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Development and Validation of Capillary Electrophoresis Method for Simultaneous Determination of Six Pharmaceuticals in Different Food Samples Combining On-line and Off-line Sample Enrichment Techniques

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Abstract A rapid and highly sensitive capillary electrophoresis method was developed combining on-line and off-line sample enrichment techniques that are capable to determine six widely used pharmaceuticals, viz. amoxicillin, carbamazepine, diclofenac, indomethacin, ibuprofen, and paracetamol from various food samples. A new sample preconcentration technique, i.e., in situ aggregated microextraction, was utilized to extract the analytes from various food samples. The process was carried out by the formation of aggregate phase in situ in the aqueous sample by ion association between oppositely charged surfactants, viz. Tiron® and cetyltrimethylammonium bromide. The separation and quantification of analytes were carried out in micellar electrokinetic chromatography mode at −20 kV. In short, the uncoated fused silica capillaries were filled with 80 mM phosphate buffer solution (pH 6.0) to act as leading electrolyte, and the samples were dissolved in 100 mM borate buffer and injected by electrokinetic mode applying −10 kV for 600 s. The method showed good linearity in between 0.06 and 50 μg/L for all six pharmaceuticals. Good repeatability and recoveries were obtained for all analytes in the range of 94.45–106.32% using optimized experimental conditions. The limit of detection ranged from 0.02 to 0.08 μg/L, and the limit of quantification ranged from 0.06 to 0.25 μg/L for six pharmaceuticals. The sensitivity of the developed micellar electrokinetic chromatography method was increased about 2500-folds compared to conventional capillary zone electrophoresis method. Finally, the developed analytical procedure was applied successfully for the detection of selected pharmaceuticals in fruits, urine, and river water samples.

Keywords Sample enrichment · River water · Pharmaceuticals · Capillary electrophoresis · Method development

Introduction

Water is indispensable to sustain the life on earth. Therefore, safe, adequate, and potable water must be available to everyone. It is the prime responsibility of water suppliers to provide good quality of drinking water all over the world (Murphy et al. 2012). The contamination of drinking water sources with pharmaceuticals and personal care products (PPCPs), endocrine disrupter compounds (EDCs), and other substances are major threat to human health and environment (Mons et al. 2013; Chevolleau et al. 2016). Among all, pharmaceutical ingredients have been found more often in surface waters of developed and developing nations. Further, the recent reports revealed that the traces of prescription drugs are also found in several fruits and vegetables (Cerqueira et al. 2014). Human and veterinary applications including inappropriate disposal, industrial use, and metabolic excretion are the major causes of pharmaceuticals present in surface waters, which end up in the

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crops and subsequently transformed to fruits and vegetables (Schulzki et al. 2017). Due to the serious human health issues even at trace level exposure, the analysis of pharmaceutical residues in several food samples has gained the researchers attention to carry out comprehensive study in the field of environmental sciences. Therefore, it felt necessary to develop novel sample pretreatment procedures and sensitive analytical methods to determine them from various food sources (Bhaskar Reddy et al. 2016).

Direct analysis of pharmaceuticals is quite tricky either using chromatography or electrophoresis, due to their lower concentrations in various sample matrices and interferences of co-extractives. Therefore, preconcentration of analytes through the extraction process is necessary prior to their analysis (Bhaskar Reddy et al. 2015). Solid-phase extraction (SPE) and liquid-liquid extraction (LLE) are familiar approaches for the preconcentration of several organic compounds from various environmental samples (Reddy Ambavaram et al. 2013; Ashri and Rehim 2011). Nevertheless, LLE demands large volume of potentially toxic solvents and the process is time-consuming, but SPE is considerably a new approach and offers many advantages for the extraction of wide range of environmental and food samples. However, SPE is still tedious, time consuming, and relatively expensive. All these disadvantages created the necessity to substitute these conventional techniques with more favorable and environmental friendly techniques which can save time, labor, and solvent consumption (Nerin et al. 2009). Hence, different kinds of sample enrichment techniques have been evolved in the past few years including cold-induced aggregation microextraction (CIAME) (Jamali et al. 2014), single drop microextraction (SDME) (Sharifi et al. 2016), solid-phase microextraction (SPME) (Pakade and Tewary 2010), hollow fiber-liquid phase microextraction (HF-LPME) (Merkle et al. 2015), cloud point extraction (CPE) (Pallabi and Sen 2014), stir bar sorptive extraction (SBSE) (Camino et al. 2014), and dispersive liquid-liquid microextraction (DLLME) (Saraji and Boroujeni 2014). High speed, low solvent consumption, and cost-effectiveness are the paramount advantages of these new techniques. However, the formation of extractant phase in situ in the sample solution and collection of analytes by dissolving it in suitable organic solvent is a new, simple, and alternative technique to the above procedures, which is called as in situ suspended aggregate microextraction (iSAME) (Tatiana 2016). It is a simple and feasible technique as the formation of extractant phase is faster, and the extraction can also be completed rapidly without the need of disperser solvents (Juan et al. 2015). In this approach, the extraction can be achieved by self-assembly of oppositely charged counter ions through ion association and the formation of a supramolecular aggregate phase. Later, the aggregate phase can be collected on the filter surface as thin film applying vacuum filtration. Next, the aggregate phase can release the analytes into organic solvent due to its instability in the organic solvents. The extraction recoveries are maximum with this procedure, as the extraction is carried out by mass transfer and analyte entrapment. Moreover, higher sample volumes can also be processed as there is no centrifugation needed during sample preconcentration (Endo et al. 1998).

Although LC is a preferable analytical technique for the analysis of organic compounds, it is often difficult to retain and separate the polar analytes on reversed-phase columns. Therefore, CE is a powerful and complimentary technique for the separation of such analytes in short run times. Further, CE is a robust analytical technique that separates the analytes with shorter analysis time, higher resolving power, and lower operational cost than advanced chromatography techniques. The considerable use of CE for pharmaceutical analysis has gained importance in recent years (Siddiqui et al. 2016). Although it has several advantages including simplicity, versatility, high efficiency, and low consumption of reagents, the lower sensitivity and small sample size are its significant limitations. Therefore, many on-line sample preconcentration strategies have been introduced to improve its sensitivity (Wojciech et al. 2016; Lee et al. 2016; Kevin et al. 2006). The key factor in all the on-line preconcentration technologies to attain higher sensitivity is the sample volume injection. The sensitivity improvements with CE-hydrodynamic injections are restricted by the volume of capillary as it is inaccessible to inject more than a capillary volume. Therefore, better sensitivity can be achieved with electrokinetic injection as it is not limited by the volume of capillary. Several studies have already confirmed the improvements in detection limits with electrokinetic injection compared to hydrodynamic injection (Amin et al. 2012). One such technique is micellar electrokinetic capillary (MEKC) electrophoresis, which combines the best features of chromatography and electrophoresis. In MEKC technique, the surfactants with concentration more than their critical micellar concentrations can be added to the BGE to form micelles, which can receive electrophoretic migration similar to other charged particles. The separation arises as a result of differential partitioning between mobile aqueous phase and micellar pseudostationary phases. In environmental analysis, MEKC procedures provide better separations for the analytes of interest. However, various types of preconcentration steps are often necessary to achieve lower detection limits for the targeted analytes in real samples (e.g., natural water). Normally, preconcentration is possible in off-line and on-line modes, which can be performed outside and inside the capillaries, respectively. However, in view of any analytical chemist, it is more interesting to develop on-line preconcentration techniques rather than off-line preconcentration techniques. In few cases, off-line and on-line preconcentration techniques have to be combined to achieve sufficient sensitivity.

