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Fiber-optic-based UV–visible absorbance detector for capillary electrophoresis, utilizing focusing optical elements

Peter Lindberg, Anders Hanning, Tommy Lindberg, Johan Roeraade*

Royal Institute of Technology, Department of Analytical Chemistry, S-100 44 Stockholm, Sweden

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Abstract

A compact and flexible fiber-optic cell for absorbance detection in capillary electrophoresis (CE) has been developed. Optical fibers with a large core diameter were combined with focusing optics and a circular aperture, the size of the aperture being matched to the I.D. and O.D. of the separation column, in order to maximize light throughput and reduce stray light. The detection limit for mesityl oxide was 1.5 μ*M* for a 100-μm I.D. column, and the linear dynamic range was larger than 1000 for columns from 100-μm I.D. down to 30-μm I.D. The design is suitable for high-speed CE, utilizing short columns.

Keywords: Detection, electrophoresis; Instrumentation; Fiber-optic-based UV–visible absorbance detection; Mesityl oxide

1. Introduction

Capillary electrophoresis (CE) is a powerful separation technique for high-speed, high-efficiency analysis of a variety of samples. However, the sensitivity of on-column absorbance detection in CE is limited due to the small optical pathlength (typically 50–100 μm). Furthermore, the narrow and rapidly moving analyte bands require a reduced axial length of the detection window and a short response time, which also limits the sensitivity [1]. Usually, a spatially incoherent light source (e.g., a deuterium lamp) is utilized. It is not easy to focus incoherent light through the inner diameter (I.D.) of the fused-silica column with sufficient intensity to minimize shot noise. These problems are also relevant to other microcolumn separation modes, such as micropacked or open-tubular HPLC. Conversions of standard UV–Vis detectors for use with CE columns have been described by several authors [2–4]. The incoming light is either focused onto the separation column by a spherical lens or directly passed through an aperture.

To obtain a more flexible coupling of light into the separation column, optical fibers have been employed [5–12]. This type of setup is advantageous when the detection cell has to be positioned close to the column outlet, as in preparative CE [12], or in applications where short separation columns are utilized, e.g. in microfabricated, chip-based systems [13]. An additional advantage of a fiber-based detector is that the detector electronics can be positioned away from the CE unit, to avoid disturbances from the high-voltage power supply. Most fiber-based detectors rely solely on optical fibers for the coupling

*Corresponding author: Tel.: +46 8 790 84 12; fax: +46 8 10 84 25; e-mail: jroe@analyt.kth.se

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of light through the separation column [7–9]. However, in this type of setup the diameter of the source fiber has to be kept fairly small in order to avoid stray light and a nonlinear response of the detector. The reduced dimensions of this fiber restrict the amount of light that reaches the photodiode, and problems with shot noise may arise.

To overcome the above limitations and improve the detector performance, the design described in this paper combines a large-diameter source-fiber with focusing optics, thereby increasing the light throughput.

A detailed description of the design of the fiber-optic cell is given. The performance of the detection cell is evaluated in high-speed separations of oligonucleotides using a short gel-filled column. Subsequently, based on the results of ray-tracing calculations, modifications in the fiber-optic cell design are carried out to further suppress stray light and extend the linear dynamic range. Experimental results on the linear range, noise and detection limits are reported for the modified cell design, utilizing columns of various O.D./I.D.

