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# Evaluation of extended light path capillaries for use in capillary electrophoresis with laser-induced fluorescence detection

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**Abstract**

An interesting development in improved capillary electrophoresis (CE) detection sensitivity is the Hewlett-Packard extended light path capillary (ELP). These capillaries have a "bubble" in the flow path at the detection zone which provides an increased optical path length. Although well characterized for use in absorbance detection, they have not been adequately assessed in the laser-induced fluorescence (LIF) detection mode. Similar advantages (e.g., improved detectability) are expected with LIF, in addition to simplifying the optical alignment. Therefore, experiments were conducted to make comparisons between conventional and ELP capillary formats. First, the detection sensitivity was compared with both formats by adjusting the background level (via fluorescence emission slit width adjustments) to a constant value. The larger dimensions of the ELP capillary allowed efficient rejection of the background with a wider slit setting, improving signal transmission several-fold. Second, alignment of the ELP capillary with the excitation beam and collection optics was significantly simplified. Clean transmission of the excitation beam was easily accomplished with both 50 and 25 $\mu$m I.D. ELP capillaries, representing a significant improvement in ease of alignment over the conventional case. To illustrate this feature further, profiles of capillary wall scatter/fluorescence were generated by linear translation of the photomultiplier assembly at a constant slit width. These profiles clearly illustrate the improved spatial characteristics relative to conventional capillaries. The rapid, high-sensitivity separation of the metabolic enzyme markers 7-ethoxycoumarin and its metabolites in a liver slice incubate is shown as a practical application.

*Keywords:* Detection, electrophoresis; Laser-induced fluorescence detection; Ethoxycoumarin; Phenylalanine; Amino acids

## 1. Introduction

Laser-induced fluorescence (LIF) has been employed in high-sensitivity separation science applications since the late 1970s [1]. This approach to detection combines the classical advantages of conventional fluorescence with the added features of high excitation beam intensity and excellent focusing characteristics. These characteristics allow the ultra-trace analysis of

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complex mixtures with absolute detection limits at the attomole level [2] and below [3]. As a result, LIF has been extensively utilized for both HPLC [1,4–8] and capillary electrophoresis (CE) [2,3,9–14] applications. Our interest in the technique centers on the high sensitivity afforded by laser excitation for the analysis of xenobiotic materials and related metabolites in biological matrices.

While LIF has significant potential for application to trace bioanalysis, it remains a technique practised nearly exclusively in the academic laboratory. One vendor (Beckman) offers an instrument designed for CE–LIF, but it is limited to excitation in the visible region of the spectrum. Two primary bottlenecks exist which are responsible for the slow evolution of LIF instrumentation. First, practical application of the technique requires excitation in the UV spectrum to allow quantification of a reasonable number of analytes without some form of derivatization. Access to laser lines in this region has classically been difficult, requiring complex instrumentation and dedicated facilities. The recent commercial availability of relatively simple UV tunable laser systems (e.g., Coherent offers the FReD system, with lines spanning 229–264 nm) greatly broadens the range of potential applications. Second, a key challenge in the development of LIF instrumentation suitable for routine analysis is the design of a rugged procedure for optical alignment of the detection flow cell with the focusing and collection optics. In academic designs, a significant amount of direct experience is required for easy alignment and, more import, optimization of instrument performance. Application of this technology to ultra-sensitive analysis in an industrial setting will require instrumentation which is simple, easy to use and highly precise in terms of intra-day performance. Thus, improvements in LIF flow cell and instrumentation design are required to make this approach more robust.

A key source of difficulty in operation of LIF instrumentation is the diminutive size of the flow cell. In the CE case, the operator must precisely probe the interior of 50 $\mu$m I.D. (and smaller) capillaries. Misalignment of the laser beam often leads to degraded detectibility owing to the collection of scatter and the generation of fluorescence from silica wall impurities [2].

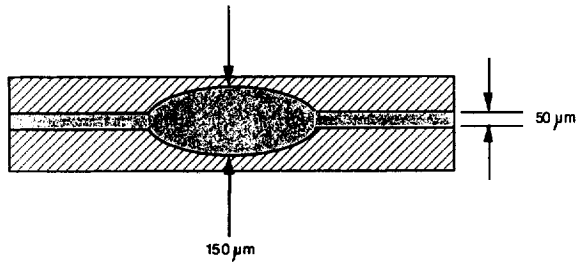


Fig. 1. Schematic diagram of bubble capillary. Expansion region dimensions are ca. 150 $\mu$m in diameter (at maximum) and 650 $\mu$m in length.

