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ENHANCED PERFORMANCE OF A LASER-INDUCED FLUORESCENCE LIQUID CHROMATOGRAPHIC APPARATUS: A SYSTEMS APPROACH

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SUMMARY

A laser-induced fluorescence liquid chromatographic apparatus has been developed, using a systems approach, resulting in enhanced overall performance. Supporting evidence will be shown concerning four factors that impact most on these types of systems: (1) increasing sample introduction efficiency; (2) achieving high chromatographic efficiency; (3) maintaining maximum illumination efficiency; and (4) obtaining the greatest possible detectivity. Specific examples include a continuously purged injection valve, columns packed with constant-packing-rate conditions, efficient optical imaging using lightguide technology and photon counting detection electronics.

INTRODUCTION

The use of laser-induced fluorescence as a detection scheme for liquid chromatography (LC) is well documented[1]. Lasers are ideal for fluorescence detection because of the well known direct proportionality between the intensities of the source and resulting fluorescent signal[2]. Other advantages of lasers include high photon flux, beam collimation and monochromaticity; the last two combine to permit the beam to be focused to a very small spot[3]. This last point is very important when considering microcolumn LC, which requires a very small flow-cell volume for optimum analytical utility.

Microcolumn LC has been developed into a highly efficient analytical technique for many separations, as illustrated in a recent review[4]. Several papers [5–7] describe the combination of microcolumns, which provide significant solute concentration enhancement, with laser-induced fluorescence, which is a highly selective and sensitive detection technique. Most of these applications are characterized by the use of expensive, high power lasers, sophisticated optics, and complicated detection/signal processing electronics. Nonetheless, minimum detectable amounts in the 10^{-18}–10^{-15} g range have been reported.

Optical interferences may be regarded as fundamental barriers to improved sensitivity in these systems. Generally, two types of interferences are seen, scattered or reflected laser light (Rayleigh scattering) and background luminescence (Raman scat-

tering and ubiquitous molecular fluorescence). Rayleigh scattering can often be rejected through the use of optical filters; but Raman bands from the solvent (or the optics) are more difficult to reduce, particularly when the scattering overlaps the emission band of the analyte[2]. After achieving the required wavelength selectivity in the emission signal (often resulting in diminished signal strength), one approach to improving detectivity is the use of a signal-to-noise (S/N) enhancement technique, such as lock-in amplification[6] or photon counting[8].

Our work will focus on improving detectability while still maintaining a very modest cost (under $10 000) for the laser, optics and all electronics (*i.e.* the detection system). We will meet this goal through improvements in injection efficiency, an improved column packing technique, employment of lightguide technologies and effective use of a novel photon counting circuit. These efforts have resulted in a very efficient LC measurement system.

EXPERIMENTAL

Sampling/injection system

The injection system consisted of an electronically actuated Valco CI4W valve, with a 60-nl internal loop (Houston, TX, U.S.A.). A constant purge device was developed, in-house, to reduce chemical contamination of the sample loop. This unit,

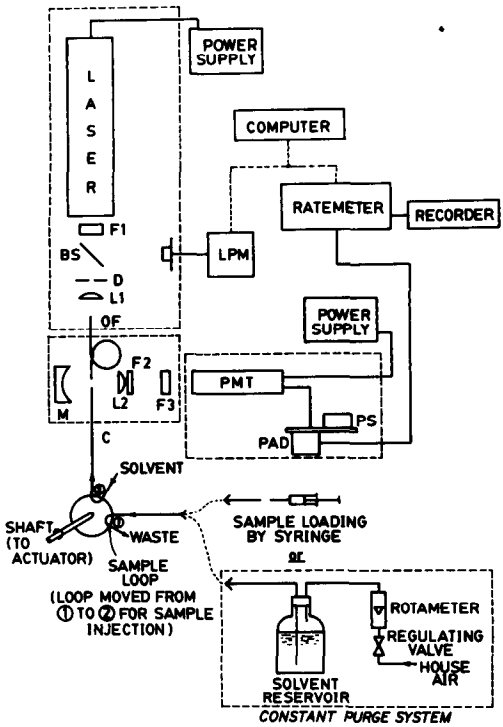

Fig. 1. Schematic diagram of the injection, separation and detection systems: F1 = liquid filter; BS = beamsplitter; D = diaphragm; L1 = planoconvex lens; OF = optical fiber; M = mirror; C = column; L2 = planoconvex lens; F2 = blocking (cut-in) filter; F3 = liquid filter; PMT = phtomultiplier tube; PAD = preamplifier/discriminator; PS = power supply; LPM = laser power meter.

