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Laser-based detectors in chromatographic analysis*

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Abstract: Advances in detection technology have been a vital part of the development of microscale chromatographic techniques. Separation techniques such as microbore liquid chromatography impose severe constraints on the permissible volume of detector cells. The use of lasers to construct a new generation of chromatographic detectors has satisfied the need for low volume detection and high sensitivity. The unique properties of laser radiation have been used to advantage in designing new approaches to the detection of optical absorbance through fluorescence emission and thermal effects. New approaches to monitoring refractive index changes and optical rotation have also been developed. Together, the combination of microscale-separation techniques and highly sensitive detection provide a powerful tool for analysing small quantities of samples. Yet, there are practical limitations arising from the cost and complexity of much of the instrumentation reported to date, as well as the difficulties of preparing small samples for analysis which have limited the wide-scale application of these methods to solve practical problems. Recent advances in laser technology such as the advent of diode lasers may be useful in overcoming some of these limitations.

Keywords: *Lasers; detectors; fluorescence; thermal lensing; photoionization; micro-chemical analysis.*

The Miniaturization of Separation Techniques and the Need for New Detectors

The development of microscale-separation techniques such as microbore liquid chromatography (μLC) [1] and capillary electrophoresis [2] has been a major emphasis in separation sciences since the mid 1970s. Advances in these areas have been based on substantial reductions in the scale of the column or electrophoresis tube. In μLC the columns are typically <0.2 mm dia. Capillary electrophoresis is typically conducted in tubes <0.1 mm dia. Separations conducted on this scale can produce significant advantages over larger scale separation techniques in terms of efficiency of separation, resolution and mass sensitivity. However, reducing the scale of the separation is not a trivial task. Pumps capable of accurately metering flow rates in the low μl min^{-1} range were needed for μLC. Very low volume sample introduction schemes are also required for these techniques. Not surprisingly, when sample introduction volumes are small, and separation efficiencies are high, peak elution volumes are correspondingly small. It is the small peak elution volumes which present a challenge to detector technology [3].

In order to preserve the advantages enumerated above for small scale separation techniques, it is important to minimize losses in separation efficiency arising from post-column band broadening. As a general rule, detector volumes needed to avoid excessive dilution of the sample as it passes through the detection cell are less than one-tenth of the smallest volume peak expected in the separation. Therefore, for μLC, detection volumes of much less than 1 μl are required. The difficulty in adapting conventional optical detection methods to these requirements is largely that the sensitivity of such measurements is highly dependent on the path length of the sample. The restriction of having to work with very small volumes generally imposes severe constraints on sample path length. Therefore, the development of microscale-separation techniques has spurred the simultaneous development of detection methods suitable for these systems. Such methods must have high mass sensitivity to enable detection of small scale samples, low cell volumes to be compatible with the separation technique, and should offer numerous and versatile modes of detection.

Taking Advantage of the Properties of Lasers

Lasers differ from conventional (broad band) light sources in several important prac-

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tical aspects [4, 5]. Laser emission is generally highly monochromatic, collimated, coherent, polarized, and can be of high peak or average power. Each of these properties is important for the development of small volume detectors.

Laser beams, like all light beams, can be thought of as a stream of photons. Certain aspects of the behaviour of photons can be described by modelling them as electromagnetic waves. When all the photons have identical wavelengths, the beam is said to be monochromatic. When all the waves are in phase with each other, the beam is coherent over time and space. Furthermore, when the planes containing the sinusoidal waves of each photon are aligned in parallel, the beam is polarized. These are properties which are highly characteristic of laser emission.

Collimation may be considered as the ability of a laser beam to maintain its diameter while transversing relatively large distances. This property is easily visualized if one compares the shape of a flashlight beam (noticeably conical) with that of a laser beam (thin and straight) propagating through a dark room. Collimation minimizes loss of intensity arising from beam divergence.

In total, lasers provide the ability to generate spectrally pure emission, of high power, which can be easily focused into small volumes. The key to laser-based detector development, then, is to utilize spectroscopic techniques which can capitalize on these properties to provide highly sensitive detection of a wide variety of analytes contained in small detector volumes.

