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REVIEW

USE OF LASER DETECTORS IN CAPILLARY LIQUID CHROMATOGRAPHY

B.G. BELENKII

Institute of Macromolecular Compounds of the Academy of Sciences of the U.S.S.R., Leningrad (U.S.S.R.)

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1. INTRODUCTION

The most important features of biomedical chromatography, the main technique of biomedical analysis, are high productivity, selectivity and sensitivity and these aspects have been intensively developed in recent years. The increase in productivity was enhanced by the automation of chromatographic analysis, including automatic sample preparation by solid- and liquid-phase (in the supercritical mode) extraction, solid-phase derivatization, automatic sample injection, the development of high-speed high-performance liquid chromatography (HPLC) and automatic processing and evaluation of chromatographic information. The choice of the correct chromatographic tactics by using multi-dimensional chromatography and the combination of a chromatographic separation of average efficiency with selective detection also played an important role. The selectivity of chromatographic analysis has been developed by a transition to the use of highly selective sorbents including chiral, affinity and immunoaffinity sorbents

and by the application of spectroanalytical detectors, including mass, UV, IR and NMR spectrometers, detectors with pre- and post-column derivatization and biospecific detectors.

The increase in the sensitivity of analysis is of particular importance in biomedical investigations. This is due not only to the necessity for the analysis of trace amounts of biologically active substances and medical metabolites in the living organism but also to the requirements for smaller volumes of investigated samples of physiological fluids and tissues, i.e., the collection of several tens of microlitres of blood from a fingertip instead of 10–15 ml of blood from a vein. This point is of particular importance in paediatrics and in routine prophylactic medical examinations. Hence it is necessary to apply to biomedical chromatography the most sensitive and economical variation of HPLC i.e., capillary liquid chromatography (CLC).

HPLC methods can be divided into four groups according to their purpose and the corresponding diameter of the chromatographic columns (Table 1). It can be seen from Table 1 that the first and second groups of methods belong to analytical chromatography and the third and fourth groups belong to preparative chromatography. The second group, belonging to conventional HPLC, is also a micropreparative group because the substances separated in the column are intended for subsequent on-line spectral analysis. Moreover, the fractions obtained can also be used for other analytical investigations, such as the automatic analysis of the sequence of amino acids and nucleotides in peptides and nucleic acids, respectively, the determination of biological activity and immune analysis. This type of HPLC may be used for multi-dimensional separations when the fractions obtained on the first column are used as samples for another type of column.

However, the most sensitive analytical modification of HPLC, the purpose of which is only to obtain information and to obtain it by the most economical method, is CLC, making it possible to carry out ultrasensitive, high-speed and superefficient analyses [1]. Nevertheless, CLC has not yet been widely used. The mass production of instruments and packed chromatographic columns is also lacking, although CLC itself has been the subject of many hundreds of papers surveyed in a number of books [1–4]. In our opinion, there are two obstacles to

TABLE 1

CHARACTERISTIC TYPES OF MODERN HPLC

1. Analytical HPLC (capillary HPLC).
2. HPLC with additional (spectrometric) analysis.
   Conventional HPLC with $d_c = 3$–8 mm, using the following additional instruments:
   (1) UV spectrophotometer with a diode array;
   (2) mass spectrometer;
   (3) IR spectrometer with Fourier transformation;
   (4) NMR spectrometer;
   (5) reactor with post-column derivatization.
3. Laboratory preparative HPLC (for chemical and pharmacological investigations), $d_c = 20$–50 mm.
4. Technological HPLC (for industrial purposes), $d_c \gtrsim 100$ mm.

the current use of CLC as the principal method of ultrasensitive analysis: (i) the absence of constructive solutions ensuring high reproducibility of retention times and sufficiently complete injection into the column of the microsample filling the injector; and (ii) the absence of highly sensitive absorbance photometric (spectrometric) detectors with measuring (sample) cell volumes of less than 1 nl, i.e., matching the columns made of flexible quartz capillaries. Verzele and Dewaele [5] are of the same opinion; they listed the following defects of micro-HPLC (CLC):

"(1) There is little or no dedicated instrumentation for micro-LC. Most pumps can be used but most require split techniques and therefore suitable splitters. There are some commercial detectors that not everybody is available to provide.

(2) There is no source of commercial columns of acceptable quality. This may change shortly but there is no decision yet.

(3) Column stability may be a delicate point. Investigations are under way but the situation is not clear.

(4) On-column UV detection, potentially the most important form of detection for micro-LC, has strange characteristics".

In fact, although columns made of flexible quartz capillaries have excellent properties, they are not commercially available and the choice of detectors employed so far with capillary columns is restricted to electrochemical detectors and those based on laser-induced fluorescence. The volumes of their measuring cells approach several nanolitres, i.e., they can be used with columns made of flexible quartz capillaries of I.D. 0.2–0.3 mm.

However, the recent achievements in the development of CLC permit us to hope that in the near future a high-quality capillary liquid chromatograph with a UV absorbance detector will be developed. The basis of this opinion is the following:

(i) Attention has been drawn to transition processes related to the increased pressure in the cylinder of the syringe pump when the column resistance or temperature varies. As it was necessary to decrease the relaxation time of transition processes, a decrease in the chamber volume and its thermostatting were required. These requirements were met by the KhZh-1309M microcolumn liquid chromatograph (Science and Technological Corporation of the U.S.S.R. Academy of Science) with 0.5 mm I.D. columns and a laser refractive index detector with a measuring cell of 0.1 μl volume and a sensitivity of $5 \cdot 10^{-8}$ RI units. This instrument ensured a relative standard deviation of retention times of $<0.2\%$ and that of the amplitude of the detector signal of $<0.5\%$ [6].

(ii) All types of HPLC with the use of capillary columns have been developed, including supercritical fluid [7] and hydrodynamic chromatography [8].

(iii) New types of high-performance capillary separation processes have been proposed. They are based on chromatography with pressure- and electroendoosmatically driven elements and on electrophoresis.

(iv) The possibility of the preparation of sorbents with submicrometre dimensions and the desirability of their application to the packing of capillary columns have been shown [5].

(v) Apart from the development of an electrochemical detector and a detector

based on laser-induced fluorescence, at the end of 1987 an absorptiometric detector based on photothermal refractometry with a detection volume of <1 pl and with the possibility of attaining a sensitivity level of 1 amol was described [9]. This detector made it possible to carry out the analysis of dabsyl amino acids on a capillary column of I.D. 250 μm and a limit of detection of a few femtomoles. This detector can evidently be combined not only with packed capillary columns of I.D. 100–200 μm but also with open-tubular capillary columns of I.D. ca. 5 μm.

Hence, at present, only one step remains in the construction of a capillary liquid chromatograph that meets all the modern requirements of analytical chemistry. There is no doubt that this chromatograph must have columns made of flexible quartz capillaries with a diameter of $\lesssim 200$ μm packed with a sorbent of submicrometre size and with an eluent driven by the pressure or electroendoosmosis. This capillary chromatograph should be supplied with detectors with a subnanolitre volume of the measuring cell on a column based on electrochemical processes, photothermal refractometry–laser-induced crossed beam thermal lens detection and laser-induced fluorescence.

Let us consider the modern approach to these problems in greater detail.

2. MODERN CONCEPT OF HPLC

If one uses the reduced value (h) of the height equivalent to a theoretical plate (HETP):

$$h = H/d_p \tag{1}$$

where H is the HETP and d_p is the particle diameter, and the reduced value (ν) of the linear flow-rate (u):

$$\nu = ud_p/D_m \tag{2}$$

where D_m is the diffusion coefficient in the mobile phase, then the dependence of h on ν is expressed by the Knox' equation [10]:

$$h = B/\nu + A\nu^{1/3} + C\nu \tag{3}$$

The universal dependence 3, shown in Fig. 1, is valid for HPLC with both (a)

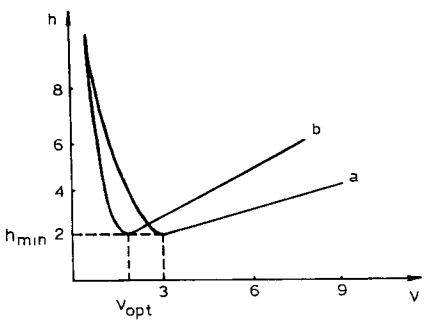

Fig. 1. Reduced HPLC (h) versus reduced rate (ν) for (a) conventional packed columns and (b) columns made of flexible quartz capillaries (according to eqn. 3).

