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A closer look at the operating definition of protein recovery in capillary electrophoresis

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Abstract

Analyte recovery is an important figure to assess protein adsorption on fused-silica capillaries. In 1991 Regnier and coworkers estimated recovery by assuming the loss of analyte from adsorption and thus the decrease in peak area measured by two detectors to be proportional to the length of the capillary section between them. In this report we closely examine this concept and its adaptation to commercial CE instruments to determine protein recovery. We hypothesize that, once a steady-state migration is reached, protein adsorption is a first order process with respect to protein concentration and surface density of adsorbing sites. This hypothesis is shown to be valid over a reasonably wide range of capillary effective length and, as a result, protein recovery decreases exponentially with the migrated distance. However, unlike the traditional recovery figure obtained through a conventional spike process, protein recovery measured by this approach does not have the same merit since it is strongly dependent from capillary dimensions and applied electric field. Nevertheless, protein recovery and the slope of the logarithmic protein peak area *vs.* length plot are useful figures to compare protein adsorption on different capillary surfaces. Several literature reports dealing with the application of Regnier concept to calculate protein recovery are discussed.

Keywords

Capillary electrophoresis; Protein recovery; Silica surface

Introduction

The advent of proteomics, one of the most hectic areas of the omics field, demands high resolution analytical tools for the separation of proteins in very complex matrices [1,2]. CE is becoming increasingly important for high throughput data generation in biological research because of its high speed, separation efficiency, selectivity and versatility, low sample size requirement and easy automation [3,4]. Unfortunately, a full exploitation of the superb separation efficiency of CE in the proteomics field has been hindered by protein adsorption on the fused silica capillary surface [5]. A variety of methods has been devised to minimize such interaction between proteins and the silica surface. One of the most common approaches involves the incorporation of suitable additives to the BGE to dynamically coat the inner surface of the capillary [6]. Such additives range from oligoamines and simple surfactants (mostly cationic, but also non-ionic and zwitterionic detergents) to neutral and cationic polymers. Most additives work as competing agents for cation exchange sites,

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mainly ionized silanol groups, on the silica surface, so that they are no longer accessible to protein interactions. Because they are cost-effective, reproducible and very easy to implement, these dynamic coatings have become increasingly popular. So, by simply masking the undesirable protein-silica interactions, dynamic coatings have significantly improved separation profiles that enable better characterization of complex proteome samples. A more elaborate but permanent approach to deal with protein adsorption is to chemically modify the inner capillary wall with, for instance, a cross-linked hydrophilic polymer. However, permanent coatings usually exhibit low hydrolytic stability that limits their useful lifetime, and high cost (money, effort, or both). While significant advances have been made, truly effective and long-lasting surface deactivation toward protein adsorption by means of dynamic or permanent covalently-bound coatings remains one of the greatest challenges in the current practice of CE.

Performance parameters such as analyte recovery, separation efficiency, peak asymmetry, electroosmotic mobility (EOM) change and baseline shifting have been used to monitoring protein adsorption in CE [6]. From an analytical point of view, method accuracy can be measured from an analyte recovery experiment [7]. A known amount of the analyte is added (“spiked”) into the sample, prior of the analysis. The amount —usually concentration— of analyte in the unspiked and spiked samples is then measured and the recovery or recovery factor, R , is determined by the expression

$$R = \frac{Q_{meas}}{Q_{theor}} \quad (1)$$

where Q_{meas} is usually the difference in concentration between the spiked and unspiked samples, and Q_{theor} refers to the known or “theoretical” concentration from the spike. Because of the extremely small surface area of the inner wall of a capillary tube, protein recovery experiments are difficult to implement and require high sensitivity detection. For instance, Righetti and coworkers used a fluorescence-labeled model protein to measure analyte loss due to adsorption on the fused silica surface [8]. After saturating the whole length of the capillary with the fluorescent protein, they desorbed it by electrophoretically flushing the capillary with an SDS-containing electrolyte. The eluted protein was then quantified by an off-line fluorescence technique. Notice that, while this method led to important conclusions regarding the adsorption process, it relies on a relatively long static protein-silica contact time to ensure reproducible data. Under regular CE conditions, however, a narrow plug of the protein sample traveling through the capillary tube experiences a much shorter contact time with the silica surface. Regnier and coworkers were the first to report an on-capillary electrophoretic method to measure protein recovery [9]. Using a home-made CE instrument equipped with two detectors in series on a single capillary tube, they estimated recovery by assuming that the loss of analyte from adsorption and hence the decrease in peak area measured by the two detectors was proportional to the length of the capillary section in between. Later on, Lucy and his research group adapted Regnier concept to commercial single-detector CE instruments [10]. They determined percent protein recoveries on the same capillary tube by comparing the peak areas before and after shortening the column. Subsequently, Taverna and coworkers simplified the experimental procedure by using the long and short portions of an intact capillary tube, *i.e.*, no cutting [11]. Unfortunately, neither Regnier and coworkers [9,12] nor Lucy and coworkers [10,13-18] provided explicit equations to calculate protein recovery. Specifically, they never mentioned which reference they used to express the decrease in peak area as a percentage, something which is essential to the recovery equation. Such lack of details has resulted in diverse interpretations of the original concept. Some authors appear to interpret the method concept in a manner presumably similar to that of the original work [19,20], while others do so in a quite different fashion, as we will discuss below [11,21-23].

