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Capillary Electrochromatography of Pyrimidine Derivatives Using UV and Mass Spectrometric Detection

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Summary. The separation of pyrimidine derivatives by capillary electrochromatography (CEC) using either UV or mass spectrometric detection is described. For UV detection an aqueous phosphate carrier electrolyte containing acetonitrile is employed. The results are compared to the analysis of the same compounds by micellar electrokinetic chromatography in terms of selectivity, migration times, linearity, and detection limits.

For the combination of CEC and mass spectrometry (MS) an inexpensive way to couple commercially available instruments is presented; the interface consists of an electrically grounded stainless steel connector (containing a stainless steel frit) serving as the electrode and coupling the CEC capillary with a fused silica transfer capillary to the MS instrument. Alternatively, a PEEK adapter combining the CEC capillary and a grounded stainless steel transfer capillary serving as the electrode is employed. To avoid the formation of hydrogen gas at the coupling piece or the transfer capillary, *p*-benzoquinone is added to the carrier electrolyte consisting of aqueous ammonium acetate and acetonitrile.

Keywords. Capillary electrochromatography; Pyrimidines; Atmospheric pressure ionization mass spectrometry; Capillary coupling.

Introduction

Capillary electrophoresis (CE) and capillary electrochromatography (CEC) have gained increasing importance in analytical chemistry during the last years. Closely connected with the progress in this area was the development of detection techniques especially designed for these applications. Nowadays, a range of different detectors is commercially available based on UV-absorption, fluorescence, conductivity, and electrochemistry. Besides these well-established techniques, mass spectrometry (MS) has also become an accepted detection method because of various advantages like selectivity and sensitivity. The combination of CE and MS has become state of the art for the determination of a wide range of analytes, whereas hyphenating CEC with MS seems to be more complex because of some limitations arising from the characteristic features of CEC. To avoid the formation of air bubbles, the application of pressure at both ends of the packed capillary is

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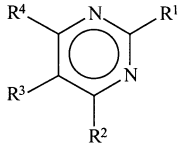
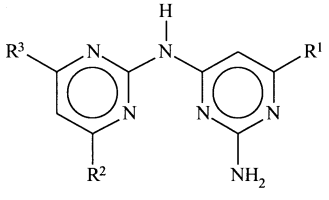
recommended for CEC separations which is not compatible with the MS interface. Another drawback is the need for rather long capillaries to cover the distance between the inlet buffer reservoir and the outlet end reaching into the MS interface. To overcome these problems, home-made CEC devices have been constructed which allow the combination with commercially available MS instruments [1–3]. With such instrumental setup the use of CEC-capillaries of usual length (up to 40 cm) is possible. Nevertheless, the application of these CEC/MS interfaces may lack sufficient automation and reproducibility necessary for modern analytical instrumentation. An interface has been reported that can decouple the electric field of a CE separation from a detector in an off-capillary position; this interface is based on a connector made of palladium [4]. In the present work, an interface based on this principle is described for the combination of CEC and atmospheric pressure ionization (API) MS made from commercially available and inexpensive parts; applications are given for the analysis of pyrimidine derivatives.

The pyrimidine derivative **1a** (Scheme 1) is a building block for the manufacturing of important herbicides and fungicides; the other compounds under investigation (**1b**–**g**, **2a**) are by-products arising during the synthesis of **1a**. They have been analyzed before by micellar electrokinetic chromatography (MEKC) using UV detection [5], but CEC has not yet been applied to this group of compounds. The aim of the work presented in this paper was the investigation of the applicability of CEC for the separation of the compounds mentioned in Scheme 1 and a critical comparison of the results with the separation obtained by MEKC. Subsequently, the combination of CEC and MS using commercially available instruments and parts is demonstrated.



