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Dynamically coated capillaries improve the identification power of capillary zone electrophoresis for basic drugs in toxicological analysis

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Abstract

In systematic toxicological analysis (STA), analytical methods should have a high identification power. This can be suitably expressed by parameters such as mean list length (MLL) or discriminating power (DP). The reproducibility of a method has a great impact on its identification power, and should be as high as possible. In this study, two separation methods based on capillary zone electrophoresis (CZE) were evaluated towards STA applications. Besides a normal phosphate buffer, the commercially available buffer CElixir was used, which is a double-layer dynamic coating system. The coating stabilizes the endoosmotic flow, is independent of the pH, and is claimed to be more reproducible and faster at low pH than with normal buffers. A test set of 73 basic pharmaceutical compounds was analyzed by the two CZE methods. The total analysis time, including rinsing steps, was 8 min when the coating was used and 18 min without the coating. Effective mobilities were calculated and the reproducibilities were a factor of 2 better when the coating was used (between-days SD 0.020 and 0.040 $m^2/V\,s$ with and without the coating, respectively). MLL and DP were calculated for the two CZE methods and for combinations with standardized liquid and gas chromatography systems. CZE with CElixir coating clearly has a high potential for STA applications, as it was shown to have a higher identification power and shorter analysis times than normal CZE.

Keywords: Coated capillaries; Toxicological analysis; Basic drugs

1. Introduction

The identification of drugs and related compounds is an important subject in analytical toxicology. Systematic toxicological analysis (STA) is the discipline that deals with the detection and identification of ''any possible'' harmful compound in a systematic approach [1–3]. Statistical procedures have been developed to calculate the identification power of analytical methods (single, combined, or hyphenated), so that their suitability to identify unknown compounds can be quantitated. Two useful parameters that can be used to express the identification power are the mean list length (MLL) [4] and the discriminating power (DP) [5], the concepts of which will be described below. MLL and DP have been mainly developed for, and applied to, toxicological analysis [6–10]. Moreover, the MLL

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approach was applied to the environmental analysis of pesticides [11].

The list length (LL_i) of substance i in a dataset with n substances ($i = 1, \ldots, n$) is the number of substances in the dataset that match with substance i, i.e. those substances that have a probability of identification larger than a certain significance level (α). The average of all LL_i values in a dataset for an analytical system give the MLL, which reaches 1.000 for ideal analytical methods. When, for instance, a MLL of 5.3 is found, an average list of 5.3 substances, coming into consideration for identification, will be obtained in data retrieval [4].

Another approach to express the identification power of a method is calculation of the DP. The DP of an identification method is defined as the probability that two substances selected at random from a test set would be discriminated using a given retrieved window by that method. DP is calculated using:

$$DP = 1 - \frac{2m}{n(n-1)} \quad (1)$$

where m equals the number of matches for two substances (in the dataset) selected at random. Two substances match when they are within a certain window. The DP always lies between 0 and 1, 1 being the optimum [5].

For both MLL and DP, the number of matches is determined by the reproducibility of the analytical value on which the identification is based (e.g. a retention time or molecular mass). With a high reproducibility the search window and thus the number of matches will become smaller, so that the identification power of the system will increase. MLL and DP values also depend on the number of compounds in the test set. A low number of test compounds will give an overestimation of the actual identification power and will be less meaningful. The larger the database, the more realistic the MLL and DP information will be.

A modern separation technique with great potentials is capillary electrophoresis (CE). Several characteristics of CE make it an attractive technique for toxicological analysis. It is a flexible technique characterized by outstanding separation efficiency, high mass sensitivity, minimal use of samples and solvents, and fast analyses. Another important aspect of CE is its separation principle, which is different from the more traditional chromatographic techniques. The most common CE mode is capillary zone electrophoresis (CZE), which is performed in a plain buffer.

However, migration times in CE have a limited reproducibility. The major cause of non-reproducibility is the change in electroosmotic flow (EOF), which is the bulk flow of liquid due to the influence of the electric field on the layer of counterions adjacent to the negatively charged capillary wall. Unstable surface conditions of the fused-silica capillary wall or small variations in buffer pH can cause small variations in the EOF, leading to variable migration times. For STA applications, the reproducibility of the parameter used for identification is of utmost importance and can be improved by the use of the effective mobility (μ_{eff}) instead of the migration time [10,12] and by the use of standards or markers [10,13–16].

