Pure & Appl. Chem., Vol. 59, No. 5, pp. 625–637, 1987.
Printed in Great Britain.

Use of luminescence techniques for sensitive and selective determinations in HPLC (high-performance liquid chromatography)

U.A.Th. Brinkman, G.J. de Jong and C. Gooijer

Free University, Dept. of Analytical Chemistry, De Boelelaan 1083,
1081 HV Amsterdam, The Netherlands

Abstract - The use of luminescence techniques for detection in HPLC has high potential. These techniques are especially useful when extremely high sensitivity and/or selectivity are required, and they can be nicely adapted to the demands of miniaturised HPLC. Three aspects are discussed in the present paper: (i) on-line post-column derivatisation in HPLC with conventional fluorescence detection; (ii) the state of the art of laser fluorescence detection for HPLC; (iii) the principles and applications of on-line chemiluminescence (peroxyoxalate system) detection in HPLC.

INTRODUCTION

In the past decade, high-performance liquid chromatography (HPLC) has gained widespread acceptance. Still, the sensitivity and selectivity of detection in HPLC often do not meet the requirements of modern trace-level determinations in biomedical and environmental samples. Among the present-day detection principles, luminescence - and, especially, its best known and most widely applied representative, fluorescence – appears to offer a high potential to achieve (sub)-ppb detection limits for analytes in complex matrices.

Since the range of compounds displaying strong native fluorescence is relatively small, derivatisation plays an important role in HPLC with fluorescence detection. Initially, pre-column derivatisation (or labelling) was the preferred technique. To-day, on-line post-column derivatisation is becoming increasingly popular. Relatively fast reactions such as ion-pair formation with fluorigenic counter ions and true chemical derivatisation with, e.g., o-phthalaldehyde, especially attract attention. An alternative approach is to use a photochemical reactor. In this case, UV-vis irradiation converts non- or weakly fluorescing analytes of interest into highly fluorescent products.

Laser excitation sources are essential to apply fluorescence detection in miniaturised HPLC. The laser beams are highly collimated, so that illuminated volumes as small as 10 nl are readily attainable. Detector design is crucial because scattered light and background luminescence limit the sensitivity. In the past, continuous lasers have mainly been applied. A recent approach to reduce scatter and to enhance selectivity utilises two-photon excitation which involves pulsed lasers with high peak power.

The limiting factor in the ultimate detectability of fluorescence is the background light, and its noise, that reaches the photomultiplier. In principle, it would therefore be ideal to be able to eliminate the light source from the detection system. One way to achieve this goal is via chemical excitation or chemiluminescence. Although extensively used in batch analyses, chemiluminescence as a method of detection in HPLC is relatively new. In the literature, most attention is devoted to the peroxyoxalate system, which has succcesfully been used to determine various types of fluorophores, fluorophore-labelled compounds as well as hydrogen peroxide.

In this paper, utilisation of the luminescence principles mentioned above as detection methods in HPLC will be discussed in general terms. Emphasis will be given to practical aspects and applications.

FLUORESCENCE: POST-COLUMN DERIVATISATION

Introduction

On-line post-column reaction detection is a well established technique to improve detection sensitivity and/or selectivity in HPLC. The general scheme of such a system is shown in Fig. 1. Some of the principal advantages of post-column reaction detectors are:

- the analytes of interest are separated in their original form, which often permits adoption of published separation procedures,
- artefact formation plays a minor role in post-column reactors, as opposed to derivatisation prior to the separation step,
- the reaction does not need to be complete and does not have to be fully defined. The only requirement is reproducibility.

Disadvantages of post-column reaction detection are the need to add reagent solution(s) which generally will require the use of additional pumps, the possibility of band broadening in the reactor which may mar the chromatographic separation, and the presence of excess reagent which may interfere with the (fluorescent) signal of the reaction products. Interesting means to reduce these disadvantages will be included in the examples presented below.

Ion-pair formation

An elegant method to minimise post-column band broadening is the use of relatively fast ion-pair formation with simultaneous extraction. The method can be used for basic and acidic analytes. Under proper pH conditions the analytes are first separated and next, a reagent solution containing a highly fluorescent counter ion is added. An ion-pair is formed and detection is based on measurement of the fluorescence of the counter ion. The main problem, of course, is that the excess of counter ion also fluoresces and has, therefore, to be separated from the ion-pair to be detected. This can be achieved by using so-called solvent segmentation, which involves rapid segmentation of the HPLC column effluent with non-miscible solvent plugs. The principle is demonstrated in Fig. 2 where basic analytes are separated in a reversed-phase system using an aqueous eluent. The fluorigenic 9,10-dimethoxyanthracene--2-sulphonate (DAS) is added as an aqueous solution of its sodium salt and a halogenated hydrocarbon such as dichloroethane is used as segmentation liquid. The non-polar organic ion-pair is extracted into this solvent, while the (large) excess of the polar DAS sodium salt remains in the aqueous phase. After phase separation, the organic solvent stream is monitored continuously in a fluorescence detector while the aqueous flow goes to waste.

Interesting applications include the determination of chloro- and bromopheniramine in urine (Ref. 1), and of drugs such as secoverine (a tertiary amine; Ref. 2), remoxipride (a substituted benzamide; Ref. 3) and a pancuronium-type compound (Ref. 4) in serum and plasma. In environmental analysis, ditallowdimethylammonium chloride, a well known detergent, has been determined in surface water samples (Ref. 5). Another interesting example involves the detection of C10-C18 linear alkylsulphonates and related sulphates with the highly fluorescent acridine-H^+as counter ion (Ref. 6). In this case, separation was achieved by linear-gradient elution (acetone-to-water), which did not adversely affect the post-column reaction/extraction procedure.

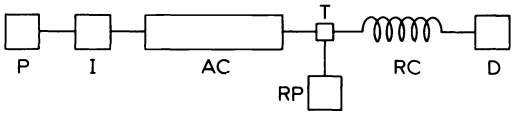

Fig. 1. General set-up of HPLC system with on-line post-column reaction detection unit. P, pump; I, injection valve; AC, analytical column; T, (mixing) tee-piece; RP, reagent addition pump; RC, reaction coil (glass, PTFE, stainless-steel); D, detector.

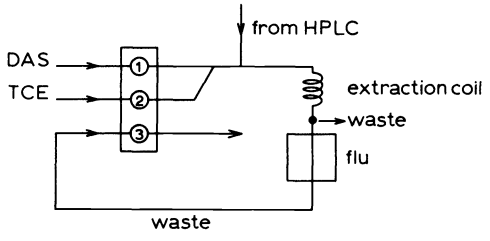

Fig. 2. General set-up of HPLC system (chemically bonded stationary phase; partly aqueous eluent) with on-line post-column ion-pair formation extraction and fluorescence detection. Conditions: DAS, aqueous DAS Na solution; TCE, tetrachloroethane; O, phase separator; Flu, fluorescence detector.