2. Experimental

2.1. Materials

A UVIS Model 200 spectrophotometer (Linear Instruments, Reno, NV, USA) was employed. Optical fibers (Model HCG-M0940T, Spectran Specialty Optics, Avon, CT, USA), with a 940-μm core diameter, a 1400-μm O.D., and a 0.22 numerical aperture, were a gift from GB Topcomp Electronics (Kista-Stockholm, Sweden). A plano-convex fused-silica lens (01LQP147), two 2-mm diameter spherical lenses (06LMS002), and 75-μm and 100-μm circular apertures (04PIP014, 04PIP015) were obtained from Melles Griot (Irvine, CA, USA). Fused-silica capillary tubing (105-μm O.D./40-μm I.D., 150-μm O.D./30-μm and 50-μm I.D., 200-μm O.D./100-μm I.D., 375-μm O.D./50-μm I.D.) was purchased from Polymicro Technologies (Phoenix, AZ, USA). In the CE experiments, in-house built equipment was utilized, consisting of a Plexiglas box with a high-voltage safety interlock and a high-voltage supply, ±0–30 kV, constructed from a Spellman CZE 100 (Plainview, NY, USA) unit. Histidine, urea, and γ-methacryloxypropyltrimetoxy-silane were obtained from Sigma (St. Louis, MO, USA). *N*,*N*-Dimethylacrylamide (DMA) and mesityloxide (>99%) was purchased from Fluka (Buchs, Switzerland) and Merck (Darmstadt, Germany), respectively. Oligonucleotides, 22–25-mers with the sequence 5′ CTC TCG CAC CCA TCT CTC TCC TTCT 3′, were a gift from Amersham-Pharmacia (Uppsala, Sweden).

2.2. Construction of the fiber optic UV-absorption cell

An outline of the fiber-optic cell design is depicted in Fig. 1. The UV-light beam from the main detection unit is picked up by the optical fiber, and is then focused by the plano-convex and the spherical lens through the separation capillary column. The outgoing light from the column is collected by the second spherical lens, picked up by the second optical fiber and then transmitted to the photo diode of the HPLC detector.

The various fittings that were necessary to hold the optical components in position were manufactured in aluminum and black Delrin polymer. Fig. 2a shows a schematic of the cross-section of the fiber cell. The cell is constructed in two subunits that can be press-fitted together, thereby enclosing the separation column. Each subunit contains holders for the lenses and optical fibers. In addition, one of the subunits has a V-shaped groove to facilitate a proper alignment of the separation column.

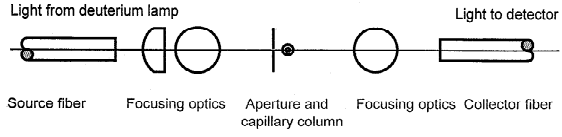

Fig. 1. Schematic drawing (not to scale) of the optical setup of the fiber-optic cell. The aperture was only used in the modified version of the cell. For details see Section 2.2.

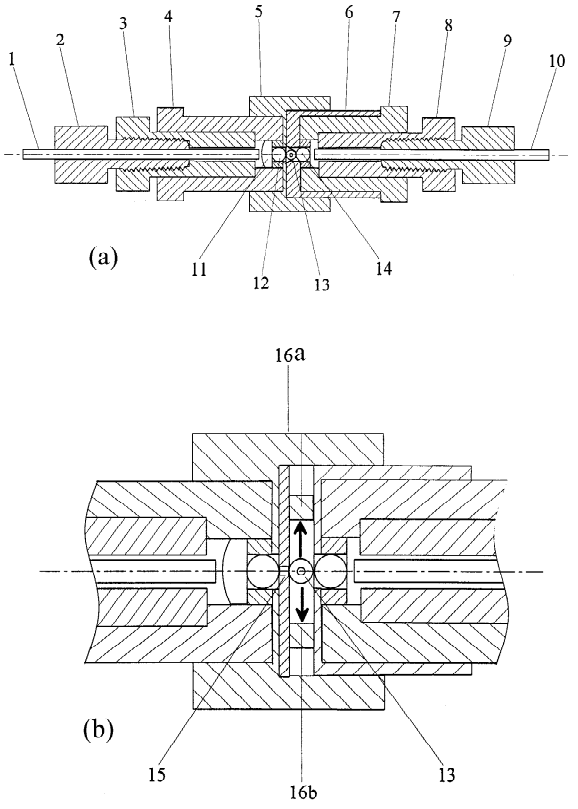

Fig. 2. (a) A schematic of the cross-section view of the fiber-optic cell. (1) Source fiber; (2–5) plastic fittings constituting the first cell subunit; (6–9) plastic fittings constituting the second cell subunit, a V-shaped groove for column alignment is milled into part (6); collector fiber (10); plano-convex lens (11); spherical lens (12); separation column (13); second spherical lens (14); (b) An enlarged cross-section schematic of the modified fiber-optic cell. (13) Separation column; (15) stainless steel shim with circular aperture; (16a and 16b) spacers. The arrows indicate possible lateral adjustment of the separation column.