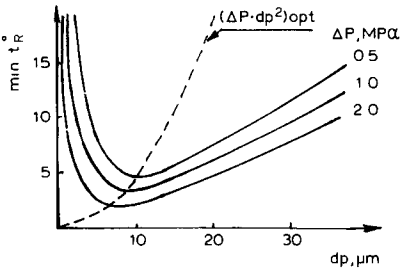

Fig. 2. Elution time of an unretained ($k'=0$) peak (t_R^0) versus sorbent particle diameter (d_p): $\Delta P=0.5$, 1.0 and 2.0 MPa [1].

a conventional and (b) a quartz capillary column. In order to carry out HPLC, several requirements should be met.

The first requirement is operation under optimum conditions. A pressure should be chosen such that $v=v_{opt}$ at which h has the lowest value ($h=h_{min}$). This should ensure the highest rate and the highest sensitivity of analysis at a given efficiency N and the highest efficiency at a given analysis time t_R. These dependences are determined by the fundamental hydrodynamic equation of HPLC:

$$(\Delta P d_p^2)_{opt} = (h_{min} v_{opt}/k_o)(D_m \eta) N \tag{4}$$

where k_o is the coefficient of hydrodynamic permeability of the column (for conventional columns $k_o \approx 1/1000$ and for columns made of flexible quartz capillaries $k_o \approx 1/400$). Hence, at a given viscosity of the eluent η and at given D_m and N, for this CLC a 2.5 times lower value may be used for $(\Delta P d_p^2)_{opt}$. Moreover, if it is taken into account that for CLC v_{opt} is also lower (Fig. 1), then $(h_{min} v_{opt}/k_o)$ is smaller by a factor of 5 for CLC than for conventional HPLC and is $1.2 \cdot 10^3$ (for conventional HPLC it is $6 \cdot 10^3$). This makes it possible to increase the column efficiency, N, 5-fold for CLC at equal $(\Delta P d_p^2)_{opt}$, to reduce $(\Delta P d_p^2)_{opt}$ 5-fold at a given N and to decrease d_p by a factor of $\sqrt{5}$, i.e., ca. 2.2-fold, at fixed ΔP and N.

The second requirement for HPLC is the necessity to decrease d_p and increase ΔP. In the limit, we have $\Delta P d_p^2 = (\Delta P d_p^2)_{opt}$. In this instance the analysis time is given by

$$t_R = \frac{N d_p^2}{D_m}\left(\frac{h}{v}\right)(1+k') \tag{5}$$

It can be seen from Fig. 2 that t_R decreases with decreasing d_p and increasing ΔP corresponding to $\Delta P d_p^2$. At a given ΔP and $\Delta P d_p^2 = (\Delta P d_p^2)_{opt}$, the use of columns made of flexible quartz capillaries makes it possible to decrease t_R by a factor of about 3 compared with its value in conventional HPLC or else, at a given t_R, to obtain N greater by a factor of 3. Eqn. 4 permits the determination of the optimum value of d_p (at given ΔP and N) for conventional HPLC:

$$d_{p_{opt}} \approx 77 (D_m \eta N / \Delta P)^{1/2} \tag{6}$$

and for HPLC with the use of quartz capillary columns:

$$d_{p_{opt}} \approx 35 (D_m \eta N / \Delta P)^{1/2} \quad (6')$$

In its turn, the decrease in $d_p = d_{p_{opt}}$ makes it possible to decrease the column length:

$$L = N h_{min} d_{p_{opt}} \quad (7)$$

and its volume

$$V_c = \pi d_c^2 d_{p_{opt}} h_{min} N / 4 \quad (8)$$

and thus reduce the consumption of the sorbent and the eluent. The rate of chromatographic analysis is determined by the productivity of the chromatograph, N / t_R. Hence, the third requirement for HPLC is the control of the productivity of analysis:

$$N / t_R \approx 10 \text{ plates/s at } N = 5 \cdot 10^3 \text{ plates} \quad (9)$$

Because for capillary columns t_R is lower and hence the productivity is higher, the condition $N / t_R \gtrsim 10$ plates/s is ensured up to high values of N.

The spreading of chromatographic zones is related to processes occurring not only in the column but also in the extra-column system. In this connection we have the fourth requirement for HPLC: the restriction of extra-column spreading by a 10% decrease in the efficiency of the chromatographic system related to the column efficiency, N_c:

$$N \gtrsim 0.9 N_c \quad (10)$$

Now let us consider the characteristics of HPLC related to the increase in sensitivity of analysis. Here the main requirement is the necessity to operate under the optimum ($h = h_{min}$, $v = v_{opt}$) conditions ensuring the minimum spreading and characterized by a volume standard deviation

$$\sigma_{v,min} = \pi \sqrt{2\pi\epsilon} d_c^2 d_p h_{min} N^{1/2} (1 + k') / 4 \quad (11)$$

where ϵ is the porosity. In this instance the height of the chromatographic peak becomes maximal. However, as can be seen from eqn. 11, the great increase in the sensitivity of the column leads to the possibility of decreasing the column diameter, d_c.

In fact, the minimum detectable mass of the substance, q_{min}, at a given minimum concentration sensitivity of the detector, C_{min}, is determined by d_c^2:

$$q_{min} \gtrsim \pi d_c^2 \sqrt{2\pi\epsilon} d_p h_{min} N^{1/2} (1 + k') C_{min} / 4 \quad (12)$$

$$q_{min} \gtrsim 0.6 d_c^2 d_p h_{min} (1 + k') N^{1/2} C_{min} \quad (12')$$

However, d_c cannot decrease infinitely. Its decrease is limited by the value of the extra-column spreading, the permissible effect of which on the efficiency of the column is determined by eqn. 10. Hence we have the fifth requirement for HPLC: the increases in the sensitivity of analyses and economy of the sorbent and the eluent are achieved by decreasing the column diameter (to the limit restricted by extra-column spreading). If the sample volume is controlled, $V_{s,lim} \gtrsim 0.5 \sigma_v^o$ ($k' = 0$), then we have

$$d_c \gtrsim 1.9\ (V_s/hd_pN^{1/2})^{1/2} \tag{13}$$

It should be mentioned that eqn. 12 is valid only when concentration-sensitive detectors are used. The application of flow detectors (e.g., a mass spectrometric or a radiometric detector) for the maintenance of sensitivity requires the stabilization of the substance flow entering the detector, $FC = \pi d_c^2 \epsilon u C/4$ where C is the concentration and F is the eluent flow-rate: $F = \pi d_c^2 \epsilon u/4$. Hence, a decrease in d_c decreases the sensitivity of the flow detector proportionally to d_c^2. Therefore, for a flow detector, a decrease in d_c of the column does not make it possible to decrease q_{min} (at best, it remains constant), because for the maintenance of constant substance flow, the sample concentration C_0 should be inversely proportional to d_c^2 (considering the economy of the sorbent and the eluent, a decrease in d_c^2 is also desirable when a flow detector is used). Apart from the lower limit of the sample mass, q_{min}, its upper limit should also be restricted because it is necessary to operate within the limits of the linear part of this sorption isotherm ($C_0 < 8\%$; for biopolymers $\lesssim 1$–2%). This limits the solute's mass (of low-molecular-mass compounds) by

$$q \lesssim 0.023\ d_c^2 d_p h N^{1/2} \rho \tag{14}$$

and the loading on the column by

$$q/d_c^2 \lesssim 0.023\ d_p h N^{1/2} \rho \tag{15}$$

which decreases with decreasing h and d_p and increases with increasing N ($\sim N^{1/2}$). Here ρ is the density of the sample solution. These restrictions should be borne in mind when the sixth requirement for HPLC is formulated: the possibility of carrying out analyses of trace amounts of the substance with a decrease in the column diameter by concentrating the sample in its upper part up to the limits expressed by the inequalities 14 and 15.

