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Migration behavior of weakly retained, charged analytes in voltage-assisted micro-high performance liquid chromatography

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Abstract

The application of voltage in micro-high performance liquid chromatography (micro-HPLC) creates a system where separation is governed by a hybrid differential migration process, which entails the features of both HPLC and capillary zone electrophoresis (CZE), i.e., chromatographic retention and electrophoretic migration. In this paper, we use our previously published approach to decouple these two mechanisms via analysis of the input data for estimation of electrokinetic parameters, such as conductivity, equivalent lengths, mobilities and velocities. Separation of weakly retained, charged analytes was performed via voltage-assisted micro-HPLC. Contrary to conclusions from data analysis using the conventional definitions of the retention factor, it is shown that our approach allows us to isolate the "chromatographic retention" component and thus, investigate the "modification" of the retention process upon application of voltage in micro-HPLC. It is shown that the traditional approaches of calculating retention factor would erroneously lead to conclusion that the retention behavior of these analytes changes upon application of voltage. However, the approach suggested here demonstrates that under the conditions investigated, most of the charged analytes do not show any significant retention on the columns and that all the changes in their retention times can be attributed to their electrophoretic migration.
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1. Introduction

Voltage-assisted micro-high performance liquid chromatography (micro-HPLC) performs similar to another analytical tool, capillary electrochromatography (CEC) that uses packed capillary columns with electrosmotically driven mobile phase at high electric field strength. These high-resolution separation techniques have gained interest due to their potential to offer selectivity different to that observed in HPLC and CZE [1–7]. Voltage-assisted micro-HPLC seeks to combine the advantages of CEC (i.e., high efficiencies, different selectivity) with that of HPLC (i.e., use of LC type stationary phases of known properties, stable operating platform and good hydrodynamic flow velocities) [8–11].

Recently, we proposed an approach to "decouple" chromatographic retention and electrophoretic migration to allow us to calculate the electrochromatographic retention factor and thus, investigate the modification of the retention process in CEC [12]. This methodology has been successfully applied for characterization of changes in the retention of neutral and charged sample components, under otherwise identical conditions of stationary and mobile phase [13] and has been used by several researchers to elucidate the separation mechanism in CEC [14–18]. Bedair and El Rassi evaluated retention of neutral and charged solutes on cationic stearyl-acrylate monoliths and the separation of water-soluble proteins and membrane proteins [14]. They found that

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while moderate and strong bases showed migration behavior dominated by their relatively strong electrophoretic mobility, separations could be achieved at low pH when minimal electrostatic interactions between proteins and the cationic sites were observed. In other recent reports, Ohyama et al. investigated the use of a novel mixed-mode stationary phase for CEC [15,16]. Their data confirmed that the separation mechanism in CEC with a 3-(4-sulfo-1,8-naphthalimido) propyl-modified silyl silica gel (SNAIP) stationary phase was a hybrid of electrophoretic migration and chromatographic retention involving hydrophobic, electrostatic as well as π–π interactions. Progent and Taverna evaluated retention behavior of peptides in CEC using an embedded ammonium in dodecacyl stationary phase [17]. Their study pointed out that the low electrochromatographic retention of peptides in CEC observed by them could not be explained by electrostatic repulsion but may be due to the vigorous electrosmotic flow (EOF).

This paper will focus on the effect of voltage on separation of weakly retained charged compounds via micro-HPLC. The above-mentioned methodology will be used to extract the electrochromatographic retention factor from the electrokinetic data. This will allow us to monitor the modification of the retention process upon application of the applied voltage. Three differing stationary phase materials were assessed—two were monolithic in nature and one was a particulate material of 12 μm particle size. All three phases were C18 derivatized and non-endcapped.