1a-h

	R^1	R^2	R^3	R^4
1a	NH_2	OCH_3	H	OCH_3
1b	NH_2	Cl	H	Cl
1c	NH_2	OH	H	OCH_3
1d	NH_2	Cl	H	OCH_3
1e	NH_2	NH_2	H	OCH_3
1f	NH_2	OCH_3	Cl	OCH_3
1g	NH_2	OCH_3	H	$N(C_2H_5)_2$
1h	OCH_3	OCH_3	H	OCH_3



2a

	R^1	R^2	R^3
2a	OCH_3	OCH_3	OCH_3

Scheme 1

Results and Discussion

Separation of pyrimidine derivatives by CEC using UV detection

Usually employed analytical techniques for the determination of pyrimidine derivatives are titrimetry for hydroxy derivatives showing acidic properties such as compound **1c** or gas chromatography (GC) and high performance liquid chromatography (HPLC) for other derivatives of pyrimidine [6, 7]. To circumvent some drawback of these techniques like decomposition of the analytes in GC or long analysis times in HPLC, the application of CEC seems to be an attractive alternative to MEKC.

First attempts to separate compounds **1a–h** and **2a** were performed using a commercially available CEC capillary (effective length 40 cm, total length 48.5 cm) packed with Hypersil particles (3 µm average particle size). Various mixtures of potassium dihydrogenphosphate and acetonitrile at different *pH* values were investigated as the carrier electrolyte and evaluated with respect to resolution and migration times of the analytes. The influence of the *pH* of the carrier electrolyte was checked with a buffer consisting of 10 m*M* phosphoric acid containing 50% (v/v) of acetonitrile and adjusted to the desired *pH* with 1 *M* KOH. Between *pH* 5 and 7, little influence of the *pH* could be observed; therefore, a *pH* of 6 was chosen for further experiments which offered a high electroosmotic flow (EOF) as well as chemical stability of the packing material and reasonable separation of the analytes. The phosphate concentration was varied between 6 and 15 m*M* resulting in minimum migration times at a concentration of 8 m*M*; selectivity hardly changed with the concentration of phosphate.

The content of acetonitrile was varied between 40 and 70% (v/v), showing a steady decrease of migration times with increasing acetonitrile content. Using a carrier electrolyte consisting of 8 m*M* potassium dihydrogenphosphate at *pH* 6, a content of 40% acetonitrile lead to migration times between 14 min (**1c**) and 55 min (**2a**). At an acetonitrile content of 70%, the analytes exhibited migration times between 11 min (**1e**) and 20 min (**2a**) but **1b**, **1d**, and **1f** could not be separated. 60% acetonitrile offered both reasonable migration times (between 11 min for **1e** and 22 min for **2a**) and sufficient resolution of all pyrimidine derivatives under investigation. A typical electrochromatogram is shown in Fig. 1. It should be pointed out that the elution order is quite different from that achieved with MEKC [5]: using MEKC, the order was **1a–1c–1h–1d–1b–1g–1f–2a**. Compound **1e** could not be separated from the EOF by MEKC under the given conditions. In CEC, **1e** is eluting after **1c** and approximately 2 min after the EOF; the elution order is **1c–1e–1a–1d–1b–1f–1h–1g–2a**. These results indicate that there are differences between the MEKC mechanism and the chromatographic mechanism, although the major principles seem to be valid for both MEKC and CEC. The polar compounds (**1c**, **1e**) have only little affinity to the pseudostationary phase in MEKC or to the reversed phase packing material in CEC, respectively, and the more non-polar compounds such as **2a** obviously have high affinity to the stationary phase. For analytes with polarities between these two extremes, the retention behaviour may strongly depend on the separation mechanism, opening up a lot of possibilities to manipulate selectivities and to develop analytical methods solving a great number of analytical problems.