displayed at the bottom of Fig. 1, was based on a compressed-air-driven gas displacement pump design. A bottle of solvent [tetrahydrofuran (THF)] was connected to the injection valve with a length of 250 μm I.D. fused-silica tubing (Polymicro Technologies, Phoenix, AZ, U.S.A.), with a small gauge (22S) needle cemented at the valve end. This needle was only removed from the valve when making an injection, hence continuous washing of the loop was provided.

Chromatographic system

The construction of the microcolumns, as well as the details of the packing study, have been described in a previous study[9]. All columns were packed and evaluated on an ISCO μLC-500 pump (Lincoln, NE, U.S.A.) using either 3-μm ODS Shandon Hypersil (Keystone Scientific, State College, PA, U.S.A.) or 5-μm Spherisorb S50DS2 (Alltech Assoc., Waukegan, IL, U.S.A.). For the test mixture and for the polynuclear aromatic hydrocarbon (PAH) mixture, we used a 750-mm fused-silica column (0.25 mm I.D.) packed with 5-μm Spherisorb.

Perylene was chosen as the standard for experiments at 442 nm, while pyrene was used at 325 nm. These standards, as well as a sixteen-component PAH mixture, were analytical grade and were purchased from Chem Service (West Chester, PA, U.S.A.). All solvents for the mobile phase were HPLC-grade and were obtained from Burdick & Jackson (Muskegon, MI, U.S.A.).

The flow-cell was a *ca.* 1-mm section of the column end connection, and was formed by acid etching of the polyimide coating with hot, concentrated sulphuric acid. This length restricts the flow-cell volume to only 75 nl, minimizing extra-column effects. The end connection was positioned inside a 0.0625-in. O.D aperture tube and was fastened inside a brass flow-cell body. The entire assembly was mounted in a Physitec (Norfolk, MA, U.S.A.) adapter plate/microbench.

Excitation and detection system

The excitation and detection system, as seen in Fig. 1, consists of six major components: (1) excitation source; (2) excitation optics; (3) emission optics; (4) detector and photon counting electronics; (5) ratemeter; and (6) recorder or computer.

A Liconix (Sunnyvale, CA, U.S.A.) 4210 NB helium–cadmium laser provided the incident radiation, and generated output powers of *ca.* 2.5 mW at 325 nm (beam diameter 1.1 mm, TEM_{01}) and *ca.* 15 mW at 442 nm (beam diameter 1.1 mm, TEM_{00}). The laser light was then transmitted through four optical components: a 10-mm pathlength liquid filter (cobalt sulfate for 325 nm, copper nitrate for 442 nm) a 0.25-mm thick quartz beamsplitter (Wilmad Glass, Buena Vista, NJ, U.S.A.), a 2-mm I.D. iris diaphragm (Physitec) and a 10-mm diameter, 19-mm focal length quartz planoconvex lens (Oriel, Stratford, CT, U.S.A.). For efficient transfer of either excitation or emission light, we chose a UV-grade optical fiber (Polymicro Technologies). This material had a core diameter of 120 μm, an O.D. of 200 μm, and provided 45–50% transmission efficiency for both 325 and 442 nm. A Model 45-PM laser power meter (Liconix) was used to make all laser power measurements as well as to provide continuous monitoring of laser output.

