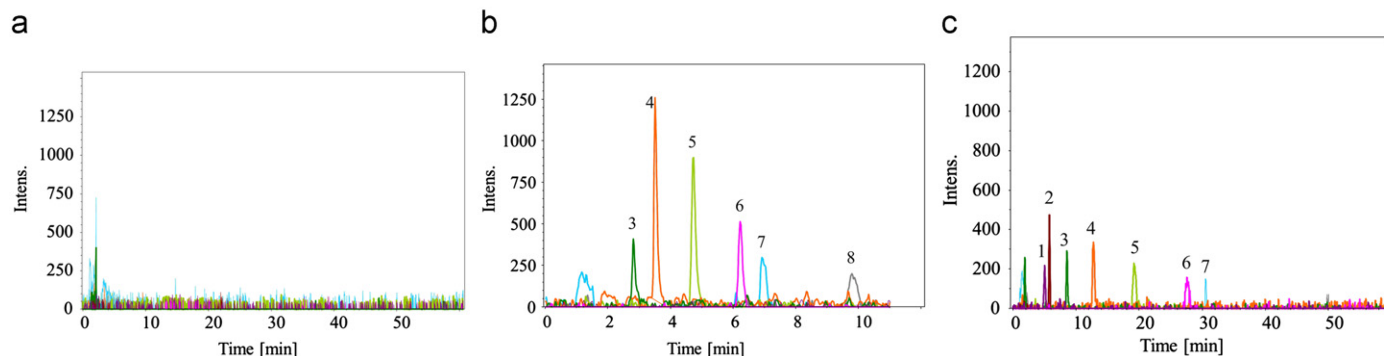

Fig. 4. Electrochromatograms of milk sample determined by on-line concentration CEC methods. Milk samples without (a) and with (b and c) spiked penicillin compounds (10 μg/L for Amp and Amo, and 4 μg/L for the rest analytes), were prepared using the procedure described in the experimental section. Sample was introduced at −10 kV injection for 90 s (a and c) and 105 s (b). All other conditions are the same as in Fig. 3. (a) mobile phase: pH3,40% ACN, (b) mobile phase:pH2,40% ACN and (c) mobile phase: pH3,40% ACN.

influence on penicillin separation. When the anionic VBSA was used as charged monomer, electrostatic repulsion with the nucleophile penicillin resulted in inefficient separation, but when the cationic VBTA charged monomer was used instead of the anionic VBSA, the positively charged stationary phase provided a weak ion-exchange interaction that led to baseline separation. Furthermore, an on-line sample concentration step of step-gradient elution combined with ASEI effectively enhanced penicillin sensitivity. This proposed CEC-MS method, especially for on-line concentration mode, which provided better detection ability and better separation for penicillin analysis compared to previous LC-MS or CE-MS methods, really possess high potential to analyze trace penicillin residues in food samples.

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Appendix A. Supporting materials

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2012.08.026.

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