The decrease in the volume of the measuring cell of a concentration-sensitive detector usually leads to a certain decrease in its sensitivity. This decrease may be compensated for by an increase in sample concentration, C_0, in particular by concentrating the sample in the upper part of the column up to the limits expressed by eqn. 12 but not higher than those indicated in eqns. 14 and 15. In this instance a decrease in d_c^2, decreasing q_{min}, completely compensates for a certain increase in C_{min} required for this measurement. Hence, the use of two procedures, a decrease in d_c and concentration of the sample in the initial part of the column (by using solvents with a high k') is essential when trace analysis is carried out by HPLC. When capillary columns are used, a problem that is negligible for conventional HPLC arises, viz., the necessity for precise and highly reproducible eluent delivery. When a syringe pump is used under these conditions, its cylinder volume considerably exceeds the column volume and, as a result, relatively long transition processes occur. These are related to changes in eluent density when the pressure increases and the temperature changes. The reproducibility of t_R is ensured by a decrease in the time of the transition process, the relaxation time τ, to a value $\tau < 0.1 t_R$. The relaxation time $\tau = V_p \beta K$, where V_p is the cylinder volume of a syringe pump, β is the compressibility of the liquid, and K is the hydraulic

resistance of the column ($K=\Delta P/F$, where ΔP is the pressure drop in the column and F is the volume flow-rate of the eluent). In order to ensure the reproducibility of t_R, it is also necessary to ensure the thermostatting of the hydraulic system of the chromatograph with ΔT determined by P, the temperature coefficient of expansion of the liquid, β, and the permissible value of $|\Delta t_R/t_R|$. Hence, one can formulate the seventh requirement for HPLC (specific for CLC): the necessity for correlation between the volumes of the syringe pump and the chromatographic column ($V_p \lesssim 10V_c$) and for the thermostatting of the pump–column system with an amplitude $\Delta T=P(\alpha/\beta)\delta$, where α is the liquid compressibility and δ is the permissible level of $|\Delta t_R/t_R|$. This shows that ultrasensitive chromatographic analysis is ensured by carrying out the chromatographic experiment under the optimum conditions and decreasing the column diameter. In this event the extra-column systems of the chromatograph decrease the efficiency of the chromatographic system and limit the minimum diameter of the column. Moreover, the sample concentration is restricted from below by the limiting sensitivity of the concentration detector and from above by the necessity to operate in the linear ranges of the sorption isotherm and the concentration sensitivity of the detector. Hence, it is evident that the requirements for efficiency and sensitivity of analysis should be correlated with the column size (d_c, L), the volume of the measuring cell of the detector and its sensitivity.

Several types of capillary columns for liquid chromatography have been described [5] (Table 2). The most promising have been found to be packed columns made of fused quartz with a particle size $d_c=100$–150 μm (micro-LC). The advantages of these columns over those for conventional HPLC are enumerated in ref. 5 (Table 3).

Let us comment on Table 3. First, one can add to the enumerated advantages

TABLE 2

TYPES OF CAPILLARY COLUMNS FOR LIQUID CHROMATOGRAPHY [5]

No.	Type of column	Material	Diameter (d_c) and length (L)
1	Packed microcolumns (packed microbore LC)	Metal, PTFE, glass	$d_c=0.5$–2.0 mm $L=3$–30 mm
2	Capillary columns (micro-LC)	Flexible quartz capillary	$d_c=0.1$–0.5 mm $L=3$–50 mm
3	Semi-packed capillary columns (SPOT LC)	Glass, flexible quartz capillary, metal drawn together with the sorbent	$d_c=25$–100 μm $L=5$–30 m
4	Open-tubular capillary columns with a porous layer on the inner wall (PLOT LC)	Flexible quartz capillary	$d_c=25$–100 μm $L=5$–30 m
5	Open-tubular capillary columns with a stationary phase (WCOT LC)	Flexible quartz capillary	$d_c=5$–25 μm $L=5$–50 m

TABLE 3

ADVANTAGES OF CAPILLARY COLUMNS (MICRO-LC) MADE OF FLEXIBLE QUARTZ CAPILLARIES [5]

1. Better hydraulic permeability ($k_o = 1/447 + 1/365$).
2. Lower ν_{opt} (decrease in ΔP at fixed N).
3. Good detection possibilities (in-column and on-column).
4. Greater possibility of detection of peaks with a high k' value (for in-column detection).
5. Good reproducibility of t_R and σ_v.
6. Chemical inertness.
7. Low sensitivity of N to packing defects (to the existence of voids).
8. Convenience of maintaining column efficiency (by periodically cutting off the upper part).
9. Economy of the sorbent and the eluent (one-drop chromatography).
10. Low cost.

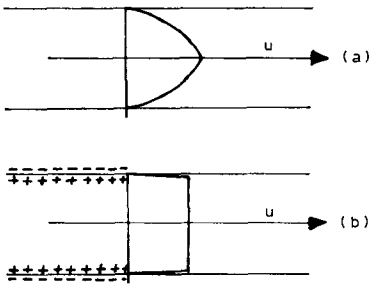

Fig. 3. Rate profile in a capillary column with eluent driven by (a) hydraulic pressure and (b) electroendoosmosis [16].

one further advantage: it is easy to prepare relatively long columns (up to 1 m) with $h = 2$ [11]. It has already been pointed out that these columns exhibit a higher hydraulic permeability and lower values of ν_{opt}. According to eqn. 6′, this fact makes it possible to decrease d_p and obtain a high N at a limited value of ΔP. Finally, an advantage of capillary columns over open-tubular columns is the close values of k' for micro-LC and conventional columns when the same separating systems are used.

A specific feature of capillary columns is also the possibility of using not only pressure-driven but also electroendoosmotically driven eluents. In the latter instance, the rate profile in the transverse direction is of the plug type and not the Poiseuille type, and the chromatographic spreading is lower, as can be seen in Fig. 3. However, the electroendoosmotic motion of the eluent leads to its undesirable Joule heating, which results in temperature distortion of the rate profile. It is possible to avoid this only by decreasing the capillary diameter to 100 μm. The same effect will be observed for capillary electrophoresis. Hence, in this instance the diameter of the capillary should also be limited to 100 μm (at a potential gradient of 500 V/cm).

Modern liquid-phase separation methods based on the use of columns made of flexible quartz capillaries are listed in Table 4. However, the transition to capillary columns with $d_c = 100$–200 μm, which are characterized by V_d (volume of

TABLE 4

CAPILLARY LIQUID-PHASE SEPARATION METHODS [8]

1. Capillary HPLC (with open-tubular and packed columns).
2. Capillary electrophoresis.
3. Micellar electrochromatography (capillary electrophoresis in micellar solutions).
4. Electroendoosmotic chromatography (electrochromatography).
5. Colloid electrochromatography (capillary electrophoresis in colloidal solutions).
6. Capillary electrophoresis of complexes.

detector cell) <0.01 μl, requires the application of optical detection systems with a small collimation volume, i.e., of laser detectors.

3. LASER DETECTORS FOR CAPILLARY LIQUID CHROMATOGRAPHY

The use of lasers for detection in liquid chromatography is not a new method and has been considered in a number of review papers [12–14] that describe the main features of the efficient use of lasers for detecting low substance concentrations in ultra-small volumes (trace analysis), i.e., in the measuring cells of detectors intended for CLC. The effective use of lasers for these purposes is based on the following features:

(i) The power of laser emission, which considerably exceeds that of other sources. However, it is more important that perfect collimation makes it possible to concentrate a high power of laser emission in a small volume. In this respect laser emission is unique.

Hence, it is possible to achieve the following effects: (a) non-linear effects: two- and multi-photon absorption, greatly changing the selectivity of detection; (b) highly sensitive photothermal refractometry based on the local change in the refractive index of the liquid upon its heating caused by the absorption of emission by the dissolved substance with a detection limit of $<10^{-18}$ mol; (c) highly sensitive laser-induced fluorescence with a detection limit of $<10^{-18}$ mol.

(ii) Monochromaticity related to time coherence. This laser property restricts the range of the Rayleigh and Raman light scattering by the eluent and thus extends the spectral range of fluorescence monitoring (Fig. 4), and also decreases the emission losses as a result of the absence of complex dispersion systems.

(iii) Low divergence of laser emission related to spatial coherence. This ensures perfect collimation (Fig. 5). It can be seen that the collimation region means the space filled with emission and extending by a distance of $\pm z$ from the focal point where z is the distance at which the diffraction-limited beam radius becomes larger than the focal radius ω_o by a factor of $\sqrt{2}$.

$$z=\pi\omega_o^2/\lambda \tag{16}$$

In this case the volume of a cylindrical detector cell V_d in which the beam does not touch the walls is given by

$$V_d=16\pi^2\omega_o^4/\lambda \tag{17}$$

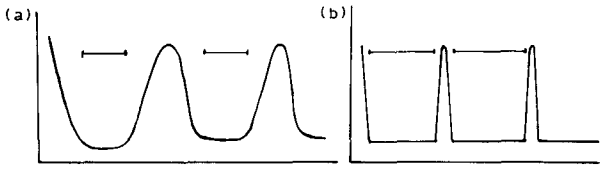

Fig. 4. Fluorescence monitoring ($\Delta\lambda$) for fluorescence excitation by (a) conventional emission sources and (b) lasers [12].