2. Theory

Migration velocity, u_m, of a charged sample component in a voltage-assisted micro-HPLC system can be expressed by the sum of the velocity of the mobile phase due to pressure driven flow, $u_{o,pr}$, velocity of the mobile phase due to electro-driven flow, $u_{o,eo}$, and the electrophoretic velocity of the migrant of interest, u_{ep}, multiplied by the retardation factor, $1/(1+k'')$, in the following manner [1,12,18,19]:

$$u_m = \frac{u_{o,pr} + u_{o,eo} + u_{ep}}{1 + k''} \quad (1)$$

where k'' is the measure of chromatographic retention under conditions of the voltage-assisted micro-HPLC experiments, i.e., the retention factor in voltage-assisted micro-HPLC. For the case of a completely packed column, Eq. (1) can be rewritten as follows [12,13,18]:

$$k'' = \frac{(L/t_o) + (\mu_{ep}(L/L_e)(V/L_e))}{L/t_m} - 1 \quad (2)$$

where L is the length of the column; μ_{ep}, the electrophoretic mobility of the analyte; V, the applied voltage; t_o, the migration time of the EOF marker and t_m, the migration time of the analyte. Further, L_e is the equivalent length of the column and can be expressed in the following manner [18]:

$$L_e = L\sqrt{\frac{\sigma_{open}}{\sigma_{packed}}} \quad (3)$$

where σ_{open} and σ_{packed} are the values of the conductivity of an open capillary and a packed column using identical mobile phase [18].

The k'' calculated from Eq. (2) in this manner estimates the magnitude of retention in voltage-assisted micro-HPLC due to reversible binding of the analyte to the stationary phase. As will be shown later and unlike in HPLC, k'' in voltage-assisted micro-HPLC is found to change with the applied electric field strength. It should be emphasized that in Eq. (2), all the parameters need to be evaluated under conditions used in the voltage-assisted micro-HPLC experiments besides the electrophoretic mobility that is obtained from separate CZE measurements using the mobile phase used in HPLC using the following expression:

$$\mu_{ep} = \frac{LL_d}{V}\left(\frac{1}{t_{m,CE}} - \frac{1}{t_{o,CE}}\right) \quad (4)$$

where L_d is the detection length of the CZE capillary; L, the total length of the CZE capillary; $t_{o,CE}$, the migration time of the EOF tracer and $t_{m,CE}$, the migration time of the analyte in CZE.

Another definition for retention factor that has been used for voltage-assisted micro-HPLC is that following the chromatographic formalism [16,20,21] and is expressed as

$$k' = \frac{t_m - t_o}{t_o} \quad (5)$$

However, since $t_m < t_o$ for basic analytes, k' calculated using Eq. (5) is negative. Also, since the k' values include chromatographic retention with the electrophoretic migration, they are also devoid of any thermodynamic significance.

3. Experimental

3.1. Chemicals and reagents

Chemicals, test analytes and reagents were obtained from sources as previously described [22], whilst the structures, pK_a and log D values of the analytes have also been previously reported [22].

A 12 μm Hypersil CEC Basic C18 material (Thermo Electron Corporation, Runcorn, UK) was packed into 0.1 mm i.d., capillaries (33.5 cm length total length and 25.0 cm effective length) as previously described [23]. The Tanaka hybrid C18 silica monolithic capillary was prepared with sol–gel technology using a mixture of equal amounts of methyltrimethoxysilane (MTMOS) and tetramethoxysilane (TMOS) [24]. The column was 0.1 mm i.d. (34.8 cm length total length, 26.3 cm effective length, non endcapped) and donated by Prof. Tanaka (Department of Polymer Science and Engineering, Kyoto Institute of Technology, Kyoto, Japan). The standard C18

silica monolithic capillary was prepared with sol–gel technology using TMOS [24]. It was 0.1 mm i.d. (34.7 cm length total length, 26.1 cm effective length, non endcapped) and donated by Dr. Lubda (Merck KGaA, Darmstadt, Germany).