In this report we closely examine the concept as well as the adaptation to a single detector of Regnier's method to assess protein recovery. Chicken egg white lysozyme is used in this work as a model analyte for adsorption. This enzyme is a small compact protein with a *pI* of about 11 which makes it positively charged at pH 4, the pH used in our study. Although the great variability in size, shape, flexibility and so forth, of different types of proteins prevents to generalize on the adsorption behavior of all proteins at the silica surface, our study should not only apply to lysozyme but also to similar globular proteins such as cytochrome C, ribonuclease A, chymotrypsinogen and the like, to mention only one group. The hypothesis that the amount of protein adsorbed is proportional to solute concentration as well as migrated distance is experimentally tested in this work using a pentamine to dynamically coat the inner wall of a capillary. The different CE-based approaches to calculate protein recovery are discussed.

Materials and methods

Materials

Fused-silica capillaries of 50 μm i.d. were purchased from Biotaq Inc. (Silver Springs, MD, U.S.). Piperazine-N,N'-bis(3-propanesulfonic acid) (PIPPS) was purchased from GFS Chemicals (Columbus, OH, U.S.); tetraethylenepentamine (TEPA), lysozyme (chicken egg white), benzyl amine and benzyl alcohol were purchased from Sigma-Aldrich (St. Louis, MO, U.S.).

Electrolyte and sample preparation

All solutions were prepared in 18-M Ω deionized water from a Millipore Corp. (Bedford, MA, U.S.) model Milli-Q system fed with distilled water. A 25-mM PIPPS buffer containing 1.5 mM TEPA and adjusted to pH 4.00 $\pm$ 0.03 with NaOH was used as BGE. A solution of 1.0 mg/mL lysozyme and 10.0 mM benzylamine was prepared in the BGE and used for protein assays.

Capillary electrophoresis

CE experiments were performed in an Agilent model 3D CE System (Palo Alto, CA, U.S.). An 8-m fused-silica capillary tube was etched with 1.0 M NaOH for 2 h by forcing the solution through with 7-bar N₂ pressure [24]. EOM determinations followed a procedure previously described [24]. A 5.0-mM solution of benzyl alcohol in the PIPPS BGE was used as neutral marker. Benzylamine was used as internal standard to correct lysozyme peak areas for injection volume variations. Protein recoveries were determined using the CE method of Regnier's modified by Lucy and coworkers [10]. In this procedure, replicate injections of the lysozyme/benzylamine mixture were performed on each of five adjacent pieces of NaOH-etched capillary with total lengths of 35.0 to 75.0 cm. Removal of a 1-2 mm section of the polyimide cladding at a fixed 8.5-cm distance from one end of the capillary was accomplished as described elsewhere [24], and tubes with effective lengths of 26.5 to 66.5 cm were obtained. Separations were carried out under constant electric field strength of 320 V/cm by adjusting the applied voltage accordingly. Likewise, injection and rinse pressures were also adjusted to allow for constant pressure gradients of 0.67 and 50 mbar/cm respectively. In all cases, the injection time was 2.0 s, and the rinse time between runs was 5 min. Prior to use, all capillaries were BGE-conditioned under high pressure for 30 min after which a plateau EOM value was obtained [24]. Again, the pressure applied during BGE-conditioning was adjusted to obtain a constant pressure gradient of 115 mbar/cm. The equations used to calculate recovery are described below. A separate 35-cm piece of the same capillary was also used to measure lysozyme peak areas after injecting the sample mix from the inlet (26.5-cm effective length) and then the outlet (8.5-cm effective length) of an intact capillary tube, according to the approach devised by Taverna *et al.* [11]. A reversed

applied potential was used in the later case. In another experiment, a 50-cm length of the same tube (41.5 cm to detection) was used to measure protein peak areas (again, referred to benzyl amine as internal standard) under various applied potentials; the experiment was repeated after cutting the tube to a 35.0-cm total length (8.5 cm to detection), according to Lucy's approach [10].

