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Flow variation as a factor determining repeatability of the internal standard-based qualitative and quantitative analyses by capillary electrophoresis

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ABSTRACT

The use of migration times and peak areas referred to another sample component – internal standard, brings many benefits in improving reliability of capillary electrophoresis. However, it is quite commonly overlooked that despite relative migration time and peak area ratio are more stable than the absolute values upon alteration in the flow rate, some shift should always be expected. The present work offers a new look at this analytically-important issue. We have derived a simple model allowing to estimate the magnitude of error for the selected pair of molecules of known mobilities upon the given flow alteration. Then, we have confronted the theoretical predictions with the experimental results obtained for the model sample separated in various flow conditions reached by the external pressure manipulation, including several internal standards of different mobilities. A good agreement has been obtained, pointing out that the magnitude of error may be large even for the seemingly "good" internal standards. Several potentially useful means have been tested to address this issue: the use of electrophoretic mobilities and electrophoretic mobility ratios instead migration times in the qualitative analysis, and performing time-correction of peak area ratios, or alternatively, transformation of electropherograms from the time-related scale into the electrophoretic mobility-related scale in the quantitative analysis. We have also considered some additional factors. The results may be of interest for all users dealing with the development and optimization of analytical methods using capillary electrophoresis.

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1. Introduction

A relatively high variation of migration times, expressed by the run-to-run relative standard deviation values (RSDs), is commonly deemed as the major weakness of the capillary electrophoresis (CE) technique [1–4]. For comparison, in high performance liquid chromatography (HPLC) the RSD values below 1% are much more common than in CE. This effect is caused by the electroosmotic flow (EOF) fluctuation which occurs when components of the sample or buffer interact and adsorb onto the inner capillary surface, changing their physicochemical characteristics. The resulting shifts in migration times, especially during long sequence of separations, reach oftentimes several minutes or even more. This hampers a correct peak identification and qualitative analysis using the absolute migration times. In addition, unlike HPLC peak areas in CE are positively correlated with migration order because the time during which the sample passes the detector depends on its velocity, which in CE is by definition different for various analytes. This rises a need for performing normalization by using the time-corrected peak areas, i.e. absolute areas divided by respective migration times [5–8].

The use of internal standards (ISs), i.e. the solutes present in the sample material to which the analyte's migration time or peak area are referred, is a popular way for enhancing precision and reliability of CE [1–4,9]. Relative migration times are independent of viscosity, and their utilization reduces also the imprecision of peak tracking following from instability of flow, electric field and temperature. Peak area ratios, in turn, eliminate the important errors related to injection repeatability, sample evaporation, alteration of the capillary's optical parameters, and many other effects [9]. Quantification based on peak area ratios is also often claimed to correct the drift in migration time and the resulting drop in the method's precision. The textbooks and manuals devoted to method developing with CE mention about a general requirement for choosing IS characterized by a similar mobility to the analyte, although virtually never indicating an acceptable margin of tolerance [1–4,9].

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This requirement is caused by the fact that any EOF variation entails disproportional shifts in migration velocity for the analyte and IS, and the following change of migration time ratio or peak area ratio increases with the difference in electrophoretic mobility of both solutes [10]. Unfortunately, this effect is rarely explained in a way appropriate for less experienced users, and maybe due to this fact it is quite commonly overlooked when applying relative migration times (identification) or peak areas (quantification). Insofar as the problem related to qualitative analysis has been effectively solved many times, e.g. by using electrophoretic mobility as the qualitative parameter independent of EOF change [10–24], a need for applying time correction of peak area ratios or other alternative ways for preventing this effect in the quantitative analysis has been reported only several times up till now [4,6,25,26]. Besides the normalization in respect to migration time, another potential method for improving precision of the absolute peak areas is the transformation of electropherograms from the conventional time-related scale into the alternative electrophoretic mobility scale. It has been studied by several research groups [11–15,22,23], but the question about its actual usefulness in the IS-based quantifications remains still open.

Although the fundamental principles of CE were intensively studied a long time ago, this paper is aimed at providing a novel look at some basic effects. Firstly, a simple model allowing to predict the magnitude of error made when utilizing migration time ratio or peak area ratio in the conditions of a changeable flow has been derived, lacking in the literature. Secondly, an original approach to the experimental investigation of this analytically-important effect has been proposed, based on the use of positive or negative external pressure of different strength to simulate flow variation. In addition, a specially designed model sample was used, containing three potential ISs of the different mobilities. This allowed us to examine the errors with regard to the wide range of flow shift, and the various distances between the analyte and IS peaks. Finally, we have investigated and critically compared various means for addressing low repeatability of relative migration times and peak area ratios: the use of electrophoretic mobility and electrophoretic mobility ratios in the qualitative analysis, and time-corrected peak area ratios and electropherograms transformed from the time scale into the electrophoretic mobility scale in the quantitative analysis. We have also studied the role of a voltage ramp time when calculating mobility and performing transformations into the mobility scale, and the positive correlation between peak area and migration time in the electrophoretic mobility scale, opposite to the situation met in the time scale. The results may be of interest for all CE users dealing with the development of new analytical methods and searching for more efficient data handling procedures.