An alternative way to enhance the reproducibility in CZE is to stabilize the EOF by coating the capillary wall. The surface can be chemically modified to minimize the effects of the variable negative silanol groups that cause the variation in EOF in fused-silica capillaries. In addition, coating procedures are regularly used to reduce wall adsorption and to eliminate or reverse the EOF. Capillaries can be permanently or dynamically coated. An advantage of dynamic coatings is that the coating can be replaced after each run, whereas a permanent coating may become irreversibly damaged after a number of runs.

CElixir (sometimes referred to as CEofix) is a patented and commercially available dynamic coating that was developed to stabilize the EOF [17]. It creates a stable, negatively charged double-layer on the wall that is independent of the pH. It is claimed that analyte–wall interactions are reduced, method robustness is improved, and analysis times at low pH are reduced, since the EOF is faster at low pH than in normal CZE. To apply the dynamic coating, the capillary is rinsed with a buffered polycation that forms a stable layer against the wall (see Fig. 1A). Then, the capillary is rinsed with a buffered polyanion at the desired pH, that forms a second layer (see Fig. 1B). Thus, a stable double-layer is formed that leaves the capillary highly negatively charged over a

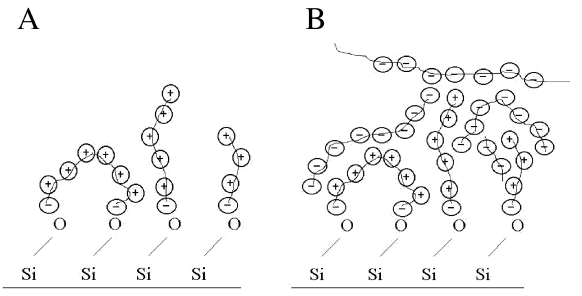

Fig. 1. Formation of the dynamic coating using CElixir. During a rinse of the capillary with the buffered polycation, a stable layer is formed against the wall (A), then the second layer is formed by a rinse with the buffered polyanion at the desired pH (B).

wide pH range. Then, the actual electrophoresis process can take place using the second solution as the run buffer. After each run, the procedure is repeated so that the coating is always in an optimal state for the next analysis.

Lurie et al. [18] describe the use of CZE with CElixir coating for the routine analysis of methamphetamine, amphetamine, MDA, MDMA, MDEA, and cocaine in seized drugs. The use of the coating at low pH shortened the analysis time by a factor of 2 and increased the reproducibility compared to a CZE method without coating.

1.1. Objectives of the study

In this study, the performance of two methods based on CZE was compared. The first method, which will be referred to as CZE1, is a standard method that is based on a normal phosphate buffer without any additives. In the second method, which will be referred to as CZE2, the dynamic coating CElixir is used as the run buffer. A group of basic pharmaceutical compounds, representing a large variety of chemical structures and drug classes, was analyzed to study the effect of the coating on the reproducibility of μ_{eff} and on the analysis time.

The identification power of both methods was determined by calculation of MLL and DP for a set of 73 basic pharmaceutical compounds. For 60 compounds, RI values were available for standardized liquid and gas chromatographic (LC and GC) STA methods [9,19], and MLL and DP were calculated for combinations of these methods with the CZE methods.

2. Experimental

2.1. Chemicals

Pharmaceutical test compounds were selected from our in-house collection of reference substances, obtained from commercial sources. Sodium dihydrogen phosphate monohydrate, sodium hydroxide, ethanol, and acetonitrile were purchased from Merck (Darmstadt, Germany). Formamide was purchased from Sigma (St. Louis, MO, USA). CElixir buffer kit pH 2.5 was obtained from MicroSolv Technology (Long Branch, NJ, USA).

The water used was demineralized and further purified with an Elga ultrapure water system (Salm & Kipp, Breukelen, Netherlands).

2.2. Equipment

Analyses were carried out on a Beckman P/ACE system 5500 capillary electropherograph (Beckman Instruments, Fullerton, CA, USA) equipped with a UV detector. Data were collected and interpreted using P/ACE System 5000 Series Software. Uncoated fused-silica capillaries were used from Composite Metal Services (Hallow, UK) and MicroSolv Technology (Long Branch, NJ, USA), for CZE1 and CZE2, respectively.

Calculations of MLL and DP values were performed using a homemade software program.