Based on the above concept, we have developed a new CE method to separate six widely used pharmaceuticals

combining MEKC-sweeping as on-line preconcentration technique and iSAME as off-line preconcentration technique (structures and pKa values of selected pharmaceuticals are stated in Fig. S1). The conditions that influenced the separation and extraction efficiencies were carefully examined. The efficacy of this developed analytical procedure was demonstrated for the detection of pharmaceuticals in different real samples including natural waters, fruit juices, and human urine samples with best analytical aspects regarding linearity, accuracy, simplicity, precision, and analysis time.

Experimental

Chemicals and Reagents

All chemicals used were of analytical grade. Selected pharmaceutical standards amoxicillin (AMX), diclofenac (DCL), indomethacin (IDC), ibuprofen (IBP), carbamazepine (CBZ), and paracetamol (PCM) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Analytical grade 4,5-dihydroxy-1,3-benzenedisulfonic acid disodium salt (Tiron®), sulfanilic acid, cetyltrimethylammonium bromide (CTAB), and sulfosalicylic acid (SSA) were procured from Sigma-Aldrich (St. Louis, MO, USA). Next, the HPLC-grade methanol, ethanol, water, and acetonitrile were received from S.D. Fine Chemicals (Mumbai, India). Further, sodium dihydrogen phosphate (NaH_2PO_4), disodium hydrogen phosphate (Na_2HPO_4), methanol (MeOH), hydrochloric acid (HCl), sodium dodecyl sulfate (SDS), boric acid (H_3BO_3), sodium hydroxide (NaOH), and disodium tetraborate ($Na_2B_4O_7$) were purchased from Merck (Darmstadt, Germany). The buffers and stock standard solutions were prepared using Milli-Q water (Millipore, Bedford, MA, USA). A set of hydrophilic filter papers with varying technical specifications were obtained from Whatman and Millipore.

Preparation of Stock Standard and Buffer Solutions

All stock solutions of CTAB, SSA, sulfanilic acid, and Tiron® were prepared at 200 mM concentration with Milli-Q water. Thereafter, the working solutions of all these surfactants were prepared at variable concentrations ranging from 1.0 to 50 mM by diluting the stock solutions in Milli-Q water.

In total, 1.0 mg/mL of analytes stock solutions was prepared dissolving appropriate amount of analytes in MeOH. Then the stock solutions were suitably diluted with 100 mM borate buffer to attain analytical concentrations. Sodium phosphate buffer with 80 mM concentration at pH 6.0 was used as BGE, and 80 mM sodium phosphate with 20 mM SDS was used as the sweeping solution. The pH strength of buffer solutions was adjusted using 0.1 M HCl and 0.1 M NaOH solutions. The sample diluents were 80 mM sodium phosphate and 100 mM boric acid, respectively, for CZE and MEKC experiments. All the samples and buffer solutions were stored at 4 °C until analysis.

CE Instrumentation

All the experiments were conducted on Agilent CE 3-D 7100 (Waldbronn, Germany) instrument supplied with a diode array detector. A Phoenix uncoated fused silica capillary with 50 μm i.d. and 50 cm effective length (total length 58 cm) was used for the analytes separation and detected at 214 nm. The temperature of the capillary was hold at 20 °C. The lift offset, which regulates the distance between capillary entrance and the tip of cylindrical electrode, was set to 4.0 mm. The new capillaries were preconditioned with MeOH, water, 1.0 M HCl, water, 1.0 M NaOH followed by water for 10 min each. During the regular analysis, the capillary was first rinsed with 0.1 M NaOH for 5.0 min followed by water and running buffer for 5.0 min each. Capillary precondition was continued between each run for 5.0 min with BGE at a pressure of 50 psi.

The experimental conditions were maintained as given below. Initially, the capillary was filled with 80 mM sodium phosphate buffer of pH 6.0. Next, the samples prepared in 100 mM borate buffer of pH 8.0 were injected electrokinetically at −10 kV for 600 s. A 20-mM SDS was added to 80 mM sodium phosphate buffer of pH 6.0 and used as the mobilization ion, and a voltage of −20 kV was applied for the analytes separation. The applied negative voltage permitted the negatively charged micelles to sweep and stack the analytes further into narrow bands. The analytes were separated in MEKC mode.

Pretreatment of Real Samples

Prior to collecting water samples, the glassware was thoroughly cleaned with a solution of 40% HNO_3 and dried at 90 °C. The raw water samples were collected into three 500-mL bottles from the inflow of local water treatment plant (SAJ, Sg. Skudai, Malaysia) during May 2016. All the collected samples were filtered using 0.45 μm nylon membranes and spiked with selected pharmaceuticals to obtain the final concentration of 1.0 μg/L.

Three blank urine samples were collected from a healthy volunteer and added 2.0% *v*/*v* of concentrated HCl as a preservative. Direct injection of urine samples into capillary may cause band broadening, owing to the high ionic strength of the sample matrix. Furthermore, the adsorption of proteins on the capillary wall may affect/decrease the reproducibility. Therefore, the urine is heated for 15–20 min prior to conduct experiments and spiked with analytes to get a final concentration of 10 μg/L (relatively higher concentrations of pharmaceuticals were spiked to urine samples assuming that

therapeutic concentrations of selected pharmaceuticals are significantly higher in human urine).

Similarly, three fresh fruit samples of each, viz. tomato, orange, and apple, were collected from the local fruit market. The fruits were partitioned into edible and inedible portions after cleaning and sectioning. The edible fractions were made as slurry and blended at room temperature followed by its storage at −4 °C until use. In total, 50 mL of each juice was vortexed for 6.0 min followed by centrifugation at 6000 rpm for 15 min. A 20-mL aliquot of each supernatant was considered for iSAME experiments. After the filtration of samples with 0.45 μm PVDF filter (Millipore, Bedford, MA, USA), they were spiked to attain a concentration of 1.0 μg/L of each analyte and used for the preconcentration as shown above.