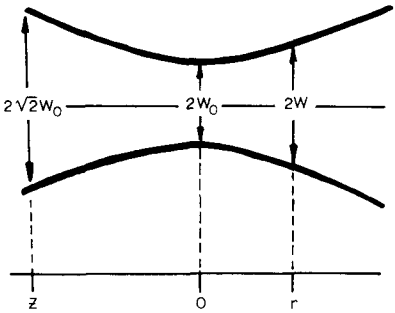

Fig. 5. Diameter of diffraction-limited laser beam near the focal point [13].

or, applying eqn. 16:

$$V_d = 16\, z^2 \lambda \tag{18}$$

As the laser beam can be easily focused with the aid of a long-focus lens into a narrow bundle with a very low value of ω_0, one can use measuring cells of nanolitre volume with a relatively long optical path of the beam. This is important for detection with the use of polarimetry, interferometry and photometry. As a result of the low divergence of the laser beam, the optical schemes for detection devices constructed with its use are simple. The optical elements consist only of planar mirrors and weak long-focus lenses, and point diaphragms for spatial filtration of scattered light.

(iv) Time resolution of exciting emission and fluorescence by using pulsed lasers with a pulse of nanosecond length. Since the fluorescence afterglow of usual substances continues for a few nanoseconds (up to 10–15 ns), then by stopping the collection of fluorescence light during the pulse (for 1–2 ns) it is possible to remove the scattered light of excitation preventing detection and to achieve selective detection on the basis of the intensity of fluorescence decay.

(v) Polarization of laser emission. As laser emission is plane-polarized, polarizing light filters can be used for the filtration of Rayleigh and Raman scattered light retaining polarization. In this instance the depolarized light from the fluorescence of molecules with small rotational relaxation times, characteristic of low-molecular-weight substances, will be detected.

The above features of laser emission show that it is ideally suitable for detection carried out in the low-volume cells used in CLC. The high collimation of laser emission and the necessity for the filtration of scattered light determine the construction of these cells (Fig. 6).

Fig. 6 shows laser detector measuring cells of the following types: (a) flow-drop

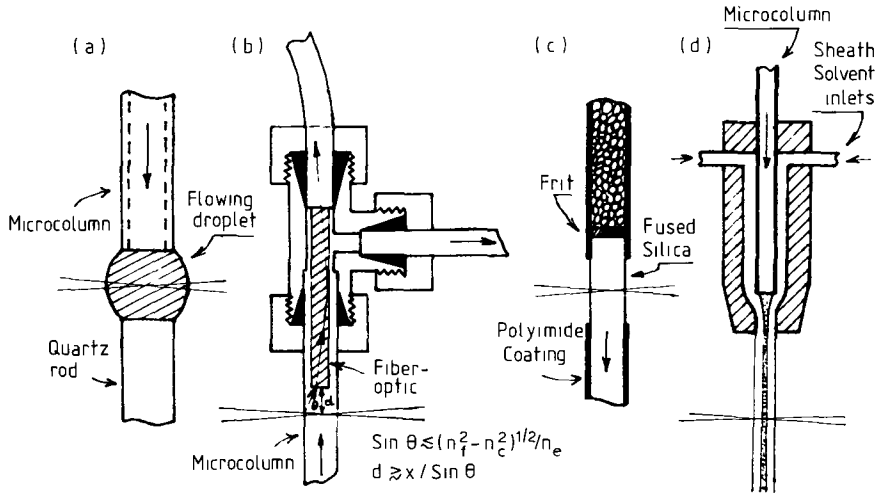

Fig. 6. Construction of flow cells for laser detection [14]. (a) Flow-drop cell; (b) cell made of flexible quartz capillary (with longitudinal excitation); (c) cell made of fused quartz capillary (with transverse excitation); (d) ensheathed cell with a laminar liquid flow.

cell (without walls); (b) cell made of a fused quartz capillary with longitudinal collection of fluorescence light (into the fibre light-guide); (c) cell made of a fused quartz capillary with transverse (at an angle of 90°) collection of fluorescence light; (d) ensheathed cells with a liquid sheath playing the role of the cell wall. In this cell, the liquid sheath (jacket) is formed by a strong flow of the sheathing liquid (with the same composition as the eluent) flowing around the end of the capillary column. As a result, the eluent flowing out of the column is compressed into a narrow stream several micrometres in size. Apart from the small size of these cells, their characteristic features include protective measures against the introduction of the scattered excitation light into the measuring channel. These features are the absence of glass (quartz) walls (a,d) which scatter light and light collection into a flexible light-guide, the end of which is located at a distance d from the focal point of the exciting laser light inside the capillary. The distance d is chosen so that the following equation should be valid:

$$d = x/\sin\theta \tag{19}$$

where θ is the angle between the intersection point of the laser beam and the inner wall of the capillary and x is the distance between this wall and the core of the fibre light-guide. In this case θ is determined by the refractive indices of the light-guide core, n_f, of its coating, n_c, and of the eluent, n_e:

$$\sin\theta = (n_f^2 - n_c^2)^{1/2}/n_e \tag{20}$$

All the measuring cells shown in Fig. 6 can be used for the measurement of the laser-excited fluorescence and those of types c and d can also be employed for thermooptical refractometry.

At present, a considerable number of detectors are used in which laser emission and small-size measuring cells permitting the use of columns with $d_c = 0.5$–1 mm

are employed [12–14]. However, as we are interested in the possibility of using highly sensitive detection in operation with capillary columns with $d_c \leqslant 0.2$ mm, only the laser detectors suitable for these purposes will be considered.

3.1. Laser fluorescence detector

The fluorescence intensity, Φ_f, in the measuring cell of the laser fluorescence detector is determined as follows:

$$\Phi_f = \Phi_e \gamma k_f (1 - e^{-\epsilon_\lambda l C}) \tag{21}$$

where Φ_e is the intensity of the laser beam collimated in the measuring cell of the detector, γ is the quantum yield of fluorescence, k_f is the intensity of fluorescence collection, ϵ_λ is the molar absorptivity of the substance, C is its concentration and l is the length of the light path in the measuring cell. At low concentrations of the substance ($\epsilon_\lambda l C \ll 1$), eqn. 21 is simplified to

$$\Phi_f = \Phi_e \gamma k_f \epsilon_\lambda l C \tag{22}$$

Eqn. 22 shows that the fluorescence intensity depends on the intensity of laser emission collimated in the cell, Φ_e, and the length of the light path in the cell, l. The increase in Φ_e can evidently compensate for a decrease in l, which makes it possible to decrease the cell volume V_d to less than 1 nl. As fluorescence is detected from the entire volume of the detector cell, a fluorescence detector should strictly be regarded as a flow detector [12]. However, at a fixed V_d value the fluorescence detector operates as a concentration detector and there are the following possibilities of using it for detecting ultra-low concentrations and ultra-small amounts of the substance in the cell which matches the capillary column in volume. The power of the laser emission Φ_e can be increased. The coefficient of collection of fluorescence light can also be increased. For these purposes, the photon count can be used with compensation of the intrinsic noise of photomul-

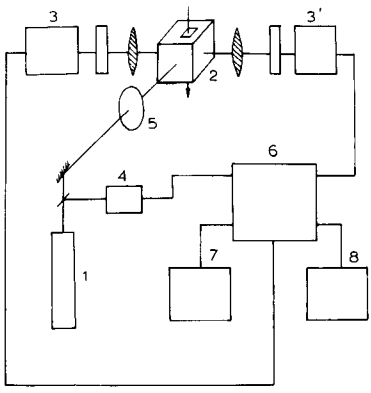

Fig. 7. Optical scheme for a laser fluorescence detector. (1) He–Cd laser, $\lambda = 325$ nm, 10 mW; (2) quartz flow cell; (3, 3′) operation channel of photomultipliers; (4) reference channel of photomultipliers; (5) focusing optics of laser beam; (6) amplifier; (7) computer; (8) recorder [15].

