Potential sources of error in capillary electrophoresis–inductively coupled plasma mass spectrometry for chemical speciation†

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The distribution concentration of chemical species in a sample is dictated by the physical and chemical properties of the matrix. As such, when a sample is pre-treated, in any way, there is a potential for redistribution of homologous species. The extent of this analyte redistribution is determined by both thermodynamic properties of species (e.g., changes in concentrations of species according to their equilibrium expressions) and kinetic properties (e.g., the rate of the reactions compared with the duration of sample preparation and analysis). The redistributions of analyte species as a function of several experimental parameters (e.g., time, solution pH, injection methods and calibration methods) are illustrated in this paper. Whereas rabbit metallothionein protein showed a stability of more than a few days under certain storage conditions, coenzyme-B12 was rapidly degraded in less than 2 h. pH studies showed that the migration of free Cd2+ ions in rabbit metallothionein was not significantly affected unless the pH of the solution exceeded the solubility limit of the metal hydroxide. However, pH-sensitive compounds such as vitamin B12 showed significant changes in the migration time and analyte composition. The injection studies suggested that electrokinetic injection may produce biased results, in favor of species that have higher electrophoretic mobility. Hydrodynamic injection will produce a result that is more representative of the initial sample composition.

Keywords: Speciation; capillary electrophoresis; elemental mass spectrometry; cyanocobalamin; rabbit metallothionein; coenzyme-B12

The rapid expansion of the chemical speciation field is due, in part, to the development of sensitive and selective coupled analytical techniques. Using modern separation procedures along with element specific detection, it is now possible to evaluate quantitatively the different forms of a specific element in a relatively complex matrix. However, for these techniques to become universally accepted, one must demonstrate measurement traceability. Traceability is an unbroken chain of calibration events that must connect the measurement process to the fundamental units; for elemental speciation, it must also be verified that the chemical species have been preserved. The errors due to coupling of separation techniques with element-specific detectors and the changes in species concentration as a function of thermodynamic stability of a sample and kinetic stabilities of a sample for different techniques have been discussed previously. Often, the new literature is based on the development of a new approach to speciation. Most of these techniques have been tested with stable chemical species within the optimum range of instrumental parameters that provide the best detection limit or signal-to-noise ratio for a given analyte (or class of compounds) in a specific matrix. The majority of the remaining new publications on speciation are focused on using a given technique for a specific analyte (or class of compounds) in a defined matrix. As the analyte of interest, compound stability or the sample matrix is changed for a given published technique, the resulting data may become suspect. Each technique may have a limitation that is specific to a given sample or an instrumental parameter. For example, Olesik et al. used ionspray mass spectrometry to evaluate the speciation of the Ni–EDTA complex. Although they were not able to observe the Ni–EDTA complex, their results indicated that the Ni2+ signal correlated with the expected free metal ion concentration in the solution (not the total metal concentration). Therefore, for this particular instrumental approach, if the analyte matrix is not known (this is the case with all real samples), the result, although reproducible, is misleading. The authors acknowledged this shortcoming by stating that the ‘...errors in quantitative analysis by electrospray or ionspray mass spectrometry may be severe...’.

If a technique for speciation involves chemical reactions, the rate or the lack of uniform reactivity towards all similar species can be troublesome. In a few instances, this limitation can be used advantageously for screening or first-order speciation. Willie used this approach for the speciation of As species that react with sodium tetrahydroborate. Although individual species could not be identified, using hydride generation atomic absorption spectrometry Willie was able to evaluate the combined concentrations of As5+, As3+, monomethylarsonic acid and dimethylarsinic acid.

The limitations of conventional chromatographic separation combined with element-specific detection for speciation have been described by Quevauviller. Capillary electrophoretic (CE) techniques have many advantages over conventional separation technologies. The lack of a stationary phase (better representation of labile analytes due to the absence of extemporaneous interactions with the materials in the stationary phase), minimal sample and buffer (mobile phase) volume requirements, ability to separate cationic, anionic and neutral species (with the use of modifiers) and high separation efficiencies are some of the attributes that make CE a potentially indispensable tool for elemental speciation. Recent advances and a better understanding of nebulizers used in inductively coupled plasma mass spectrometry (ICP-MS) have led to a series of functional interface designs for CE–ICP-MS applications. Interestingly, the use of CE separation with ICP-MS...
detection for speciation has brought to light several parameters that may contribute to misleading results. Because fused-silica columns used in CE have small internal diameters (25–100 μm), real samples have to be pre-treated to some extent to enhance sensitivity, facilitate separation or prevent physical damage to columns (e.g., clogging). This pre-treatment can be relatively simple (e.g., filtration, adjustment of pH or ionic strength, extraction, preconcentration and fractionation) or extremely complex (e.g., derivatization, chemical labeling, enzyme digestion and denaturation).

