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Short communication

Practical method for evaluation of linearity and effective pathlength of on-capillary photometric detectors in capillary electrophoresis

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Abstract

The optical characteristics of on-capillary photometric detectors for capillary electrophoresis were evaluated and five commercial detectors were compared. Plots of sensitivity (absorbance/concentration) versus absorbance obtained with a suitable testing solution yield both the linear range and the effective path length of the detector. The detector linearity is a crucial parameter when using absorbing electrolytes, such as for indirect photometric detection, and especially for highly absorbing electrolyte probe ions. The upper limits of the linear ranges (determined as 5% decline in sensitivity) for five commercial detectors ranged from 0.175 to 1.2 AU. The effective pathlength reflects the quality of the optical design of the detector and is equal to the capillary internal diameter only for a light beam passing exactly through the capillary centre, but becomes progressively shorter for imperfect optical designs. The determined effective pathlength for the five investigated detectors ranged from 49.7 to 64.6 μm for a 75 μm I.D. capillary.

Keywords: Detection, electrophoresis; Photometric detection; Indirect detection; Instrumentation

1. Introduction

Photometric detection is the most commonly used method of detection in capillary electrophoresis and can be performed in two modes. Firstly, direct photometric detection can be used to detect absorbing analytes against a non-absorbing background. This approach relies on the analyte containing a suitable chromophore and its sensitivity is limited due to the small path length (50–100 μm) inherent to on-capillary detection in capillary electrophoresis. Alternatively, indirect photometric detection in which the absorbance of a strongly absorbing co-ion (termed the probe) added to the electrolyte is monitored [1,2] can provide universal detection which can be also more sensitive than direct absorbance detection for many analytes. In contrast with electrolytes used for direct detection where the separation current is the sole limiting factor which determines the electrolyte concentration, the background absorbance of the electrolyte becomes an additional limiting factor of the probe concentration in indirect detection. For reliable quantitative results, the background absorbance must remain within the linear range of the detector. Hence there is a clear need to know the detector linearity and as most manufacturers do not give reliable data, it has to be determined experimentally.

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A number of approaches to determine detector linearity are possible [3–6]. However, it has been shown [3] that the best method of evaluating the linearity is by carefully measuring the response (absorbance) caused by a series of known probe concentrations so that the sensitivity (response/concentration) can be calculated. A plot of sensitivity versus concentration can then be constructed to show when the detector linearity limit is reached, usually by determining the concentration at which the sensitivity falls below its maximum value by a defined amount (such as a 5% decline from maximum sensitivity). This approach shows more clearly when the detector linearity is exceeded than by estimating when a simple plot of absorbance versus concentration begins to deviate from a straight line [7]. Importantly, the plot of sensitivity versus absorbance is strictly an instrumental characteristic which should be independent of the absorptivity of the probe, thereby eliminating any need for further linearity measurements for different probes. Such plots can also provide useful information on comparisons regarding sensitivity and linearity between various detectors and instruments. A comparison of the detection sensitivity achieved with various probes can also be gained by this technique, and an estimate of effective pathlength can also be calculated.

Unlike in rectangular cells where all pathlengths are equal, in cells possessing a variety of possible individual pathlengths, such as a cylindrical cell in on-capillary detection in CE, an effective pathlength is defined as the single pathlength of a hypothetical rectangular cell which would yield the same absorbance as that measured through the illuminated part of the cylindrical cell [6]. Effective pathlength is dependent on the geometry of the light beam incident on the capillary window. The effective pathlength would equal the capillary inner diameter if only the centre of the capillary would be illuminated and the ray of a collimated beam could travel through the full length of the inner diameter of the capillary. For beams further away from the central axis, the distance travelled through the capillary will be shorter and finally approaching zero for a ray travelling through the fused-silica capillary but outside the inner channel of the capillary. Depending on the width of the aperture defining the illuminated part of the capillary, all those individual pathlengths will combine to produce the final effective pathlength. In reality, the effective pathlength will be always smaller than the inner capillary diameter. Bruin et al. [6] presented a theoretical model for calculation of the effective pathlength, which may be useful for approximate estimations. However, this model is based on parallel light beams passing through the cylindrical capillary and this model is therefore not applicable to detectors used in practice. Therefore, it is desirable to have an experimental method for the determination of effective pathlength. Macka et al. [3] fitted curves to experimentally measured sensitivity plots and derived the effective pathlength from the fitted curves. Whilst this method would be optimal for detectors exhibiting very poor linearity, it is very time consuming. Therefore here we propose a simple and fast method, based on a calculation of the effective path length for a known probe absorptivity and the measured sensitivity in the linear range of the detector.