2.3. Capillary gel electrophoresis of oligonucleotides

Gel columns[1] consisting of 14%-T linear poly-(DMA) were prepared as previously described [14]. A 100 m*M* histidine, 7 *M* urea electrophoresis buffer [15] was utilized in order to minimize the electrical power consumption in the high field-strength electrophoresis experiments. In the preparation of a mixed 22–25-mer oligonucleotide sample, lyophilized 22–25-mer oligos were dissolved in deionized water to a total concentration of 20 μg/ml, or 5 μg/ml per oligo. The sample was injected electrokinetically into the gel column at 170 V/cm for 3 s. Detection was performed at 260 nm, utilizing the initial design of the fiber-optic based detector cell described in this paper. The output signal from the detector was recorded on a PC utilizing a chromatography evaluating software (EZChrom, Scientific Software, San Ramon, CA, USA).

2.4. Evaluation of the modified fiber-optic cell design

The fiber-optic cell was modified by the addition of an aperture. As can be seen in the enlarged cross-section schematic in Fig. 2b, the aperture was inserted after the focusing optics, in front of the separation column. To verify that the aperture was correctly positioned in the optical axis, a visual inspection was performed utilizing a microscope. Some additional modifications of the cell design were carried out compared to the initial design presented in Fig. 2a. The precision of the V-shaped groove turned out to be insufficient for a proper alignment of the column to the aperture (and the optical axis), so the groove was removed. Instead, two spacers (16a) and (16b) were positioned on one of the cell subunits, so that the column was not in a permanent position after it was mounted in the cell, but could be adjusted laterally by means of an external micromanipulator. During the alignment step, the column was filled with a strongly absorbing solution (mesityloxide). The position of the column was adjusted while the signal from the detector was monitored. When the absorbance signal reached a maximum value, it was assumed that the column was correctly aligned.

To evaluate the performance of the fiber cell, solutions of mesityloxide in water were pumped through the separation column in a continuous mode. Tests were performed with several column I.D. and O.D. combinations (see Section 2.1.) using 75-μm

[1] T=(g acrylamide+g *N,N′*-methylene bisacrylamide)/100 ml solution.

and 100-μm apertures, at a wavelength of 240 nm. The time constant of the measurements was 1 s and the spectral bandwidth 6 nm. The detector output was recorded on a PC, as previously described in Section 2.3. OPTICAD optical software for PC (Focus Software, Tucson, AZ, USA) was utilized in the construction of ray-tracing diagrams for various combinations of column dimensions and apertures.

3. Results and discussion

3.1. High speed capillary gel electrophoresis of oligonucleotides

Synthetically manufactured oligonucleotides are widely utilized in molecular biology, e.g. as primers for DNA sequencing. Anti sense oligonucleotides, which possess a DNA-sequence complementary to a specific site on a gene, are rapidly gaining interest as possible future drug candidates. Reliable methods for the assessment of purity of these compounds that are faster than the present HPLC and CE protocols are needed, in order to reduce analysis time and cost. The flexibility and small dimensions of the fiber-optic cell facilitate the use of short columns, since the detection unit can be positioned close to the column outlet. In addition, the use of optical fibers allows the detector electronic circuitry to be positioned away from the CE setup, where it is then less disturbed by the high-voltage power supply.

In our experiments, an effective column length of 12 cm was utilized. Using the maximum 30-kV voltage of the power supply, this yielded an electrical field-strength of 2000 V/cm. To keep the current low under these conditions (in order to avoid disturbances due to Joule-heating effects), a low conductivity buffer containing 100 m*M* histidine and 7 *M* urea was employed [15]. Also, due to the strong dependence of the current on the column inner radius, a 150-μm O.D./50-μm I.D. column was utilized despite the lower detection limit and larger linear dynamic range that could have been obtained with a larger I.D. column. A 14%-T linear poly-(DMA) gel matrix was employed. The chains of this polymer are stiffer than those of poly(acrylamide) due to the substituted methyl groups on the amide nitrogen of the DMA monomer. The increased stiffness of the polymer chain leads to an improved sieving of DNA fragments [16].