In general, the detectors discussed in this review are based on two broadly differing mechanisms by which the analytes interact with a laser beam to produce a detectable signal. One class of detectors is based on photoabsorbance. The other broad class of detectors is based on changes in the refractive index (RI) within the flow cell arising strictly from the presence of the analyte.

The photoabsorbance process, and some of the physical events which can follow it, are illustrated in Fig. 1. In conventional absorbance spectrophotometry, the absorbance of a photon is detected by comparing the intensity of the excitation beam before and after it interacts with a sample. The magnitude of the detected signal is directly proportional to the path length of the cell. Thus, for very small path lengths, this measurement approach requires that increasingly small differences between two large signals must be accurately measured to detect a constant concentration of the analyte. Clearly, this is a disadvantage for detection in microscale-separation techniques. Thus, the most successful approaches have been those which utilize measurements which are relatively independent of the sample path length. The fluorescence, thermal relaxation and ionization events shown in Fig. 1 are examples of physical phenomena which can be used to detect photoabsorbance based on zero blank measurements. Such measurement processes can be performed relatively independently of sample path length because the signal is proportional to excitation power.

In zero-blank measurements, the detected signal approaches zero as the sample concentration decreases. Compared with conventional absorbance measurements in which one must determine ever smaller differences between two large signals (sample and reference

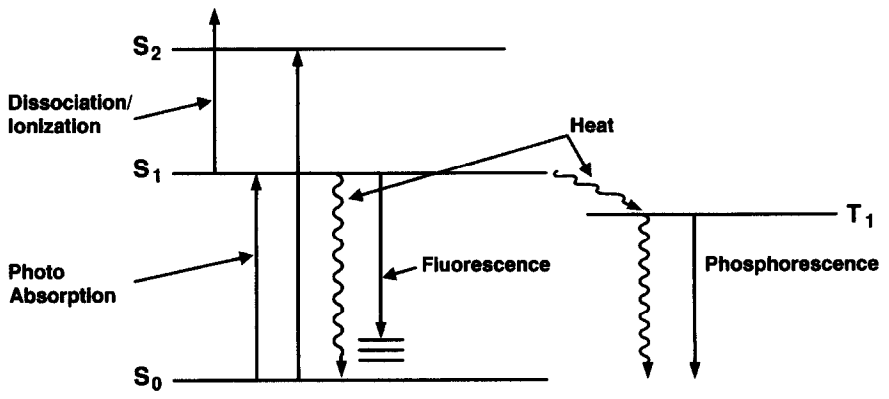

Figure 1
Jablonski diagram illustrating the processes of photoexcitation and relaxation.

beams) as the sample concentration decreases, this is a more favourable measurement situation.

Laser-induced Fluorescence

As illustrated in Fig. 1, fluorescence is the rapid emission of a photon which sometimes occurs as a molecule relaxes from a higher energy state (which it reached by photoabsorbance) to the ground state. The measurement of fluorescence emission is based on the detection of photons emitted from a fluorescing sample. The intensity of fluorescence is directly proportional to the number of fluorescing species and the intensity of the excitation source. Hence, the sensitivity of the measurement can be increased by increasing the intensity of excitation. Since the intensity of fluorescence is measured relative to the signal (usually very small) produced in the absence of any fluorescing species, this is a zero-blank measurement. However, in a real measurement, the background signal is never truly zero. Moreover, the intensity of the background signal is also proportional to the laser power. Therefore, simply increasing excitation intensity does not yield the maximum possible return in decreasing detection limits achievable with laser-induced fluorescence (LIF). To minimize detection limits, contributions to the fluorescent blank [6] must be minimized. This is generally achieved through the use of high purity solvents and by careful design of flow cells.