As an illustration, we shortly discuss the semi-automated procedure developed for the selective dopamine antagonist remoxipride in biological fluids (Ref. 3). The set-up is essentially the same as that shown in Fig. 2. However, additionally a disposable pre-column unit was inserted to permit the direct injection of 1:1 diluted plasma and of 1:9 diluted urine. The HPLC separation is carried out on a C18-bonded silica column deactivated with cetrimide, with aqueous acetonitrile buffered to pH 3 as the eluent. On the reaction detection side, an aqueous DAS solution is used for ion-pair formation, while 1,2-dichloroethane serves as the organic extraction solvent. A new sandwich-type phase separator with an internal volume of only 30-40 µl helps to keep band broadening very low. Its upper half is constructed of stainless-steel, and the lower half of PTFE. With this separator, which is based on the wetting principle and which does not contain a membrane, a purely organic phase can be obtained. Some typical results for the analysis of plasma samples are summarized in Table I.

Table I. Analytical data for remoxipride determination in plasma.

Parameter		Condition	Results
Calibration plot		over 2 orders	r = 0.9999
Detection limit		signal/noise, 3/1	1 ng/ml
Recovery		spiked plasma	88%
Repeatability	: one series	200 ng/ml	3.5% RSD
	: day-to-day	200 ng/ml	3.5% RSD

Chemical derivatisation

An excess of reagent can easily be tolerated when it is only the reaction product formed, and not the reagent itself, which is fluorescent. A good example is provided by o-phthalaldehyde (OPA). In alkaline solution and in the presence of a strong reducing agent such as 2-mercaptoethanol, the non-fluorescent OPA reacts with primary amino groups of amines, amino acids and amino sugars to yield highly fluorescent derivatives (see Fig. 3). OPA is best known (Ref. 7) for the determination of amino acids in biological fluids at, typically, the picomol level. Other applications involve the determination of therapeutic agents such as aminoglycosides (gentamicin, amikacin) and ß-lactam antibiotics (penicillin, cephalosporin).

In the field of environmental analysis, N-methylcarbamates can be quantitated via a dual-reactor approach (Ref. 8). After their separation on an alkyl-bonded silica column, the analytes are hydrolysed on-line in a 4-6 cm long stainless-steel column packed with an anion-exchange resin and heated at a temperature of about 100°C. Next, the methylamine-containing reactor effluent is mixed, via a tee-piece, with an OPA solution, and the fluorescent derivative is formed in the second reactor, a coiled stainless-steel capillary (residence time, 20-30 sec). The detection limits of the six carbamates tested are between 0.1 and 1 ng. For real trace-level analysis, the method has been combined with on-line precolumn trace enrichment (Ref. 9). Using a 20-ml surface water sample spiked with carbaryl, a detection limit of about 20 ppt can be obtained (see Fig. 3).

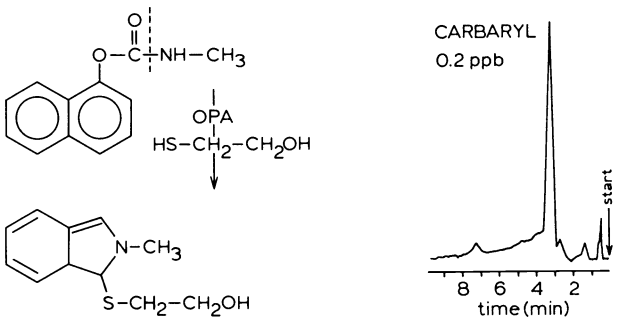

Fig. 3. Catalytic hydrolysis of the N-methylcarbamate carbaryl, its reaction with OPA, and the trace-level determination of 0.2 ppb of carbaryl in a 20 ml canal water sample. See Ref. 9.

Photochemical reactor

Next to the catalytic reactor mentioned in the preceding section, the photochemical reactor is an example of a so-called pumpless reactor (Ref. 10). In the simplest case, photons are the only reagents added. If we limit ourselves to the area of fluorescence detection, the irradiation with UV light converts the non- or weakly fluorescing analytes of interest into reaction products which are highly fluorescent. A photochemical reactor essentially consists of a high- or medium-power mercury or xenon-mercury lamp, a PTFE reaction coil—which, somewhat surprisingly, shows a light transmission in the 200-300-nm region comparable to that of quartz—and a reactor housing which accommodates a cooling device. The temperature should be kept rigidly constant, because temperature fluctuations will affect flow rates, reaction kinetics and/or reaction pathways and, consequently, will mar the optimisation procedure. Most photochemical reactions utilised to produce fluorescent products for HPLC analyses require reactor residence times of some 10-60 s only. Detection limits often are 50-500 pg and selectivity is high. A number of applications is reported in Table II. With the exception of the determination of clomiphene, they all deal with reversed-phase-type separations, i.e., with the use of organic-aqueous eluents.

Table II. Applications of HPLC with on-line photochemical reaction detection*

Cannabinol	Demoxepam	Methotrexate
Chlorophenols	Diethylstilbestrol	Phenothiazines
Clobazam	LSD	Tamoxifen
Clomiphene	Metal ions	Vitamin K_1

*For references, see Refs. 10 and 13. Most applications are for urine, blood, and/or surface water samples.

An early example of photochemical reaction detection is shown in Fig. 4 which deals with the determination of the anti-anxiety drug clobazam and its main metabolite desmethylclobazam in human serum (Refs. 11 and 12). Neither of the two compounds displays native fluorescence (Fig. 4 left), but at the optimum irradiation time of 15 sec both compounds show up nicely (Fig. 4 right) with detection limits of 20 pg (clobazam) and 50 pg (desmethylclobazam), respectively. Uihlein and Schwab (Ref. 12) use a very elegant knitted reactor, with the UV light source being mounted on a sledge so that it can easily be moved further into or out of the reactor in order to vary the time of irradiation. Recent applications deal with the determination of methotrexate and its metabolites in body fluids (Ref. 14) and of chlorophenols in surface water and urine samples (Ref. 15). In the former case, a small amount of hydrogen peroxide has to be present and optimum irradiation takes less than 4 s. In the latter case, the analytes are separated as their dansyl derivatives, with post-column irradiation releasing the higly fluorescent dansylsulphonic acid.

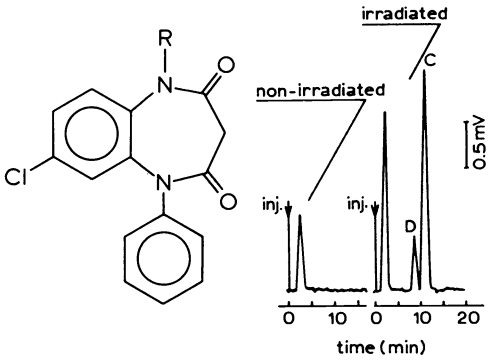

Fig. 4. Structure of clobazam (C; R = CH_3) and desmethylclobazam (D; R = H) and their determination in human serum (0.7 ppm each) using a C18/methanol-water (1:1) HPLC system. Fluorescence detection without (left) and with (right) UV irradiation is shown. Irradiation time, 15 sec. See Ref. 11.