2. Experimental

2.1. Instrumentation

Absorbance measurements were recorded on four different CE instruments. These were Applied Biosystems 270A-HT (Perkin-Elmer, San Jose, CA, USA), Waters Capillary Ion Analyser (Milford, MA, USA), Agilent Technologies ^{3D}CE (Waldbronn, Germany) and P/ACE System MDQ (Beckman Instruments, Fullerton, CA, USA). The Applied Biosystems 270A-HT was fitted with a deuterium lamp with variable wavelength detection. The Waters CIA was fitted with a deuterium lamp with detection at 254 nm. The Agilent Technologies ^{3D}CE system was fitted with a deuterium lamp with a photodiode array detector. A blue alignment interface designed for use with 75 μm I.D. capillaries was used for this work. The P/ACE System MDQ was used with two different detection systems, a deuterium lamp with a fixed filter at 254 nm used for UV measurements, and a 256 element diode array photodiode array detector. A 100×800 μm slit width aperture was used with both systems. The different instrumental configurations are listed in Table 1 and for con-

Table 1
Upper limits of detector linearity (corresponding to a 5% drop in sensitivity) and the effective pathlength values derived from the data plotted in Figs. 1 and 2[a]

Instrument No.	Instrument brand	Detector linearity upper limit (AU)	Effective pathlength (μm)
1	Agilent Technologies ^{3D}CE	1.2	64.6
2	Applied Biosystems 270A-HT	0.75	60.5
3	Waters CIA	0.175	49.7
4	Beckman MDQ PDA	0.55	54.9
5	Beckman MDQ UV	0.30	53.6

[a] Conditions: capillary I.D. 75 μm, wavelength 254 nm, for other conditions see Experimental.

venience in this paper are referred to as Instruments 1–5.

A new capillary for each instrument was cut to suitable length from a 60 m spool of fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA) of 75 μm I.D.×365 μm O.D. The I.D. reported by the manufacturer for the start of the spool was 75 μm, and at the end of the spool was 74 μm). Detection windows were prepared by burning off a small section of polyimide coating using a butane torch. The detection window was cleaned with a tissue moistened with methanol. Spectrophotometric measurements were conducted using a Cary UV–Vis–near IR (NIR) spectrophotometer (Varian Australia) with 1-cm pathlength quartz cells.

Finally, it should be pointed out that as different detectors used with the same capillary will produce different linearity and effective pathlength values, different capillary diameters (both I.D. and O.D.) will result in somewhat different linearity values, and also in different effective pathlength values. It can be generally anticipated that smaller capillary diameters will result in relatively poorer photometric detection.

2.2. Reagents

Sodium chromate (LR grade, Ajax Chemicals, Sydney, Australia) was used to prepare a series of aqueous chromate standards solutions. Tartrazine (Fluka, Switzerland) was purified [1] and used to prepare a series of tartrazine standards. Water treated with a Millipore (Bedford, MA, USA) Milli-Q water system was used to prepare and dilute standard solutions.

2.3. Procedures

A series of standards was prepared by serial dilution by a factor of two of a stock solution. Chromate standards were prepared in 50 m*M* sodium hydroxide to ensure the presence of chromate rather than dichromate. Absorbance measurements at 254 nm (chromate) or 426 nm (tartrazine) were performed by flushing the capillary with water or the desired standard solution (approx. 10 capillary volumes), then stopping the flow and measuring the absorbance under static conditions. The absorbance of each test solution was measured in triplicate. Absorbances were measured in order of increasing concentration standards to minimise possible carryover errors.