The electropherogram in Fig. 3 shows a separation of a mixture of 22–25-mer oligonucleotides that have identical DNA sequences and differ only in DNA fragment-length. The oligonucleotides are nearly base-line resolved in less than 70 s, demonstrating that the detection cell works well for rapid separations on short columns. However, in the purity control of oligonucleotides, where trace impurities must be determined in the presence of bulk components, a linear dynamic range in the order of 1000 is likely to be required. Initial investigations were performed, utilizing aqueous mesityloxide solutions of various concentrations, which indicated that the linear range of the detection cell was not satisfactory (data not shown). In view of this, the present cell design was modified by adding an aperture and by

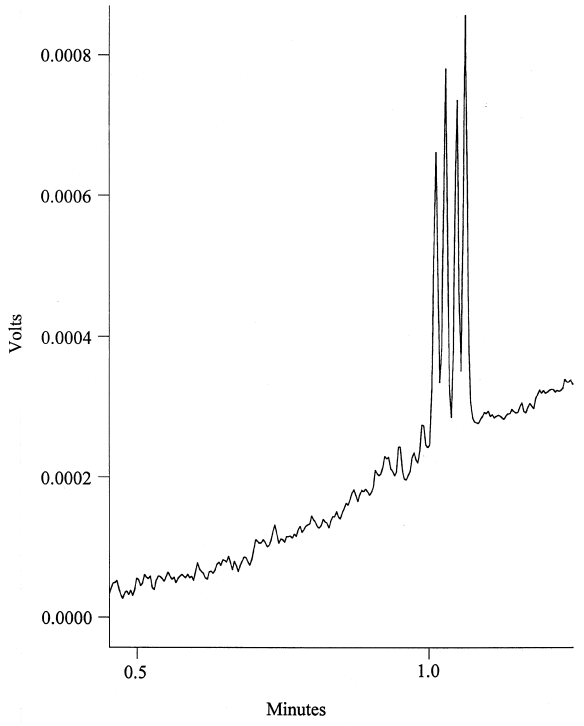

Fig. 3. Electrophoresis of 22–25-mer oligonucleotides on a 150-μm O.D./50-μm I.D., 14%-T poly(DMA) gel column at 2000 V/cm. Injection: 100 v/cm for 5 s. Effective column length: 12 cm. Total column length: 15 cm.

optimizing the optical system. These modifications are described in Section 3.2.

3.2. Optimization of the dynamic range of the modified fiber-optic cell

The modified absorption cell is an optical system consisting of four main elements: the optical fibers, the focusing optics, the aperture and the capillary column. All these elements must be carefully designed in order to optimize the linear dynamic range of the total system. The lower linear limit is determined by the detector noise. In order to minimize this noise, light throughput must be maximized. The upper linear limit is determined by stray light, since this causes a deviation from the linear Beer's law behaviour of the cell at high absorbances. Stray light also impairs the sensitivity (expressed as measured absorbance units per concentration unit) of the system at low absorbances.

Thus, the source fiber should transport as much light as possible to the absorption cell. The ability of a fiber to transport light is governed by the diameter and the numerical aperture of the fiber. A large diameter facilitates the coupling of light into the fiber, since it does not put excessive demands on the focusing of the light source. However, if the diameter of the source fiber is larger than the I.D. of the capillary (usually $\leq$100 μm), a large fraction of the light will not pass through the capillary I.D., but rather through the quartz wall of the capillary or outside the capillary, giving rise to stray light. A large numerical aperture enhances the collection of light into the fiber. The drawback in this case is that the light leaving the fiber will be divergent, so even the use of a source fiber smaller than the capillary I.D. will give rise to stray light. In this work, a large diameter fiber with an intermediate numerical aperture was found to be a satisfactory compromise.