Sample Enrichment by iSAME Procedures

A brief experimental procedure for iSAME preconcentration step is depicted in Fig. 1. The measured volumes of 1.0 mL of 1.5×10^{-2} M Tiron® and 0.5 mL of 2.0×10^{-3} M CTAB solutions were added in a sequence to 20 mL aqueous standard solution or real water samples already fortified with 1.0% (*w*/*v*) NaCl to adjust the ionic strength. The mixture was agitated on a magnetic stirrer for 15 min to facilitate the dispersion. The cloudy solution gradually turned out to form an aggregate phase, which was filtered through a Millipore AP-40 glass fiber filter paper with suction filtration at ~ 400 mbar, and then the aggregate phase was collected as a thin film on the filter surface (thickness ≤ 0.1 mm). The vacuum was extended for a few minutes to eliminate any water content remained. Next, 2.0 mL of methanol was passed through the filter to dissolve the thin film and to extract the analytes, and the vacuum was further steadily increased to collect the solvent into a dry flask. Negligible retention of analytes on the filter was noticed, considering their high solubility in methanol. The extracts were filtered through 0.22 μm nylon mesh, and aliquots were then injected for CE-DAD analysis. The extracts were evaporated under a gentle nitrogen stream, and the residues were reconstituted in 0.2 mL of methanol to enhance the sensitivity. This step was possible since no losses of pharmaceuticals were found by the evaporation.

Method Validation

A series of experiments have been conducted to validate the developed analytical method regarding its linearity, limits of detection (LOD), and limit of quantification (LOQ), precision and enhancement factors (EF). The linearity was evaluated by preparing and analyzing six point calibrations ranging from 0.06 to 50 μg/L. Calibration graphs were drawn, taking the analytesconcentration on X-axis and their peak areas on Y-axis. The least squares linear regression equation was used to determine their slope, intercept, and regression coefficient values. The detection limits were determined considering S/N ratio of 3.0 by electrokinetic injection at −10 kV injected about 600 s for the six analytes. Further, the precision of the method was calculated for two different concentrations using six determinations and expressed as relative standard deviation (%RSD). CZE (sample prepared in deionized water and injected by hydrodynamic mode at 0.5 psi, 10 s) was compared to the MEKC-sweeping method to determine the actual sensitivity enhancements. Finally, the matrix effects were

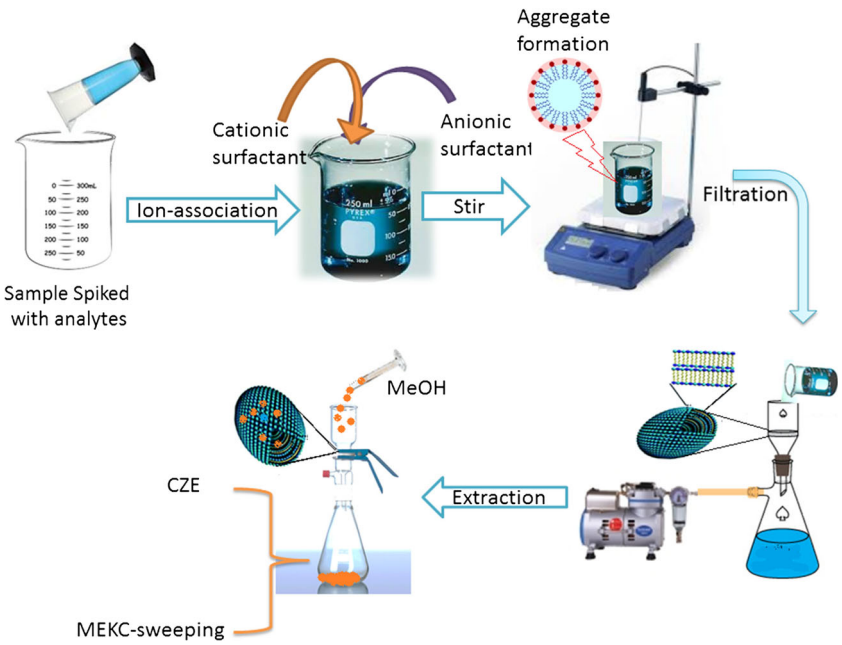

Fig. 1 A schematic representation of iSAME procedure for the preconcentration of selected analytes from various food samples

determined for all samples as some biological and food samples can suppress the sensitivity of analytical method due to its complexities.

Results and Discussion

Development of iSAME for Off-line Preconcentration

Based on the previous reports, CTAB and benzenesulfonic acid derivatives were selected as the respective cationic and anionic surfactants, in which long chain trimethylammonium surfactants have the ability to form aggregates through ion association with benzene sulfonic counter parts (Wang and Wang 2014; Khaledi et al. 2013). In order to select the suitable counter-anionic surfactant, different concentrations of sulfanilic acid, Tiron®, and sulfosalycilic acid (SSA) were inspected for their effectiveness to extract the selected analytes from 20 mL of aqueous standard solutions in combination with CTAB.

Based on average recovery data of all analytes (Tiron® > SSA > sulfanilic acid) and signal repeatability (Tiron® > sulfanilic acid > SSA), Tiron® was found to provide consistent recoveries with good repeatability than SSA and sulfanilic acid. Particularly, 1.0 mL of 1.5×10^{-2} M Tiron® and 0.5 mL of 2.0×10^{-3} M CTAB composition has provided very good extraction efficiencies and consistent recoveries for 20 mL of sample containing all six analytes (Fig. 2). Therefore, the same composition was chosen for further work. Both the selected surfactants, Tiron® and CTAB, have formed transparent and optically isotropic low viscous solutions in water at 25 °C. However, upon mixing their solutions, an optically isotropic turbid solution was formed, indicating the formation of aggregate phase. The ion association between sulfonate groups of Tiron® and the quartenary amine of CTAB caused the formation of this aggregate phase. This interaction can be understood from the fact that Tiron® solution is acidic due to the dissociation of its sodium sulfate groups (Kim et al. 2006). Therefore, the two remaining acid protons of Tiron® (−OH: pKa > 11.0) are already protonated and cannot associate with CTAB. These ion pairs can mobilize to form various bilayer-type assemblies as a function of the molar ratios and molecular structures of their monomer components, which are classified as catanionic aggregates (Mataa et al. 2005). In order to explicate the extraction mechanism, first the aggregate phase was formed in the blank aqueous solution, then the standard analytes solution was added to the aqueous sample and the mixture was equilibrated under stirring for 15 min. The range of average extraction recoveries for this experiment was found between 35.54 ± 2.0 and 55.61 ± 1.5%, which is distinctly lower