Flow-cell designs in LIF are of primary importance because the optical properties of the cell have a significant impact on the level of background signal contributed by the cell walls and the interface between cell walls and sample solvent. The importance of these contributions in small volume cell technology was recognized by Diebold and Zare [7] who published an early report on the application of LIF to achieve sensitive detection of chromatographically separated aflotoxins. Their windowless flow cell design (see Fig. 2) was intended to eliminate possible noise contributions from the cell walls, but suffered from the disadvantage of instability of the optical properties of the solvent droplet. Other windowless flow cells have also been applied to LIF detection in chromatography [8–10]. Many of these cells have been difficult to utilize in applications for which low column effluent rates are required, such as in μLC.

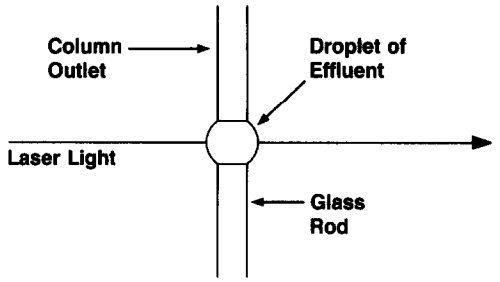

Figure 2
Depiction of a hanging droplet, windowless flow cell as first reported by Diebold and Zare. Adapted with permission from ref. 7 (© American Association for the Advancement of Science, 1977).

Sypaniak and Yeung [11] first reported the use of a flow cell incorporating optical fibre wave guides to reduce the noise contribution from cell walls. More recently, the same design has been utilized by Roach and Harmony [12] who have reduced the volume of the cell to be compatible with microscale separations. A representation of this type of flow cell is shown in Fig. 2. In principle, the flow cell will preferentially transmit fluorescence which originates in the central region of the flow cell rather than scattering and fluorescence from the cell walls. In this way, the detector is optically shielded from contributions to the fluorescent blank arising from the cell wall, yet the problems associated with maintaining consistent optical properties in windowless flow cells are avoided. Attomole detection limits have been achieved with these cells.

Fluorescence is a highly selective process. Only a small number of compounds which undergo photoabsorbance can fluoresce. This can be of advantage in detecting a fluorescent analyte contained in a complex matrix of non-fluorescent compounds. In order to increase the selectivity of the fluorescence process, some researchers have utilized two-photon excited fluorescence as a detection scheme [13, 14]. This approach is only practical when a high-powered laser is used as the excitation source because the probability of a multi-photon absorption event occurring is much lower than the probability of absorption of a single photon. In addition to providing added selectivity to fluorescence detection, this approach also provides a means of lowering the fluorescent blank. The excitation wavelengths used by Pfiffer and Yeung [14] were in the

visible region of the spectrum, yet, because the energies of the multiple-absorbed photons are additive, the emission from their analytes was in the UV region. Therefore, scattering and Stokes-shifted fluorescence was easily rejected from the background signal. A detection limit in the femtogram range was achieved for aryl substituted oxadiazoles.

From a slightly different perspective, the high selectivity of fluorescence detection can be a disadvantage when the compound of interest is not fluorescent. This problem is exacerbated by the relatively small number of wavelengths accessible with most lasers. For example, although tryptophan-containing peptides and proteins can be detected by fluorescence when broad band excitation sources are used, many lasers cannot produce wavelengths suitable for excitation of the tryptophan residues. Therefore, chemical derivatization is often needed before peptides can be detected by LIF. The derivatization is commonly performed pre-column [12], but post-column derivatization with *o*-phthaldehyde for the analysis of amino acids via open-tubular chromatography has been reported [15] with femtogram detection limits.

Thermal Lensing Techniques

The selectivity of fluorescence arises because many compounds which undergo photo-absorbance do not relax by emission of light. As illustrated in Fig. 1, the most common alternative to fluorescence is thermal relaxation. The net result of this event is an increase in the temperature of the solution surrounding an excited-state molecule. A change in the temperature of a solution is associated with a change in the RI of that solution. Detection of this change in RI of the solution forms the basis of thermal lens techniques.