3. Results and discussion

Sensitivity data were calculated from the measured absorbances and plotted against chromate concentration as shown in Fig. 1. The concentration at which sensitivity declined by more than 5% was used to define the upper limit of detector linearity. From Fig. 1 it can be seen that Instrument 1 provided the highest sensitivity and greatest linearity. Linearity (at 95% of maximum sensitivity) was maintained up to a concentration of ~80 m*M*, far in excess of the typical background electrolyte of 5 m*M* chromate used for indirect detection. An even more universal comparison of the instruments and their detector linearity can be gained by plotting sensitivity versus absorbance (Fig. 2). This plot is independent of the absorptivity of the probe and the detection wave-

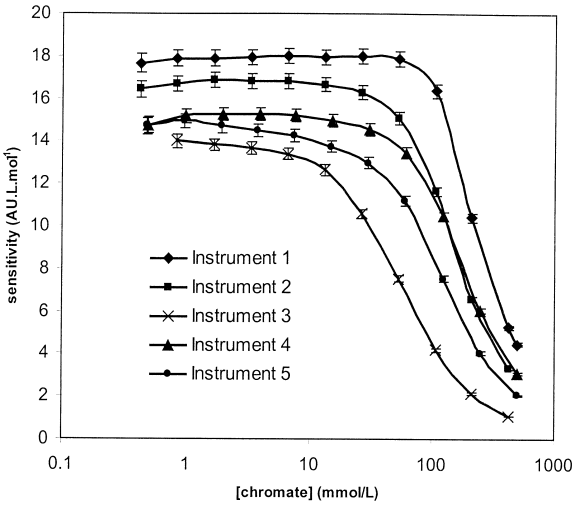

Fig. 1. Sensitivity versus concentration plots for chromate at 254 nm. For other conditions see Experimental.

length. For Instrument 1 linearity is maintained up to 1.2 AU. It should be pointed out that the linearity limits for all instruments exceed background absorbances typically used (~0.1 AU) for indirect detection in capillary electrophoresis when using moderately absorbing probes such as chromate, the concentration of which is limited by the separation current.

The use of highly absorbing probes, such as dyes, to improve sensitivity has been well documented [1,2]. Measurement of detector linearity using a highly absorbing probe such as tartrazine (ϵ=21600 L mol^{-1} cm^{-1} at 426 nm, pH =8 [1]) can illustrate the increased sensitivity of such probes and also provide a guide to the concentration at which they should be present in the background electrolyte. It is desirable that the probe be present at as high a concentration as possible so that the calibration plot for analytes can be extended and also to provide significant benefits in gaining better sample stacking upon sample injection. However, this then leads to potential problems of calibration linearity if the background absorbance of the electrolyte exceeds the limit for detector linear range. A plot of sensitivity versus absorbance for tartrazine on Instruments 1 and 2 is shown in Fig. 3, along with chromate data for the same instruments. This plot demonstrates two important features. First, it highlights that tartrazine is significantly more sensitive (about 7 times higher) than chromate and this translates into improved detection limits [1]. Second, detector linearity followed the same trend as evident from the chromate data, with sensitivity decreasing at approximately the same absorbance. This shows that detector linearity was independent of the probe, which means that the detector linearity can be characterised by the measurement of just one probe. The concentration of a

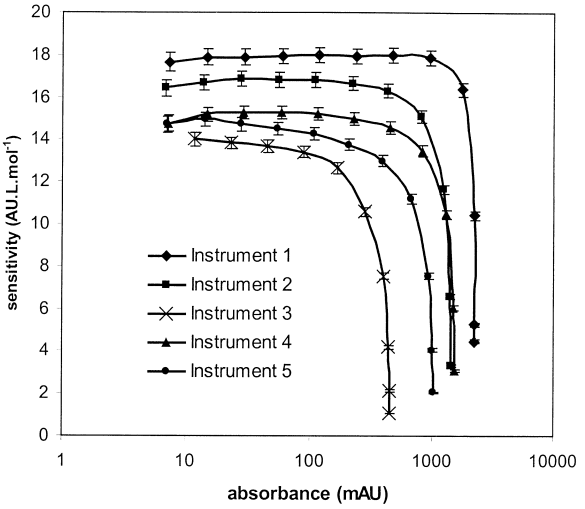

Fig. 2. Sensitivity versus absorbance plots for chromate at 254 nm. For other conditions see Experimental.