3.2. Instrumentation

For micro-HPLC, voltage-assisted micro-HPLC and CE separations, an Agilent ^{3D}CE instrument was used with ChemStation v. 6.04 CE(C) software (Agilent Technologies, Cheadle, UK). The ^{3D}CE instrument has the capability of pressurising the inlet and outlet vials to 1.2 MPa (provided by a N_2 cylinder).

3.3. Buffer and mobile phase preparations for micro-HPLC, voltage-assisted micro-HPLC and CE separations

KH_2PO_4 (20 mM), pH 2.7 was prepared by dissolving the appropriate quantity of buffer salt in ~900 mL of pure water before adjusting the pH of the solution using orthophosphoric acid as required. The volume was then made to 1000 mL before a mobile phase composition of 1:1 (v/v) ACN: 20 mM buffer was prepared for the separations.

3.4. Capillary electrophoresis conditions

The electrophoretic mobility of bases and acids were determined on fused silica capillaries (0.1 mm i.d., 64.3 cm length total length, 55.8 cm effective length) from Composite Metals Ltd. (Hallow, Worcestershire, UK). The capillaries were preconditioned by flushing with 1 M NaOH for 10 min, then H_2O for 10 min, followed by 0.1 M NaOH for a further 10 min. Conditioning between injections during a run comprised of flushing with 0.1 M NaOH for 2 min, then with mobile phase for 3 min. Mobile phase conditions consisted of 1:1 (v/v) ACN:20 mM KH_2PO_4, pH 2.7, an applied voltage of 20 kV, cartridge temperature of 20 °C, detection wavelength of 210/254 nm and samples were hydrodynamically injected onto the capillaries (0.005 MPa/5 s). Thiourea was used as the EOF marker.

3.5. Capillary packing and evaluation conditions

The particulate capillaries were packed, equilibrated and tested (acceptability based upon linear velocity and efficiency of designated peaks) as reported previously [23]. Both monolithic capillaries were equilibrated by applying sequential voltages of 5, 10 and 15 kV for 30 min before commencing the experiments.

3.6. Capillary window fabrication

To ensure a high signal to noise ratio for UV detection, the polyimide coating on the fused silica capillaries was removed. This was achieved for bare fused silica capillaries by using a window burner. For the detection through the packed/monolithic bed of the capillaries the coating was removed using a plastic tubing filled with aqueous ammonia solution (37%, w/v), which was sealed with rubber bungs; the capillary was pierced through the tubing before filling so that the polyimide coating of the capillary was exposed to the ammonia solution within the 2 mm i.d. of the tubing. After around 24 h, a suitable window was formed.

All capillaries used had their polyimide coating removed at their inlet and outlet ends of the capillary prior to analysis to avoid polyimide swelling. This was especially important when the mobile phase contained a high proportion of organic modifier.

3.7. Conditions used in micro-LC and voltage-assisted micro-LC

Separations utilising the pressure mode with the Agilent ^{3D}CE instrument with packed capillaries were called micro-LC separations. The inlet vial was pressurized at 1.0 MPa thus "pushing" the analytes through the capillary. Detection, injection, temperature and other conditions were similar to those described in CZE experiments.

Voltage-assisted micro-LC combined pressure flow with electrodriven flow through packed capillaries. The inlet vial was pressurized at 1.0 MPa and in addition voltages were applied with increasing 1 kV steps from 1 to 10 kV.

4. Results and discussion

4.1. Measurement of electrokinetic parameters

Experiments were performed as described above and several parameters were monitored during the separations. These included capillary dimensions, applied voltage, current, pressure drop, temperature and time of migration for the analytes and for an inert and neutral marker. Experiments were performed in duplicate and the average was used for the calculations presented in the following sections.