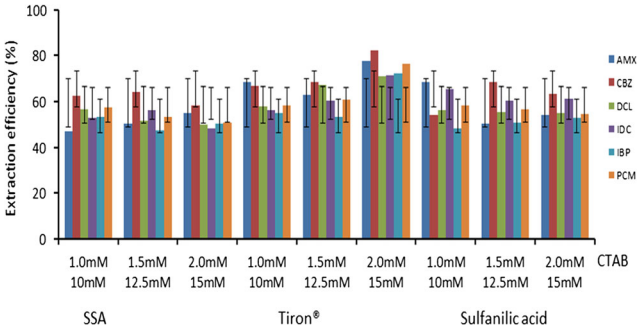

Fig. 2 Percentage recoveries of six analytes at 1.0 μg/L concentration with different concentrations of CTAB, SSA, sulfanilic acid, and Tiron®

than the recoveries found for in situ aggregate formation. Based on these evidences, it is suggested that in situ formation of the aggregate phase is primary extraction mechanism, which involves the entrapment of analytes inside the hydrophobic domain of the bilayer during the spontaneous self-assembly of the vesicles. A conceivable route of performance for this extraction process is illustrated in Fig. S2.

Optimization of Experimental Variables

The major extraction parameters have been optimized considering 1.0 μg/L each of analytes mixture for 20 mL of sample volume. Several parameters have been optimized including vacuum pressure over the range of 200–400 mbar, selection of dissolution solvent, and surfactants concentration. The optimal conditions were chosen considering the extraction efficiencies (EE) of analytes, which is calculated from the percentage recoveries of analytes to that of aqueous standard solution. However, the filter selection was not considered as the previous studies have already reported that the paper thickness does not affect the extraction efficiency (Juan et al. 2015). Based on this, glass fiber filter (Millipore AP 40, thickness 475 μm) was selected as the solid support for the extraction.

Effect of Filtration Rate

The influence of filtration on the analyte recoveries was examined by changing the applied vacuum pressure. The formed multilamellar vesicles (MLVs) are found to be strong and were not deformed on filtration (Nicolini et al. 2006). Hence, high vacuum pressures (i.e., higher suction speed) have given a positive approach on the method performance. On the other hand, lower extraction efficiencies were observed on decreasing vacuum pressures, which can be clarified from the fact that the vesicles undergo budding of small vesicles, (e.g. 200–300 mbar) (Table 1) (Pavlovic et al. 2007). Therefore, the leakage of aggregate phase was responsible for the loss of analytes during low vacuum filtration. Based on these facts,

Table 1 Effect of filtration, surfactant concentration, sample pH, and elution solvent on the recoveries of selected analytes through iSAME procedure

Parameter	(%Recoveries + RSD)[a]					
	AMX	CBZ	DCL	IDC	IBP	PCM
Vacuum pressure						
200 mbar	46.50 ± 1.80	58.21 ± 2.11	56.82 ± 3.25	48.58 ± 1.52	60.81 ± 1.88	63.02 ± 2.54
300 mbar	60.18 ± 2.25	71.55 ± 3.60	60.23 ± 2.78	56.81 ± 1.60	64.66 ± 1.93	66.00 ± 3.18
400 mbar	75.20 ± 2.82	88.05 ± 1.96	72.10 ± 2.44	72.40 ± 2.28	69.81 ± 2.54	79.15 ± 3.68
Ion-pair reagent conc.						
1.0 mM CTAB +10 mM Tiron	68.50 ± 3.32	66.28 ± 2.96	61.25 ± 2.66	58.40 ± 3.61	54.30 ± 2.80	58.80 ± 3.14
1.5 mM CTAB +12.5 mM Tiron	63.81 ± 2.50	60.80 ± 2.35	64.61 ± 2.10	59.44 ± 2.64	56.00 ± 2.62	62.54 ± 2.15
2.0 mM CTAB +15 mM Tiron	79.28 ± 1.85	83.50 ± 1.83	72.88 ± 3.05	73.21 ± 1.81	70.54 ± 1.95	78.22 ± 2.75
pH selection						
3.0	50.20 ± 2.86	69.11 ± 1.72	60.21 ± 3.91	56.45 ± 2.70	64.15 ± 3.26	70.32 ± 3.41
6.0	68.82 ± 2.92	80.32 ± 2.48	68.96 ± 2.67	68.30 ± 3.28	73.19 ± 3.65	76.54 ± 2.56
9.0	61.26 ± 2.05	64.90 ± 2.12	67.36 ± 2.10	69.18 ± 3.22	59.50 ± 2.88	72.81 ± 3.08
Elution solvent						
Acetonitrile	63.50 ± 0.94	67.30 ± 2.15	58.16 ± 2.68	54.15 ± 1.88	65.60 ± 1.50	59.81 ± 3.22
Methanol	74.22 ± 2.58	79.93 ± 3.35	72.50 ± 2.76	69.36 ± 2.32	72.65 ± 2.56	78.95 ± 3.30
Ethanol	68.41 ± 1.99	70.60 ± 1.32	64.11 ± 3.52	59.92 ± 3.81	68.21 ± 1.25	72.80 ± 3.21
2-Propanol	66.23 ± 2.14	71.35 ± 1.94	65.10 ± 2.81	64.17 ± 2.95	59.28 ± 3.81	65.20 ± 2.16

[a] Calculated from three determinations

a relatively high vacuum pressure of 400 mbar was adopted and it also supports the high sample throughput.

Effect of Ion-Pair Reagents Concentration

The optimum concentrations for Tiron® and CTAB were achieved at 1.5×10^{-2} M SSA and 2.0×10^{-3} M CTAB, respectively. A decrease in EEs and consistencies was noticed on changing the concentrations of Tiron® and CTAB above or below the optimum values. Hence, a particular molar concentration of Tiron® and CTAB favored the formation of supramolecular assemblies to give high recoveries with good consistency. When that composition was altered, either the process does not support the extraction or the analytes are not adequately remained on the filter paper surface (Table 1). It is remarkable that EEs are virtually maxima at the optimal ion-pair concentrations excluding for DCL, which is comparatively more polar.