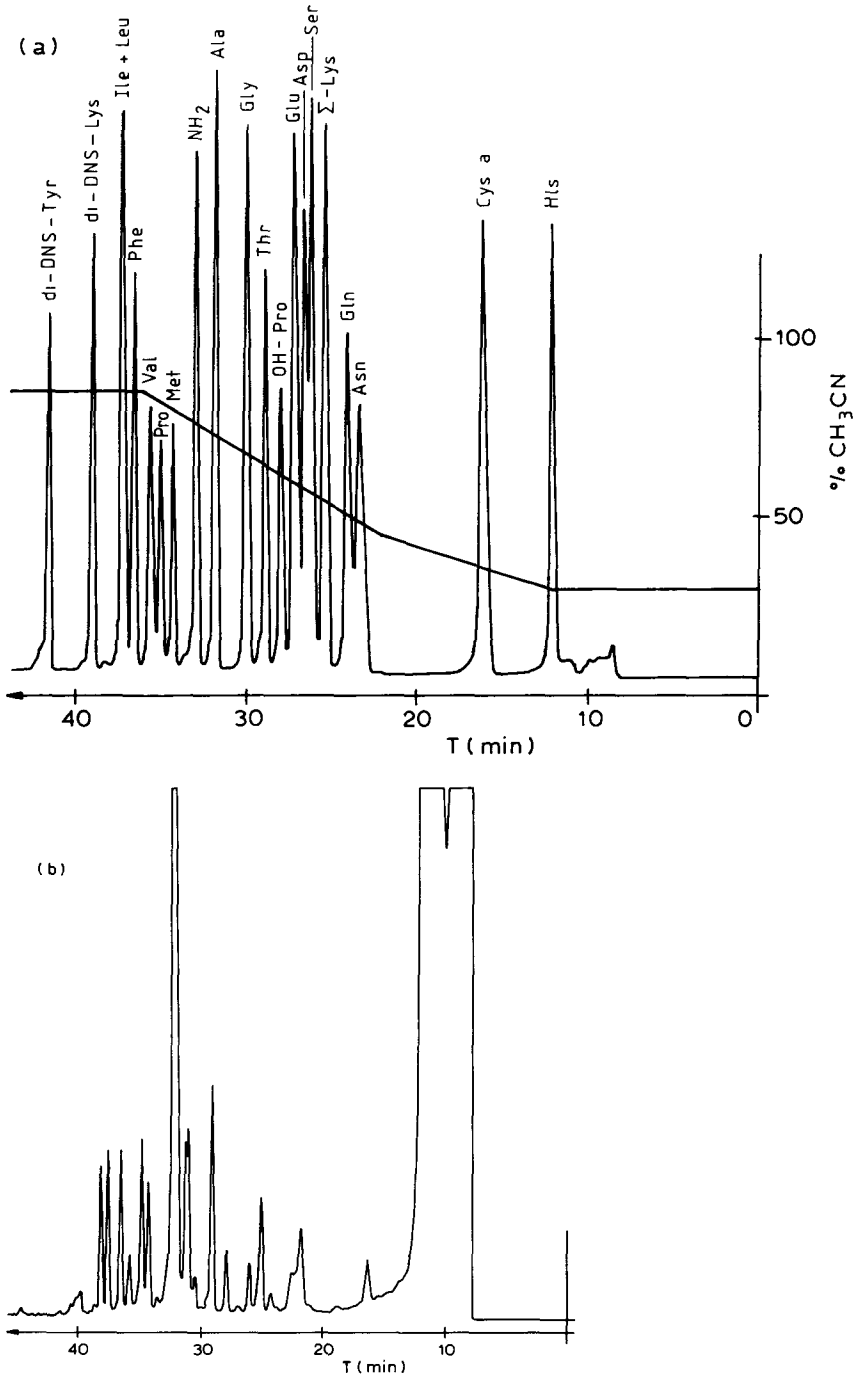

Fig. 8. Ultrasensitive analysis of DNS-amino acids: (a) standard and (b) analysis of amino acids in serum with detection by laser-induced fluorescence. Sample, $5 \cdot 10^{-16}$ mol of each DNS-amino acid; column, 300 mm × 0.5 mm I.D.; sorbent, Silasorb-sphere C_{18}, $d_p = 5$ μm; elution rate, 5 μl/min. The acetonitrile gradient in a 0.01 *M* sodium formate buffer at pH 3.5 is shown. Sample volume, 1 μl.

tipliers (coincidence scheme), and the fluctuations of laser emissions can be taken into account with the aid of the reference channel. This is the scheme according to which the laser fluorescence detector [15] used by us for analyses of DNS derivatives of amino acids including the determination of amino acids in serum and prostaglandins was designed (Fig. 7). The chromatograms of these substances are shown in Figs. 8 and 9. This chromatographic system makes it possible to determine $\lesssim 10^{-15}$ mol of the substance with a detection limit of $5 \cdot 10^{-17}$ mol of DNS derivatives. In these experiments, the cell volume was 0.1 μl but could easily be decreased to 10 nl. In this instance, the detector sensitivity drops, but this drop may be compensated for by an increase in the intensity of the laser emission or by a transition to the method of intra-column detection [5] (Fig. 10). The latter method makes it possible to decrease the mass of the substance by a factor of 50, as can be seen from Fig. 11, which shows the chromatograms of polynuclear aromatic hydrocarbons obtained on a 200 mm $\times$ 0.32 mm I.D. capillary column packed with 5-μm ROSIL-C_{18}-D. The laser detection of the fluorescence of DNS-amino acids has also been used for their separation by high-voltage capillary electrophoresis [16] on a 1000 mm $\times$ 0.075 mm I.D. capillary column at 30 kV. In both instances in the analysis of DNS derivatives, a helium–cadmium laser with emission at 325 mm was used.

Many papers have been published on the use of laser-excited fluorescence of amino acids labelled with fluorophores. An interesting result has recently been achieved in the chromatography of fluorescent amino acid derivatives obtained with naphthalene dialdehyde (NDA derivatives). They fluoresce at 490 nm with excitation by an argon-ion laser at 457.9 nm with a power of 900 mW [17]. A chromatogram of NDA-amino acids is shown in Fig. 12. In this work a detector was used with the measuring cell described above (Fig. 6b) supplied with a fibre-optic light-guide. At an inner diameter of the capillary of $d_c = 320$ μm and a diameter of the fibre-optic light-guide of 200 μm, the detection limit was 100 amol at a signal-to-noise ratio of 2. In this work, an increase in the sensitivity of the laser detector compared with the conventional fluorescence detector of 1–2 orders of magnitude was reported.

Detection based on laser-induced fluorescence may be developed in several directions: first, by the determination of fluorescence spectra [18]; second, by investigating the role of the fluorescence drop after the pulsed excitation, as is shown in Figs. 13 and 14; and finally, by using two-photon-excited fluorescence. The last phenomenon is interesting, not only making it possible by using excitation at a wavelength λ to obtain fluorescence excited by a shorter ($\lambda/2$) emission (Fig. 15), but also because it leads to some useful effects. First, as a result of shorter-wave fluorescence it is easy to filter off the scattered light at a wavelength λ; second, being a less frequent phenomenon than fluorescence excited by a one-photon absorption, it ensures the possibility of the selective detection of substances that absorb two photons (e.g., polynuclear aromatic hydrocarbons and substances that contain a long chain of π-conjugation) and makes it possible to observe the fluorescence of substances on the background of admixtures capable only of one-photon absorption (simple solvents, protein blood and urine components). It also permits the preparation of simpler chromatograms than those

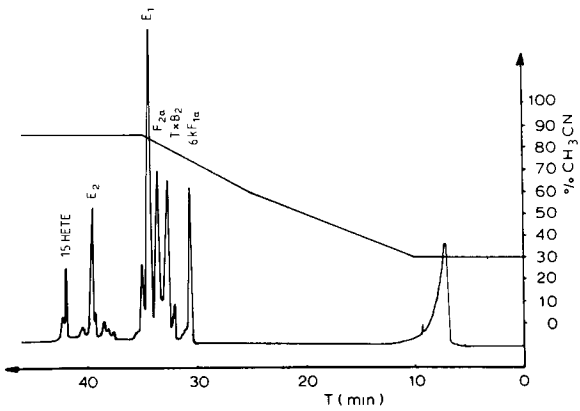

Fig. 9. Ultrasensitive analysis of DNS-prostaglandins. Sample, $5 \cdot 10^{-16}$ mol of each DNS-prostaglandin; column, 300 mm×0.5 mm I.D.; sorbent, Silasorb-sphere C_{18}, $d_p = 7$ μm; elution rate, 6 μl/min. The acetonitrile gradient in water is shown. Sample volume, 1 μl.