As a first approach to alleviating these difficulties, an alternative capillary geometry was assessed, namely the extended light path (ELP) capillary (Hewlett-Packard). ELP capillaries show promise as an advancement in detection technology because they are constructed with a 3–5× expansion (bubble) in the detection zone (Fig. 1). This bubble has been used to improve absorbance detection sensitivity by increasing the optical pathlength [15,16]. Owing to the improved detection zone dimensions, it was postulated that the ELP design could potentially be used to ease optical alignment constraints and improve sensitivity in LIF. This paper outlines experiments aimed at the quantitative assessment of this postulate.

## 2. Experimental

### 2.1. Instrumentation

The optical layout has been described previously [14], and will only be outlined here. Fluorescence excitation was provided by an Omni-Chrome (Chino, CA, USA) He–Cd laser, 20 mW, 325 nm, Model 3074-20M (used for xenobiotic materials), or a Spectra-Physics (Mt. View, CA, USA) argon ion laser, 15 mW, 488 nm, Model 162A (used for fluorescein). Excitation

and emission beams were collected/focused with standard quartz $f/1$ lenses. Fluorescence was isolated with an adjustable optical slit (Edmund Scientific, Barrington, NJ, USA), appropriate interference filters and measured with an RP-1 type photomultiplier (Hammatsu, Bridgewater, NJ, USA). Signals were processed with an analog photometer and displayed/collected with a PE Nelson Model 1020 integrator.

### 2.2. Alignment procedure

Alignment of capillaries was performed with a dual-lens detection system, incorporating an $X$–$Y$–$Z$ capillary cell holder, two adjustable mirrors and an adjustable optical slit mounted to a photomultiplier tube on a translational stage. First, the excitation optics are aligned. The laser beam is passed through the center of the excitation $f/1$ lens and its position relative to the capillary is adjusted to provide a clean focus through the inner channel of the capillary column. Clean focus is determined by placing a white card in the far field and observing a crisp oval-shaped image. The second step in the procedure involves aligning the emission optics. The capillary is filled with a (visible) fluorescent dye and the colored image is positioned. In our optical arrangement, the emission lens was adjusted to provide a ca. twenty-fold magnification of the image emanating from the dye in the capillary. The slit–PMT assembly is positioned in the center of this magnified image. At this point, the capillary is flushed with mobile phase and the alignment fine tuned by observing spectral scatter. This step can be easily understood with respect to the structure of the magnified scatter image. In the case of a 365 $\mu$m O.D./50 $\mu$m I.D. capillary, the far-field image is composed of four "spots" distributed over ca. 7 mm. The "spots" occur as a result of scatter from the inner and outer capillary walls. Proper adjustment of the slit position and width results in efficient rejection of specular scatter and transmission of signal.

Slit translation experiments followed the above procedure with the exception that the slit–PMT assembly was incrementally translated across the magnified image while maintaining a fixed slit width.

### 2.3. Separation capillaries and conditions

Conventional 50-$\mu$m in addition to 25- and 50-$\mu$m I.D. ELP capillaries were obtained from Hewlett-Packard (Wilmington, DE, USA). All ELP capillaries used in these studies had a nominal 363 $\mu$m O.D. Conventional 25-$\mu$m capillaries were purchased from Polymicro Technologies (Phoenix, AZ, USA). Capillaries were cut to length with a sapphire scoring tool and cemented into a suitable holder, as described elsewhere [13,17]. Prior to optical alignment or test compound injection, each capillary was washed for ca. 10 min with 0.1 $M$ NaOH followed by a 5-min rinse with 0.05 $M$ disodium phosphate buffer (pH 9).

Separation runs for the capillary performance studies were carried out using CE in the free zone mode for Dns-phenylalanine and sodium fluorescein studies and in the micellar electrokinetic capillary chromatographic (MECC) mode for 7-ethoxycoumarin (7-EC) separations. The exact mobile phase and separation conditions are specified in the figure and table legends. All sample injections were made via hydrostatic injection.

### 2.4. Preparation of liver slice incubates

Precision-cut liver slices were prepared as described by Barr et al. [18]. Human liver tissue was obtained from the International Institution for the Advancement of Medicine (Exton, PA, USA). Tissue cores were sliced to 10–20 mg wet mass with a modified Krumdieck slicer [19]. Slices were pre-incubated in Krebs–Henseleit buffer for 90 min at 37°C in a roller incubator prior to incubation with 100 $\mu M$ 7-EC. Slices were incubated for 4–6 h with 100 $\mu M$ 7-EC at 37°C prior to reaction termination and analysis. Samples were stored in a conventional freezer at 0°C. Samples were diluted 1:1 with mobile phase before CE analysis.