The fluorescence signal was then passed through the emission optical components. A quartz planoconvex lens, 10 mm diameter and 15 mm focal length (Physitec) was placed adjacent to the flow-cell. Next, a glass, cut-in filter (Oriel) was used to

isolate the emission signal from the laser radiation. A liquid filter (copper nitrate for 325 nm, copper chloride–calcium chloride for 442 nm) was placed between the glass filter and the transducer for additional isolation of the two signals.

The resulting collimated fluorescence beam was directly imaged onto a miniature photomultiplier tube (PMT) (Hamamatsu, Middlesex, NJ, U.S.A.), Model R-1635-02. This transducer, operating at a bias of −1250 V, provides a quantum efficiency of 14.2% at 420 nm (pyrene emission) and 7.45% at 500 nm (perylene emission)[10]. Virtually all of the 8-mm diameter photocathode was illuminated. The output of the PMT was connected to an Amptek (Bedford, MA, U.S.A.) Model A-101 preamplifier/discriminator assembly, which we used as our photon counting circuit. This device is a unique, hybrid microelectronic charge-sensitive preamplifier/discriminator, all incorporated into a single transistor package. The adjustment of discriminator level in this circuit has been described[8]. The pulse output of this circuit was directed to the TTL input of a Model PRM-100S ratemeter (Modern Instrumentation Technology, Port Matilda, PA, U.S.A.), which had both analog and digital (8-bit parallel) outputs. The analog output signal was connected to a Type LS recorder (Linseis, Princeton-Jct, NJ, U.S.A.), and the digital signal was sent to a Leading Edge Model D computer (Canton, MA, U.S.A.). We chose the recorder for all our detector Figure-Of-Merit determinations, and we used a 10-s analog output filter time constant on the ratemeter. More importantly, the ratemeter gate-time was fixed at 100 ms.

We evaluated two different optical configurations to achieve optimum detection system performance. Configuration 1, where the optical fiber carries the laser light to the flow-cell, is shown in Fig. 1. This alignment includes a 22-mm diameter, 8-mm focal length mirror to retro-reflect any fluorescence from the flow-cell. Configuration 2, where the fiber carries the fluorescent signal to the PMT, is shown in Fig. 2 and is similar to a design used by Sepaniak and Yeung[11]. The excitation optics are similar to those in configuration 1, but the placement of the flow-cell is different. Here, the laser is directly imaged into the flow-cell, and the subsequent emission is transmitted through the fiber to the planoconvex lens, cut-in (blocking) filter and solution filter of the emission optics. Therefore, the role of the fiber is reversed in configuration 2 from that of configuration 1. The laser power meter was used to make the power distribution measurements.

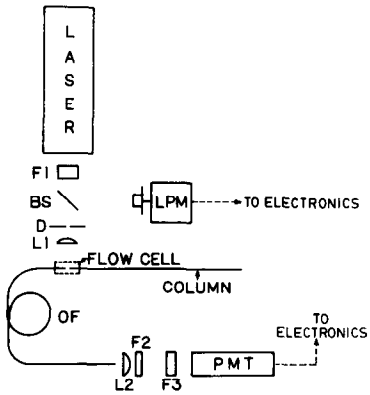

Fig. 2. Optical configuration 2: abbreviations as in Fig. 1.

RESULTS AND DISCUSSION

Evaluation of the sampling/injection system

We have recently utilized several procedures to enhance the ease of injection, as well as improve injection efficiency. Referring again to the constant purge device in Fig. 1, we have observed dramatic reductions in the washout problem associated with these valves when injecting non-aqueous solutes. For example, we found only negligible carryover with a blank injection that was preceded by an injection of 10^{-4} *M* perylene. Prior to using this device, we experienced washout problems that persisted for as long as 24 h for both pyrene and perylene[12].