Most of the lasers commonly employed in chromatographic detection can be tuned so that the laser beam will have a Gaussian-intensity profile. This is illustrated in Fig. 3 which shows the relationship between intensity and distance from the centre of a Gaussian beam. The RI of a solution through which the laser beam is allowed to pass is dependent on the excitation energy. Since the excitation energy is spatially dependent on the distance from the centre of the laser beam, the RI changes resulting from heat produced by interaction with the laser beam will also depend on the distance from the centre of the beam Fig. 3).

The net result of the heating and RI changes described above is the formation of a lens within the flow cell. Because the RI will be lower in the centre of the lens, a diverging lens system is established (Fig. 4). The simplest means of detecting this phenomenon is to monitor the divergence of a laser beam as it passes through a sample [16]. To perform this measurement on a chromatographic time scale, a modulated laser beam can be used with lock-in detection [17]. However, superior detection limits are most commonly obtained with a dual beam, pump-probe arrangement [18, 19]. In this approach, two laser beams are used; one to form the thermal lens and the other to detect

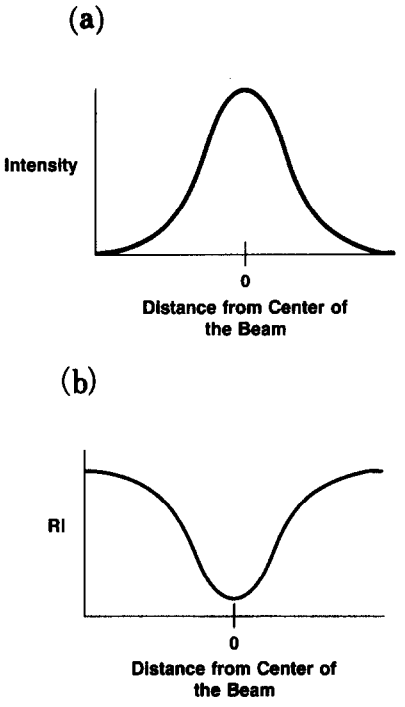

Figure 3
Laser intensity (a) and refractive index of a sample solution (b) as a function of distance from the centre of a Gaussian laser beam.

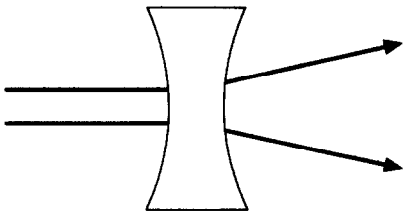

Figure 4
Illustration of the optical properties of a diverging, thermal lens.

it. As in fluorescence spectroscopy, flow-cell design is critical. Excessive turbulence in the flow cell and pulsations from the chromatographic pumps can cause degradation of the thermal lens, thereby lowering the signal-to-noise (S/N) ratio.

Although thermal lens techniques do not provide detection limits as low as those attainable with fluorescence, this is a more sensitive detection scheme compared with conventional absorbance spectrophotometry. A dual beam apparatus was used by Yang *et al.* [18] to detect absorbances as low as 10^{-5} in an 8 mm flow cell for LC detection. Nolan and Dovichi [19] have reported a dual-beam apparatus for μLC detection. They were able to achieve femtogram detection limits for derivatized amino acids.

Photoionization Detectors

The detection schemes discussed thus far have been based on detecting the photoabsorbance by observing how the excited molecule returns to the ground state. As illustrated in Fig. 1, another possible approach is to introduce enough energy into the system to ionize the analyte and then detect this relatively long lived excited state species. Typical ionization energies for organic molecules in the liquid phase range from 6 to 8 eV. To achieve this excitation energy, absorption of two photons is required. Therefore, the high intensity of laser emission is very helpful in achieving a significant analytical signal from this process. The use of photoionization as a detector for LC analysis was first reported by Voigtman and Winefordner [20] who used pulsed N_2 and XeCl eximer lasers as an excitation source. The approach was found to be applicable to the analysis of many polynuclear aromatic hydrocarbons and a wide variety of drugs. However, the detection limits achieved in this study were no better than those attainable with conventional UV absorbance detectors. In later studies, flow-cell volumes had to be scaled down to allow application to μLC, and detection limits have been enhanced to be comparable and occasionally superior to detection by conventional UV absorbance [21, 22]. For example, a detection limit of 8 fmol was obtained for pyrene by photoionization, which compares favourably with the UV detection limit of 31 fmol observed under the same chromatographic conditions [22]. Comparative chromatograms for the two detection techniques are presented in Fig. 5.