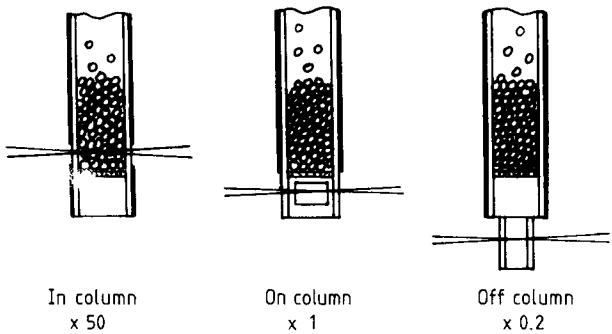

Fig. 10. Capillary liquid chromatography. Methods of fluorescence detection [5].

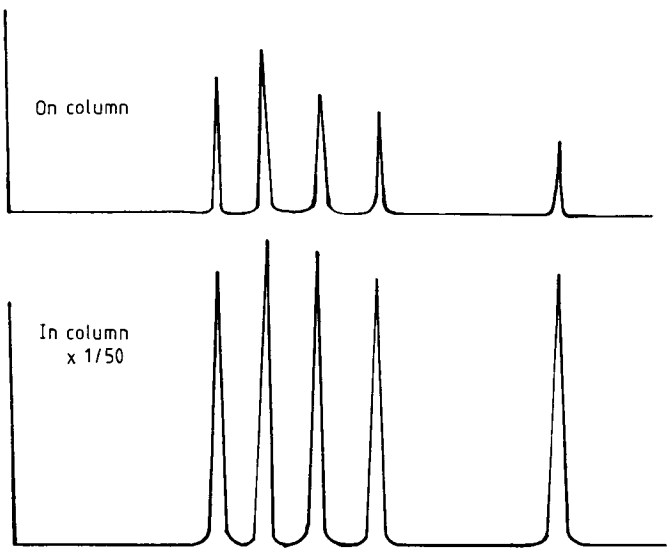

Fig. 11. Chromatograms obtained with the in- and on-column fluorescence detection (325/400 nm) on a quartz capillary column (200 mm×0.32 mm I.D.) packed with a ROSIL-C_{18} (5 μm) sorbent [5].

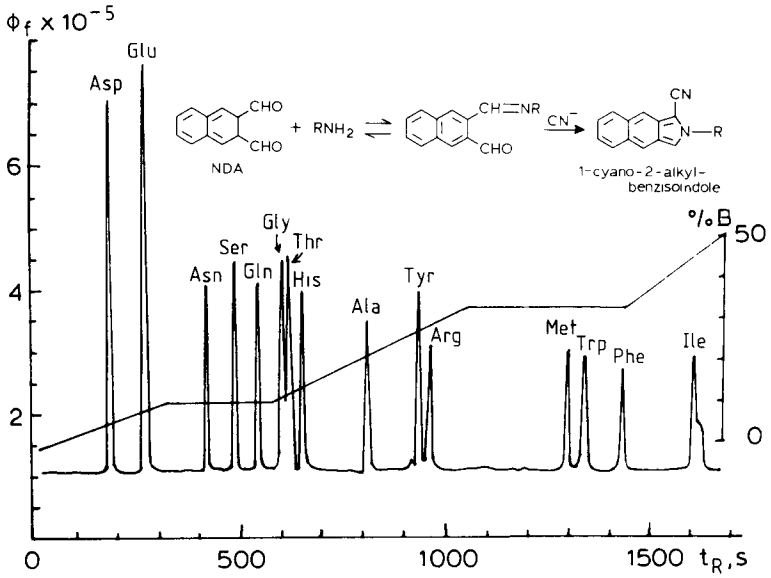

Fig. 12. Chromatogram of a mixture of sixteen NDA derivatives of amino acids with detection by fluorescence induced by an ionized argon-ion laser ($\lambda = 457.9$ nm, 900 mW). Sample, $5 \cdot 10^{-13}$ mol of each NDA-amino acid. The acetonitrile gradient with 15→40% of 0.05 *M* phosphate buffer at pH 6.9 is shown. Column, 150 mm × 4.6 mm I.D. packed with Hypersil ODS, $d_p = 5$ μm [17].

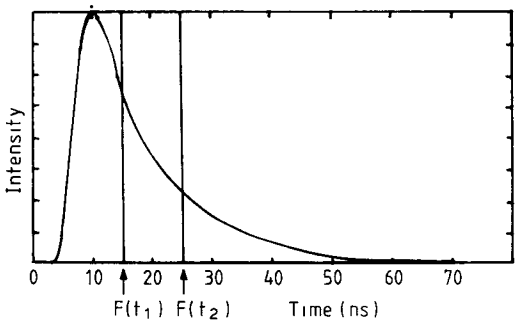

Fig. 13. Measurement of fluorescence decay at two points, $F(t_1)$ and $F(t_2)$; $\Delta t = t_2 - t_1 = 10$ ns [19].

detected from one-photon fluorescence. The latter fact is illustrated in Fig. 16 [20], which shows the chromatograms of a coal extract obtained on a reversed-phase column with detection from UV absorption at 254 nm, with the aid of one-photon-excited fluorescence (by an ionized argon-ion laser, $\lambda = 488$ nm) at 540 nm and with the aid of two-photon-excited fluorescence at 375 nm. In reversed-phase chromatography, t_R increases with increasing molecular mass of the substance. It is clear that UV absorption reveals the low-molecular-mass sample components present in large amounts and two-photon-excited fluorescence reveals only some of the fluorescent components of those present in the mixture.

3.2. Thermooptical detector

When light is absorbed by the substance and the absorbed energy is dissipated by the non-radiative path, the solution is heated. This heating may be made local

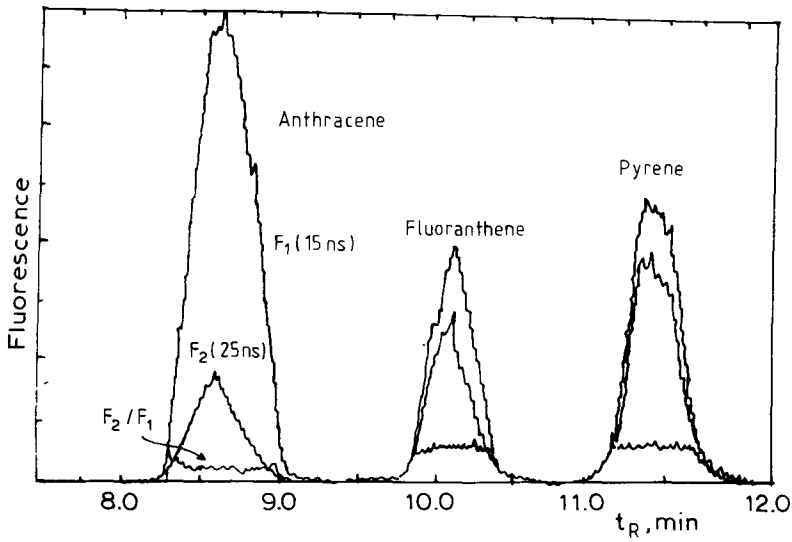

Fig. 14. Chromatogram with a 10-ns interval between fluorescence pulses, $F(t_1)$, $F(t_2)$, induced by a pulsed laser and the $F(t_1)/F(t_2)$ ratio for three standards of polynuclear aromatic hydrocarbons [19].

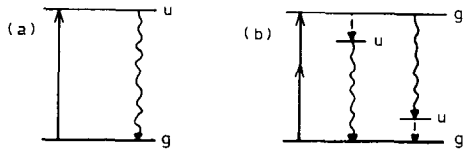

Fig. 15. Fluorescence with (a) one-photon and (b) two-photon excitation. →, Absorption; -->, relaxation; ~ ~ >, emission; *g* and *u* are symmetry types [12].

and sufficiently intense by using laser emission. Hence, the solution density changes within the region heated by the laser beam. As a result of the Gaussian energy distribution in the laser beam, the heated region forms a thermal lens in which the refractive index differs from that in the solution volume. The volume of the thermal lens is <1 pl. The light of another (probe) laser passing through the thermal lens is focused at a certain distance from the thermal lens of

$$f=\frac{\pi k\omega^2\lambda}{2.303P(\mathrm{d}n/\mathrm{d}T)A} \tag{23}$$

which changes the focusing and, hence, the light intensity in the optical system of the detector. Here ω is the radius of the laser beam in the cell, k is the thermal conductivity coefficient of the eluent, P is the laser power, $\mathrm{d}n/\mathrm{d}T$ is the temperature coefficient of the refractive index and A is the absorbance. If we designate $P(\mathrm{d}n/\mathrm{d}T)/(1/91)\lambda k \equiv E$, then the relative change in the light intensity ($\Delta I/I$) in the centre of the laser beam after passing the thermal lens is given by

$$\Delta I/I=2.303EA+0.5(2.303EA)^2 \tag{24}$$

where at low substance concentration $A=\epsilon_\lambda lC$.