2. Materials and methods

2.1. Materials

All compounds: 3-hydroxycoumarin, 4-hydroxycoumarin, 6,7-dihydroxycoumarin and 7.8-dihydroxy-4-phenyl-coumarin were supplied by Sigma-Aldrich (St. Louis, MO, USA). All other chemicals were supplied by Avantor Performance Materials Poland. S. A. (Gliwice, Poland). All solutions were prepared in the deionized water (MilliQ, Merck-Millipore Billerica, MA, USA) and filtered through the 0.45 μm regenerated cellulose membrane, then degassed by centrifugation. 4-hydroxycoumarin was used as the analyte (AN), 3-hydroxycoumarin was used as the first internal standard (IS1), 6,7-dihydroxycoumarin was used as the second internal standard (IS2), while 7.8-dihydroxy-4-phenyl-coumarin was used as the third internal standard (IS3). The concentration of AN in the sample was 100 μg/mL, whereas the concentration of all ISs 50 μg/mL. They were dissolved in the methanol mixed with the borate buffer ($Na_2B_4O_7{\cdot}10H_2O$/HCl) of pH 9.5 and 50 mM ionic strength, in the 1:1 ratio (v/v). Dimethyl sulfoxide (DMSO) was used as the electroosmotic flow (EOF) marker to enable calculation of electrophoretic mobility, its final concentration in the sample was 0.2% (v/v).

2.2. Experimental conditions

Experiments were performed on the P/ACE MDQ Capillary Electrophoresis (CE) System (Beckman-Coulter, Brea, CA, USA) equipped with a diode array detector (DAD). The detection wavelength was 200 nm. The unmodified bare fused-silica capillary was used. It was of 60.1 cm total length, 50.1 cm effective length, 50 μm internal diameter and 365 μm external diameter. Between runs the capillary was rinsed with 0.1 M NaOH for 2 min, and background electrolyte (BGE) for 2 min. Before the first use of the capillary at a working day: methanol for 5 min, 0.1 M HCl for 3 min, deionized water for 3 min, 0.1 M NaOH for 10 min, and BGE for 10 min were applied. For the fresh capillary conditioning, the latter sequence was used but the duration of each individual step was doubled. Sample injection was conducted using the forward pressure of 3.45 kPa (0.5 psi) for 5 s. During separations the separation voltage of 30.0 kV (normal polarity) was applied, using the external pressure equaling to +3.45, +1.38, 0, −1.38 and −2.76 kPa (+0.5, +0.2, 0, −0.2 and −0.4 psi). The values of pressure applied were low enough to maintain the peak shape despite the different profiles of the pressure- and voltage-driven flows. The current values were much below 50 μA, minimizing the impact of Joule heating on the measured mobilities. The temperature of cooling was set at 25 °C. Each measurement was repeated 6 times. BGE was composed of the borate buffer ($Na_2B_4O_7{\cdot}10H_2O$/HCl) of 50 mM ionic strength and pH 9.5. Taking into account the pK_a values determined experimentally in our previous work [27], at this pH all compounds are totally ionized and the potential minor variation of pH does not affect their mobilities. Function fitting was performed using the Origin 9.1. software (OriginLab Corporation, Northampton, MA, USA).

2.3. Electrophoretic mobility calculation

The electrophoretic mobility values (μ_{ep}) were calculated using two approaches. The first conventional approach employed Eq. (1):

$$\mu_{ep} = \frac{L_{tot}L_{eff}}{U_{nom}}\left(\frac{1}{t_{obs}} - \frac{1}{t_{eof}}\right) \quad (1)$$

where μ_{ep} is the electrophoretic mobility ($m^2V^{-1}s^{-1}$), L_{tot} and L_{eff} are the total and effective capillary lengths (m), U_{nom} is the nominal (programmed) separation voltage (V); t_{obs} is the observed migration time of analyte (s); while t_{eof} is the time measured for the neutral marker of electroosmotic flow (EOF).

Eq. (1), despite used very commonly in the literature, gives mobility values burdened with the error resulting from the voltage ramping effect [28–30], i.e. a gradual increase of voltage at the beginning of separation, lasting around 0.1–0.2 min, which causes a deviation of the average electric field strength from its nominal value used in Eq. (1). This effect can be easily overcome by using the second approach to electrophoretic mobility calculation [27,28], based on Eq. (2):

$$\mu_{ep(ramp)} = \frac{L_{tot}L_{eff}}{U_{nom}}\left(\frac{1}{t_{obs} - 0.5t_{ramp}} - \frac{1}{t_{eof} - 0.5t_{ramp}}\right) \quad (2)$$

where $\mu_{ep(ramp)}$ is the ramping-corrected electrophoretic mobility, and t_{ramp} is the voltage ramp time set up in a software. In this experiment t_{ramp} was 12 s (0.2 min).

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