2.3. Procedures

For CZE1, conditions were taken from a standard STA method for basic drugs in our laboratory [20]. The buffer consisted of 50 m*M* sodium phosphate buffer at pH 2.5. The capillary was 57 cm (50 cm to the detector)×50 μm I.D., and a voltage of 30 kV (ramp time 2.9 kV/s, current 42 μA, electric field strength 52.6 kV/m) was applied. At the beginning of each day, capillaries were rinsed with 0.5 *M* sodium hydroxide for 5 min followed by a rinse of 5 min with water. Before each run, the capillary was rinsed for 3 min with buffer. Overnight, capillaries were stored in water.

For CZE2, conditions were used as recommended by the manufacturer of CElixir. The run buffer was CElixir accelerator solution, consisting of 75 m*M*

phosphate and a polyanion at pH 2.5. The capillary was 47 cm (40 cm to the detector)×50 μm I.D., and a voltage of 25 kV (ramp time 0.4 kV/s, current 50 μA, electric field strength 53.2 kV/m) was applied. At the beginning of each day, capillaries were rinsed with 0.1 *M* sodium hydroxide for 5 min followed by a rinse of 2 min with water. Before each run, the capillary was rinsed for 0.5 min with 0.1 *M* sodium hydroxide, 0.5 min with CElixir initiator solution, and 1 min with CElixir accelerator solution. Overnight, capillaries were stored in water.

For both methods, samples were hydrodynamically injected at 0.5 p.s.i. (1 p.s.i.≈7 kPa) for 5 s. UV detection took place at 214 nm and the capillary was thermostated at 25°C. Compounds were analyzed at a concentration of ca 25 μg/ml in 2.5% ethanol in water.

3. Results and discussion

3.1. Effects of the dynamic coating CElixir

Fig. 2 shows the electropherograms of a test mixture of eight compounds and a reference compound (paraquat, PQ) analyzed by the standard method, CZE1, and by the method using the dynamic coating, CZE2. The fast migration times of CZE2 are immediately apparent. Migration times are below 6 min for CZE2, whereas they are up to 12 min for the same compounds analyzed by CZE1. This difference cannot be caused by the somewhat higher electric field strength used for CZE2 (53.2 vs. 52.6 kV/m for CZE1) and results from the faster EOF. The total analysis time, including rinsing steps, was 18 min for CZE1 and 8 min for CZE2.

Since the migration time window in minutes becomes smaller when the dynamic coating is used, it is important that the peak efficiency remains high and that the selectivity is not reduced. The number of theoretical plates was between 115 000 and 145 000 plates for CZE1 and between 115 000 and 175 000 plates for CZE2. All peaks in the test mixture were baseline separated in both methods with resolutions of 2.5 or higher. The same migration order was obtained.

For the test group of 73 compounds, the effective mobility, μ_{eff}, was calculated for each compound in

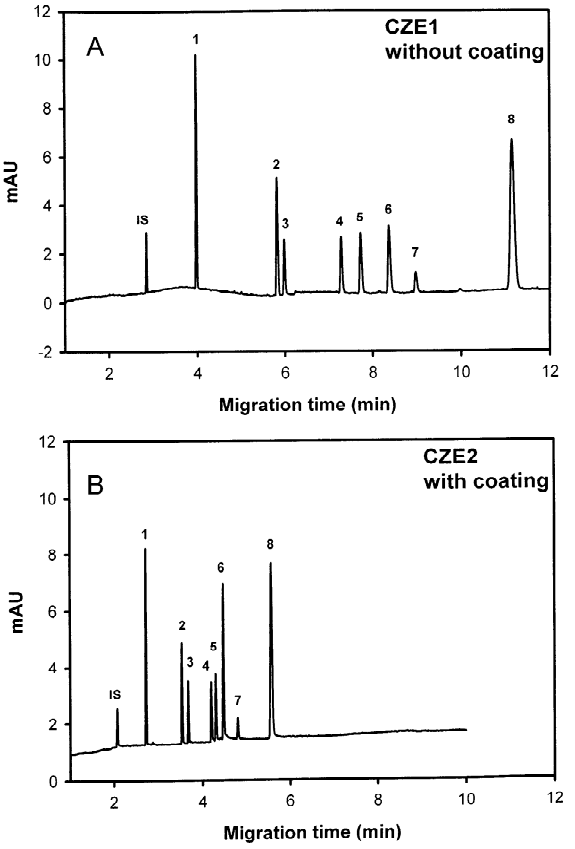

Fig. 2. Electropherograms of the standard mixture analyzed by CZE without (A) and with (B) the CElixir coating. PQ: paraquat, 1: metformin, 2: amphetamine, 3: acetophenazine, 4: butacaine, 5: phencyclidine, 6: fluoxetine, 7: timolol, 8: nitrazepam, all at 25 μg/ml in 2.5% ethanol in water.