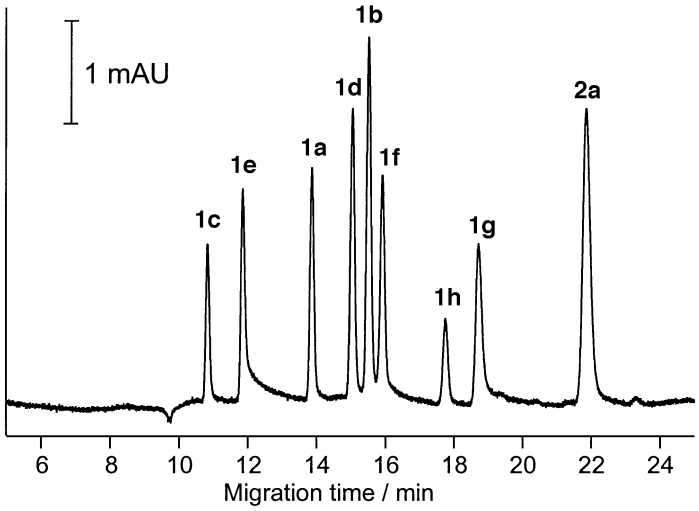


Fig. 1. Separation of pyrimidine derivatives **1a–h** and **2a** by CEC using UV detection; carrier electrolyte: 8 m*M* KH_2PO_4, *pH* 6, 60% (v/v) acetonitrile; electrokinetic injection: 5 kV, 20 s; separation voltage: 20 kV; pressure: 8 bar at the inlet and outlet side; UV detection: 230 nm; concentration: 10 mg/l each

Table 1. Linearity data and detection limits for the separation and detection of pyrimidine derivatives by CEC and MEKC, respectively

Analyte	Detection wavelength (nm)	CEC		MEKC [5]	
		Linear range (mg/l, $R^2 \geq 0.998$)	Detection limit (mg/l)	Linear range (mg/l, $R^2 \geq 0.998$)	Detection limit (mg/l)
1a	230	0.8–54	0.8	–[a]	–[a]
1b	230	0.6–62	0.6	1.5–17	1.5
1c	230	1–50	1.0	2.7–4.8	2.7
1d	230	0.6–58	0.6	1.4–15	1.4
1e	230	0.7–54	0.7	–	–
1f	240	0.6–57	0.6	1.4–14	1.4
1g	215	0.7–50	0.7	1.1–17	1.1
1h	240	0.9–64	0.9	1.9–14	1.9
2a	230	1.2–52	1.2	1.6–14	1.6

[a] Because **1a** represented the main compound in technical products investigated in this work, the linearity and detection limit were not established

To compare the CEC to the MEKC method, linearity and detection limits were determined using a signal-to-noise criterion of 3. The results are given in Table 1 for the optimum detection wavelengths. For all analytes the detection limits are better for CEC, and the linear range is higher relative to the MEKC separation

which may be due to the bigger inner diameter of the CEC capillary (100 µm) compared to the MEKC capillary (50 µm). The application of a 100 µm I.D. capillary in the MEKC method would not be possible because the electrical current would be too high, thus resulting in excessive *Joule* heating followed by evaporation of the carrier electrolyte and breakdown of the current. Drawbacks of the CEC method are the long time necessary for equilibration after changing the carrier electrolyte, somewhat longer migration times, and the higher price of the CEC capillary. Nevertheless, CEC seems to be an attractive complementary technique to MEKC.

Combination of CEC and MS

As can be seen from the preceding results, UV detection may exhibit a sufficient sensitivity, but in certain cases a more selective detection may be desirable. The hyphenation of CEC and MS raises a number of problems which are not known from the combination of other separation techniques with MS. There is no possibility to apply pressure at the outlet side of the CEC capillary with an atmospheric pressure ionization (API) interface; furthermore, a certain distance between the inlet side of the capillary in the CEC instrument and the outlet side in the MS interface has to be covered; last but not least, an MS compatible carrier electrolyte has to be employed. Solutions for these problems with special attention being paid to the practical realization are presented in the following.