Results and discussion

The fundamental concept of Regnier method relies on the assumption that the decrease in peak area measured by the two detectors is solely proportional to the capillary length separating them. In contrast, while protein adsorption on silica substrates is an extremely complex and controversial subject, the simplest model depicts a bimolecular process involving attachment of the protein to the surface active sites [5,25]. Accordingly, we assume that protein adsorption is a first order process with respect to its concentration as well as the surface concentration of adsorption sites, and that the surface composition of the inner wall of a fused-silica capillary is uniform. Under steady-state migration conditions, as a sample plug travels a distance dl along the column its peak area decreases in an amount dA which should be proportional to the concentration of protein (hence its peak area, A) and to the number of active adsorption sites:

$$-dA \propto A \quad dn_{ads} \quad (2)$$

where dn_{ads} represents the surface sites available for adsorption. As the protein plug reaches the detector, the total number of adsorption sites to which it has been exposed is

$$n_{ads} = 2\pi r l \Gamma_{ads} \quad (3)$$

where r is the capillary inner radius, l is the travel distance to the detection point (the effective capillary length) and Γ_{ads} is the surface density of adsorption sites. Hence, the number of adsorption sites per unit length of capillary is given by

$$\frac{dn_{ads}}{dl} = 2\pi r \Gamma_{ads} \quad (4)$$

Therefore,

$$dA = -2\pi r \Gamma_{ads} A dl = -k A dl \quad (5)$$

This equation integrates to

$$\ln \frac{A}{A_0} = -kl \quad (6)$$

Here, the intercept A_0 ($l = 0$) corresponds to the peak area of the protein plug sample before any adsorption takes place. In other words, A_0 is the peak area the protein sample would have had at the capillary inlet. The slope, k , is proportional to the surface density of adsorption sites and the tube id (Eq. 5).

When Eq. 1 is applied to protein recovery in CE, Q_{meas} stands for the number of protein molecules that reach the detector (*i.e.*, recovered after traveling a distance l through the capillary), a quantity which is directly proportional to its peak area A . Q_{theor} represents the original number of protein molecules that are injected at the capillary inlet which, in turn, is proportional to the theoretical or "initial" peak area A_0 .

$$R = \frac{Q_{meas}}{Q_{theor}} = \frac{A}{A_0} \quad (1a)$$

Combined with Eq. 6, this expression leads to

$$R = e^{-kl} \quad (7)$$

Notice that, according to Eqs. 5 and 7, recovery is not only a function of the surface density of active adsorption sites but also of capillary dimensions. This is a matter of significant importance. Strictly speaking, protein recovery as calculated from Eq. 7 (based on a CE profile experiment) is not an intrinsic feature of the inner surface of the capillary only and, consequently, does not have the same universality as the analyte recovery figure calculated from Eq. 1 (typically, a spike experiment), as generally thought. Unless $k = 0$, the percent recovery value obtained will never, in principle, be equal to 100% (l , in practice, can never be equal to zero) and hence capillary dimensions as well as CE conditions must be fully reported. Naturally, a k -value not significantly different from zero would be a strong evidence of an adsorption-free CE surface. Nevertheless, protein recovery based on the equations presented here is still a valid figure to compare, for instance, different BGE additives or varying capillary chemistries run on the same system and under the same conditions.

In contrast with the operating definition of analyte recovery (Eq. 1), there is no spiking in this case and, therefore, R can only be obtained indirectly. A formal way to do this is by measuring the peak areas on several pieces of capillary tube (or a single long piece shortened several times), followed by linear fitting of $\ln A$ vs. l data to Eq. 6. Fig. 1 (curve with blue circles) shows that, within the range of effective length considered, a plot of the logarithm of protein peak area vs. migrated distance is indeed a straight line:

$$\ln A = \ln A_0 - kl \quad (6a)$$

Linear regression furnishes the slope and recovery figures shown in Table 1.