Two other procedures have resulted in reductions of extra-column effects at the injection valve: (1) inserting the column into the pump channel of the valve (instead of the column channel); and (2) using a moving injection technique. Fig. 3 is a cutaway drawing of the Valco CI4W valve. Noting that the pump channel is 0.006 in. wider than the column channel, the column may be inserted through the wider channel (Fig. 3B). A Teflon sleeve is used to position the column in this channel, as shown. The column inlet is extended to within *ca.* 0.003 in. of the loop. The result of the new connection is that the particles at the column head are much closer to the loop than the previous arrangement (Fig. 3A), producing minimal unswept volume and more efficient solute transfer. This column connection (Fig. 3B) was developed with the aid of several consultations with scientists at Valco[13] and Brigham Young University[14].

Presently, an electronic actuator is being used to facilitate injection, in a "moving loop' technique. Timing of the injection cycle, combined with a 2-s injection period, has nearly eliminated exponential washout, a common feature of these valves. Based on a paper by Harvey and Stearns[15], we estimate a 30% injection of the total loop volume (18 nl for a 60-nl loop).

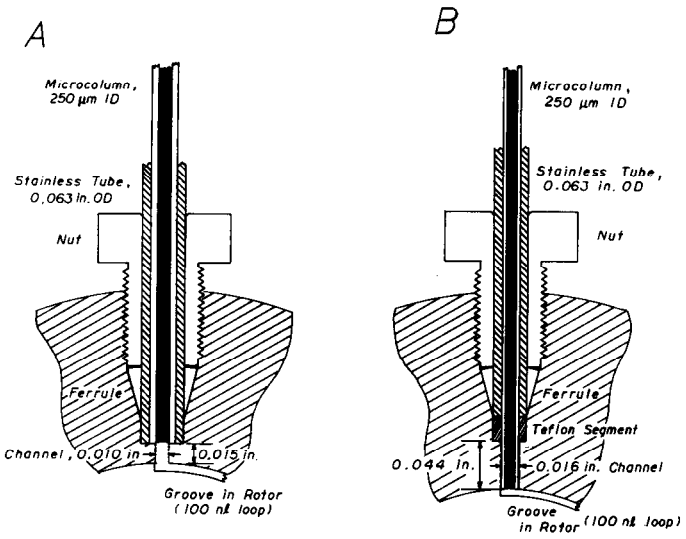

Fig. 3. Detail of column–valve connections: (A) column port attachment, end of column extends to port opening; (B) pump port attachment, end of column extends through port opening to rotor.

Optimization of the chromatographic system

Most recent efforts have focused on optimizing the pump pressure *vs.* packing (filtration) rate programs. These programs were employed by manually increasing the pump pressure control by a fixed amount every 30 s, simultaneously measuring bed height with a meter stick. This constant time interval enabled the packing rate to be determined, in a straightforward manner. A linear pressurization regime will likely result in a more uniform, more efficient bed. Following the technique proposed by Andreolini *et al.*[16], we obtained a constant packing rate (using 5-μm Spherisorb) after the first minute, but showed non-linearity prior to the first minute. However, we achieved minimum reduced plate heights (h_{min}) of 2.63–2.67. Although most of these packing studies were completed some time ago[9], the constant filtration rate work is still in its early stages and is a topic for a separate paper[17]. We hope to achieve more complete control of the pressure-time profile by computer interfacing the pump, thus enabling us to determine relationships between filtration rate, longitudinal bed uniformity and chromatographic efficiency.

Detector performance

Table I displays a comparison between detector performance at 325 and 442 nm for the two different optical configurations. The power distrubution data show that the two configurations were similar in their ability to deliver a respectable power level to the flow-cell. These measurements do not reflect the actual spot diameters, however. Therefore, it is reasonable to expect that the flow-cell power level for configuration 2 was actually *ca.* ten times that for configuration 1, since the lens (configuration 2) provided a much smaller spot diameter than the optical fiber (configuration 1). We used an equation, given by Scott[18], to determine detector minimum detectable quantities (MDQs). Use of this equation assumes a Gaussian profile, with the solute concentration at the peak maximum taken to be twice the average concentration. Lower detection limits were seen for 442 nm, which can be partially attributed to the higher power density at this wavelength. The fact that perylene has a higher quantum efficiency than pyrene (Φ_F for perylene = 0.94 and Φ_F for pyrene = 0.32[19]) certainly contributed to the results. The improvement in MDQ at 442-2 (442 nm excitation line, configuration 2) compared to 442-1 (442 nm excitation line, configuration 1) is partially due to the fact that a more efficient column was used for the configuration 2 measurements. However, it is interesting that the background count