4.2. Calculation of electrophoretic mobility

Electrophoretic mobility was calculated using the expression shown in Eq. (4) and data obtained from CZE measurements. Experiments were done with mixtures of a single analyte and the inert marker at one time and the results are shown in Table 1. As expected, the conductivity of the open capillary, which is a function of the mobile phase that was kept identical for all the experiments, is constant for the different experiments. However, some run-to-run variability (approximately ±10%) is seen in the EOF velocity and mobility measurements amongst these experiments. It is seen that the analytes are a mixture of positively and negatively charged components. Further, it is seen that most of

Table 1
Calculation of electrophoretic mobilities of the various analytes using Eq. (4) and data from CZE measurements

Parameter (units)	Nortriptyline	Procainamide	Benzylamine	Diphenhydramine	Remacemide	Terbutaline	4-Chlorobenzoic acid	2,5-Dihydroxybenzoic acid	4-Hydroxymandelic acid
Cond open ($\Omega^{-1}\,m^{-1}$)	0.085	0.085	0.085	0.085	0.085	0.085	0.083	0.083	0.083
EOF velocity ($10^{-3}\,m\,s^{-1}$)	0.67	0.57	0.55	0.66	0.55	0.62	0.61	0.61	0.61
EOF mobility ($10^{-8}\,m^2\,s^{-1}\,V^{-1}$)	2.14	1.83	1.76	2.11	1.77	1.99	1.96	1.96	1.96
Electrophoretic mobility ($10^{-8}\,m^2\,s^{-1}\,V^{-1}$)	2.31	2.34	3.46	2.47	2.18	1.97	−0.12	−1.35	−0.36

Experimental conditions: total capillary length: 64.3 cm; detection length: 55.8 cm; capillary diameter: 100 μm; applied voltage: 20 kV; pressure applied: 0 MPa; EOF marker: thiourea; temperature: 20 °C; mobile phase: 50:50 (v/v) ACN: 20 mM KH_2PO_4 buffer pH 2.7.

the sample components are very strongly charged for example $\mu_{ep} \approx 2\mu_{eo}$ for benzylamine, $\mu_{ep} \approx 1.5\mu_{eo}$ for diphenhydramine, $\mu_{ep} \approx 1.4\mu_{eo}$ for nortriptyline and procainamide, $\mu_{ep} \approx 1.3\mu_{eo}$ for remacemide and $\mu_{ep} \approx 1.2\mu_{eo}$ for terbutaline. The three acids showed a negative charge of about $\mu_{ep} \approx -0.7\mu_{eo}$ for 4-chlorobenzoic acid, $\mu_{ep} \approx -2.3\mu_{eo}$ for 4-hydroxymandelic acid and $\mu_{ep} \approx -0.8\mu_{eo}$ for 2,5-dihydroxybenzoic acid.

4.3. Calculation of electrical conductivity and equivalent length

Electrical conductivity and equivalent length of the various columns was estimated using the current and flow measurements performed using voltage-assisted micro-HPLC and Eq. (3). The data is presented in Table 2. The issue of Joule heating in CE and voltage-assisted micro-HPLC is known to have a significant impact on current and flow measurements and it is important to take these effects into account when performing calculations such as these [25]. For this reason, electrical conductivities were measured at 1, 10, 20 and 30 kV. In absence of Joule heating, electrical conductivity should remain constant [25]. We found that the Joule heating was significant at 20 and 30 kV. However, between 1 and 10 kV Joule heating was minimal as confirmed by the linear Ohm's plot and hence for the calculations in Table 2, data obtained at 10 kV were used.

As expected, the current is much lower for packed columns in comparison with the open capillary. This is due to the lower porosity of the columns resulting in the ions following a more tortuous path while traveling through the column [18]. This translated to a lower conductivity of the packed columns. It is seen that the Hypersil CEC C18 Base stationary phase has approximately half the conductivity as compared to the Merck Standard C18 monolithic stationary phase, with the Tanaka Hybrid C18 monolithic stationary phase in the middle. The equivalent lengths of the packed columns follow the same sequence for the three stationary phases, namely Merck Standard C18 Monolith (40 cm), Tanaka Hybrid C18 Monolith (44 cm), and Hypersil CEC C18 Base (55 cm).