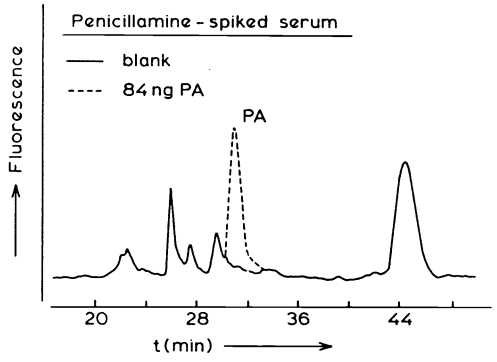

Fig. 5. HPLC chromatogram of a penicillamine-spiked sample. Separation in a cation exchanger/aqueous pH 2.2 buffer HPLC system. Reactor residence time, 20 min at 55°C. For air segmentation, fluorescence detection, etc., see Ref. 16.

Ligand exchange

A final application is reported to illustrate that even ligand exchange with its relatively slow kinetics can successfully be used in a post-column reactor.

The trace-level determination of organosulphur compounds such as ETU, penicillamine and low-molecular-weight thiols and thioethers can be carried out on the basis of an HPLC separation followed by an exchange reaction between the non-fluorescent (quenching!) 1:1 Pd(II)-calcein complex and the analytes which displace an equivalent amount of the highly fluorescent calcein from the reagent, the released calcein being the actual species to be detected (Ref. 16). There is no need to introduce an extraction step from the water-rich (pH around 7) mobile phase, because the excess of Pd(II)-calcein does not fluoresce. However, air (or solvent) segmentation is necessary in order to suppress extra-column band broadening: a large-size reaction coil is required because the reaction is rather slow, with a 10-15 min residence time in the reactor at a temperature of 50-60°C often being used. As an illustration, data on the analysis of a penicillamine-spiked serum sample are shown in Fig. 5.

LASER FLUORESCENCE DETECTION

Introduction

In a review paper published in 1980, Yeung and Sepaniak (Ref. 17) clearly outlined the high potential of laser fluorescence detection in HPLC, especially in view of the existing trend to miniaturise HPLC systems. In 1984, Dovichi (Ref. 18) demonstrated laser-induced fluorescence of flowing samples to be an approach to single-molecule detection in liquids. Nevertheless, despite its extraordinary inherent sensitivity, to date relatively few papers on microbore and/or open-tubular HPLC combined with laser fluorescence detection have been reported (Refs. 19-21). This is rather surprising, since (i) miniaturisation is becoming increasingly important in HPLC, and (ii) in this field of research detection still is a weak point because extremely small detector cell volumes are required - a problem that can at least partly be solved by employing lasers with their high brightness and favourable collimation properties. Of course, the simple replacement of a conventional light source by a laser does not guarantee improved detectability, since background radiation plays a crucial role - especially with very small cell volumes. However, the main reason that relatively little effort has been spent in the area under dicussion presumably is that lasers are considered highly expensive and complicated instruments, and therefore not amenable to routine analysis.

Excitation in small detector cells

The detector cell volume in an HPLC system must be small enough to prevent unacceptable additional band broadening of the chromatographic peaks. This implies that in microbore HPLC—with a typical column I.D. of 1 mm, and flow rates ranging from 20 to 100 µl/min—a detector cell of around 0.5 µl is allowed. In open-tubular HPLC (OTLC), the column I.D. is as small as 10-25 µm and the flow rates are only 10-500 nl/min; consequently, detector cell volumes of 0.1-10 nl are required. Obviously, OTLC demands highly sophisticated detection techniques to achieve relevant sensitivities. However, adequate detection also is a point of concern in microbore HPLC. It has been shown (Ref. 21) that, in comparison with conventional HPLC (3-4.6 mm I.D. columns), the concentration detection limits attainable with classical UV-vis absorbance and fluorescence detection show an unfavourable increase (5-fold) in microbore HPLC.

The preceding paragraphs suggest that in miniaturised HPLC the most important property of the laser is its directionality. Because the angle of radiation is very small—typically 0.5-1 mradian—the photon flux arriving at the detector cell is high despite the fact that the power of the laser usually is much lower than that of a classical light source. To quote an example, the frequently used He-Cd laser emits only 1-10 mW at 325 nm. Nevertheless it produces a higher intensity in a small detector cell used for OTLC than does a 100-W mercury lamp at 254 nm. The explanation is that the laser can be focussed down to 5 to 50 µm without much loss of photons, whereas the mercury lamp spreads out in all directions so that efficicient light collection is hardly possible and the minimum spot size can not be smaller than 1 mm (Refs. 16, 19).

Another important aspect is the reduction of unwanted stray light in fluorescence detection. Here, two aspects favour the use of laser excitation. Firstly, the Rayleigh and Raman bands are much sharper because of their monochromaticity and, hence, their effect can be more easily minimised. Secondly, the scattering pattern produced by a laser is virtually planar, whereas the pattern obtained with an arc lamp is more omnidirectional. Hence, it is relatively simple to reduce the background due to laser scatter by careful positioning of the light source, the detector cell and the photomultiplier collecting optics (Refs. 22, 23).

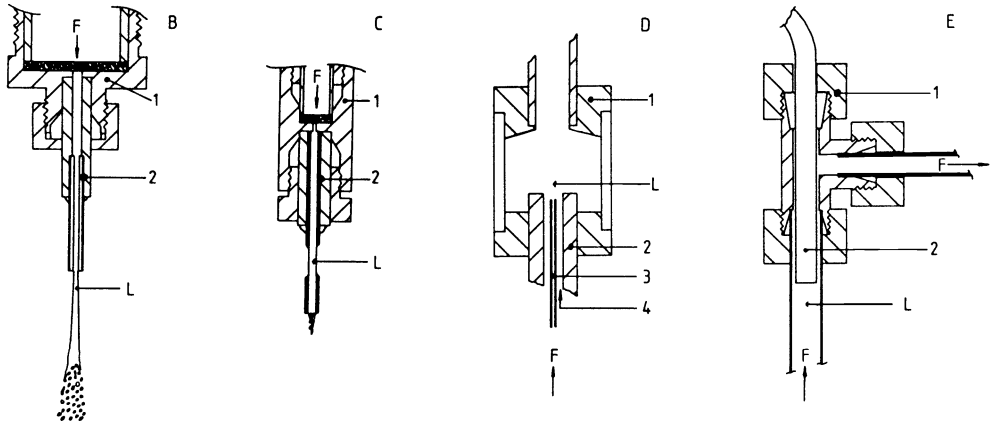

Fig. 6. Cell designs for laser fluorescence detection. (B) Free falling jet. 1, HPLC column; 2, fused silica capillary glued in stainless-steel tubing. (C) Fused silica cell. 1, Column; 2, fused silica capillary, with polyimide layer removed over short distance. (D) Sheath flow cell. 1, PTFE holder for quartz cuvet; 2, outer tube; 3, last part of OTLC column or connection to outlet of packed column; 4, sheath flow. (E) Optical fiber cell. 1, Modified tee-piece; 2, optical fiber. F, flow direction; L, laser light. See Ref. 21.