Effect of pH on Sample Solution

The change in pH of sample solution has revealed minor impact on the efficacy of extraction method. The pH alteration over the range of 3.0–9.0 has shown considerable effect on the EEs of many analytes (Table 1). The analytes are primarily in their neutral form when the sample solution pH is maintained in between 3.0 and 5.0, but the analytes are predominantly anionic when the sample solution pH increased from 5.0 to 9.0. The EEs of highly ionizable compounds such as AMX (pKa = 2.4, 7.4, 9.6), CBZ (pKa = 13.9), and PCM (pKa = 9.5) at this pH were lower due to the increased hydrolysis. The same kind of results has been reported in other studies for mixed pharmaceuticals (polar, neutral, and non-polar) with the pH variations during analyte extraction (Ferhi et al. 2016; Wainright 1990). This notion supports the fact that the ionizable character of the analytes is only responsible for the lower EEs at elevated pH values, but not the properties of extraction medium. It should also be emphasized that filtration became more unfavorable at higher pH values from the fact that suction rate has been declined. Further, it is also explained that the deprotonation of hydroxyl groups in Tiron® molecules introduced more negative charge to the bilayer membrane and caused the membrane swelling because of electrostatic repulsion among adjacent molecules in the bilayer domain. Based on these findings, all the experiments were conducted without adjusting the solution pH.

Selection of Elution Solvent

A variety of organic solvents with different polarities including acetonitrile, methanol, ethanol, and 2-propanol were tested to dissolve the aggregates and to recover the selected analytes quantitatively. Based on the EEs, methanol was selected as dissolution solvent for the extraction of

analytes (Table 1). This is because there are fairly polar and non-polar compounds present in the selected pharmaceuticals, for which methanol has a polarity index of 5.1. It is the best suitable solvent for the extraction of analytes with high polarity and also for certain non-polar analytes. Hence, the total analytes were released quantitatively in a single step with 2.0 mL of methanol by adding it on the top of the filter for 2.0 min prior to apply the vacuum. The EEs were maxima for all selected analytes using methanol as eluent. Particularly, no carryover was detected after adding the solvent three times repeatedly on the same filter paper and implied that the analytes were already recovered in the first step. Further, the leftover aqueous solution was also tested to confirm the presence of any analytes, but no analyte peak was found during the analysis with CE.

Effect of Incubation Time and Temperature

The incubation time after adding ion-pair reagents and working temperature of the sample solution on EEs of selected pharmaceuticals were investigated in between 2.0–12.0 min and 20–60 °C, respectively. The results showed that the reaction process is kinetically convinced in 6.0 min at room temperature. At longer incubation times, the aggregate phase became stiffer, turbidity was increased, and therefore the filtration rate was declined. Further, the decrease in EEs was noticed with increasing temperature, particularly the solution became transparent at temperatures higher than 45 °C due to thermal perturbations in the aggregate structure, apparently by involving a transition from vesicles to worm-like micelles. Hence, the experiments were conducted at optimal temperature of 25 °C.

Selectivity and Matrix Effects

Previous studies have been revealed that iSAME is highly favorable for the extraction of hydrophobic analytes rather than hydrophilic analytes. As a result, the selectivity of the procedure was evaluated by taking a series of fairly hydrophilic pharmaceuticals, viz. AMX, CBZ, DCL, and PCM from 20 mL aqueous standard solutions containing 1.0 μg/L of each analyte (Table 2). No distinguishable impurity peaks were found for any of the hydrophilic compounds even though the analytes examined containing at least one aromatic ionizable group, which could also bind to CTAB and Tiron®. Furthermore, the unadjusted pH of the aqueous solution could inhibit their ionization in favor of their protonated (non-ionized) forms. Hence, the analytes are attachable to the hydrophobic aggregate core by non-polar interactions. Based on these findings, the hydrophobic organic compounds with low water solubility slightly favor this extraction.

Development of CE Method

The electrophoretic mobility difference between phosphate and borate buffers makes them useful for the on-line preconcentration technique (Reijenga et al. 1996; Girona et al. 2010). Boric acid (pKa = 9.24) served as the terminating ion in MEKC preconcentration step, and phosphoric acid (pKa = 2.16, 7.21, 12.67) acted as the leading ion. A brief MEKC-sweeping procedure proposed for this study is illustrated in Fig. S3. Initially, the fused silica capillary was loaded with phosphate buffer of pH 6.0. The electroosmotic flow (EOF) has risen due to the spontaneous charge formation at the liquid-solid interface, but it was found to be small due to the suppressed dissociation of silanol groups. Further, the analytes with pKa values from 3.23 to 9.50 would thoroughly dissociate in alkaline sample matrix (borate, pH 8.0). The analytes with negative charge receive an electrokinetic injection when applied with a suitable voltage. Meanwhile, a pH junction was also established between the sample matrix and background electrolyte. A zone with less conductivity was created at the boundary because of the neutralization reaction. Because of different dissociation states in both electrolytes, the mobility levels of

Table 2 Average recovery and matrix effects data of analytes in various food samples

Analytes	Concentration (μg/L)	%Recovery[a]	CV%	%Matrix effect[b]	CV%
AMX	1.0	87.24	2.81	95.81	3.81
CBZ	1.0	91.56	4.18	98.44	4.21
DCL	1.0	96.32	3.44	102.61	2.60
IDC	1.0	104.50	2.80	98.42	1.55
IBP	1.0	92.60	2.48	102.50	2.10
PCM	1.0	106.89	3.66	98.20	2.88

[a] %Recovery calculated from the ratio of mean peak area of analytes spiked into real samples before extraction to that of after extraction

[b] Matrix effect calculated as ratio of mean peak area of analytes in post-extraction spiked samples to that of neat standard solution peak area

the analytes gradually decreased when they entered the capillary. Besides, the mobility difference between borate and phosphate ions has created a transient isotachophoresis state. As a result, the analytes were then concentrated. Following accumulation, the acidic mobilization buffer (phosphate buffer at pH 6.0) containing anionic micelles (SDS) was positioned at both ends of the capillary. When applied a negative voltage, the anionic micelles began to enter the capillary, mobilized the analytes, and further stacked and swept the sample to form narrow bands.

Optimization of the MEKC-Sweeping Conditions

Various parameters were explored and optimized to achieve better resolution and higher sensitivity including the concentrations and pH of borate and phosphate buffers, SDS concentration, electrokinetic injection time, and separation voltage. In the following experiment, 100 mM borate buffer was used to attain an adequate buffer capacity. Since the analytes have different pKa values ranging in between 3.23 and 9.50, different alkaline pH values (8.0, 9.0, and 9.5) were tested to observe the effect of pH on analytes sweeping and stacking. It was noticed that the sample matrix ingredients might be the opponents for sampling on electrokinetic injection. The pKa value of boric acid is 9.23, and therefore, the degree of dissociation was increased with increasing pH from 8.0 to 9.0. Accordingly, a huge quantity of borate but fewer analytes were introduced at higher pH values. As a result, the peak heights decreased with increasing pH, as depicted in Fig. 3a. Hence, the pH of borate buffer was finalized as 8.0. Further, the phosphate buffer was picked as the leading electrolyte in this procedure. The leading electrolyte influences the stacking efficiency as well as the magnitude of EOF on which the separation of analytes depends. Therefore, different concentrations (40–120 mM) and pH values (6.0–8.0) of the phosphate buffer were examined. The concentrations higher than 80 mM produced broad peaks with decreased peak height intensities. Further, the pH of phosphate also revealed a remarkable effect on the separation. As depicted in Fig. 3b, a pH value of 8.0 produced broad signals with prolonged analysis time, which is caused by the opposite migration of EOF. At pH range 6.0 to 7.0, good baseline separation was achieved, but the higher analytes response