Potassium dihydrogenphosphate in the carrier electrolyte used with UV detection was replaced by ammonium acetate yielding a *pH* of 6 without any adjustment. The acetonitrile content was the same (60% (v/v)). With the commercial instruments employed, the minimum length of the capillary was 60 cm which is generally too long for a reasonable CEC capillary. We therefore focused our attention on the coupling of two capillaries: the CEC capillary at a usual length (up to 40 cm), and a capillary covering the rest of the distance, in the following called transfer capillary. In our first design we used an electrically grounded stainless steel connector to connect the CEC capillary with the transfer capillary. The coupling piece contains a stainless steel frit at the end of the CEC capillary to hold the packing material in place (Fig. 2). In addition, the grounded connector serves as the second electrode (cathode) in the electrophoretic process. This setup avoids the necessity of sintering a frit at the outlet side of the CEC capillary which should help to prevent the formation of air bubbles often occurring at sintered frits when voltage is applied to the capillary [8–12].

One thing that had to be taken into account was the formation of hydrogen gas due to reduction of protons at the cathode which led to an unacceptable noise of the baseline of the MS detection. To overcome this problem, the use of an easily reducible compound as additive to the carrier electrolyte was investigated. *p*-Benzoquinone, for example, is known as a strong oxidant being able to react with nascent hydrogen forming *p*-hydroquinone (standard potential: $E^0 = 0.699$ V). The minimum concentration of *p*-benzoquinone necessary in the carrier electrolyte was estimated in dependence of the velocity of the EOF, the volume of the capillary, and the electric current. With these parameters, a quinone concentration of approximately 10 m*M* was calculated; assuming that not all of the benzoquinone

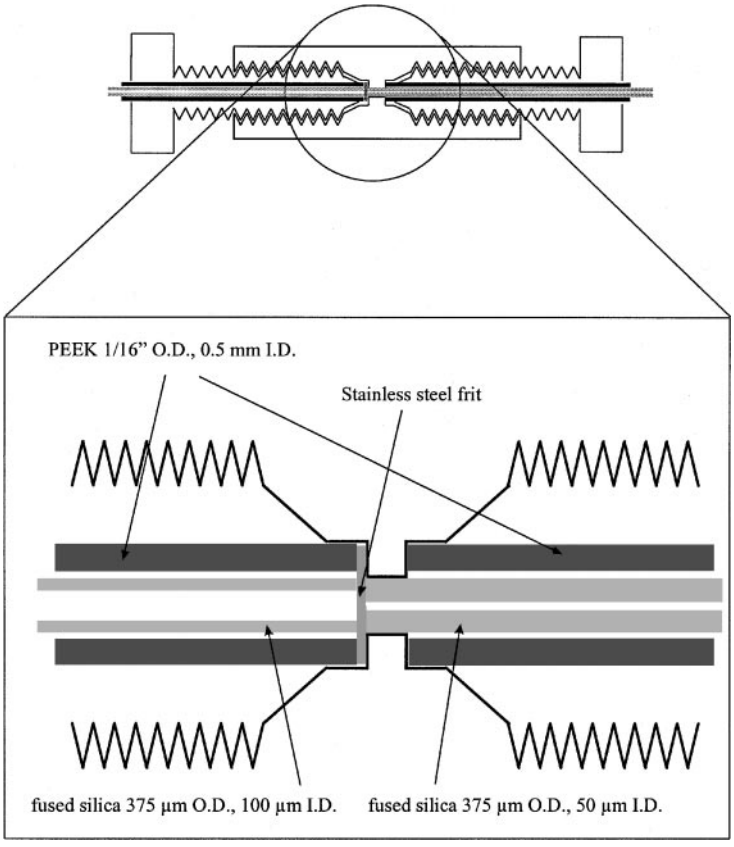


Fig. 2. Stainless steel capillary connector for the coupling of a CEC capillary with a fused silica transfer capillary

reacts at the electrode, a higher concentration was chosen (20 m*M*). As a result, the formation of hydrogen gas could be avoided.

The possible introduction of a dead volume by the coupling piece was checked by CE/MS experiments using either a traditional CE capillary with a length of 60 cm (100 µm I.D.) or the same capillary cut into to parts (35 cm and 25 cm, respectively) and reconnected with the coupling piece. The comparison was performed applying the voltage at the inlet side of the separation capillary and the outlet side of the transfer capillary (which means that the steel connector was not grounded in this experiment). As a test sample, *tris*-(hydroxymethyl)-amino-methane was injected, and the resulting number of theoretical plates was calculated as the criterion for the quality of the interface. The number of theoretical plates was approximately 9580 without and 9490 with interface. This indicates that almost no additional dead volume was introduced by the interface which was an important and encouraging result for further investigations.