It should be pointed out that application of the method assumes that essentially irreversible adsorption occurs, which —within the present context— means that any adsorbed protein can be completely removed by simple rinsing between runs with protein-free BGE. In this manner, a renewed (and reproducible) capillary surface should be obtained and reliable peak area measurements can be made. If, on the other hand, truly irreversible protein adsorption takes place, the tube can no longer be used and no useful data result [12]. Indeed, since application of the method depends on peak area integration measurements, protein profiles with moderate to severe tailings would not provide reliable results. This is why we used a fused-silica capillary dynamically modified with TEPA as opposed to an untreated one. It is also worth mentioning that, within the experimental uncertainty, benzyl amine exhibited insignificant adsorption on the silica surface and, hence, its use as internal standard did provide a better precision of the lysozyme peak areas when compared to the non-ratioed values. See Supporting Information for details.

Obtaining the regression parameters from Fig. 1 may seem a time-consuming and expensive process. A simpler approach to estimate protein recovery could be a two-point straight line approximation by which peak areas are measured either before and after cutting the capillary (according to Lucy *et al.*) [10,13-18], or after separately injecting the sample at both ends of a single and intact capillary (according to Taverna *et al.*) [11,21,22]. In either case, the slope k can be approximated to

$$k = \frac{1}{l-s} \ln \frac{A_s}{A_l} \quad (8)$$

where A_l and A_s are the measured peak areas for the effective lengths l and s respectively, and s represents either the effective length of the capillary after cutting, or the shorter segment of the uncut tube. In this case $s = L - l$, where L is the total length of the capillary, and it is implicit that $s < l$ and $A_s > A_l$. Recovery can be estimated from

$$R = \left(\frac{A_l}{A_s} \right)^{\frac{l}{l-s}} \quad (9)$$

It appears that this two-point-straight line approximation has been the approach used by all researchers in the past [10,11,13-23].

When we attempted to apply Taverna's approach using the long and short portions of an intact capillary tube to estimate protein recovery, interesting results arose. Our CE instrument can accommodate capillaries with a minimum total length of 35 cm, with a fixed distance (8.5 cm) from the point of detection to the outlet. This represents an effective length of 23.5 cm under "regular" CE conditions (this is, injection at the anodic inlet). Contrarily to what is expected from a backward extrapolation of the fitted line in Fig. 1, the lysozyme peak area obtained after separation from the short ($s = 8.5$ cm) end of the capillary was smaller than that from the longer end ($l = 23.5$ cm), as shown by the red square data points in Fig. 1. This means that apparent recoveries above 100% were obtained (typically $107 \pm 2\%$), as calculated from Eq. 9. Clearly, Eq. 6 does not apply for lysozyme at such short effective length on our system. There are several facts relevant to the experimental remark in question. First, adsorption of strongly cationic proteins such as lysozyme occurs preferentially at the capillary inlet [12]. Second, such initial protein adsorption process occurs immediately after injection, typically within micro- to milli-seconds and, most likely, as multilayered aggregates [5]. Finally, desorption of the reversibly bound protein molecules from the silica surface follows, until a steady state migration is reached [5,25]. Of course, such desorption starts right after voltage application: as the protein plug starts moving along the tube, it is continuously preceded and followed by protein-free BGE, which results in surface rinsing during which a net desorption occurs. Notice that protein peak area is undoubtedly increasing in the length interval between 8.5 and 26.5 cm; which means that desorption prevails at $l = 8.5$ cm. Shortly after (definitely before the upper end of this length interval), a new condition is reached under which adsorption dominates and Eq. 6 applies. So, one possible qualitative interpretation of our unusual observation is that after just 8.5 cm of protein traveling, desorption has not yet counteracted the initial adsorption effect that led to a substantially decreased peak size; *i.e.*, a steady-state migration condition has not been reached at this point. In contrast, at 26.5 cm and beyond, such steady state has already been attained and the delayed protein molecules originate a second wave that manifests itself as significant peak tailing, a higher baseline after the passage of the peak, or even a secondary peak [26]. The effect is illustrated in Fig. 2 for a typical pair of electropherograms from the short and long portions of a single capillary tube. The highly symmetrical lysozyme peak coming out from the short effective length (asymmetry factor = 1.01 ± 0.01) deteriorates significantly at the long effective length (asymmetry factor = 1.87 ± 0.05) owing to the tailing that results, at least in part, from the delayed initial desorption. A direct inference from this observation is that the minimum effective length to which Eq. 6 applies must be determined for a CE system prior to calculate protein recovery from this method. When we applied Lucy's experimental approach consisting of repeating peak area measurements after shortening the capillary tube total length from 50 to 35 cm, no anomalous behavior was

observed. This alternate two-point straight line experiment gives k and R values which are moderately comparable with those from the regression (see Table 1). Estimated precision, however, is poorer for the two-point straight line approximation, particularly for k . Precision data were estimated from conventional random error propagation analysis. Details are given as Supporting Information.