Detector design

Despite the above, the current major limitations in miniaturised HPLC with laser fluorescence detection are still centered on the detector design. Four different principles are of special interest, viz. the use of (i) sheath flow, (ii) optical fibers, (iii) the free falling jet, and (iv) a fused silica capillary. They will be shortly discussed below (See Fig. 6).

Sheath-flow. A sub-microliter flow-through cell based on the sheath-flow principle was first presented by Hershberger et al. (Ref. 24). The HPLC column effluent is surrounded by a second flow, the sheath flow, which has the same composition as the column effluent. If the flows are laminar, no mixing will occur. By adjusting the relative flow-rates of the two solvent streams, the diameter of the inner sample stream can be adjusted and, consequently, different cell volumes can be obtained. The applicability of the cell in conventional and micro HPLC (Ref.24) and in OTLC (Ref. 21) has been demonstrated.

Optical fibers. With this detection principle, an optical fiber — which nowadays is available with diameters down to 10 μm — is inserted into the fused-silica capillary column. The fiber entrance is located close to the illuminated volume of the detector cell although at a distance where cell-wall interferences such as scattered and reflected light can be neglected. The luminescence collection efficiency is rather high and the optical fiber can be directly connected to a monochromator without the use of additional optics. Gluckman et al. (Ref. 25) used this approach to obtain a laser-illuminated volume as small as 3 pl, while the actual cell volume was 98 nl. Unfortunately, even that volume is still too high for use in OTLC (cf. above).

Free-falling jet. In this set-up, the HPLC effluent is led through a fused silica capillary to increase its linear velocity and obtain a jet stream. That is, luminescence detection is performed on a liquid eluent cylinder with a smooth and fresh surface which is optically well defined. It should, however, be realised that a zone of intensely reflected light is generated in the laser beam plane, because the liquid cylinder acts as an optical quasi-lens. The collecting optics must therefore be placed outside this area.

Recently, the possibility of producing free-falling jets with flow-rates compatible with microbore HPLC columns has been studied by Folestad et al. (Ref. 23). Experimentally it was observed that a 12-μm I.D. quartz capillary produced a suitable jet with pure methanol at a flow-rate of 45 μl/min. However, so far the jet cell seems not to be suitable for the extremely low eluent flow-rates of around 0.1 μl /min used in OTLC for which no stable jet can be formed (Refs. 19, 21).

Fused silica capillary. An interesting alternative is to construct a cell by simply burning away the protective polyimide coating from a fused silica capillary. Alternatively, the last part of an OTLC column can be transformed into the detector cell itself, which allows on-column detection. Unfortunately, small-bore capillaries of 5-10 µm I.D. have relatively thick walls and, thus, produce a high background signal. The signal-to-noise ratios are comparable with those of the free-falling jet detector (Ref. 23). On-column detection in OTLC has been reported in Refs. 22 and 23.

Summary. At the present moment, an ideal cell for miniaturised HPLC with fluorescence detection does not exist and, besides, as yet no final conclusion can be given regarding the best type of design. Still, the fused silica capillary cell is the most simple and practical detector. The absence of any connection between HPLC column outlet and detector cell inlet should make the fused silica capillary the preferred detector cell for OTLC. The main challenge for OTLC is to improve the detection limits in units of concentration which still are two orders of magnitude higher than in conventional HPLC with 3 µl fluorescence flow cells (Ref. 23).

Applications

As was already stated above, relatively few applications of laser fluorescence detection for HPLC have been reported so far (for references, see Refs. 19, 21). In most cases, a low-cost He-Cd laser, with its two weak (1-10 mW) lines at 325 and 442 nm, is used. In some papers, a high-power Ar ion laser has been employed. This laser provides several strong lines in the visible part of the spectrum - such as the 1-10 W lines at 458, 476, 488, 496, 514 and 529 nm and a series of weaker UV lines at 334, 351 and 364 nm (0.1-1 W). The He-Cd and the Ar ion laser both are continuous, but the latter can also be operated in a pulsed mode.

One way to use more fully the potential of laser fluorescence detection is, of course, to install a tunable dye laser. However, as far as we are aware, this has not been done in miniaturised HPLC . Generally, an alternative approach is preferred viz. derivatisation of the analytes of interest to obtain products with fluorescent characteristics compatible with the available laser wavelengths. Derivatisation is generally done in the pre-column mode; recently, post-column derivatisation has also been applied (Ref. 21). Gluckman et al. (Ref. 25) have shown the great potential of using He-Cd (325 nm) laser fluorescence labelling for biochemically important compounds, such as prostaglandins, steroid hormones and bile acids. They used reagents containing a coumarin moiety which has an absorption maximum at 317 nm. The authors achieved a minimum detectable quantity of 8.4 fg of coumarin at a laser excitation power of 6.45 mW. With a 66 cm x 250 µm I.D. packed fused-silica HPLC column, the steroid peaks in Fig. 7 correspond to 50 pg of injected compound. Guthrie et al. (Ref. 22) used OTLC separation columns with 16 µm and 25 µm I.D., and applied the He-Cd laser directly on column. The collection optics were arranged off the laser-scattering plane. They achieved a detection limit of 35 fg of riboflavin (injected amount) at the 442-nm excitation line of the laser.

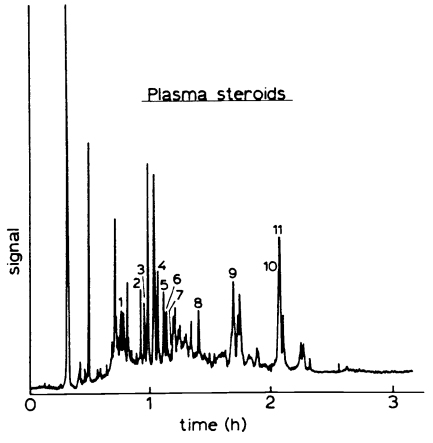

Fig. 7. HPLC of solvolysed plasma steroids; about 50 pg of each steroid injection. Peak numbers (tentative): 5α-androstan-3α, 11β-diol-17-one; 2, 5β-androstan-3α, 11β-diol--17-one; 3, 5β-pregnane-3α, 11β, 17α, 21--tetrol-20-one; 4, 5β-pregnane-3α, 17α, 20β, 21-tetrol-11-one; 5, 5β-pregnane-3α, 11β, 17α, 20β, 21-pentol; 6, 5β-pregnane-3α, 17α, 20α, 21-tetrol-11-one; 7, 5β-pregnane-3α, 11β, 17α, 20α, 21-pentol; 8, 5α-androstan-3α-ol-17-one; 9, androstene-3β-ol-17-one; 10, 5β-pregnane-3α, 20α, 21-triol; 11, 5β-androstan-3α, 17β-diol. See Ref. 25

Until now, pulsed lasers have been used only occasionally for fluorescence detection in miniaturised HPLC. These lasers possess the inherent advantage of reducing the background signal by means of time-resolved fluorescence. In addition, some pulsed lasers deliver very high peak powers, which makes them suited for two-photon excitation. The potential of the latter technique in micro HPLC has recently been evaluated by Pfeffer and Yeung (Ref. 26). The simultaneous absorption of two photons is determined by other selection rules than is the conventional single-photon absorption process; in other words, additional selectivity is provided. Besides, it suffices to use long-wavelength excitation, because fluorescence emission occurs at shorter wavelengths than does excitation. As a consequence, the background signal is virtually negligible.