The situation may be improved by using focusing optics between the source fiber and the capillary. The role of the focusing optics is to increase the fraction of light passing through the capillary I.D., thereby decreasing stray light. However, it is very difficult to focus all the light down to $\leq$100 μm, mainly for the following reasons: the deuterium lamp is not an ideal point source, the light is incoherent, the source fiber has a finite diameter and numerical aperture, and the focusing optics will give rise to imaging errors.

The role of the aperture is to block the stray light emanating from imperfect focusing, but it is important not to use too small an aperture, since this will decrease the light throughput. The quartz capillary itself will also act as a focusing element, the focal length of which is determined by the capillary O.D. [17]. Thus, in order to obtain the best overall performance, it is necessary to make an interactive optimization of the aperture diameter, the capillary O.D., and the capillary I.D. As a general rule, the aperture diameter should be as large as possible, but not as large as to introduce excessive stray light. The capillary O.D. should be as small as possible, but it must be as least as large as the aperture. The choice of O.D./I.D. ratio may also be influenced by practical problems of handling thin-walled capillaries. The optimal ratio of these three parameters may be determined theoretically or experimentally.

A theoretical optimization of the system was carried out by ray-tracing simulations. Examples are shown in Figs. 4 and 5. In both cases, a 100-μm aperture is used. The light from the aperture is assumed to be parallel, which is a somewhat simplified assumption. In practice, most of the light is slightly convergent, but there is also a divergent fraction. However, ray-tracing simulations with convergent and divergent light (not shown) show the same qualitative result as simulations with parallel

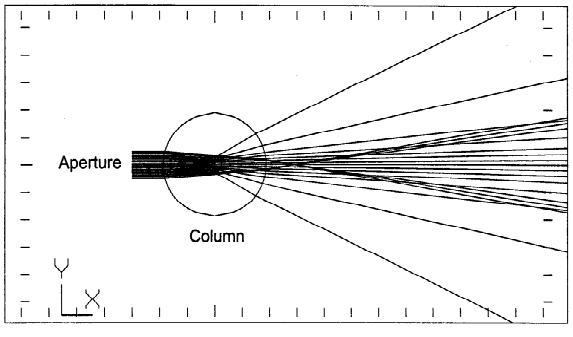

Fig. 4. Ray-tracing simulation. Parallel light from a 100-μm aperture passing through a capillary column with O.D. 375 μm and I.D. 50 μm.

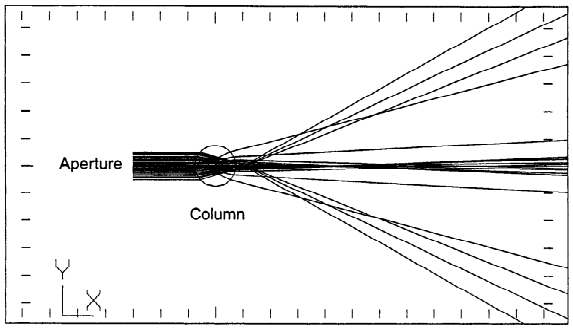

Fig. 5. Ray-tracing simulation. Parallel light from a 100-μm aperture passing through a capillary column with O.D. 150 μm and I.D. 50 μm.

light. Both examples utilize a capillary with 50-μm I.D. The refractive indices are set to 1.00 (air), 1.46 (quartz), and 1.33 (interior of capillary). The refractive index values are for visible light (590 nm), but the general principle is also valid for UV light. In Fig. 4, the capillary O.D. is 375 μm, corresponding to a commonly used dimension. As can be seen, the light is slightly focused by the capillary, but a considerable number of rays do not pass through the capillary I.D. The rays that do pass the I.D., as well as most of the ones that do not, travel approximately along the optical axis when leaving the capillary, and thus both categories of rays reach the collecting optics. Therefore, this design leads to excessive stray light. In Fig. 5, the capillary O.D. is decreased to 150 μm, while all other parameters are unchanged. The light is now strongly focused by the capillary. As a consequence, the rays that do not pass the capillary I.D. are deflected to such an extent that they do not reach the collecting optics. This simulation demonstrates that the amount of stray light can be very low, even when the aperture is much larger than the capillary I.D. Further ray-tracing calculations (not shown) indicated that stray light rejection is even more efficient with capillaries of reduced dimension, e.g., 40-μm I.D. and 105-μm O.D.