4.4. Effect of applied voltage on flow velocity

Fig. 1 shows a plot of residence time of the inert and neutral marker (thiourea) with increasing applied voltage for the three columns. In general, it is seen that the EOF is minimal in comparison to the pressure driven flow suggesting that the stationary phase particles are weakly charged under the experimental conditions chosen. Only for the case of the Hypersil column, it is found that the EOF is significant ($u_{o,eo} \approx 0.3u_{o,pr}$). The two monolithic phases would be expected to exhibit minimal ion exchange interactions with charged analytes ($u_{o,eo} \approx 0.0u_{o,pr}$) and the dominant separation processes would be partitioning and electrophoretic in nature. In view of these results, we decided to account

Table 2
Calculation of electrical conductivities and equivalent lengths for the three CEC columns

Parameter (units)	Open capillary	Merck Standard Monolith C18	Hypersil CEC Base C18 12 μm	Tanaka Hybrid Monolith C18
Total length (cm)	64.3	34.7	33.5	34.8
Detection length (cm)	55.8	26.1	25.0	26.3
Capillary diameter (μm)	100	100	100	100
Applied voltage (kV)	20	10	10	10
Current (μA)	20.80	14.50	7.55	11.80
t_o (inert, neutral) (min)	14.025	7.304	10.937	12.833
Conductivity open (Ω^{-1} m^{-1})	0.085			
Conductivity packed (Ω^{-1} m^{-1})		0.064	0.032	0.052
Conductivity ratio		0.815	0.440	0.679
Equivalent length (10^{-2} m)		40.004	54.472	44.409

Applied voltage: 10 kV. Rest of the conditions same as in Table 1.

for the changes in t_o for the calculations in the following sections.

4.5. Measurement of electrochromatographic retention factor for voltage-assisted micro-HPLC

Separation of basic and acidic compounds by CEC has been studied by several authors recently [13,14,17,26–30]. Nakagawa et al. recently investigated the effect of alternating voltage on the separation of organic acids by CEC [28]. They found that the retention factor varied according to both the amplitude and the frequency of an applied alternating voltage and the variation greatly depended on the kinds of sample solutes and packing materials. In this section, we attempt to present an approach to elucidate the effect of applied voltage on the separation of analytes by voltage-assisted micro-HPLC. Progent and Taverna found that the retention factor of three peptides decreased with increasing voltage and hypothesized that ion-exchange is not the predominant retention mechanism [17]. Ru et al. investigated the separation of eighteen amino-acid derivatives by pressurized gradient CEC and reported that an increase in applied voltage resulted in improved sensitivity and resolution of the separation, as well as changes in the order of elution [29]. They suggested that the results could be explained by adsorption of amino acids on the porous C18 column that was used for separation. Recently, Fu et al. published separation of peptides in hydrophilic interaction CEC [30]. They found that the retention of all the peptides decreases with increasing applied voltage, although the elution order remained the same. They concluded that the decrease in retention could result from the Joule heating that occurred in the system. In the following, we evaluate the effect of applied voltage using the methodology that has been described in Section 2.

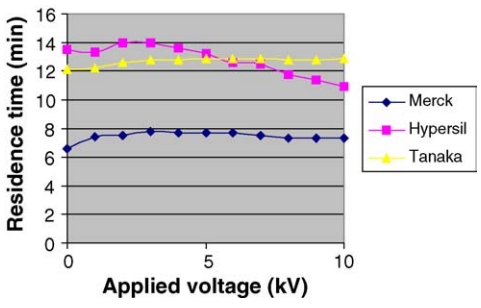

Fig. 1. Changes in residence time of the inert and neutral marker (thiourea) with increasing applied voltage. Experimental conditions as listed in Tables 1 and 2.