## 3. Results and discussion

### 3.1. System alignment

In addition to improving sensitivity, the increased capillary diameter at the detection zone provides a larger "target" when attempting to focus the excitation beam cleanly through the flow cell. In these studies, the ELP capillaries were found to be much easier to align than conventional capillaries. The increased size of the ELP flow cell image allows the use of a larger optical slit size while maintaining efficient rejection of optical background (capillary wall scatter and fluorescence). To illustrate this point further, background signal levels were mapped relative to detector position (Fig. 2). This plot was obtained by translating the PMT assembly with a narrow slit across the far-field image (see Experimental). As is evident from the plot, the conventional image is three times narrower than the ELP capillary. Vertical lines mark the slit positions used in the constant background experiment (see below). Clearly, a larger percentage of the signal can be collected while maintaining efficient background rejection with the ELP.

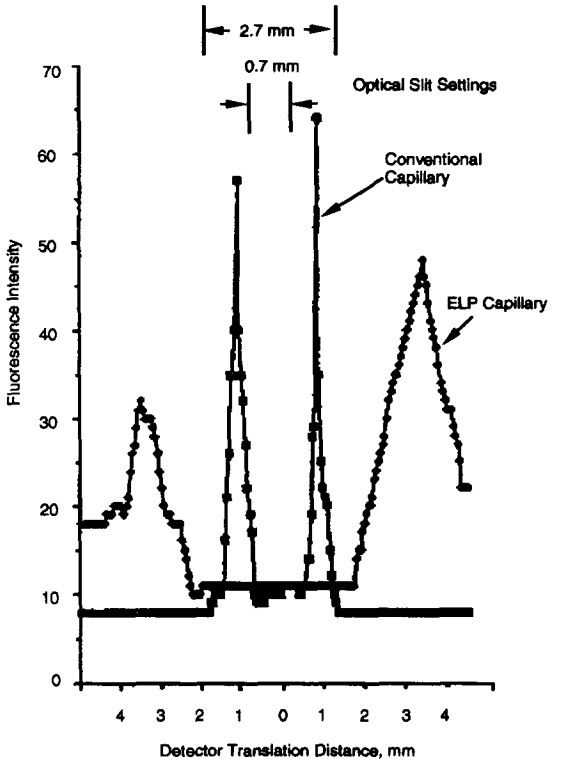


Fig. 2. ELP and conventional capillary wall fluorescence maps. Slit settings illustrate spatial region collected and are not to scale with $x$-axis.

The wider "target" provided by the ELP concept allows the use of simplified instrumentation. Complex optical configurations are not required for clean transmission through the cell or for efficient and selective collection of emission signal. Owing to the relaxed instrumentation and alignment constraints, it is expected that use of ELP cells will add a significant degree of ruggedness to this type of LIF configuration. However, care must be exercised in centering the excitation beam on the bubble.

### 3.2. Comparison of capillary performance

Optimization of LIF detection sensitivity requires maximum collection of the signal coupled with careful rejection of scatter and background fluorescence. Capillaries of conventional dimensions require relatively sophisticated focusing/collection configurations and efficient rejection of capillary wall fluorescence to achieve high sensitivity [2,14]. As an important goal of these studies is to avoid overly complex instrumentation, ELP and conventional capillary formats were compared using relatively simple optics and an adjustable slit. Use of the adjustable slit was key in these experiments in that it provided variable light rejection and the ability to measure the slit widths accurately.

The first experiment involved comparison of the conventional and ELP capillaries with the optical slit adjusted to values which yield equivalent background signals. After alignment of each capillary, the slit width was increased to a distance which gave a background signal value of ca. 40 mV. In this case, the larger dimensions of the ELP detection zone allowed the use of wider slits and, hence, a longer effective path length. Fig. 3 shows electropherograms of Dns-phenylalanine obtained for each capillary under these conditions. The ELP signal was a factor of ca. seven greater relative to the conventional capil-