Compared to single-photon absorption, two-photon absorption is a much less probable process. This probability is, on the other hand, proportional to the square of the photon density. That is, only pulsed lasers can be employed, because during the pulses the power can be extremely high. Pfeffer and Yeung showed a copper vapour laser to be an appropriate choice. It has excitation lines at 510 and 578 nm, the average power is 3 W, the pulse duration 30 ns, the pulse repetition frequency 5000 Hz and the peak power 2 x 10^4 W. The excitation lines effectively excite at 255 and 289 nm, which permits one to select from among a large number of chromophores. As an example, the detection of two phenyloxadiazoles is shown in Fig. 8.

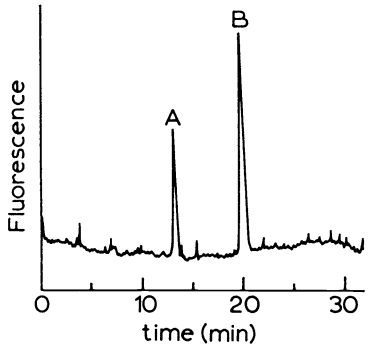

Fig. 8. Two-photon-excited fluorescence HPLC chromatogram of 1 µl of (A) 0.2 µM 2,5-diphenyl-1,3,4 -oxadiazole, and (B) 0.01 µM 2-(4-biphenyl)-5-phenyl- -1,2,3-oxadiazole. Conditions: C18-bonded phase/ acetonitrile-water (9:1) at 30 µl/min. Detection limit of B, 250 pg injected. See Ref. 20.

SEMI OFF-LINE FLUORESCENCE DETECTION

Fluorescence detection has the inherent selectivity that two types of spectra can be recorded, viz. emission spectra (at a fixed excitation wavelength) and excitation spectra (at a fixed emission wavelength). Despite several on-going attempts, to-day one cannot enjoy the advantages of this selectivity using commercially available equipment for HPLC. Rather, the fluorescence signal is monitored with fixed - or, at best, programmable - excitation and emission wavelengths using 5 - 10 nm band widths.

Recording complete spectra on-line requires the use of optical imaging devices, which allow for the simultaneous detection of a wide spectral region. In UV-vis absorption detection, linear photodiode arrays have become widely accepted for this purpose (Ref. 27); however, for fluorescence detection, no comparable equipment has been marketed. This can be readily understood: in emission spectrometry, very low light levels have to be monitored, which is not possible with a conventional diode array. Instead, the rather expensive intensified linear photodiode array is needed. Recently, such a device has successfully been employed by Gluckman et al. (Ref. 28) for the detection of several polyaromatic hydrocarbons in micro HPLC with a fiber optic flow cell.

One approach studied in our laboratory by Hofstraat et al. is to immobilise the effluent from a micro HPLC separation on a (slowly moving) thin-layer (TLC) plate. This can be done without serious additional band broadening (Refs. 29,30). Complete fluorescence emission and excitation spectra can now readily be obtained using a conventional spectrometer equipped with an accessory for the analysis of solid samples. As an example, Fig. 9 shows the emission and excitation spectra of benz[k] fluoranthene immobilised on a silica TLC plate. For the HPLC-TLC procedure, the detection limit of the polyaromatic compound is 3 pg, which satisfactorily agrees with the data in Ref. 28. The linear dynamic range is three decades.

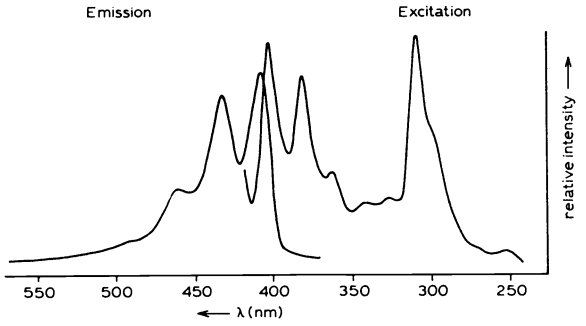

Fig. 9. Fluorescence emission and excitation spectra of 2.5 ng benz(k)-fluoranthene on a silica TLC plate recorded at room temperature. Virtually identical spectra are obtained in methanolic solution. See Ref. 30.

The immobilisation approach also allows one to use fluorescence line narrowing (FLN) spectroscopy. This technique requires extremely low sample temperatures of around 10 K and can, therefore, not be used for on-line detection in HPLC. The high selectivity of FLN spectroscopy is based on the fact that the inhomogeneous broadening of the spectral bands in fluorescence analysis - which is mainly responsible for the lack of structure in the spectra - can largely be removed by selective laser excitation at sufficiently low temperatures. Several studies (Refs. 31, 32) demonstrate that TLC plates are suitable immobilisation materials for FLN spectroscopy. As an example, in Fig. 10 the FLN spectrum of 3.1 ng of tetracene excited by the 476.5 nm line of an argon ion laser at a laser power of only I mW is shown; a C18-modified silica TLC plate was used for immobilisation. One should note that only vibronic transitions are observed, because the spectrum can only be recorded starting at 483 nm. Even under these unfavourable conditions, and when working with low-intense laser light and with a test compound which has a low fluorescence quantum yield, the detection limit is 30 pg. Further exploration of HPLC with off-line FLN spectroscopic detection obviously is indicated.

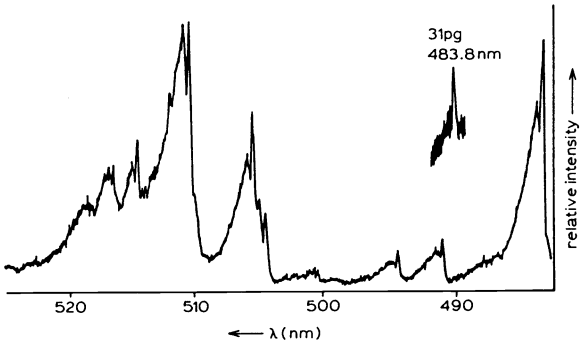

Fig. 10. FLN spectrum of 3.1 ng tetracene on a C18-bonded TLC plate; Ar ion laser (1 mW) excitation at 476.5 nm; temperature, 9.3 K. The insert shows the 483.8 nm emission line recorded for 31 pg tetracene. See Ref. 30.