CZE1 and in CZE2 (see Table 1). Since EOF markers elute much later than the analytes at low pH, the quaternary ammonium ion paraquat (PQ) was used as a reference compound to estimate the EOF. PQ migrates in front of all the analytes tested due to its high positive charge. Reference values of μ_{eff} of PQ were used, that had been determined for both methods by repeated analysis of the compound in the presence of the EOF marker formamide. The reference values are $5.393 \cdot 10^{-8}$ and $5.035 \cdot 10^{-8}$ $m^2/V\,s$ for CZE1 and CZE2, respectively.

The so-called analytical window can be expressed by the difference between the highest and lowest value of μ_{eff}. A large window is advantageous for the identification power of the method. The window

Table 1
Effective mobilities ($\cdot 10^{-8}$ $m^2/V\,s$) of 73 compounds for normal CZE (CZE1) and CZE using CElixir coating (CZE2)

	CZE1	CZE2		CZE1	CZE2
Acebutolol	1.243	1.565	Loxapine	2.677	2.536
Acetophenazine	2.500	2.455	MDMA (XTC)	2.164	2.373
Amoxapine	2.899	2.734	Medazepam	1.926	1.992
Amphetamine	2.575	2.598	Mepivacaine	1.739	1.897
Atenolol	1.643	1.688	Methaqualone	0.771	0.919
Atropine	1.882	1.878	Methoxamine	2.098	2.077
Baclofen	1.965	2.138	Methformin	3.820	3.652
Bambuterol	1.422	1.375	Methamphetamine	2.456	2.483
Benzocaine	0.939	1.283	Metoprolol	1.432	1.717
Bisoprolol	1.186	1.788	Minoxidil	1.798	2.023
Brompheniramine	3.192	3.016	Morphine	1.856	1.864
Bupivacaine	1.675	1.855	Nadolol	1.351	1.597
Bupronion	1.878	1.906	Nitrazepam	1.298	1.319
Buspiron	1.594	1.676	Oxprenolol	1.773	1.767
Butacaine	2.032	2.048	Papaverine	1.642	1.939
Chloroquine	2.955	2.915	Phencyclidine	1.917	1.968
Chlorpheniramine	3.040	3.148	Pheniramine	3.468	3.275
Cimetidine	1.949	1.901	Phenylephrine	2.198	2.220
Clozapine	3.007	2.875	Prazosin	1.530	1.644
Cocaine	1.936	1.960	Procainamide	2.752	2.687
Codeine	1.842	1.877	Procaine	2.269	2.443
Dextromethorphan	1.978	1.982	Propranolol	1.628	1.869
Diazepam	1.384	1.697	Psilocin	2.280	2.306
Diltiazem	1.494	1.575	Pyridoxine	2.362	1.789
Diphenylhydramine	1.922	1.993	Quinidine	3.035	2.925
Doxepin	1.897	2.027	Salbutamol	1.755	1.713
Ephedrine	2.317	2.278	Tiotixene	2.427	2.425
Fluoxetine	1.766	1.854	Terbutaline	1.732	1.749
Fluvoxamine	1.688	1.747	Tetracaine	2.250	2.270
Haloperidol	1.576	1.555	Timolol	1.640	1.664
Heroine	1.705	1.645	Tolazoline	2.684	2.673
Hydrazaline	2.737	2.711	Trazodone	1.614	1.588
Hydroxyzine	1.760	1.728	Triamterene	2.001	2.065
Ketamine	1.911	1.660	Trifluoperazine	2.638	2.535
Ketazolam	1.592	1.593	Trimipramine	1.836	1.937
Labetalol	1.553	1.734	Verapamil	1.324	1.452
Lidocaine	1.920	1.974			

was somewhat larger for CZE1 than for CZE2 (3.049 and $2.733 \cdot 10^{-8}$ $m^2/V\,s$, respectively).