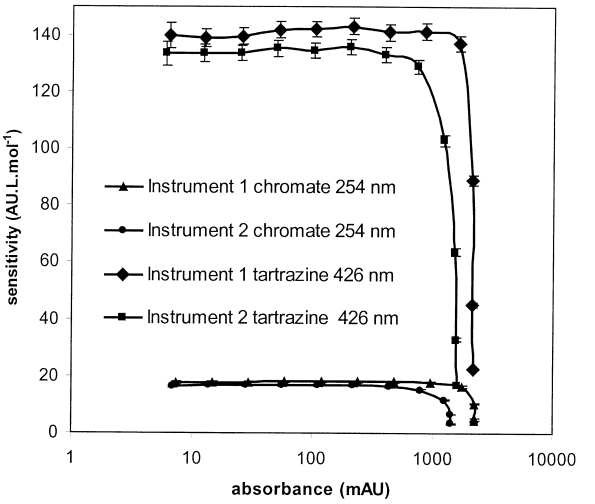

Fig. 3. Sensitivity versus absorbance plots for chromate at 254 nm and tartrazine at 426 nm. For other conditions see Experimental.

different probe required to produce this absorbance can then be easily determined. A tartrazine concentration of 8 and 6 m*M* can be used with Instruments 1 and 2, respectively, while still retaining 95% of the maximum sensitivity and remaining in the linear range of the detector.

Effective pathlengths were calculated by rearranging the Beer–Lambert law to give the ratio of sensitivity to probe absorptivity. The sensitivity value chosen was at an absorbance of ~0.05 AU which is well inside the linear range of all five instruments. It is vital that such a calculation is performed in the linear absorbance range to provide a true estimate of the effective pathlength. This highlights the importance of knowing the detector linearity range. Observed effective pathlengths ranged from 49.7 (Instrument 3) to 64.6 μm (Instrument 1) for a capillary of 75 μm I.D. Effective pathlength can be used to judge and compare the quality of the detector optics. Most importantly, it is well known that detection pathlength inhomogeneity, similarly to incident light wavelength inhomogeneity (polychromatic light), will result in detector nonlinearity [8]. This effect will cause a departure from linearity across the whole absorbance range, also affecting the low-absorbance region, in contrast to stray light, which mostly affects the high-absorbance regions [3,8]. It is important to note that this method of measurement of effective pathlength does not need any prior knowledge of the detector geometry, and it allows a general judgment on the geometry based on the determined effective pathlength value. For instance, using this method the effective pathlength could be easily evaluated for irregularly shaped cells such as the Agilent Technologies Extended Light Path Capillary [9] and the Agilent Technologies High Sensitivity Detection Cell. Finally, it should be pointed out that as different detectors used with the same capillary will produce different linearity and effective pathlength values, different capillary diameters (both I.D. and O.D. would play a role) will result in somewhat different linearity values, and, of course in different pathlength values. It can be generally anticipated that smaller capillary diameters will result in relatively poorer photometric detection.

4. Conclusions

The evaluation of detector linearity in capillary electrophoresis instruments provides vital information regarding the upper linearity limit of the instrument, the sensitivity of probes used for indirect detection, and the maximum concentration at which a probe may be used in background electrolytes. From this work it can be clearly seen that some instruments have superior optical properties which can lead to improved results. It is also clear that background electrolyte concentrations of most probes can be markedly increased whilst still working in the linear range of the detector. This is particularly important for highly absorbing probes, the concentration of which is limited by the background absorbance rather than by the separation current. Increasing the background electrolyte concentration of such a probe is essential for gaining better sample stacking. The effective pathlength is another important instrumental parameter which is determined quickly and easily from the approach described in this work. In addition, judgements can be made on the quality of detector optics of on-capillary absorbance detectors and the concentration of the probe used for indirect detection methods can be optimised.

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