CHEMILUMINESCENCE DETECTION

Peroxyoxalate reaction

The beneficial effects of substituting chemical excitation for photoexcitation, thus achieving chemiluminescence, have been mentioned in the introduction to this paper. Probably, the peroxyoxalate reaction is the most efficient and versatile chemiluminescence system available today. It can be used for the excitation and , thus, determination of many classes of fluorophores, and also for the detection of small amounts of hydrogen peroxide produced by photochemical or enzymatic reactions. The peroxyoxalate reaction was discovered two decades ago (Ref. 33); the first paper on its application for HPLC detection stems from I980 (Ref. 34). The mechanism reaction generally proposed is shown in Fig. 11. An energy-rich intermediate, postulated to be 1,2-dioxetanedione, is generated by the reaction of hydrogen peroxide and an aryl oxalate such as bis (2,4,6-trichlorophenyl)oxalate or bis (2,4-dinitrophenyl) oxalate, abbreviated TCPO and DNPO, respectively. The intermediate excites the fluorophore and the excited fluorophore returns to the ground-state via emission of light. Lechtken and Turro (Ref. 35) have reported that the peroxyoxalate reaction can not transfer more than 105 kcal/mol. This corresponds with an excitation wavelength of about 270 nm in photoexcitation. For high CL intensities the excitation energy should preferably be low (Refs. 35-37).

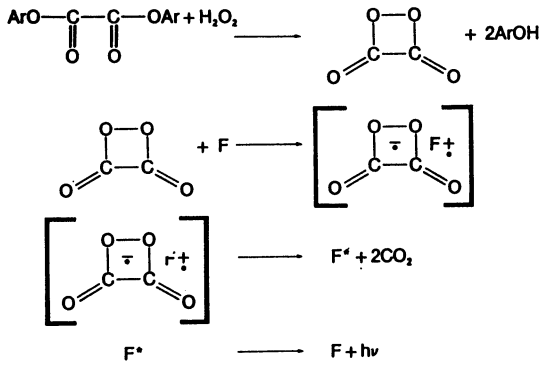

Fig. 11. Peroxyoxalate reaction scheme

As is shown in Fig. 11, a charge-transfer complex is formed between the fluorophore and 1,2-dioxetanedione. Electron transfer from the fluorophore to the intermediate occurs during this step and fluorophores with low oxidation potentials are indeed found to be good CL energy acceptors. For example, Sigvardson et al. (Ref. 38) observed a rather nice correlation between the CL intensity (corrected for the fluorescence quantum yield) and the oxidation potential for several aromatic hydrocarbons, and Honda et al. (Ref. 37) obtained comparable results for a series of heterocyclic compounds. In other words, the peroxyoxalate reaction is a so-called chemically induced electron-exchange luminescence reaction.

The existence of the dioxetanedione intermediate is still under discussion. For example, the dependence of the reaction kinetics and the CL intensity on the nature of the aryl groups of the oxalates appears to contradict with a common intermediate for all oxalates studied. Catherall and co-workers (Refs. 39, 40) have, therefore, suggested an alternative mechanism, in which only one of the two aryl groups is eliminated from the oxalates. On the basis of CL intensity vs. time curves, Alvarez et al. (Ref. 41) have recently concluded that the fluorophore is excited by at least two intermediates. Further research in this area is required because a better insight into the reaction mechanism may well improve the analytical potential of this reaction.

The role of various parameters

A significant difference exists between most other reaction detection principles used for HPLC, and CL reactions. The latter type of reaction displays a luminescence growth curve followed by a decay of the signal intensity that is caused by the exhaustion of the light-generating agent(s). In a flow system, the half-life of the CL signal is a very important parameter; for given values of the various flow rates, the dead volume between the mixing tee and the flow cell, and the volume of the flow cell itself, the CL half-life determines the percentage of the emitted light that will be measured (Ref. 42). Honda et al. (Ref. 43) have demonstrated that the half-life is highly dependent on the aryl oxalate structure. The relatively short CL half-life of the DNPO as compared with the TCPO system has also been commented upon in other papers (Refs. 44, 45). For a given aryl oxalate, the half-life is dependent on the (final) solvent composition, and invariably decreases with increasing pH (Refs. 42, 43, 46) and with an increase of the water content (Refs. 42, 46). The lifetime of the CL signal is a function of the concentration of catalyst such as imidazole. A large imidazole concentration however impairs the background signal (Refs. 47, 48).

Another problem encountered when using the peroxyoxalate CL system concerns the proper solvent selection. In most published papers, TCPO is the aryl oxalate used; esters and ethers are the best solvents for this reagent. However, because ethers react with oxygen to form peroxides, TCPO decomposes rather rapidly if dissolved in an ether. As for esters, most common esters are not too readily miscible with water. In others words, CL monitoring of partly aqueous solutions—which is what most HPLC eluents are—requires the presence of a third solvent to create an homogeneous system. In practice, ethyl acetate is often selected as solvent for TCPO, and hydrogen peroxide is dissolved in tetrahydrofuran or acetone. Conventionally, the two reagent solutions are mixed on-line, and the mixture is then added to the HPLC effluent. Occasionally, TCPO and hydrogen peroxide are premixed; however, the sensitivity of such a system does not remain constant for over one day.

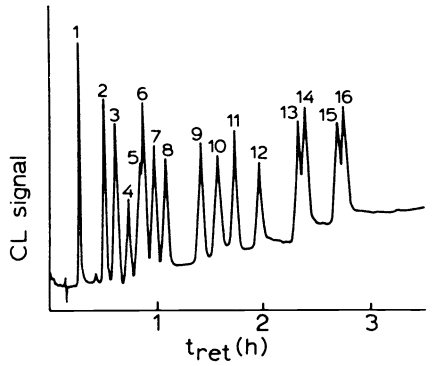

Fig. 12. Gradient-elution HPLC of dansylated amino acids (100 fmol each) with CL detection. Peak numbers 1 to 16: Asp, Asn, Gln, Ser, Gly, Thr, Ala, Pro, Lys, Val, Arg, Met, Ile, Leu, Trp, Phe. Conditions: C18-bonded phase/imidazole nitrate-acetonitrile gradient at 0.3 ml/min: CI-reagent (TCPO in ethyl acetate-hydrogen peroxide in acetone) at 1.8 ml/min. See Ref. 51.