The detection limits attainable with photoionization are limited primarily by background contributions from the solvent. The ionization potential of water is approximately 6 eV in the condensed phase. Because this is very close to the ionization potential of many analytes of interest, the solvent contributes a high level of background signal in reversed-phase separations. Less polar solvents, which have higher ionization potentials, also tend to allow recombination of ions before they can reach the detector electrode. Vapour-phase detection would seem to be advantageous based on these physical limitations, however, vaporization of the mobile phase followed by ionization and collection of ions is technically difficult [23].

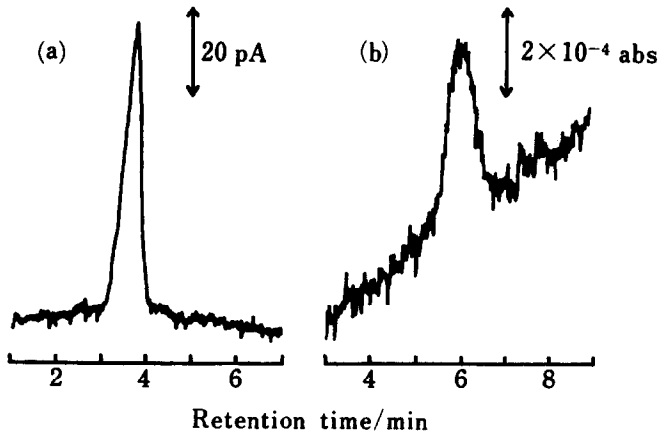

Figure 5
Comparative chromatograms demonstrating photoionization (a) and UV (b) detection of pyrene after separation on a microbore column. Taken with permission from ref. 22.

Detectors Based on Refractive-index Changes

In addition to the approaches discussed above for the detection of the photoabsorption event, many attempts have been made to develop more sensitive detectors for RI changes based on lasers. In contrast to methods based on photoabsorption, RI measurement does not require that the wavelength of the laser be matched to an absorption band of the analyte. Since the presence of virtually any solute will cause a change in RI, detection of these changes is a much less selective measurement process than any of the measurements based on photoabsorption.

The primary advantage of using lasers as light sources in RI detection is a reduction of the flow-cell volume. Detection limits achievable with laser-based RI detectors are roughly equivalent to those attainable with commercially available RI detectors when the comparison is based on ΔRI. Either approach can measure ΔRI in the region of RI = 10^{-7} [24–27]. The primary limitation to achieving superior detection limits with RI measurements is fluctuations in the RI of the sample due to temperature changes and pressure changes arising from pump pulses and other sources.

Refractive-index detectors have been made small enough to use with capillary column LC. Dovichi and co-workers [26] have reported the detection of nanogram quantities of sugars using this technique. Taking this as representative of the state of the art, it is apparent that RI detection is still at least six orders of magnitude less sensitive than fluorescence.

Recalling the earlier discussion of thermal lens phenomena, it can be hypothesized that the sensitivity of these measurements could be greatly enhanced by using a pump-laser beam to excite analytes as they pass through the flow cell, thereby causing a thermally induced change in RI. Indeed, many of the reports of RI detectors include just such an approach to measuring photoabsorbance [24, 27]. Absorbances of approximately 10^{-6} can be detected in this manner, which is comparable to the detection limits achieved with thermal lensing.

It is well known that optically active compounds retard left circularly polarized light differently than they retard right circularly polarized light, thereby giving rise to optical rotation (OR) of plane polarized light. Yeung *et al.* have reported the development of a laser-based OR detector for use with HPLC [28]. This detector has been miniaturized to allow application in μLC [29], and detection limits are comparable to those attainable with RI detection. A representative chromatogram from this work is reproduced in Fig. 6. The ability to determine optical purity by coupling this detector with chromatographic analysis can be expected to be particularly important to analytical chemists working in the pharmaceutical industry.