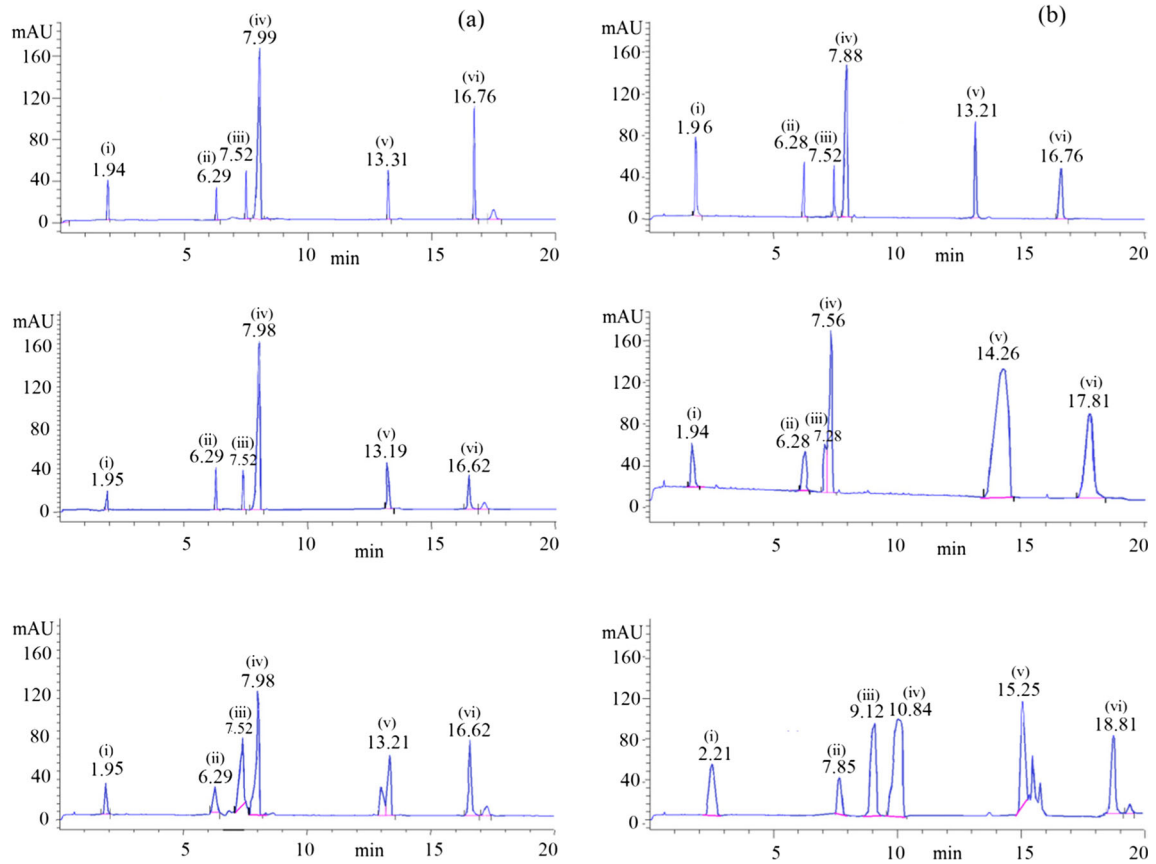

Fig. 3 Electropherogram of analytes at different pH values of **a** borate buffer pH 8.0, pH 9.0, pH 9.5 and **b** phosphate buffer pH 6.0, pH 7.0, pH 8.0 at the following experimental conditions: sample concentrations 1.0 μg/L; CE conditions: leading electrolyte 80 mM phosphate; sample electrolyte 100 mM borate; mobilization electrolyte 80 mM phosphate with 20 mM SDS; sample injection −10 kV for 600 s; separation voltage −20 kV

and sharp peaks were found at pH 6.0. The phosphate buffer concentration of 80 mM and pH value of 6.0 were selected for further study, taking into consideration the sensitivity and analysis time. The pseudo-retention factor of the analytes in MEKC mode is considered equivalent to the micelles concentration. Therefore, the preliminary investigations were conducted without using sodium dodecyl sulfate (SDS) surfactant, but the required resolution for the studied analytes had not been achieved. A complete separation of all studied analytes was achieved with the addition of 20 mM SDS to the micellar system. Here, SDS served as stacking and mobilization agent, which does carry the analytes while moving towards the detector end. When the amount of micelles is inadequate, the effective electrophoretic mobility of the analytes is expected to be diminished, and the velocity difference between analytes will also be reduced. Various concentrations of SDS (10 to 90 mM) have been tested to obtain the higher sensitivity. As expected, the total analysis time was decreased with increasing SDS concentration and the peak heights were also raised to certain extent. However, the high concentration of SDS did not allow separations and leads to unacceptably high running currents. Further, it caused to capillary clogging and makes the analysis difficult in CE-MEKC mode. Hence, 20 mM SDS was considered as the optimum concentration with 80 mM phosphate buffer.

Although the longer sample injection times can increase the introduction of analyte concentration, it can cause band broadening and declines the peaks resolution. To optimize the sample injection time, the electrokinetic injection was varied between 300 to 1200 s. The analyte peak heights were increased with increasing injection time, but the variance in the injection period was not observed, which can cause a significant impact on the migration time or peak width (Fig. S4). Based on peak height and total analysis time, 600 s was selected as the optimal sample injection time for this procedure. The sample solution was replaced after every injection to maintain the good repeatability. When higher voltages were applied for the electrophoretic analysis, the electrophoretic velocity of the analytes increased, and therefore, the analysis time was reduced by suppressing the dispersion and increasing the efficiency. Nevertheless, a higher voltage provided a higher current and led to change in the local viscosity of the buffer and resulted to peak broadening, poor separation, and repeatability. As shown in Fig. S5, higher separation voltages shorten the separation times. The total analysis time was reduced by 5.0 min. Nevertheless, a current over 160 μA was achieved when the applied voltage reached to −20 kV. Therefore, the applied voltage was fixed at −20 kV, taking into consideration of the current and analysis time. A standard electropherogram of six selected analytes obtained by the optimized separation conditions is shown in Fig. 4. Finally, the sensitivity enhancements were achieved because of high affinity of the analytes with the anionic SDS micelles through electrostatic attraction and micellar solubilization.