In the CEC mode (with grounded connector), the separation of **1a**, **1e**, **1g**, **1h**, and **2a** was possible, although the long-time stabilities of both the CEC current and the migration times were not satisfactory. Compounds **1b**, **1c**, **1d**, and **1f** were not included in further investigations; their detection by MS was impossible because

their low basicity did not allow protonation under these conditions, and therefore no charge necessary for MS detection could be introduced.

Efforts to improve the stability of the current and migration times led to the construction of a modified design of the interface. Because the stainless steel frit as the electrode was assumed to be subject to changes of its surface (maybe causing changes of its porosity and electric conductivity due to deposition of polymerized *p*-benzoquinone), we tried to separate the frit from the electrode. The new interface consisted of a Fingertight PEEK adapter and Fingertight fittings connecting a CEC capillary and a stainless frit (for retaining the packing material), insulated from the electrode (a stainless steel transfer capillary) by a piece of a solvent filter made of poly-(vinylidene fluoride). With this setup, the electrochemical reactions took place at the grounded transfer steel capillary, and no changes of the frit were observed during the application of the voltage, thereby increasing the stability of the current and the migration times. The effect of the parobolic flow profile in the steel capillary on peak broadening was determined by CE/MS experiments in two ways: (*i*) using a fused silica capillary as the transfer capillary and applying the separation voltage at the outlet side of this capillary (thereby generating an EOF in the transfer capillary), and (*ii*) using the steel capillary as the transfer capillary (no EOF in the transfer capillary). For a CE capillary with a length of 35 cm and a transfer capillary with 25 cm, a decrease of the number of theoretical plates by about 10% could be observed.

The separation of **1a**, **1e**, **1g**, **1h**, and **2a** by CEC and MS detection in the Selected Ion Monitoring (SIM) mode using the described interface is shown in Fig. 3. The sheath liquid consisted of 2-propanol/water (80:20) containing 1% (v/v)

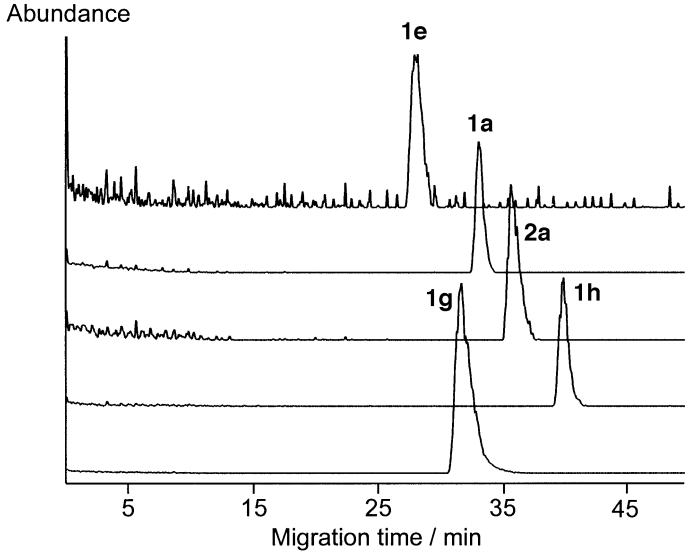


Fig. 3. Separation of pyrimidine derivatives **1a**, **1e**, **1g**, **1h**, and **2a**, by CEC using MS detection; carrier electrolyte: 8 m*M* ammonium acetate, *pH* 6, 60% (v/v) acetonitrile, 20 m*M* *p*-benzoquinone; electrokinetic injection: 5 kV, 20 s; separation voltage: 20 kV; pressure: 2 bar at the inlet side; MS detection; concentration: 20 mg/l each