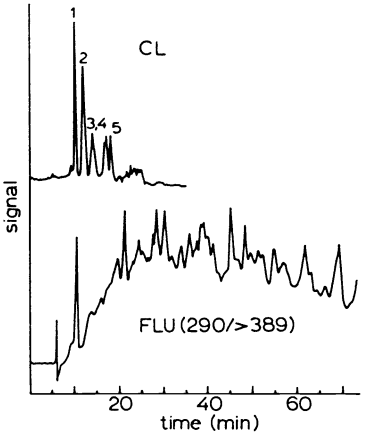

Fig. 13. HPLC of fractionated shale oil extract with CL and fluorescence detection. Peak numbers: 1, aminonaphthalenes; 2, C_1-aminonaphthalenes; 3, aminophenanthrenes; 4, aminoanthracenes; 5, aminopyrenes. Conditions: C18-bonded phase/acetonitrile-water (3:1) at 0.7 ml/min. See Ref. 38.

The maximum allowable concentration of TCPO in ethyl acetate is about 0.01 M. DNPO is more polar and can be dissolved in acetonitrile to reach a maximum concentration of about 0.02 M. A higher maximum concentration, in principle, means higher detection sensitivity. Therefore, the search for an aryl oxalate having a still higher solubility in a suitable solvent, is important (Ref. 49). The hydrogen peroxide concentration usually is relatively high, i.e. 0.1-1 M, because of high sensitivity and in order to reduce the half-life of the CL signal (Refs. 34, 48).

Applications

The potential of the peroxyoxalate system for HPLC-CL has been demonstrated for, e.g., dansylated amino acids (Refs. 34, 44, 50-52) and dansyl derivatives of a secondary amine-type drug, steroids and a number of primary amines (Refs. 45, 53, 54) Dansylated compounds often display a CL detection limit which is 25-100-fold lower than the fluorescence detection limit. Miyaguchi et al. (Ref. 52) reported detection limits of around 200 attomol for a series of dansylated amino acids in a microbore HPLC system. These authors also used gradient elution HPLC-CL, as is shown in Fig. 12. Kobayashi and coworkers (Ref. 55) successfully detected femtomol amounts of fluorescamine-labelled catecholamines.

Amino-substituted polycyclic aromatic hydrocarbons are among the analytes that are most sensitively detected by CL. Trace-level determination is successful even if large amounts of other types of polycyclic aromatics are present. In Fig. 13, this is illustrated for the detection of amino-substituted aromatics in a shale oil extract (Ref. 38) and compared with fluorescence detection. Upon the pre- or post-column insertion of a zinc column, the same system can be used for the sensitive detection of nitro aromatics (Ref. 38). As a further extension, amino-substituted aromatics can be used to convert other analytes into CL-detectable products. Honda et al. (Ref. 37) were able to detect 100 attomol of 3-aminoperylene derivatives of carboxylic acids, and Mann and Grayeski (Ref. 56) have demonstrated the potential of labelling aldehydes and ketones with 3- aminofluoranthene.

Miniaturisation. It has repeatedly been demonstrated that the peroxyoxalate system can well be used in miniaturised HPLC (Refs. 42, 48, 52, 57). Because of its short half-life, DNPO is the preferred oxalate for such work. However, it is not easy to construct a properly designed post-column mixing cum detection unit. For one thing, the decay of the CL signal is so rapid (half-life often less than I s) that even a very small dead time between the mixing of the reagent solution with the HPLC effluent and the detector cell inlet will result in a considerable loss of emitted light. This problem can be solved by carrying out the mixing within an integrating sphere which also efficiently collects (nearly) all the emitted light. It is noteworthy that even with a relatively large 450-µl glass coil as flow cell (Ref. 42), additional band broadening is almost negligible, because the CL signal completely decays in a small part of this cell. For a full discussion of this so-called chemical band narrowing effect, one should consult the literature (Refs. 42, 45).

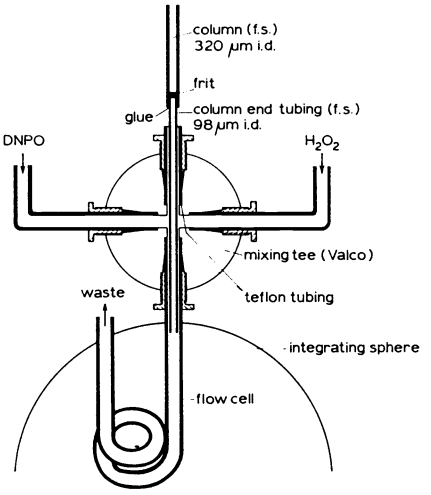

Fig. 14. Mixing of LC eluent from packed capillary column and CL detection reagents (DNPO and H_2O_2; added by syringe pump). Mixing takes place in a 70-µl (0.3 mm I.D.) Teflon flow-cell placed in an integrating sphere. See Ref. 48.

Recently, the same principle has been applied (Ref. 48) for HPLC with 320-µm I.D. packed capillary columns. The detection system is shown in Fig. 14 and explained in the legend. With several dansylated compounds, detection limits of about 400 attomol were obtained.

Hydrogen peroxide. Finally, it should be mentioned that, in the presence of an excess of fluorophore, the peroxyoxalate reaction can be used to determine trace level amounts of hydrogen peroxide. The system can be combined on-line with enzyme reactions which produce hydrogen peroxide. Honda et al. (Ref. 58) have used a post-column reactor containing immobilised choline oxidase and cholinesterase, plus the addition of a buffer and a TCPO- and perylene-containing solution to detect low-pmol amounts of choline and acetylcholine in standard solutions. Van Zoonen et al. (Ref. 59) have studied the same enzyme system with a more simple design, viz. using solid TCPO and 3-aminofluoranthene chemically bonded to glass beads; these beads were packed in the detector flow-cell in front of the photomultiplier (Ref. 60). The authors were able to determine choline and acetylcholine in urine and serum, thereby demonstrating the high selectivity of the combined use of enzyme reactors and peroxyoxalate CL detection.

REFERENCES

1. R.W. Frei, J.F. Lawrence, U.A.Th. Brinkman and I. Honigberg, J. High Res. Chromatogr. Chromatogr. Commun. 1, 11 (1979).
2. R.J. Reddingius, G.J. de Jong, U.A.Th. Brinkman and R.W. Frei, J. Chromatogr. 205, 77 (1981).
3. C. de Ruiter, U. A. Th. Brinkman and R.W. Frei, J. Liq. Chromatogr. (1987) in press.
4. J.H. Wolf, C. de Ruiter, U.A.Th. Brinkman and R.W. Frei, J. Pharm. Biomed. Anal. 4, 523 (1986).
5. C. de Ruiter, J. Hefkens, U.A.Th. Brinkman and R.W. Frei, in preparation.
6. F. Smedes, J.C. Kraak, C.E. Werkhoven-Goewie, U.A.Th. Brinkman and R.W. Frei, J. Chromatogr. 247, 123 (1982).
7. A.T. Rhys Williams, Fluorescence Detection in LC, Perkin-Elmer, Beaconsfield, U.K., 1980.
8. L. Nondek, R.W. Frei and U.A.Th. Brinkman, J. Chromatogr. 282, 141 (1983).
9. Low Kun She, U.A.Th. Brinkman and R.W. Frei, Anal. Letters, 17A, 915 (1984).
10. J.W. Birks and R.W. Frei, Trends Anal. Chem. 1, 361 (1982).
11. A.H.M.T. Scholten, U.A.Th. Brinkman and R.W. Frei, Anal. Chim. Acta, 114, 137 (1980).
12. M. Uihlein and E. Schwab, Chromatographia, 15, 140 (1982).
13. I.S. Krull and W.R. LaCourse, in I.S. Krull (ed.), Reaction Detection in Liquid Chromatography, M. Dekker, New York, 1986, p. 303.
14. J. Salomoun and J. Frantisek, J. Chromatogr. 378, 173 (1986).
15. J.F. Bohle, C. de Ruiter, J. Haslova, J.C. Gluckman, U.A.Th. Brinkman, G.J. de Jong and R.W. Frei, in preparation.