TABLE I

DETECTION SYSTEM PERFORMANCE DATA

	Perylene		*Pyrene*	
	442-1	*442-2*	*325-1*	*325-2*
Power at laser (mW)	14.0	15.5	3.10	2.05
Power through filter (mW)	10.0	11.3	2.08	1.65
Power at flow-cell (mW)	6.20	6.65	1.55	1.10
R_b (kHz)	500–600	0.1–0.2	1000–1100	0.1–0.3
MDQ detector (fg)	10	1.7	540	180

rate (R_b) in the second configuration was nearly 10^4 *lower*. Experiments using the 325-nm excitation line yielded similar results: 325-2 gave almost a 10^4 decrease in R_b compared to 325-1, and 325-2 gave somewhat better detector performance as well. The high background count rate for configuration 1 can not be attributed to varying chromatographic conditions, because open tube measurements were performed for both configurations (at 442 nm), yielding the same background rates as for the packed column experiment. It has been thought that for best performance, photon counting needed to be accomplished at low count rates, yet our data show that we achieved somewhat lower MDQs with the high background configuration. This can be attributed to the large bandwidths of both the preamplifier/discriminator (4 MHz) and the ratemeter (100 MHz) as well as the fast rise-time of the PMT (0.8 ns, which corresponds to a bandwidth of *ca.* 4 GHz). More work must be done to completely characterize the kinetic performance of the photon counting electronics.

These data in Table I prompted us to investigate the source of the high background count rate for configuration 1. A test mixture containing acetone, sodium chloride and pyrene was formulated in order to study the optical response characteristics of the flow-cell. This mixture was chosen because acetone absorbs strongly at 325 nm, sodium chloride can function as a refractive index (RI) probe, and pyrene was previously used as a fluorescent analyte for excitation at 325 nm. As shown in Fig. 4, peak 1, occurring at *ca.* 3 min, was mainly due to the RI change of the sample solvent and sodium chloride. Peak 2, the acetone peak, eluted just after 3 min and generated only a refractive index change. Upon elution into an emission detector, a UV-absorbing solute should generate an inverted peak, provided that the excitation and background wavelengths are the same. Since acetone displayed only an RI

Fig. 4. Chromatogram of flow-cell performance test mixture. Components: acetone, 1%; sodium chloride, 250 ng/ml; pyrene, 1000 ng/ml in acetonitrile–water (65:35). Mobile phase, 100% acetonitrile.

change, reflected laser light, occurring outside the flow-cell in the emission optics, may be reaching the PMT. We can not conclude that the flow-cell is relatively immune to RI effects because sodium chloride has a similar RI to acetonitrile. It is interesting that only positive-going peaks were seen for sodium chloride and acetone. This may be due to the unique optical arrangement of configuration 1 compared to absorption-type detectors, which generate characteristic positive/negative RI peaks. Peak 3 was the usual pyrene peak, eluting at *ca.* 7 min.

We investigated the possibility that reflected/scattered light, arising from the optical fiber, was contributing to the high background of configuration 1. Reflected laser light, that exits from the optical fiber without passing through the flow-cell, may be collected by the emission optics. This light could originate in cladding or buffer modes or in a poorly-cleaved fiber end-face. We coiled the fiber in a small-diameter loop and meticulously cleaved and cleaned the fiber end-face, producing both improved transmission efficiency and a slight reduction in background count rate. The mirror-lens combination in configuration 1 produces nearly a 1.5 stearadian collection angle compared to the small 25.4 degree acceptance cone of the optical fiber in configuration 2. Therefore, the emission optics of configuration 2 are much more selective than those of configuration 1. However, it is unlikely that collection efficiency alone would account for the large background count rate. Additionally, Raman scattering that originates within the fiber may be reaching the PMT. Though we have made preliminary measurements of the background emission spectra for configuration 1, the results are inconclusive. Raman scattering from solvent within the flow-cell has been a complicating factor, as well as the difficult optical alignment necessary to carry out these measurements. We are exploring these aspects in more detail.