Validation of the Analytical Method

The quantitative applicability of the developed analytical procedure was examined for its linearity, LOD, LOQ,

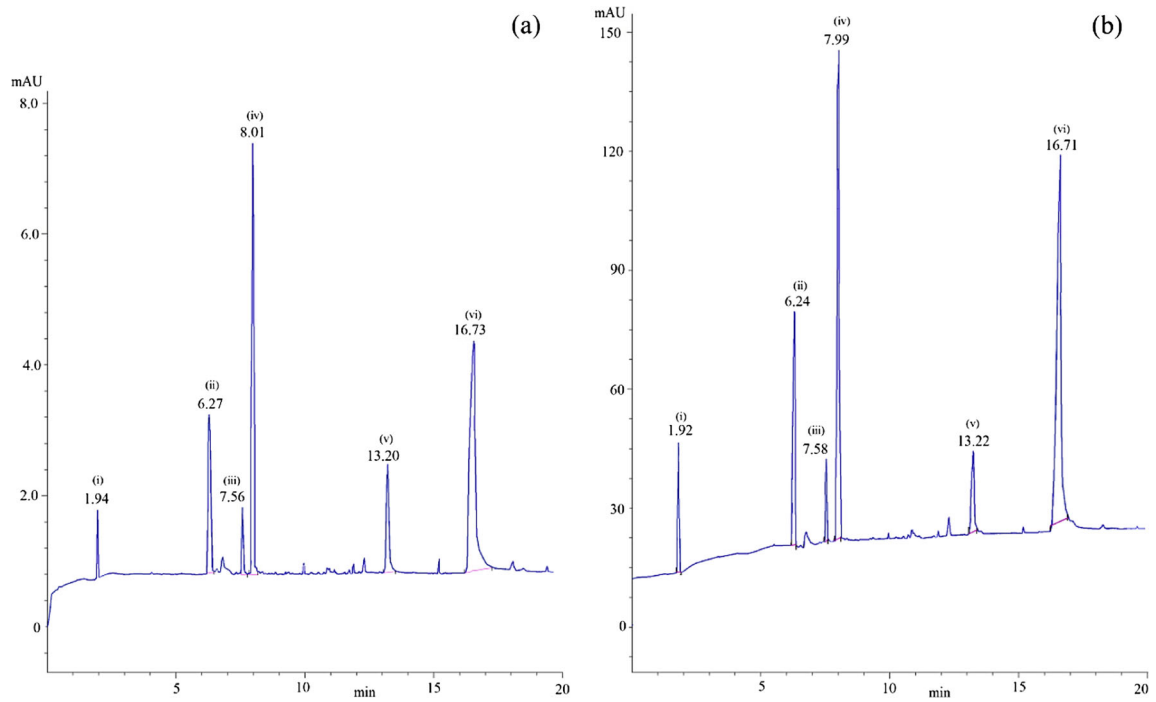

Fig. 4 Typical electropherogram of six selected analytes in **a** CZE and **b** MEKC mode under optimized conditions

Table 3 Linearity data, correlation coefficients, limits of detection (LODs), and enhancement factor (EF) values for six selected analytes

	AMX	CBZ	DCL	IDC	IBP	PCM
Linearity range (μg/L)	0.06–50	0.20–50	0.25–50	0.20–50	0.15–50	0.10–50
Correlation coefficient (R^2)	0.9987	0.9983	0.9987	0.9989	0.9989	0.9987
LOD (μg/L)	0.02	0.07	0.08	0.07	0.05	0.03
LOQ (μg/L)	0.06	0.20	0.25	0.20	0.15	0.10
Precision at LOD[a] (%RSD)	0.91	1.23	1.84	2.33	2.82	1.20
Precision at LOQ[a] (%RSD)	1.23	1.40	2.80	3.10	1.96	1.45
Repeatability[b] (1.0 μg/L)						
Mean recovery (%)	89.43	92.64	102.10	90.92	104.63	105.86
%RSD	2.41	1.58	2.89	3.50	2.50	4.25
Intermediate precision[b] (1.0 μg/L)						
Mean recovery (%)	87.30	94.50	100.85	95.46	106.83	103.28
%RSD	2.90	1.68	0.91	3.28	4.88	3.89
EF[c] (folds)	2489	1735	1645	1480	2080	2350

[a] Calculated from triplicate injections of LOD, LOQ solutions

[b] Calculated from five determinations

[c] Calculated from LOD ratios of CZE and MEKC-sweeping

precision, accuracy, and enhancement factors. The linearity of the method was determined by injecting six different concentrations of each analytes and calculated their peak area ratios. Calibration curves were drawn, taking analyte concentration on the X-axis and peak area ratios on the Y-axis. The linearity range, correlation coefficients, LODs, LOQs, and EF data of the developed method under optimized conditions are shown in Table 3. The correlation coefficients of each plot ($R^2 \geq 0.9987$) revealed high linearity between peak areas and analyte concentrations over the selected range. The limit of detection ($S/N = 3.0$, electrokinetic injection −10 kV, 600 s) was found to be in the range of 0.02–0.05 μg/L for six analytes detected at 214 nm. The precision and accuracy of the method were evaluated in terms of relative standard deviation (%RSD) of three determinations ($n = 3$) of each analyte at three different concentrations. The resulted %RSD values below 4.88% indicates the good precision and accuracy of this method. Further, the CZE mode (sample prepared in deionized water and applied via hydrodynamic injection at 0.5 psi, 5.0 s) was compared with MEKS-sweeping method to calculate the appropriate sensitivity improvements of this method. The results revealed that the EF of the proposed method is as high as 2500-folds for all six analytes. This analytical approach has an absolute advantage in terms of EF, sensitivity, and analysis time when compared with the methods that have recently reported for the analysis of pharmaceuticals (Aranas et al. 2011; Hamidi and Abolghasem 2015).