Eqn. 24 shows that the sensitivity of absorption detection by the thermal lens method increases with increasing laser power, temperature coefficient of the refractive index and optical density and decreases with increasing λ and coefficient

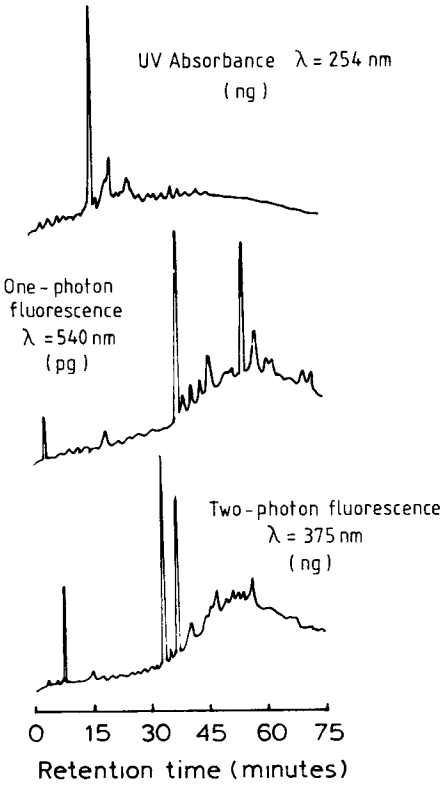

Fig. 16. Chromatogram of a coal extract. Information from three detectors: UV absorption ($\lambda = 254$ nm); one-photon fluorescence ($\lambda = 540$ nm); two-photon fluorescence ($\lambda = 375$ nm) [20].

TABLE 5

THERMOOPTICAL PROPERTIES OF SOLVENTS FOR THERMAL LENS ABSORPTION [21]

Solvent	k (mW cm^{-1} K^{-1})	$10^4 dn/dT$ (K^{-1})	E/P (mW^{-1})
Carbon tetrachloride	1.03	−5.9	4.7
Cyclohexane	1.24	−5.4	3.6
n-Heptane	1.26	−5.0	3.3
Dioxane	1.39	−4.6	2.7
Isobutanol	1.52	−3.9	2.1
Methanol	2.02	−4.2	1.7
Water	6.11	−0.8	0.1

of thermal conductivity, k. Hence, the detector signal is proportional to the absorption of the substance, i.e., to its concentration and the laser energy. It also depends on the solvent properties, dn/dT and k. Table 5 lists these values and also the specific value of E/P per milliwatt. It is clear that the absorption detection by the thermal lens method is carried out with the highest sensitivity in nonpolar solvents (tetrachloromethane and cyclohexane) and with the lowest sensitivity in water. It has been proposed [21] to use for the chromatography of

water-soluble substances a solution of reverse micelles in a non-polar solvent or ion-pair agents.

The detection of thermooptical phenomena related to light absorption by a solution in a capillary may also be carried out by other methods: with the aid of "thermoprism" [22], from the change in optical rotation [23] and the diffraction of the passing light [24]. The construction of a detector based on the last principle is shown in Fig. 17. This detector has two measuring channels: an absorption channel with illumination of the capillary by a modulated beam of the pump laser and a refractometric channel (without this illumination). It is evidently possible to use the third, fluorimetric, channel if the light is collected at a certain angle to the direction of the pump laser beam. Table 6 lists the limiting sensitivities of absorption and refractive index detection with the use of capillaries 50, 100 and 500 μm in diameter. It is clear that the limiting sensitivities are independent of the capillary diameter and that in its adsorption sensitivity this detector exceeds

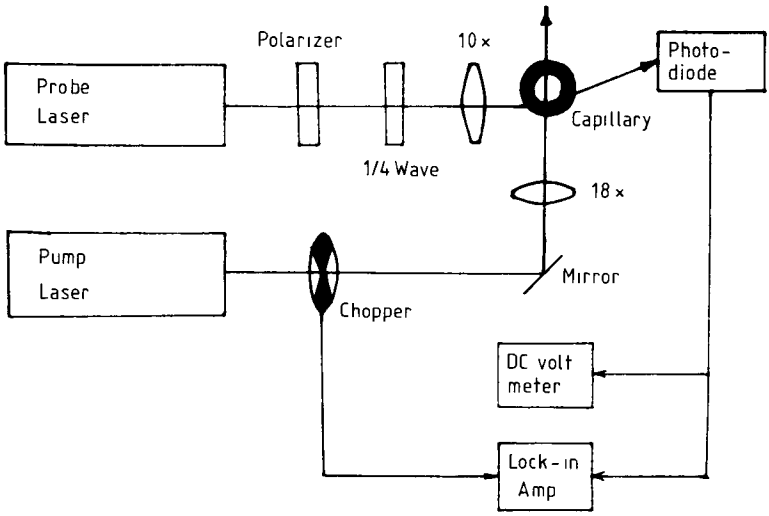

Fig. 17. Capillary thermooptical laser detector. Simultaneous determination of refractive index and absorption in a capillary. $d_c = 50$, 100 and 500 μm; He-Cd pump laser, 2.8 mW, $\lambda = 442$ nm, modulation 12.5 Hz; He-Ne probe laser, $\lambda = 632.8$ nm, 1 mW [24].

TABLE 6

DETECTION LIMITS OF ABSORPTIVITY AND REFRACTIVE INDEX (He-Cd, LASER, $\lambda = 442$ nm, 2 mW) [24]

Diameter of measuring cell (μm)	Detection limit		
	Refractive index (ΔRI)	Absorptivity (A)	Absorptivity per unit optical path length (cm^{-1})
50	$4 \cdot 10^{-6}$	$6 \cdot 10^{-6}$	$1.2 \cdot 10^{-3}$
100	$3 \cdot 10^{-6}$	$9 \cdot 10^{-6}$	$9.0 \cdot 10^{-4}$
500	$3 \cdot 10^{-6}$	$6 \cdot 10^{-5}$	$1.2 \cdot 10^{-3}$

the best UV photometers based on differential photometry. At the end of 1987, papers were published that described the designs of capillary laser adsorption detectors with characteristics such that they may be used in CLC [9,25]. These constructions exhibit all the parameters required for this purpose.

The scheme for the absorption detector described in ref. 9 is shown in Fig. 18. The beam of an argon-ion laser, $\lambda = 457.9$ nm with a power of 150 mW modulated at a frequency of 90 Hz, illuminates the microvolume of the eluent in a capillary with a square cross-section of 80×80 μm. The thermal lens is detected by a helium–neon beam of 1 mW of the probe laser at 632 nm located at an angle of 90° to the beam of the pump laser. The beams of both lasers intersect inside the capillary cell of the detector in the plane normal to the eluent motion. The volume of this intersection region (the optical volume of detection) is less than 0.2 pl. This detector has been used for detecting the eluent on a column with $L = 80$ cm and $d_c = 250$ μm packed with 5-μm Spherisorb C_{18} and supplied with an injector of volume $V_s = 60$ nl. The elution rate was 1 μl/min. This capillary liquid chromatograph was used for the separation of dabsyl-amino acids. The chromatogram of 75 fmol of eighteen dabsyl-amino acids is shown in Fig. 19. A good chromatogram of dabsyl-amino acids can also be obtained by the injection of 10 fmol of the analyte, which corresponds to its concentration in the sample of $2 \cdot 10^{-7}$ M. Fig. 19 gives an idea of the possibilities of applying this detector. Moreover, in the volume of optical detection ($\lesssim 0.2$ pl) only 60 molecules of the analyte are present at the maximum of the chromatographic peak. The optical detection volume of 0.2 pl permits the application of this detector not only to packed quartz capillary columns but also to open-tubular capillary columns with $d_c \approx 5$ μm. In this instance the detection limit will be much lower than 1 amol.