Recently, we published on retention of basic compounds in HPLC and capillary electrochromatography [13]. Some of these compounds were strongly retained on the stationary phase with k' values up to >6. The chromatographic and electrochromatographic retention factors (k' and k'') for these compounds in HPLC and CEC are plotted in Fig. 2. It was observed that there were significant differences in selectivity between the two modes and this was due to an interplay of the effect of the organic solvent and the electric field on the analyte (and its physicochemical properties) and the structure, organization and partitioning with the stationary phase. In this paper, we focus on the migration behavior of the weakly retained, charged analytes in voltage-assisted HPLC.

Figs. 3–5 present plots showing the effect of applied voltage on electrochromatographic retention factor measured according to Eq. (2) (k'') and that estimated according to the chromatographic formalism using Eq. (5) (k') for the Merck Standard C18 Monolith, Hypersil CEC Base C18, and Tanaka Hybrid C18 stationary phases, respectively. It must be pointed out that due to the errors in measurements of the current and migration times, the calculations of the k'' are only approximate and these errors can sometimes lead to slightly negative k'' values. Several key trends, however, can be seen from a comparison of the k'' and k' plots for the different stationary phases.

For the case of basic analytes, the k' values are increasingly negative with greater applied voltage for most of the analytes and do not yield any useful insight into the separation process [13,14]. In contrast, the k'' values are positive and close to zero for most cases. It can be concluded that these analytes do not show very significant retention under the chosen conditions and the differences in retention time is almost solely due to the differences in their electrophoretic mobilities.

For the case of acidic analytes, the k' values are positive and for the case of 2,5-dihydroxybenzoic acid, k' increases with applied voltage. It appears as if the acidic analytes