In this paper, a series of experiments are presented that illustrate the above principles as they relate to chemical speciation. Sampling bias, chemical stability and temporal degradation of chemical species were investigated using CE–ICP-MS.

Experimental

Instrumentation

ICP-MS

A Perkin-Elmer SCIEX (Thornhill, ON, Canada) Elan 6000 instrument was used for the ICP-MS work. In all experiments, the ICP was operated at 1000 W with an Ar plasma flow rate of 15.0 l min⁻¹ and an auxiliary Ar flow rate of 0.860 l min⁻¹. The carrier flow rate (nominal 0.7 l min⁻¹) was changed according to the experimental requirements.⁶ The ICP-MS data collection was initiated manually, immediately after the separation voltage was turned on. The rate of data collection for each electropherogram was set to 0.5, 1 or 2 Hz depending on the experimental requirements. For each electropherogram either one, two or three channels, corresponding to different m/z ratios, were interrogated (¹¹²Cd, ¹⁴Cd, ⁶³Cu, ⁶⁵Cu, ⁶⁴Zn, ⁶⁵Zn and ⁶⁰Co).

CE

For the CE–ICP-MS interface, a laboratory-made instrument was favored over the commercial instrument because of the total flexibility in the selection of column lengths and sample injection methodologies. This interface is described in detail elsewhere.⁶ Briefly, the laboratory-made CE system consisted of a 110 cm long (50 μm id, 363 μm od) fused-silica capillary (Polymerics Technologies, Phoenix, AZ, USA). The separation potential was produced by a Series 230 high voltage power supply from Bertan (Hicksville, NY, USA). A new capillary was turned on. The rate of data collection for each electropherogram was set to 0.5, 1 or 2 Hz depending on the experimental requirements. For each electropherogram either one, two or three channels, corresponding to different m/z ratios, were interrogated (¹¹²Cd, ¹⁴Cd, ⁶³Cu, ⁶⁵Cu, ⁶⁴Zn, ⁶⁵Zn and ⁶⁰Co).

Reagents

TRIS buffers were made by dilution of appropriate amounts of crystalline acids and their corresponding conjugate bases. The dilutions were made using appropriate amounts of 18 Ω cm ultra-pure water, produced with a Milli-Q water purification system (Millipore, Bedford, MA, USA). All chemicals, including the proteins and metal salt standards, were purchased from Sigma (St. Louis, MO, USA) at the highest purity available.

Results and discussion

Owing to the lack of a stationary phase, CE is the least invasive separation technique for chemical speciation. Stationary phases in various chromatographic techniques can interact with labile species and ultimately disturb the original distribution of species. The analytical results on chemical speciation can be significantly biased depending on how the experiment was conducted, how long the sample has been stored, whether the sample was pre-treated and what type of separation procedure was employed. The following discussion focuses on a few of the most common factors that can give rise to erroneous results for chemical speciation. We should emphasize that most of the potential errors highlighted in this paper are not unique to CE techniques.

Sample storage and aging

Sample aging begins when the sample is removed from its native environment. In biological systems, organisms actively function to maintain a balance between various chemical components (chemical species). Once the sample is removed from its native environment, kinetic and thermodynamic factors will dictate the new distribution of species. This is also true for environmental samples. Fig. 1 illustrates the effect of aging on a rabbit metallothionein (MT) solution. Metallothionein is a cysteine rich protein with an average molecular mass of 6–7 kDa. These proteins are believed to be synthesized as a direct response to metal (Cd, Cu, Zn, Ag and Hg) exposure by the biological organisms. A typical 1 mg ml⁻¹ solution of rabbit Cd-MT solution will generate two major peaks when separated by CE (with UV absorption detection) in a 50 mm TRIS buffer (pH 9.1).¹⁷ The CE–ICP-MS electropherogram (Cd ion intensity) of fresh 1 mg ml⁻¹ rabbit Cd-MT, injected electrokinetically at 10 kV for 5 s and separated at 30 kV, as a function of migration time is shown in Fig. 1(a). As expected, two major Cd peaks are observed for this sample, corresponding to the different isoforms of the rabbit MT. When this sample was allowed to sit for 2 weeks without refrigeration, the CE–ICP-MS electropherogram in Fig. 1(b) was obtained. Metallothioneins have a tendency to degrade under unfavorable conditions, and as is apparent from the lack of well defined individual components in Fig. 1(b), this sample has degraded. It is important to note that the lack of separation resolution is not due to poor adjustment of the CE conditions but rather a result of sample instability.