In spite of the slight performance advantage of configuration 2, as evidenced in Table I, it is unclear which configuration is preferred when considering linear dynamic range performance. We have observed a linear dynamic range of two orders of magnitude for configuration 1, whereas for configuration 2 linearity was observed for one decade of concentration, at best. Some of these features may be attributed to photophysical phenomena, such as excimer emission, delayed fluorescence or E-type (eosin) fluorescence, as described by Parker[20], that contribute to different extents in the two configurations. Probably these small linear calibrations are limited at high concentration by primary absorption, as defined by Christman *et al.*[21]. Wider linear dynamic ranges will result from a combination of several factors: (1) reduction in R_b; (2) identification of the source of detrimental photophysical effects; and (3) improved detectability through optical/electronic improvements.

Evidence of an overall highly efficient LC apparatus is presented in Fig. 5, which shows a PAH separation in which thirteen of the sixteen components were detected in 15 min (30 pg injected). A subsequent injection of only 3 pg of the same mixture resulted in detection of several peaks. For the pyrene peak, this would represent an MDQ below 200 fg, a very good result considering the low excitation power level and modest total cost of *ca.* $10 000 for the detection system. Another noteworthy aspect of Fig. 5 is the efficiency of the peaks, reflected in their widths. High efficiency chromatography demands fast, accurate detection electronics; and it is apparent that the photon counting system was adequately following the solute elution profile, characteristic of an efficient separation. We have not explicitly evaluated the

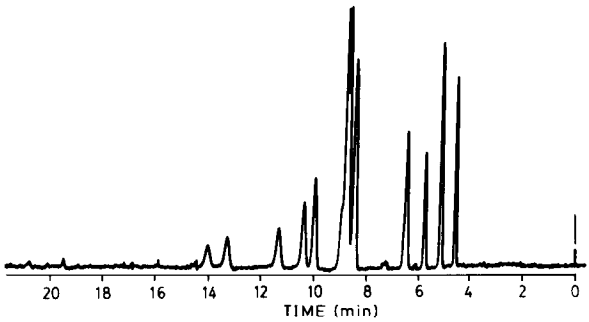

Fig. 5. Chromatogram of polynuclear aromatic hydrocarbon mixture: 30 pg injected amount for each of sixteen components. Mobile phase, acetonitrile–water (92:8).

temporal response characteristics of the detector. However, it must be remembered that the detection technique is digital and therefore not subject to the same distortion that RC-analog systems impart to fast signals. Thus, our photon counting system is quite appropriate for signals on the chromatographic time-scale. This aspect of detector operation will be included in a future paper.

CONCLUSIONS

We have demonstrated good overall system performance of our laser-induced fluorescence LC apparatus by critically evaluating the sampling/injection, chromatographic and detector systems. Modifications in our injection valve have resulted in two improvements: (1) almost no carryover from previous injections (no memory effects); and (2) more efficient solute transfer due to a near-zero dead volume column connection. Improvements in the chromatographic system, through a constant packing rate, have resulted in more uniformly packed columns and better efficiencies. In addition, we have obtained very good detector MDQs for both optical configurations, while operating under the constraints of low cost and low laser power. Most importantly, we have shown that a systems approach to instrument optimization, where improving each subsystem in the apparatus with constant consideration of the interrelationships between them, will enhance overall performance.

Some of our future work will include spectrometer measurements that identify the source of the high background count rate for configuration 1, and a critical evaluation of the small linearity in configuration 2. Because photon counting is most advantageous at low light levels, the configuration which will perform best is the one that exhibits both a low background count rate and wide linearity. We will also improve detector performance through greater utility of photon counting. One of our goals is to make this unique detection technique more useful for analytical separations.

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