Application of Method for Real Sample Analysis

To demonstrate the applicability of the developed analytical procedure, it has been applied for the determination of selected pharmaceuticals from real water, urine, and fruits samples. For this, we have considered the blank samples of water, urine, and fruits and were spiked with pharmaceuticals to attain a final concentration of 1.0 μg/L for water and fruits and 10 μg/L for urine samples. The

Table 4 Recovery results for the determination of six analytes in various food and biological samples

Analyte	Spiked conc. (μg/L)	% Recovery[a]		Spiked conc. (μg/L)	% Recovery[a]
		River water	Fruit samples[b]		Urine samples
AMX	1.0	90.10 ± 2.50	89.25 ± 2.89	10	94.62 ± 1.85
CBZ	1.0	97.26 ± 1.55	94.21 ± 1.94	10	90.69 ± 1.82
DCL	1.0	100.85 ± 1.22	93.68 ± 2.89	10	94.82 ± 2.90
IDC	1.0	88.15 ± 4.32	92.68 ± 3.75	10	96.55 ± 1.85
IBP	1.0	95.60 ± 1.87	100.61 ± 2.00	10	104.50 ± 2.66
PCM	1.0	101.56 ± 2.66	103.39 ± 1.92	10	93.20 ± 1.05

[a] (mean ± %RSD) calculated from three determinations

[b] Considered the average value of three different fruit varieties viz., tomato, orange, and apple

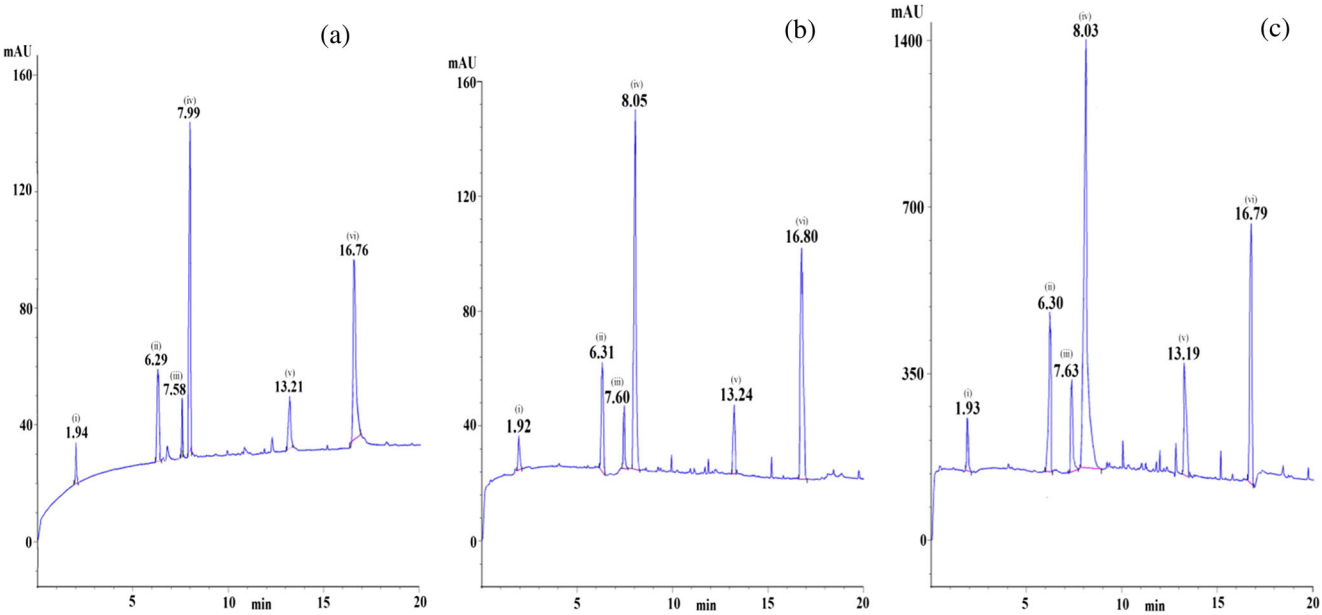

Fig. 5 A characteristic electropherogram of analytes spiked in **a** river water, **b** tomato sample, and **c** urine samples

analyte peaks were identified by means of migration times, standard additions, and UV spectra. Furthermore, there were few unidentified peaks found in the electropherogram of fruit samples followed by fewer in urine samples. Good recoveries were obtained for all samples over the range 88.15–101.56%, 89.25–103.39%, and 90.69–104.50% for water, fruits, and urine, respectively. The %RSD value was calculated from triplicate determinations of each sample, and it was found less than 4.0 in all cases (Table 4). Further, the matrix effects of the real samples were studied by adding 1.0 μg/L standards to fruit samples and 10 μg/L standards to urine samples and calculated the analytes recoveries. The relative recoveries of all analytes were found between 95.81 and 102.61%, which indicated the good accuracy and absence of matrix interferences from the samples. A characteristic electropherogram of a fortified water, fruit, and urine sample is depicted in Fig. 5. In view of the potential health effects of the unnecessary pharmaceuticals, the exact quantification can be an important factor. The method could be useful for the researchers and enforcers to examine the studied analytes for commercial juices and fruits.

Conclusions

A sensitive and rapid MEKC-sweeping online CE procedure is proposed for the simultaneous determination of six pharmaceuticals in various food samples. The in situ formation of suspended aggregate phase was introduced as extraction approach for the off-line preconcentration of selected pharmaceuticals and is a new concept in sample preparation. In this aspect, a dual extraction mechanism, i.e., entrapment and mass transfer of the analytes from the aqueous phase to supramolecular phase, was supported for the analytes recovery. The iSAME is a new, simple, and alternative technique to the conventional sample preconcentration methods. The best extraction recoveries were obtained using this approach, and it was possible to process high volume of samples. Previous studies have been reported that iSAME is highly favorable for the extraction of hydrophobic analytes rather than hydrophilic analytes. However, the selectivity of the procedure was evaluated by taking a series of fairly hydrophilic pharmaceuticals, viz. AMX, CBZ, DCL, and PCM, and achieved good recoveries after optimization. Further, the use of MEKC-sweeping as online preconcentration technique helped remarkably to obtain lower detection limits, i.e., 0.02–0.05 μg/L for all six analytes. A sensitivity enhancement about 2500-folds of magnitude was achieved when compared with conventional CZE methods. After the method optimization, it has successfully applied for the quantification of selected analytes in real water, fruits, and urine samples. The developed analytical method has demonstrated several advantages in sample extraction such as its simplicity, rapid analysis time, cost-effectiveness, and also provided best analytical figures of merit in terms of linearity, precision, accuracy, and recoveries for selected analytes. The method could be applicable for the determination of a large variety of pharmaceutical pollutants in a wide range of polarity. Mainly, the integrity of the complete procedure and the low cost associated with its application proved this method as a good substitute for the most preferable LC techniques in routine use.

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Compliance with Ethical Standards

Conflict of Interest A. Vijaya Bhaskar Reddy declares that he has no conflict of interest. Zulkifli Yusop declares that he has no conflict of interest. Jafariah Jaafar declares that she has no conflict of interest. Nur Hidayati Jamil declares that she has no conflict of interest. Z. A. Majid declares that he has no conflict of interest. Azmi B. Aris declares that he has no conflict of interest.

Human and animal rights This article does not contain any studies with human or animal subjects.

Informed Consent Informed consent is not applicable.

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