Another example of sample aging can be seen in Fig. 2, which illustrates a time-dependent Co ion intensity electropherogram for coenzyme-B₁₂, generated by CE–ICP-MS, as a function of migration time. In this case, a pure solid sample of coenzyme-B₁₂ was removed from the refrigerator and dissolved in a deoxygenated buffer to provide a 1 mg ml⁻¹ solution. However, the sample solution was allowed to sit uncapped, in an ambient environment, for 810 days. The peak intensity at Co (10.5 min) is reduced, and a second unknown peak (13.5 min) is present. The analytical results on chemical speciation can be significantly biased depending on how the experiment was conducted, how long the sample has been stored, whether the sample was pre-treated and what type of separation procedure was employed. The following discussion focuses on a few of the most common factors that can give rise to erroneous results for chemical speciation. We should emphasize that most of the potential errors highlighted in this paper are not unique to CE techniques.

![Fig. 1](image-url)  
Fig. 1 Cd ion intensity in CE–ICP-MS of rabbit metallothionein in TRIS buffer (pH 9.1). a. Fresh preparation of metallothionein solution; b. an unrefrigerated, 2 week old preparation of rabbit metallothionein.
environment, and a CE–ICP-MS profile was obtained for this solution about every 30 min. As the oxygen dissolved in the buffer solution, the coenzyme-B$_{12}$ began to oxidize. Two peaks were observed in the CE–ICP-MS electropherogram only 10 min after the solution was prepared. The peak appearing later (approximately 17 min) was due to coenzyme-B$_{12}$ and the peak appearing earlier (approximately 15 min) was indicative of the oxidation product of coenzyme-B$_{12}$. The broad shoulder immediately after the oxidized coenzyme-B$_{12}$ is due to incomplete separation of the oxidation products during the migration (i.e., coenzyme-B$_{12}$ continues to be oxidized in the separation capillary). The intensity of the oxidized coenzyme-B$_{12}$ becomes nearly three times as large as that of the unoxidized coenzyme at 43 min after the solution is prepared, and by 108 min after the preparation of the coenzyme solution the majority of the cobalt species are in the form of an oxidized analog of coenzyme-B$_{12}$.

**Sample injection parameters**

Sample injection is seemingly one of the simplest operations for elemental speciation; however, in CE techniques, the type of injection and the injection duration are two critical parameters that may bias the results and lead to poor separation and irreproducible migration times. Because the inner diameter of the CE column is very small (i.e., low tolerance for sample loading), longer injection durations can severely degrade the resolution of electropherograms. This column overloading is demonstrated in Fig. 3 for both electrokinetic (sample injection achieved using a potential difference) and hydrodynamic (sample injection achieved using a pressure difference) injections. In these experiments, rabbit Cd-MT was injected on to the column and separated in 50 mM TRIS buffer (pH 9.1) at a 25 kV running potential. Different durations of hydrodynamic injection [Fig. 3(a)] were performed by suction (6200 Pa) at the cathodic end of the capillary. The electrokinetic injections [Fig. 3(b)] were done by imposing a 3 kV potential difference between the sample (anode) and the cathodic end of the capillary. In Fig. 3(a) (hydrodynamic injection), it can be seen that with shorter sample injection durations (e.g., 5 s injection), near baseline resolution is obtained for different cadmium containing components. As the injection duration becomes longer, the column becomes overloaded and the resolution is degraded to the point that for a 60 s injection duration it is difficult to discern the number of components present. When using electrokinetic injection, the separation resolution is preserved for longer injection durations because during the injection period the sample is actually undergoing separation. As such, the band overlap is not as severe as it is for hydrodynamic injection. Nonetheless, because of this pre-separation, during the injection period, the migration times for a given component with different injection periods will not be the same.