acetic acid; the flow rate was 4 µl/min and the drying gas temperature was kept at 150°C at a flow rate of 2 dm^3/min. Data acquisition was performed in the positive ion mode at *m/z* values of 141 for **1e**, 156 for **1a**, 279 for **2a**, 171 for **1h**, and 197 for **1g**. Although the same packing material was employed for manufacturing of the home-packed CEC capillary used for CEC/MS experiments, the elution order is different from that obtained with the commercial CEC capillary and UV detection; with CEC/MS method, compound **1g** is eluting before **1a** (after **1h** with UV detection), and **2a** before **1h** (the last to elute with UV detection). An explanation for this behaviour could be small differences of the packing materials in terms of degree of end-capping and similar properties; detailed investigations on this phenomenon have not been carried out. Detection limits were calculated using a signal-to-noise criterion of 3. Due to a higher noise of the baseline at the *m/z* value of **1e** the detection limit for this compound is relatively poor (2.5 mg/l). **1h** and **2a** exhibit approximately the same detection limits (1.0 mg/l and 1.3 mg/l, respectively), and **1a** together with **1g** show better detection limits (0.4 mg/l and 0.1 mg/l) compared to UV detection. Although improvements in migration times and peak widths would be possible, the results obtained from these experiments clearly indicate that the combination of CEC and MS using an interface as described is possible with the same instrumental equipment employed for CE/MS.

Experimental

Instrumentation and packing procedures

A HP ^{3D}CE system (Hewlett-Packard, Palo Alto, CA, USA) was used for CEC experiments with UV detection; a Crystal 310 (Thermo CE, Franklin, MA, USA) was employed for CEC/MS experiments. MS detection was performed on a quadrupole system HP 5989B using a pneumatically assisted electrospray ionization interface HP 59987A (Hewlett-Packard) equipped with a CE-probe and a radio-frequency-only hexapole (Analytica of Branford, Branford, CT, USA). The sheath liquid (2-propanol/water (80:20) containing 1% (v/v) acetic acid) was delivered by a syringe pump (Model 22, Harvard Apparatus, South Natick, MA, USA). A CEC capillary for the use with UV detection was obtained from Hewlett-Packard (40 cm effective length, 48.5 cm total length, 100 µm I.D., 3 µm Hypersil).

Capillaries for the combination of CEC and MS were home-packed using a pneumatic pump. In the first design, the outlet end of the CEC capillary (100 µm I.D.) was attached together with a stainless steel frit to a stainless steel connector with the aid of a PEEK capillary (0.5 mm I.D.) and a stainless steel fitting and ferrule (see Fig. 2); in the second design, a PEEK Microtight Adapter (10/32 to 6/32) and a PEEK Fitting were used instead of the stainless steel connector and the stainless steel fitting. The other end of the CEC capillary was connected to a reservoir (120×4.6 mm) protruding about 1 cm into it. Hypersil 3 µm particles (Hypersil, Cheshire, UK) as the stationary phase were suspended in methanol (1% suspension) and filled into the reservoir. The suspension was then packed into the capillary at a constant pressure of 400 bar using methanol as the packing liquid. After the desired length of the capillary had been filled, the flow was stopped; when the pressure had dropped to zero, the CEC capillary was detached from the reservoir and flushed with water for about one hour. 4 cm before the end of the packed zone a frit was sintered using an electric heating element made from a few turns of resistance wire. The conditioning procedure was performed using the CE instrument at an applied pressure of 1.5 bar at the inlet side and a voltage of 5 kV against ground.

Chemicals and materials

Ammonium acetate, acetic acid, acetonitrile, 2-propanol, methanol, and potassium dihydrogenphosphate were purchased from Merck (Darmstadt, Germany). Fused silica capillaries with 375 µm O.D. were obtained from Polymicro Technologies (Phoenix, AZ, USA). A stainless steel capillary (190 µm O.D., 50 µm I.D.) was obtained from Hamilton (Reno, NV, USA), and a PEEK Microtight Adapter (10/32 to 6/32) as well as Micro Fingertight Fittings and PEEK sleeves from Upchurch Scientific (Oak Harbor, WA, USA).

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