16. C.E. Werkhoven-Goewie, W.M.A. Niessen, U.A.Th. Brinkman and R.W. Frei, J. Chromatogr. 203,165 (1981).
17. E.S. Yeung and M.J. Sepaniak, Anal. Chem. 52, 1465A (1980).
18. N.J. Dovichi, Trends Anal. Chem. 3, 55 (1984).
19. S. Folestad, B. Galle and B. Josefsson, J. Chromatogr. Sci. 23 , 273 (1985).
20. W.D. Pfeffer and E.S. Yeung, Anal. Chem. 58, 2103 (1986).
21. H.P.M. van Vliet, Laser Induced Fluorescence Detection in Microbore and Open Tubular Liquid Chromatography, Thesis, University of Amsterdam, (1986).
22. E.J. Guthrie, J.W. Jorgenson and P.R. Dluzneski, J. Chromatogr. Sci. 22, 171 (1984).
23. S. Folestad, R. Johnson, B. Josefsson and B. Galle, Anal. Chem. 54, 925 (1982).
24. L.W. Hershberger, J.B. Callis and G.D. Christian, Anal. Chem. 51, 1444 (1979).
25. J.C. Gluckman, D.C. Shelly and M.V. Novotny J. Chromatogr. 317, 443 (1984).
26. W.D. Pfeffer and E.S. Yeung, Anal. Chem. 58, 2103 (1986).
27. D.G. Jones, Anal. Chem. 57, 1057A (1985); 57, 1207A (1985).
28. J.C. Gluckman, D.C. Shelly and M.V. Novotny, Anal. Chem. 57, 1546 (1985).
29. J.W. Hofstraat, M. Engelsma, R.J. van der Nesse, C. Gooijer, N.H. Velthorst and U.A.Th. Brinkman, Anal. Chim. Acta, 186, 247 (1986).
30. J.W. Hofstraat, M. Engelsma, R.J. van der Nesse, U.A.Th. Brinkman, C. Gooijer and N.H. Velthorst. Anal. Chim. Acta, (1987) in press.
31. J.W. Hofstraat, M. Engelsma, W.P. Cofino, G.Ph. Hoornweg, C. Gooijer and N.H. Velthorst, Anal. Chim. Acta, 159, 359 (1984).
32. J.W. Hofstraat, H.J.M. Jansen, G.Ph.Hoornweg, C.Gooijer and N.H. Velthorst, Anal. Chim. Acta, 170, 61 (1985)
33. M.M. Rauhut, L.J. Bollyky, B.G. Roberts, M. Loy, R.H. Whitman, A.V. Jannotta, A.M. Semsel and R.H. Clarke, J. Am. Chem. Soc. 89, 6515 (1967).
34. S. Kobayashi and K. Imai, Anal. Chem. 52, 424 (1980).
35. P. Lechtken and N.J. Turro, Mol. Photochem. 6, 95 (1974).
36. P.A. Sherman, J. Holzbecher and D.E. Ryan, Anal. Chim. Acta, 97, 21 (1978).
37. K. Honda, K. Miyaguchi and K. Imai, Anal. Chim. Acta, 177, 111 (1985).
38. K.W. Sigvardson, J.M. Kennish and J.W. Birks, Anal. Chem. 56, 1096 (1984).
39. C.L.R. Catherall, T.F. Palmer and R.B. Cundall, J. Chem. Soc. Faraday Trans. 2 80, 823 (1984).
40. C.L.R. Catherall, T.F. Palmer and R.B. Cundall, J. Chem. Soc. Faraday Trans. 2 80, 837 (1984).
41. F.J. Alvarez, N.J. Porekh, B. Matuszewski, R.S. Givens, T. Higuchi and R.L. Schowen, J. Am. Chem. Soc. 108, 6435 (1986).
42. G.J. de Jong, N. Lammers, F.J. Spruit, R.W. Frei and U.A.Th. Brinkman, J. Chromatogr. 353, 249 (1986).
43. K. Honda, K. Miyaguchi and K. Imai, Anal. Chim. Acta, 177, 103 (1985).
44. K. Honda, J. Sekino and K. Imai, Anal. Chem. 55, 940 (1983).
45. G.J. de Jong, N. Lammers, F.J. Spruit, U.A.Th. Brinkman and R.W. Frei, Chromatographia, 18, 129 (1984).
46 R.Weinberger, J. Chromatogr. 314, 155 (1984).
47. K. Imai, K. Miyaguchi and K. Honda, in K. Van Dijke (ed.) Bioluminescence and Chemiluminescence: Instruments and Applications, CRC Press, Cleveland, U.S.A. Vol. 11,1986, p. 65.
48. G.J. de Jong, N. Lammers, F.J. Spruit, C. Dewaele and M. Verzele, Anal. Chem. (1987) in press.
49. K. Imai, Analyst, 111, 209 (1986).
50. G. Melbin, J. Liq. Chromatogr. 6, 1603 (1983).
51. K. Miyaguchi, K. Honda and K. Imai, J. Chromatogr. 303, 173 (1984).
52. K. Miyaguchi, K. Honda and K. Imai, J. Chromatogr. 316, 501 (1984).
53. T. Koziol, M.L. Grayeski and R. Weinberger, J. Chromatogr. 317, 355 (1984).
54. G. Melbin and B.E.F. Smith, J. Chromatogr. 312, 203 (1984).
55. S. Kobayashi, J. Sekino, K. Honda and K. Imai, Anal. Biochem. 112, 99 (1981).
56. B. Mann and M.L. Grayeski, J. Chromatogr. 386, 149 (1987).
57. M.L. Grayeski and A.J. Weber, Anal. Lett. 17, 1539 (1984).
58. K. Honda, K. Miyaguchi, H. Nishino, H. Tanaka, T. Yao and K. Imai, Anal. Biochem. 153, 50 (1986).
59. P. Van Zoonen, C. Gooijer, N.H. Velthorst, R.W. Frei, J.H. Wolf, J. Gerrits and F. Flentge, J. Pharm. Biomed. Anal. (1987) in press.
60. G. Gübitz, P. Van Zoonen, C. Gooijer, N.H. Velthorst and R.W. Frei, Anal. Chem. 57, 2071 (1985).