From the above experiments, one may conclude that electrokinetic injection is superior to hydrodynamic injection for chemical speciation. However, because electrokinetic injection uses a potential difference for sampling, the analytes with higher electrophoretic mobility will be preferentially loaded on to the column. Hence, depending on the difference between the electrophoretic mobilities of the components, substantial sampling bias may be introduced in the analysis. This concept is shown in Fig. 4, where the peak heights for two of the cadmium containing isoforms are illustrated. The hydrodynamic injections in these experiments were performed using a 3200 Pa pressure difference and the electrokinetic injections using a 3 kV injection potential. In Fig. 4(a), it can be
seen that the intensity of the cadmium peak for the two different isoforms increases linearly as a function of the injection period. Furthermore, the ratio of these two components remains nearly constant for all injection periods. The peak intensity of the cadmium containing components is not a linear function of the injection duration and the ratio of the two peaks varies significantly for electrokinetic injection [Fig. 4(b)]. In general, electrokinetic injection can provide better resolution for longer injection periods and can lead to the preconcentration of certain analytes (better detection limits). However, these improvements are at the expense of sampling accuracy. By using electrokinetic injection, severe sampling bias will result in analytical data which are counter to the fundamental principle of speciation.

**Influence of pH**

pH plays an important role in CE separations and in the chemical distribution of pH sensitive species. For example, by adjusting the pH of a biological sample to a slightly basic value, the analyst can greatly simplify the separation and identification of different isoforms of cadmium metallothionein. However, any relevant information concerning free metal and weak-ligand complexes of cadmium is lost owing to formation of hydroxide precipitates; $K_{sp}$ for Cd(OH)$_2$ is $5.9 \times 10^{-15}$. The Cd ion intensities obtained from the CE–ICP-MS electropherogram for a 1 mM cadmium chloride solution at different pHs are shown in Fig. 5(a). According to the solubility constant for Cd(OH)$_2$, at pH values less than 8, more than 6 mM of free Cd can be present in the solution without significant hydroxide formation. Subsequently, for CE–ICP-MS runs at pHs 4.5, 6.0 and 7.0 we see Cd ion intensities of similar magnitude. The reason for the earlier appearance of the Cd peak at increasing pH values is that the electroosmotic flow becomes more pronounced as the pH increases. At pH 8.5, the Cd ion intensity becomes much smaller and broader than in previous runs. At this pH, according to the solubility constant, only 0.59 mM of Cd can exist as free ions. At pH 9.0 only 59 µM of free Cd can be present in the solution (at pH 9.0 the Cd signal generated by CE–ICP-MS was small, but detectable).

The influence of pH becomes more problematic if the analytes present in the sample can inter-convert into one another. This is demonstrated by analyzing a pharmaceutical preparation of vitamin B$_{12}$ by CE–ICP-MS. Szpunar$^{18}$ has shown that the pharmaceutical preparation of vitamin B$_{12}$ is composed of three cobalt containing components. Furthermore, it is known that vitamin B$_{12}$ is most stable in the pH range 4.5–5.19 The Co ion intensities obtained from the CE–ICP-MS electropherogram for a 1 mg ml$^{-1}$ solution of vitamin B$_{12}$ at different pHs are shown in Fig. 5(b). At pH 4.5, as reported by Szpunar,$^{18}$ three cobalt containing peaks are observed for the vitamin B$_{12}$ solution. These peaks are assigned to free cobalt (early peak), hydroxycobalamin and adenosylcobalamin. At pH 6.0, these three peaks are shifted towards later times. This shift is most likely due to deprotonation of the phosphate moiety on the parent ring system (i.e., changing the electrophoretic mobility) and coordination of free cobalt with some hydroxides. At pH 7.0, the electroosmotic flow becomes strong enough to early shift the peaks, while the central band is lost. At pH 9.0 it is likely that the Co is removed from the porphyrin structure in order to form Co(OH)$_2$.

**Conclusion**

The combination of CE with ICP-MS is an extremely valuable tool for elemental speciation. As with any sophisticated instrumental technique, the users must be aware of the possible analytical difficulties that may be associated with sample handling and/or each of the individual techniques (CE and ICP-MS) in addition to the combined technique (CE–ICP-MS). Interestingly, none of the potential sources of error are unique to CE–ICP-MS. A good protocol for sample preparation, storage and calibration is needed to avoid sample aging and facilitate quantitative analysis. The pH influence on the analyte can be minimized by running CE with a pH buffer that is similar to that of the sample. Fortunately, CE can operate over a wide pH range (2–11). Column overloading is also a common problem with all separation techniques. Employing smaller sample volumes and using peak shapes as a guide, overloading of the column can be avoided. The only potential source of error that was unique to CE is the sampling bias observed during electrokinetic injection. This bias can be easily eliminated if the hydrodynamic injection technique is used for CE–ICP-MS analysis. With careful preparation of samples, and judicious selection of sampling techniques and analysis conditions, CE–ICP-MS can become a benchmark technique for elemental speciation.

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