The μ_{eff} of some compounds seems to be affected by the use of the dynamic coating (see Table 1). However, before comparing μ_{eff} values for both methods, it must be noted that the phosphate concentration was 50 m*M* for CZE1 but 75 m*M* for CZE2. In a simple experiment, μ_{eff} of several compounds was determined by both the 50 m*M* phosphate buffer at pH 2.5 of CZE1, and a 75 m*M* phosphate buffer at pH 2.5. The μ_{eff} values were all some 2.5% higher in the latter buffer. Therefore, for comparison of μ_{eff} of CZE1 and CZE2, only differences larger than 5% were assumed to arise from the presence of the polyanion. A group of 21 compounds had a μ_{eff} of 5–50% higher in CZE2, for instance bisoprolol, diazepam, and nadolol. On the other hand, six compounds had a μ_{eff} of 5–25% lower in CZE2, for instance ketamine, pheniramine, and loxapine. From these results, it is clear that there are some interactions between these compounds with the polymer at the wall and/or in the buffer solution. In some cases, this results in changes in the migration order. A significant change in selectivity was also

Table 2
Mean effective mobility ($\cdot 10^{-8}$ m^2/V s) and standard deviation (SD) of eight test compounds for normal CZE (CZE1) and CZE using CElixir coating (CZE2) ($n=7$ days)

	CZE1		CZE2	
	Mean	SD	Mean	SD
Metformin	3.802	0.020	3.650	0.021
Amphetamine	2.587	0.027	2.590	0.010
Acetophenazine	2.492	0.029	2.451	0.015
Butacaine	2.014	0.033	2.031	0.009
Phencyclidine	1.908	0.033	1.958	0.012
Fluoxetine	1.750	0.035	1.844	0.012
Timolol	1.615	0.037	1.653	0.013
Nitrazepam	1.243	0.041	1.316	0.017

observed by Lurie et al. [18], who suggested that this may result from ionpairing or hydrophobic interactions.

The reproducibility of μ_{eff} was determined for both methods by analyzing the mixture of eight compounds shown in Fig. 1 five times daily for 7 days. Mean values and standard deviations (SD) of μ_{eff} are shown in Table 2 and Fig. 3 for CZE1 and CZE2. SDs were significantly lower for CZE2 than CZE1 ($P=0.000$). The maximum SD obtained for CZE1 was 0.041 ($\cdot 10^{-8}$ m^2/V s). The maximum SD obtained for CZE2 was $0.021 \cdot 10^{-8}$ m^2/V s, which is a factor two better than for CZE1.

For CZE1, a trend is observed, as the SD of μ_{eff} increases with decreasing mobility, i.e. with increasing migration time. This trend, and the absence of a similar trend for CZE2, could imply that the EOF in CZE1 changes during the run, which is not completely corrected for by the calculation of μ_{eff}. Jumppanen and Riekkola [13] already showed that the EOF changes during an analysis in a non-linear way. In CZE2, no trend is observed and the EOF is probably more stable during the run.

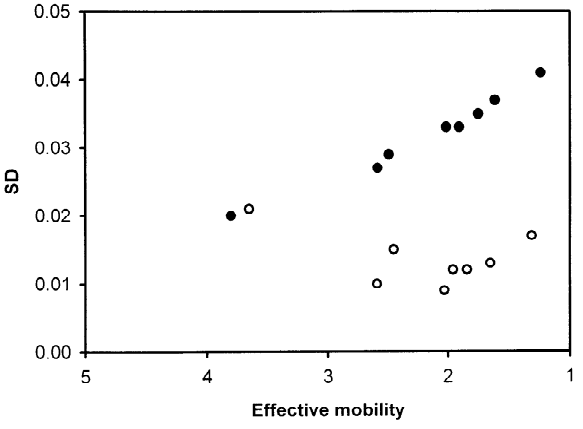

Fig. 3. Standard deviation (SD) plotted against the effective mobility ($\cdot 10^{-8}$ m^2/V s) for normal CZE (black spots) and CZE with CElixir coating (white spots); $n=7$ days.

3.2. Identification power

The identification power of the CZE methods was expressed by calculation of MLL and DP values. For the calculations, μ_{eff} values were taken from Table 1. SDs were based on the results of the test mixture, and were set at $0.040 \cdot 10^{-8}$ m^2/V s for CZE1 and $0.020 \cdot 10^{-8}$ m^2/V s for CZE2. For MLL calculations, the significance level, α, was set at 0.01. From the results in Tables 3 and 4, it is clear that MLL and DP values show similar results. Therefore, in this section, only the results of MLL calculations will be discussed.