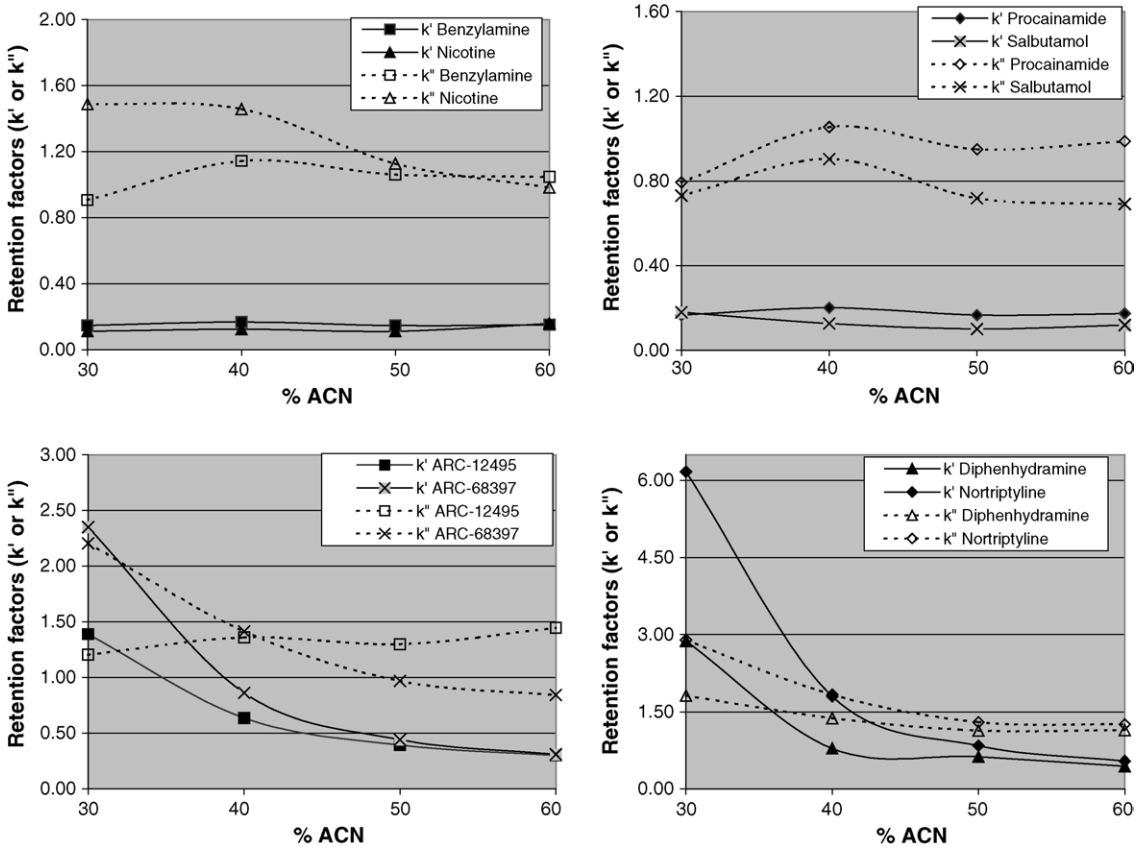

Fig. 2. Retention behavior of basic solutes in HPLC and capillary electrochromatography (CEC). Adapted from Ref. [13].

are retained on the columns. However, once again after the calculation of the k'' values, it is seen that for most cases chromatography retention under the chosen conditions is not significant and the differences in retention time is almost solely due to the differences in their electrophoretic mobilities. The only exception to this is 4-chlorobenzoic acid, which shows positive chromatographic retention with nearly all phases. Also, the retention is seen to be stronger with the Hypersil CEC C18 Base and the Tanaka Hybrid C18 monolithic stationary phases versus the Merck Standard C18 monolithic stationary phase.

It must be emphasized that for all the three cases illustrated in Figs. 3–5, the k' plots using retention factor measured using the chromatographic formalism of Eq. (5) do not provide any insight into the separation process and the trends seen could be misleading.

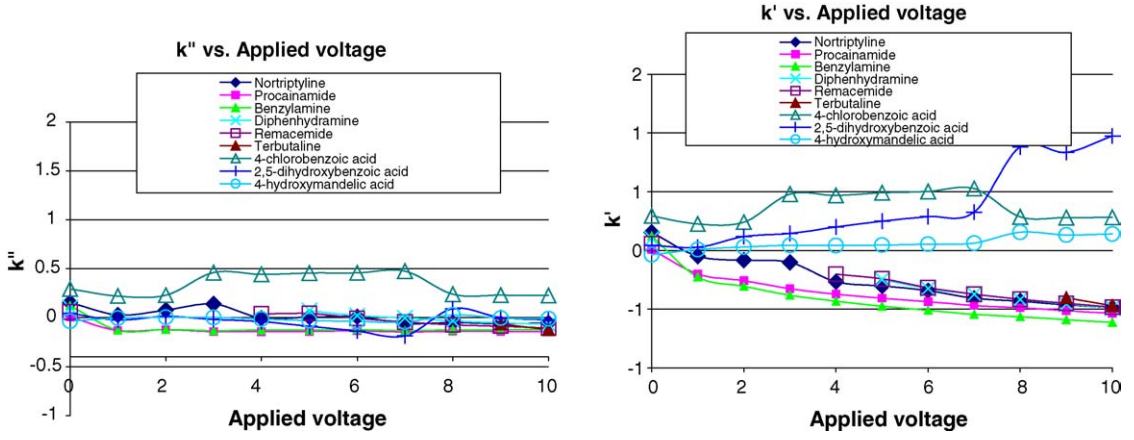

Fig. 3. Effect of applied voltage on the electrochromatographic retention factor measured according to Eq. (2) (k'') and that according to the chromatographic formalism shown in Eq. (5) (k') for the Merck Standard C18 Monolith stationary phase.