Electric field effects

The dependence of recovery on the extent of protein exposure to the silica surface suggests that a brief exposure may lead to an improved recovery. Besides using a short effective length, the residence time of the protein in the capillary can be further shortened by increasing the applied electric field strength. In this work we use PIPPS, a zwitterionic buffer whose low conductivity enabled us to apply high voltage gradients without significant overheating. As shown in Supporting Information, Ohm's law applies fairly well up to 500 V/cm. The percent recovery of lysozyme increases rapidly at low to moderate strengths, while the effect is progressively less pronounced at higher field strengths, as illustrated in Fig. 3. Needless to say, this result emphasizes our former suggestion that all CE conditions must be fully documented in a protein recovery report.

Comments on selected literature reports

Regnier and coworkers as well as Lucy and his research group determined protein loss on a capillary tube by subtracting peak areas either between two detectors (Regnier) or between an original and shortened capillary (Lucy). To the best of our knowledge, they never provided an explicit equation for their calculations. More recently though, Liu *et al.* [19] and afterward Jurcic and Yeung [20] applied Regnier concept to assess protein recovery in coated capillaries while clearly stating that recovery, R' , was calculated from the peak area ratios obtained from the long (A_l) and the short (A_s) capillaries:

$$R' = \frac{A_l}{A_s} \quad (10)$$

Unlike Eq. 1a, this expression should result in a biased protein recovery value. In order to present a more meaningful comparison, let us express R' in terms of R by combining Eq. 10 with Eq. 9 and rearranging:

$$R' = R^{\frac{L-s}{L}} \quad (11)$$

Clearly, Eqs. 9 and 10 should deliver different results, except in the extremes cases where $R=0$ or $R=1$. Otherwise R' is always greater than R , the “true” value, as shown in Fig. 4 (continuous red curve).

Furthermore, Taverna *et al.* made a different interpretation of Regnier concept [11,21,22]. They acknowledged the necessity of including the theoretical A_0 parameter as a reference in their recovery percentage calculation:

$$\frac{A_0 - A_s}{A_0} = \frac{A_s - A_l}{A_s} \quad (12)$$

They arrived at a different expression for recovery, R'' :

$$R'' = \left(\frac{A_l}{A_s} \right)^2 \quad (13)$$

Again, when Eq. 13 is combined with Eq. 9 and rearranged, one obtains

$$R'' = R^{\frac{2(l-s)}{l}} \quad (14)$$

This equation leads to the dot-dashed green curve of Fig. 4. It appears that Eq. 13 exhibits a similar bias, although to a larger extent, in comparison to Eq. 10.

Yet, another interpretation of Regnier method can be found in the recent literature. Descroix and coworkers apparently assumed the decrease in peak area to be proportional to the migrated distance alone (that is, they applied Regnier original concept) but somehow utilized Eq. 5 instead, and ended up with an expression equivalent to Eq. 9 [23]. Although these authors had previously reported this equation, a systematic description of the underlying mechanism was still missing from their paper.

Interestingly, when one tests the original Regnier assumption that the decrease in peak area was proportional to the effective capillary length only [9], a reasonably straight line (within the experimental uncertainty) also turned out, as shown in Fig. S-5 of the accompanying Supporting Information. This experimental “evidence” of a zero-order for the absorption process with respect to protein concentration would mean that protein peak area decreases at a constant rate as the plug travels along the capillary:

$$dA = -kdl \quad (15)$$

Whereas Eq. 5 makes more physical sense, let us for a moment go along with Eq. 15 and allow it to lead us to the following equation for recovery:

$$R''' = \frac{A}{A_0} = \frac{A_0 - kl}{A_0} \quad (16)$$

After applying the two-point straight line approximation, one obtains

$$R''' = \frac{A_l}{A_0} = \frac{(l-s)A_l}{lA_s - sA_l} \quad (17)$$

Finally, the following equation yields R''' in terms of R , as illustrated by the dashed blue curve in Fig. 4:

$$R''' = \left(\frac{lR}{l-s+sR} \right)^{\frac{l}{l-s}} \quad (19)$$

Notice also that, for a sufficiently long capillary, R moves asymptotically toward zero (Eq. 7) as expected, while the R''' figure can become negative. On the other hand, at recoveries above 80%, R''' -values closely approach those of the true value, a fact that is consistent with the apparent linearity of the peak area vs. effective length plot. Nevertheless, such presumed proportionality should not justify the use Eq. 16 or 17, which rely on an unproven hypothesis for protein adsorption.