Figure 6
Separation of chiral components by HPLC with optical activity detection. Component F = fructose, component R = raffinose. Taken with permission from ref. 28.

Future Prospects

As stated in the introduction to this review, the driving force behind the development of laser-based detectors for chromatographic analysis has been the need to provide a new generation of detectors with properties suitable for use with microscale separation techniques. Researchers in the field have been quite successful in achieving this goal. Why then has the application of these techniques been limited to a few laboratories that specialize in the use of lasers for chemical analysis?

One reason may be the cost and complexity of the instrumentation used in these detectors. Helium–cadmium lasers are relatively inexpensive (comparable in cost to a xenon arc lamp with housing and power supply) but offer very limited wavelengths (325 and 442 nm) and low powers, and suffer from high maintenance costs. Another class of lasers which is very simple to operate and relatively inexpensive is the diode laser [30]. Unger and Patonay [31]

have reported the development of an absorbance detector in which the sample cell forms part of the laser cavity, thereby allowing a very sensitive intracavity absorbance measurement. The simplicity of the instrument design is apparent by inspection of Fig. 7. Detection of 10^{-5} absorbance units was reported, making this approach competitive with the more elaborate instruments discussed earlier in this review. Ishibashi and co-workers have reported a small volume flow cell suitable for application to μLC with diode LIF detection [32]. They were able to detect femtogram quantities of fluorescent dyes, which is comparable to the detection limits attained with other LIF instruments. The practical limitation to the use of diode lasers at the present time is the very limited wavelength range available. Very few analytes have significant absorbance or fluorescence when exposed to 700–800 nm light. Expanded applications of this technology are likely to be dependent on the availability of diode lasers with shorter wavelengths of emission or the development of suitable derivatizing reagents. Nonetheless, this is an excellent example of the development of simple and inexpensive instruments that can broaden the applications of laser-based detectors.

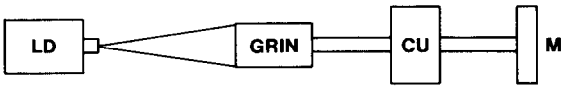

Figure 7
Schematic of a laser diode intracavity absorbance detector. LD = laser diode, GRIN = gradient refractive index lens, CU = cuvette, M = mirror. Taken with permission from ref. 31.

In addition to considering the complexity and cost of laser-based instruments, one must also consider the need for such instruments. While the mass sensitivity offered by μLC with laser-based detectors is quite impressive, these detection limits do not always translate to outstanding concentration sensitivity. The mass sensitivity is due in large part to the extremely small sample volumes injected on column in μLC and capillary electrophoresis. Thus, the instruments discussed in this review are most likely to be of use in situations where analyte is in very limited supply, but concentration can be maintained at high levels in small volumes. In the pharmaceutical industry, this situation is often encountered in the development of synthetic peptides and proteins produced by recombinant technology. The analysis of very small volumes of such samples is an area of growing interest [33, 34]. The cost of laser-based instruments pales in comparison with the cost of these compounds, so that any savings in compound which can be realized by the use of instruments with high mass sensitivity can quickly compensate for the high initial cost of the instrumentation. However, achieving these savings in the amount of compound required per analysis may not be as straightforward as it appears because the use of instruments with high mass sensitivity may not provide the optimum reduction in sample usage unless it is carefully coupled with microscale sample preparation and handling techniques, thereby yielding a high degree of mass utilization throughout the analytical measurement procedure.

The 1980s were years in which tremendous efforts were directed toward development of novel laser-based detection systems. This work was very successful in providing tools for highly mass sensitive analysis. The publications from this phase of research emphasized the development and testing of the detectors themselves, with only a minor emphasis on the application of these techniques to solve real problems. It is anticipated that the 1990s will be a decade in which the most successful of these approaches will be applied to a variety of analytical problems, with a variety of publications appearing in which laser-based detectors are used as a tool to achieve an end.

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