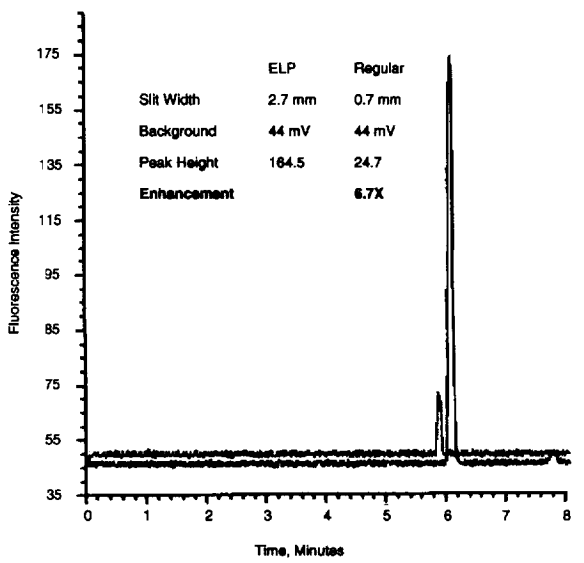


Fig. 3. Sensitivity comparison for conventional and ELP capillaries. Conditions: 0.01 *M* sodium phosphate buffer; applied voltage, 20 kV; 50 $\mu$m I.D. capillaries, 56 cm total length; $\lambda_{ex} = 325$ nm, $\lambda_{em} = 540$ nm; sample, $10^{-5}$ M Dns-phenylalanine.

lary. The inset in Fig. 3 summarizes the results for this series of experiments. The difference in slit widths (a factor of 3.8) is close to three-fold, as expected from increased capillary dimensions. However, the observed gain in signal exceeds the expected value (3.8-fold) stemming from the increased pathlength. The following factors could contribute to the higher than expected enhancement of the signal. First, the simple dependence of the fluorescence signal on pathlength assumes a uniform pathlength for the entire cross-section of the excitation beam. In this work, the focused laser beam spot size (estimated to be 30–40 $\mu$m) is appreciable relative to the regular capillary diameter, thereby diminishing its effective pathlength (i.e., moving radially from its center, the laser beam intersects the capillary channel over diminishing distances, hence the average pathlength is less than the capillary diameter). Second, centering of the emission slit relative to the far-field signal image is also more difficult to accomplish with the smaller (regular) capillary, so light transmission is less than optimal. Finally, the focusing effect provided by the curvatures of the outer and inner surfaces of the capillaries is expected to influence the beam intensity differently owing to differences in curvature. In fact, the higher than expected signal enhancement with the ELP capillary underscores the simplicity of alignment.

To assess these effects more carefully, calibration plots were generated for injections [five concentrations spanning the range from the limit of detection (LOD) to $5 \cdot 10^{-7}$ *M* in triplicate] of sodium fluorescein in the capillary zone electrophoresis mode. Under carefully optimized conditions, the ELP capillary exhibited a 4.25-fold improved LOD relative to the conventional case. Table 1 compares the analytical figures of merit for the two formats based on three calibration plots.

In addition to improving sensitivity, a decrease in efficiency is attributed to the ELP expansion region. In these studies, the efficiency loss is roughly 30% (estimated from Fig. 3), presumably owing to movement of the band into the larger volume region of the ELP. Experiments aimed at a more quantitative assessment of this effect are underway and will be the topic of a later paper.

The use of capillaries with even smaller dimensions (<50 $\mu$m) is attractive for application to rapid CE separations [9,12,19,20]. However, the alignment considerations outlined above become increasingly serious as the capillary diameter is reduced. A 25-$\mu$m diameter capillary with a 5× expansion is commercially available and it exhibits similar advantages (see below). Relative to

Table 1
Calibration data for 50-$\mu$m I.D. conventional and ELP capillaries

| Parameter | Conventional | ELP |
|---|---|---|
| Slope | 1.056 | 3.096 |
| LOD (*S*/*N* 2) | $2.7 \cdot 10^{-10}$ *M* | $6.4 \cdot 10^{-11}$ *M* |
| Amount injected (1 nl) | $2.7 \cdot 10^{-19}$ mol | $6.4 \cdot 10^{-20}$ mol |
| Correlation coefficient | 1.000 | 1.000 |

Conditions: Analyte, sodium fluorescein; 50 $\mu$m I.D. capillaries, 37 cm total length, 15 kV applied; $\lambda_{ex} = 488$ nm, $\lambda_{em} = 510$ nm cut-off filter; 10 m*M* sodium phosphate buffer (pH 6.0); slit width, constant at 2.7 mm; dynamic range, ca. 2.5 orders of magnitude in injected concentration.

the work reported herein, it is also important to note that problems with the rejection of optical background are likely to be more significant when smaller fluorescence Stokes shifts are involved and/or at shorter wavelength excitation which can produce greater silica capillary fluorescence.