The theoretical predictions were also experimentally verified. The performance of the fiber optic cell was evaluated by passing water solutions of mesityloxide, with concentrations ranging from 10 μ*M* to 100 m*M*, through fused-silica capillaries, while monitoring the resulting absorbance and noise levels. Capillaries of various dimensions were utilized with 75-μm or 100-μm circular apertures. The results are summarized in Table 1, which presents

Table 1
Summary of performance evaluation of the fiber-optic cell. For details see Section 3.2

	Aperture diameter 100 μm			Aperture diameter 75 μm			
	Capillary			Capillary			
	100-μm I.D. 200-μm O.D.	50-μm I.D., 150-μm O.D.	30-μm I.D., 150-μm O.D.	100-μm I.D. 200-μm O.D.	50-μm I.D., 375-μm O.D.	50-μm I.D. 150-μm O.D.	30-μm I.D., 105-μm O.D.
Limiting absorbance (mA)	1070	830	410	>1100	220	970	>1000
Stray light (%)	8	15	39	<7	60	11	<10
Noise (mA)	0.04	0.05	0.06	0.07	0.06	0.06	0.06
Sensitivity (AU/M)	79	39	16	81	18	39	32
Detection limit (μ*M* mesityloxide)	1.5	3.6	11.9	2.7	10.4	4.8	5.8
Upper linear limit, 10% deviation (mA)	610	320	200	730	100	560	690
Upper linear limit, 10% deviation (m*M* mesityloxide)	8.5	9.3	13.5	10.0	6.2	15.8	24.0
Linear dynamic range	5500	2600	1100	3700	600	3300	4200
Upper linear limit, 5% deviation (mA)	230	160	55	260	30	220	210
Upper linear limit, 5% deviation (m*M* mesityloxide)	3.0	4.3	3.6	3.4	1.9	5.8	6.8
Linear dynamic range	1900	1200	300	1300	180	1200	1200

data obtained on the limiting absorbance, the percentage stray light, the noise level, the sensitivity, the detection limit, the upper linear limit, and the linear range for each tested column and aperture combination. The stray light was determined as the light reaching the photodiode under conditions of almost total absorbance, utilizing a strongly absorbing mesityloxide solution. Only light that did not pass the column I.D. was measured under these conditions. Noise was defined as the pooled standard deviation during 10 s for the seven lowest concentrations. The sensitivity was defined as the slope of the calibration curve, in absorbance units per molar (AU/M), in the linear region at low concentrations. The detection limit was defined as the concentration corresponding to three times the noise. The upper linear limit was calculated from absorbance data, and defined as the absorbance value where the sensitivity had changed 10% from the constant value obtained at low concentrations [18]. For comparison, calculations were also carried out by fitting a second order polynomial to the calibration curve, and defining the upper linear limit as the absorbance value where the polynomial deviated 5% from the extrapolated line obtained by fitting a straight line to the calibration curve at low concentrations [3]. The linear range was defined as the ratio between the concentration corresponding to the upper linear limit and the detection limit.

Some general observations can be made regarding the results presented in Table 1. The linear dynamic range is larger than 1000 for 30–100-μm capillaries, allowing the detection of trace compounds (0.1%) in the presence of bulk components. Low stray light levels (approximately equal to or less than 10%) are obtained when using apertures as large as 2× the capillary I.D. Even apertures as large as 3× the capillary I.D. provide acceptable results. These findings confirm the theoretical predictions.

The lowest detection limit, 1.5 μ*M* mesityloxide, and the largest linear dynamic range, 5500, are, as expected, obtained with the 200-μm O.D./100-μm I.D. column in combination with the 100-μm aperture. When the aperture is reduced to 75 μm for the same column, the stray light is only slightly reduced, indicating that most of the light already passes through the I.D. of the column when the 100-μm aperture is utilized. The only effect of the 75-μm aperture is an increase of the noise level and the detection limit due to decreased light throughput.