In Table 3, MLL and DP of CZE1 and CZE2 are shown for the group of 73 basic pharmaceuticals. MLL was 8.79 for CZE1 and 5.58 for CZE2. The lower MLL of CZE2 is caused by the lower SD of the method. To show the great influence of the reproducibility, the MLL was also calculated for CZE2 using the SD of CZE1, $0.040 \cdot 10^{-8}$ m^2/V s. The MLL increased to 10.12, which is even higher than the value of CZE1 itself. Therefore, when SDs of CZE1 and CZE2 would have been equal, MLL would be better for CZE1, which can be explained by the larger analytical window in CZE1. The large impact of SD on MLL emphasizes the importance of a high reproducibility method. With a smaller SD,

Table 3
Mean list length (MLL) and discriminating power (DP) for normal CZE (CZE1) and CZE using CElixir coating (CZE2) based on a data set of 73 compounds

	SD	MLL	DP
CZE1	0.040[a]	8.79	0.883
CZE2	0.020[b]	5.58	0.933
CZE2	0.040[a]	10.12	0.861

[a] SD of CZE1.
[b] SD of CZE2. Data are calculated for two standard deviations (SD, $\cdot 10^{-8}$ m^2/V s) for CZE2.

Table 4
Mean list length (MLL) and discriminating power (DP) for effective mobilities (μ_{eff}) of normal CZE (CZE1) and CZE using CElixir coating (CZE2), and retention indices (I) of liquid chromatography (LC) and gas chromatography (GC), and for combinations of methods based on a data set of 60 compounds

	SD[a]	MLL	DP
CZE1	0.040	7.67	0.878
CZE2	0.020	4.72	0.936
LC	3[b]	3.53	0.957
GC	5[b]	1.70	0.989
LC–GC		1.03	0.999
CZE1–LC		1.32	0.990
CZE2–LC		1.13	0.994
CZE1–GC		1.13	0.996
CZE2–GC		1.03	0.998
CZE1–CZE2		2.45	0.954

[a] SD values of methods cannot be compared, since μ_{eff} in CZE, I in LC, and I in GC have completely different analytical windows.
[b] Estimated intralaboratory SD.

the identification power of a method can improve substantially. However, the MLL does not approach 1.00 for CZE1 or CZE2. Therefore, methods can be combined to obtain lower MLLs.

For 60 of the 73 compounds, retention indices (I) of LC [9] and GC [19] were available from the literature, obtained with standardized STA methods. For these 60 compounds, MLLs were calculated for CZE1, CZE2, LC, or GC alone, and for each combination of two methods (see Table 4). When MLLs of the CZE methods in Tables 3 and 4 are compared, it is clear that the number of compounds in the data set influences the MLL of a method. DPs are independent on the number of substances, and deviations in DPs of the CZE methods in Tables 3 and 4 are caused by random variance.

The combination with chromatographic methods resulted in a substantial improvement of the identification power. For all combinations, MLL values below 1.32 were obtained. The best MLL was obtained for the combinations CZE2/GC and LC/GC, resulting in an MLL of 1.03.

To investigate the effect of analyte–polymer interactions as described in Section 3.1., data for CZE1 and CZE2 were also combined for the calculation of MLL. The latter improved to 2.45, which indicates that the analyte–polymer interactions influence the separation mechanism to the extend that a higher identification power is obtained.

4. Conclusions

In this study, CZE using the CElixir coating (CZE2) was found superior to normal CZE (CZE1) for identification purposes of basic drugs. CZE2 was found to have a higher reproducibility and identification power, and an analysis time that was a factor two shorter than CZE1. Although the methods are both based on CZE, it was shown that the CElixir polymer interacts with some compounds resulting in a slightly different selectivity. The above-mentioned properties of the CElixir coating provide a high potential for application in STA.

The methods presented here are suitable for the screening of basic compounds. For the screening of acidic compounds, CZE methods should be compared with and without the CElixir coating at high pH. For the screening of neutral compounds by CE, micellar electrokinetic chromatography (MEKC) is useful. Micelles cannot be simply added to the CElixir buffer used in this study, but a CElixir buffer for MEKC is under development.

In general, a higher identification power of CZE could be accomplished by combining the effective mobility with diode-array or even mass spectra. Finally, the influence of biological samples on the migration behavior and reproducibility of CZE with and without the CElixir coating is currently being investigated.

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