Concluding remarks

We have experimentally confirmed that the hypothesis considering the amount of protein adsorbed to be proportional to protein concentration as well as the migrated distance is a valid one over a reasonably wide range. The lack of an explicit equation for protein recovery during its initial stage has resulted in different interpretations of Regnier concept. Protein recovery, as determined by this method, does depend on the capillary dimensions as well as the applied electric field; consequently, it is not an intrinsic property of the capillary silica

surface, as usually thought. Thus, such protein recovery does not have exactly the same worth as the established analyte recovery figure obtained from sample spiking. Nevertheless, the slope k should be a useful figure to comparatively assess protein adsorption on different capillaries or BGE additives. Moreover, it is important to experimentally determine the minimum effective length to which Eq. 6 applies for a given CE instrument prior to quantify protein adsorption. This is a consequence, at least in part, of the delayed protein desorption that occurs at the beginning of the capillary tube.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

EOM	electroosmotic mobility
PIPPS	piperazine-N,N'-bis(3-propanesulfonic acid)
TEPA	tetraethylenepentamine

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Figure 1.

Logarithm of peak area of lysozyme as a function of migrated distance. CE conditions: BGE, 25 mM PIPPS buffer containing 1.5 mM TEPA, adjusted to pH 4.00 ± 0.03 ; sample, 1.0 mg/mL lysozyme and 10 mM benzylamine (internal standard) in BGE; injection, 2 s at 0.67 mbar/cm; applied electric field, 320 V/cm for 10-20 min; 5-min BGE-rinse between runs at 50 mbar/cm; detection at 200 nm. Blue circles: Separate capillary tubes with varying effective lengths and injections at the inlet. Error bars represent ± 1 SD ($n = 10$). Red squares: A single 35.0-cm piece of the same capillary tube after injection of the same sample mix at the inlet ($l = 26.5$ cm), and then at the outlet ($s = 8.5$ cm) with reversed applied potential. Error bars represent ± 1 SD ($n = 5$).

Figure 2.

Typical pair of electropherograms obtained from a benzyl amine/lysozyme sample injected at the short ($s = 8.5$ cm, blue curve) and then the long ($l = 26.5$ cm, red curve) length of a single capillary tube. A negative voltage was applied in the later case. Peaks: (1) benzyl amine, (2) lysozyme. The inset visually compares the lysozyme peak profiles on an expanded time scale.

Figure 3.

Percent recovery of lysozyme as a function of applied electric field strength. Five replicate injections were performed on a 50.0-cm ($l = 41.5$ cm) capillary. The tube was then shortened to 35.0 cm ($s = 26.5$ cm) and the injections were repeated; rinse and injection pressures were shortened accordingly. Other experimental conditions as in Fig. 1.

Figure 4.

“Apparent” percent recoveries from three different interpretations of Regnier's procedure to evaluate protein adsorption, as a function of the true recovery values. The dotted line indicates unbiased results, according to Eq. 9 (this work); the continuous red, dot-dashed green and dashed blue curves represent percent recoveries results in terms of the true recovery value, according to eqs 11, 14 and 19, respectively. In all cases $l = 41.5$ cm and $s = 26.5$ cm.

Table 1

Comparison of analytical parameter values based on the modified Regnier method as calculated by linear regression and by a two-point linear approximation.

	Five-point linear regression	Two-point linear approximation
Slope, k	$0.0108^a (3.0\%)^b$	$-0.0038^c (27\%)$
%Recovery ($l = 26.5$ cm)	$75.2^d (0.9\%)$	$90^e (3\%)$

^aEstimated from linear fitting to Eq. 6.

^bPercent relative standard deviation in parenthesis.

^cEstimated from Eq. 8 with $l = 41.5$ cm and $s = 26.5$ cm.

^dFrom Eq. 7 with $l = 26.5$ cm (the shortest effective length attainable with our CE instrument).

^eCalculated from Eq. 9 with $l = 41.5$ cm and $s = 26.5$ cm.