When decreasing the column I.D., using the 100-μm aperture, the noise increases due to reduced light throughput. Also, the stray light increases. This leads to a deterioration of the detection limit, larger than that predicted by Beer's law. On the other hand, when a 75-μm aperture is used, the decrease in column I.D. from 100 μm to 50 or 40 μm does not lead to any increase in noise or stray light, and the theoretical Beer's law behaviour is fully obeyed.

The significant influence of the I.D./O.D. ratio is also illustrated in Table 1 for a 50-μm I.D. column used with the 75-μm aperture. When the O.D. is increased from 150 μm to 375 μm, the stray light increases from 11% to 60%. This increase in stray light impairs the linear dynamic range in two ways: the upper linear limit decreases and the sensitivity decreases. The total decrease in linear dynamic range is a factor of about 6.

3.3. Comparison of the modified fiber-optic cell with the designs of previous workers

The present detector cell may be compared with previously described designs. Svensson et al. [9] described a fiber-optic cell without any aperture or focusing elements. Source fibers with a rather small diameter, in the order of 200 μm, were used, which reduces the light throughput. Since the source fiber diameter was substantially larger than the 50-μm I.D. of the capillary being used, and since the unfocused light from the fiber the will be divergent, this design can be expected to yield a considerable amount of stray light. Further, the focusing properties of the capillary were not fully exploited. Although the collector fiber, which was in the order of 200–400 μm, is likely to act as a limiting aperture, it will still collect a significant amount of stray light. The obtained linear range was about 1500 with a 50-μm I.D. capillary (using the 10% sensitivity change method). The linear range of our detector cell is at least twice as large, even for capillaries of 40-μm I.D. Further, since the diameter of the beam hitting the capillary is less than 100 μm, the axial

length of the detection cell and the detection volume are reduced in the present detector cell. This minimizes extra column band-broadening, which is of importance in high-efficiency separations and when using short separation capillaries [1,3].

Also Bruno et al. [7,8] described a cell without an aperture or focusing elements. Optimum source fiber diameters were calculated for given values of capillary I.D.s and O.D.s. Very small source fibers were recommended, e.g., a 40-μm source fiber for a 50-μm I.D. capillary column. Such small source fibers would lead to very low light throughput. No experimental test data using capillary separation columns were presented.

Several different cell designs, not based on optical fibers, were described and evaluated by Bruin et al. [3]. Some of these designs included focusing optics and apertures. The upper linear absorption limit of our design is equal to or higher than the best designs evaluated by Bruin et al. However, the total linear dynamic range of our fiber-based design is inferior, which is caused by a higher noise-level. This is mainly due to the fact that the fiber cell is based on a commercial detector, to which several extra optical elements are added. Since every added element impairs the coupling of light, the total light throughput through the fiber cell is lower, resulting in increased noise. Further, the axial detector length of the designs evaluated by Bruin et al. [3] was significantly larger, in the order of 1 mm, leading to increased light throughput and less noise, but also to increased detection volume.

4. Conclusions

The main features of the fiber-optic detection cell are its flexibility and small size, making it possible to locate the detection window near the column outlet to facilitate fraction collection, or to utilize a very short column as well as a high electrical field strength, which is demonstrated in this work. Also of significance is the ease with which the detection cell can be clamped onto or removed from the CE-column.

In the modified design of the cell, the combination of large-core optical fibers with focusing lenses and an aperture that is carefully matched to the separation column I.D./O.D., leads to a maximized dynamic range and optical throughput while stray light is minimized.

It should be possible to further decrease the noise, e.g., by decreasing the number of optical components and/or using anti-reflex coated optics, by using a more intensive light source, or by using a rectangular slit.

The column adjustment procedure described in Section 2.4 is not practical in applications where an in situ polymerized gel-matrix is used, since the column cannot be flushed with a highly absorbing solution. Instead, an arrangement for accurate direct alignment (*e.g.* a precision V-groove) should be utilized, thereby avoiding the need for column adjustments. This is presently under development.

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