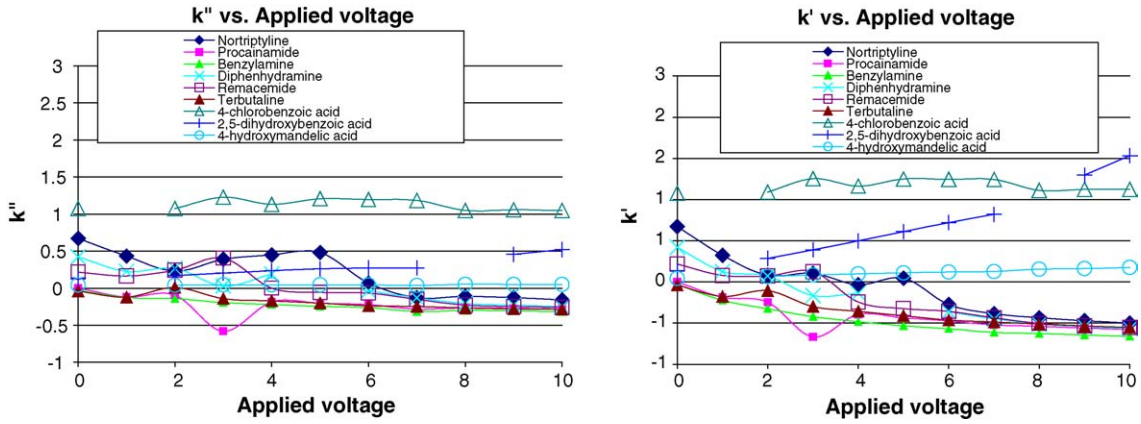

Fig. 4. Effect of applied voltage on the electrochromatographic retention factor measured according to Eq. (2) (k'') and that according to the chromatographic formalism shown in Eq. (5) (k') for the Hypersil CEC Base C18 12 μm stationary phase.

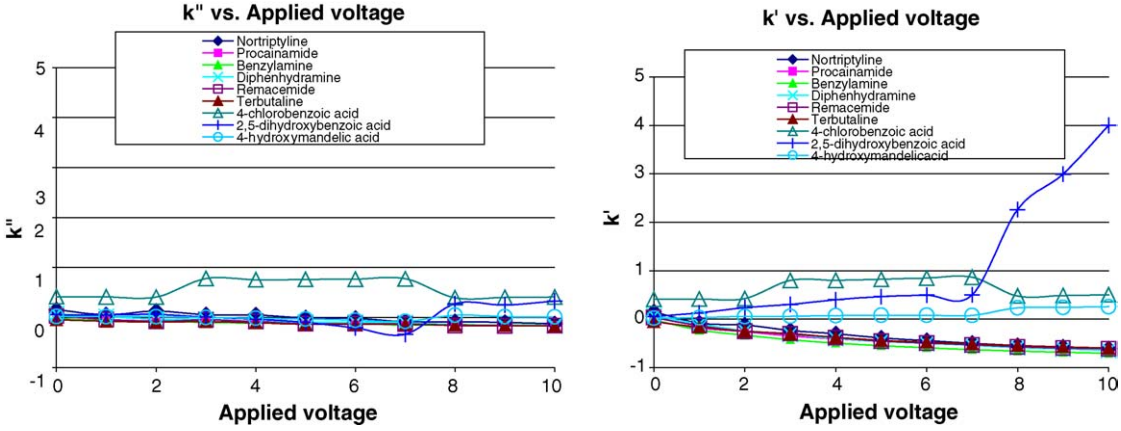

Fig. 5. Effect of applied voltage on the electrochromatographic retention factor measured according to Eq. (2) (k'') and that according to the chromatographic formalism shown in Eq. (5) (k') for the Tanaka Hybrid C18 Monolith stationary phase.

5. Conclusions

In this paper, we use our previously published approach to decouple chromatographic retention from electrophoretic migration for a variety of weakly retained, charged analytes on different columns used in voltage-assisted micro-high performance liquid chromatography. It is shown that the calculation of the electrochromatographic retention factor, k'', is very useful in characterization of the separation system. This approach also allows us to investigate the "modification" of the retention process in voltage-assisted micro-high performance liquid chromatography. It is found that under the conditions investigated, most of the charged analytes do not show any significant retention on the columns and that all the changes in their retention times can be attributed to their electrophoretic migration.

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