### *3.3. Determination of 7-ethoxycoumarin and metabolites from in vitro preparations*

CE–LIF is well suited for the analysis of in vitro-generated samples owing to the high sensitivity and speed of separation possible with this combination. The nature of this approach to the study of metabolism often allows the rapid generation of large numbers samples. As a result, analysis time becomes an issue as this step is often rate-limiting. Most conventional in vitro chromatographic assays are based on HPLC and require gradient elution due to the presence of endogenous material, the complex mixtures generated and/or the wide disparity in polarity between parent compound and metabolite(s). The high resolving power of CE and the selective nature of fluorescence detection alleviate many of these difficulties.

Liver slices are useful in the study of drug metabolism in that they provide insight to both Phase I (e.g., simple hydroxylation) and Phase II (conjugation) metabolism. Since the cellular architecture is preserved in these systems, few endogenous interferences are observed. However, multiple metabolites are produced, requiring resolution from one another for accurate analysis. In the case of 7-EC, three metabolites are produced: 7-hydroxycoumarin (7-HC), the 7-glucorinide and the 7-sulfate.

Conventionally, these analytes are analyzed via ion-pair gradient HPLC with UV absorbance detection [20]. This approach requires a total analysis time of ca. 30 min. Although improvements employing short HPLC columns have recently been made [21], the total analysis time still remains at ca. 10 min. In the MECC mode, the CE separation of solutes with wide variations in polarity which are poorly retained and separated can be easily addressed without the use of gradient elution or lengthy run times. Fig. 4 shows an overlay of MECC chromatograms of liver slice media run in 25 $\mu$m I.D. capillaries. Here, the ELP expansion is fivefold at the detection region. While all three major metabolites are known to be present in this sample (via HPLC–UV), only 7-HC is detected. In this case, the conjugated metabolites could not be determined owing to their presence at low levels and the fact that they have significantly different excitation/emission wavelengths. Clearly, the increased response observed in the ELP capillary provides sufficient enhancement to allow quantification at lower levels. Relative to the conventional format, the ELP provides a ca. fivefold increase in response for these analytes, underscoring their utility in industrial bioanalysis.

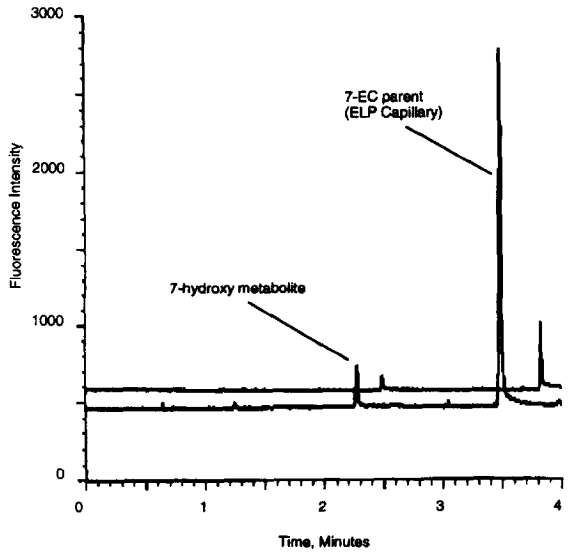


Fig. 4. Separation of 7-EC and metabolites in liver slice incubation media. Conditions: 0.01 *M* sodium phosphate buffer–0.05 *M* SDS; 37 cm total capillary length; applied voltage, 20 kV; 7-HC, $8 \cdot 10^{-8}$ *M*; 7-EC, $1 \cdot 10^{-5}$ *M*; slit width, 1.67 mm.

## 4. Conclusions

The use of ELP capillaries for LIF detection in CE results in improved detection characteristics relative to conventional capillaries. Direct com-

parisons between the two formats shows a substantial improvement in sensitivity for the test compound used. This improvement is attributed to a combination of increased optical pathlength coupled with improved transmission of emission signal. In addition to performance characteristics, the increased probe volume in the detector cell eases difficulties with system alignment and allows the use of simplified instrumentation. Incorporation of ELP capillaries into CE–LIF configurations can be expected to improve system ruggedness and precision. In addition to applications involving CE, the ELP capillary format may prove useful for HPLC separations. Microbore and microcapillary separations have been interfaced to LIF instrumentation via fused-silica capillaries [8], and suffer from similar limitations to those outlined above for CE. The use of ELP capillaries should theoretically improve the detection characteristics in these techniques.

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