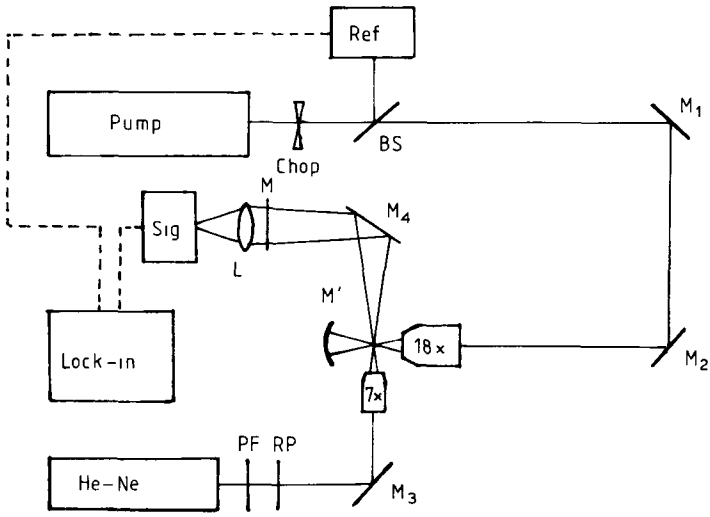

Fig. 18. Crossed-beam thermal lens detector for CLC. M_1–M_4 = mirrors; L = lens; $f = 50$ mm, 7× and 18× = microscope objectives; PF = polarizing light filter; RP = 1/4-wave plate; M = diaphragm; M′ = light trap; Sig = signal photodetector; Chop = mechanical chopper, 90 Hz; BS = beam splitter, Ref = reference photodiode [9].

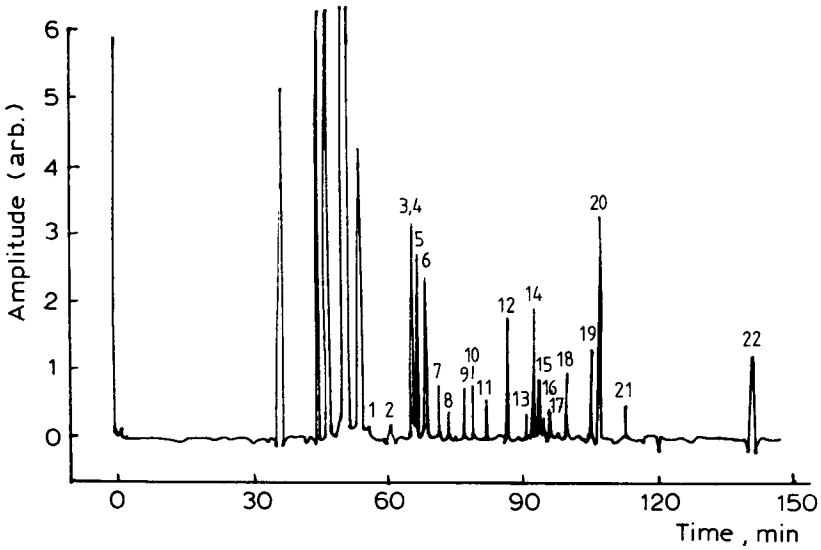

Fig. 19. Chromatogram of 75 fmol of dabsyl-amino acids. 1 = Cys; 2 = Ser; 3, 4, 8, 18 = reagent; 5 = Asp; 6 = Thr; 7 = Glu; 9 = Gly; 10 = Arg; 11 = Ala; 12 = Pro; 13, 14, 15 = Phen; 16 = Thr; 17 = Trp; 19 = His, Leu; 20 = Ileu; 21 = Tyr; 22 = Lys. Column: $L = 80$ cm, $d_c = 250$ μm, C_{18}, 5 μm. Elution rate, 1 μl/min. Eluent programme: acetonitrile–sodium acetate buffer from 40:60 to 75:25 [9].

It should be noted that this analysis of amino acids is the most sensitive of those described in the literature. Moreover, this result was not obtained with the aid of a detector based on highly sensitive laser-induced fluorescence (here the record figures were attained in the analysis of 25 fmol of 9-fluorenyl derivatives of amino acids in a column with $d_c = 0.25$ mm [26] and 0.5 fmol of DNS derivatives of amino acids in a column with $d_c = 0.5$ mm [15], but with the aid of an absorption detector).

It is also noteworthy that the amino acid analysis on a quartz capillary column with crossed-beam thermal lens detector is characterized by a sensitivity higher by many orders of magnitude than that of the conventional chromatographic procedure of amino acid analysis [27].

Another microabsorption detector described in 1987 [25] (Fig. 20) is the crossed-beam thermal lens detector using a pulsed dye laser for pumping (23 Hz, mean power 0.6–1.0 mW) at 400 nm, which in turn is pumped by an excimer laser. As in the preceding case, a helium–neon laser was used for the detection of the thermal lens. The detector in combination with a column of $L = 62$ cm, $d_c = 200$ μm packed with 5-μm CPS-Hypersil sorbent was used for the analysis of nitropyrenes. Kettler and Sepaniak [25] reported a relatively low sensitivity of this analysis with the maximum detectable concentration higher by two orders of magnitude than that attained by using a constant pump laser, the power of which also exceeded by two orders of magnitude the integral power of the pulsed laser used. However, when the power of the pump laser is increased, the powerful pulse burns a hole in the cell wall. In order to solve this problem, an ensheathed measuring cell may be used (Fig. 6d). Its walls are formed by a rapid laminar liquid flow. The advantages of the thermal lens detector with a pulsed pump laser are

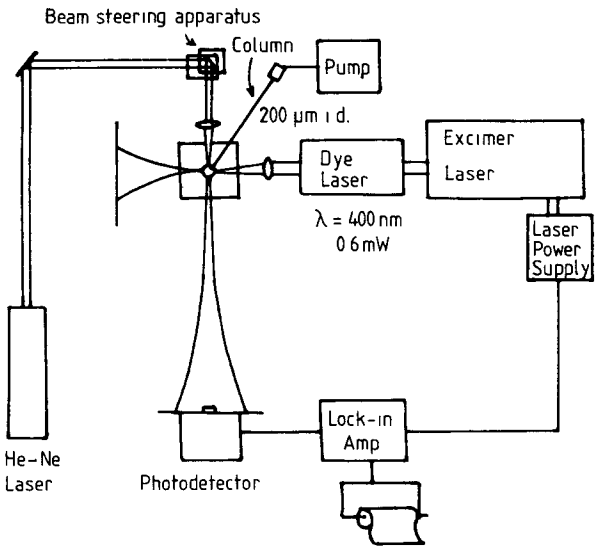

Fig. 20. Detector for photothermal refractometry (absorptiometry) based on pulsed laser for CLC. Pump laser: dye laser with pumping from an excimer laser ($\lambda = 308$ nm). Pulse: $\lambda = 400$ nm, 30 μJ, 23 Hz. Probe laser: He-Ne laser, $\lambda = 632.8$ nm [22].

also emphasized, in particular its higher stability and lower sensitivity to fluctuations in the elution rate.

4. CONCLUSIONS

Laser detection systems characterized by high-concentration sensitivity and ultra-small detection volumes have been successfully applied to CLC, meeting its requirement for the minimization of extra-column volumes. The use in CLC of columns made of flexible quartz capillaries makes it possible to prepare high-performance separating systems with sorbents of submicrometre dimensions. Hence, at present three principal elements of a capillary liquid chromatograph, capillary quartz columns, submicrometre-size sorbents and ultrasensitive laser fluorimetric and absorption detectors, have been developed. Their combination makes it possible to design a capillary liquid chromatograph for the analysis of femtomole and attomole amounts of substances. Further, the difficulties in developing the systems of gradient elution and the injection of nanolitre sample volumes can be avoided by operating without programming the eluent composition (fifteen to twenty components can be separated on a capillary column under isocratic conditions) and by applying the split technique. Consequently, the effective combination of three modern achievements of HPLC, columns made of a flexible quartz capillary, submicrometre-size sorbents and laser detectors, should permit the development in the near future of a commercial capillary liquid chromatograph with record analytical possibilities.

5. SUMMARY

The main relationships of high-performance liquid chromatography (HPLC) are considered. It is shown that the optimum conditions of ultrasensitive trace analysis should be achieved by using packed capillary columns manufactured from flexible quartz capillaries with $d_c \lesssim 0.2$ mm. The main features of these columns ($\nu_{opt} = 0.6\ \nu_{opt}$ of that for conventional HPLC columns with double the hydraulic permeability) make it possible to obtain two or three times higher plate numbers for the same analysis time and column pressure characteristic of conventional HPLC, as a result of using a submicrometre sorbent. The main features of laser detection in capillary liquid chromatography (laser-induced fluorescence and cross-beam thermal lens absorption detectors) are considered. The requirements that should be met by a modern capillary liquid chromatograph based on using flexible quartz capillary columns with a submicrometre sorbent and laser detectors are formulated. Examples of using these systems for femtomole and attomole analyses of biological samples (amino acids and prostaglandins) are given.

6. ACKNOWLEDGEMENT

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