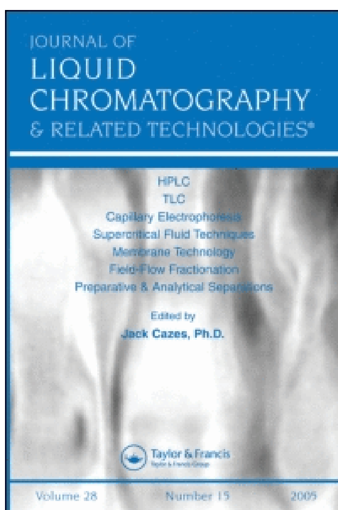
This article was downloaded by:
On: *24 January 2011*
Access details: *Access Details: Free Access*
Publisher *Taylor & Francis*
Informa Ltd Registered in England and Wales Registered Number: 1072954 Registered office: Mortimer House, 37-41 Mortimer Street, London W1T 3JH, UK



Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

A Chemiluminiscence Detector for Trace Determination of Fluorescent Compounds

G. Mellbin[a]
[a] Technical Analytical Chemistry, Chemical Center, Lund 7, Sweden

To cite this Article Mellbin, G.(1983) 'A Chemiluminiscence Detector for Trace Determination of Fluorescent Compounds', Journal of Liquid Chromatography & Related Technologies, 6: 9, 1603 — 1616
To link to this Article: DOI: 10.1080/01483918308064878
URL: http://dx.doi.org/10.1080/01483918308064878

PLEASE SCROLL DOWN FOR ARTICLE

Full terms and conditions of use: http://www.informaworld.com/terms-and-conditions-of-access.pdf

This article may be used for research, teaching and private study purposes. Any substantial or systematic reproduction, re-distribution, re-selling, loan or sub-licensing, systematic supply or distribution in any form to anyone is expressly forbidden.

The publisher does not give any warranty express or implied or make any representation that the contents will be complete or accurate or up to date. The accuracy of any instructions, formulae and drug doses should be independently verified with primary sources. The publisher shall not be liable for any loss, actions, claims, proceedings, demand or costs or damages whatsoever or howsoever caused arising directly or indirectly in connection with or arising out of the use of this material.

JOURNAL OF LIQUID CHROMATOGRAPHY, 6(9), 1603-1616 (1983)

A CHEMILUMINISCENCE DETECTOR FOR TRACE DETERMINATION OF FLUORESCENT COMPOUNDS

G. Mellbin
Technical Analytical Chemistry, Chemical Center
P.O.B. 740, S-220 07 Lund 7, Sweden

Downloaded At: 17:36 24 January 2011

ABSTRACT

A detector making full use of the advantages offered by chemiluminiscence has been constructed. Light measurement was performed with modern electronic equipment using photon counting and the system was tested using dansyl derivatives of adrenaline, noradrenaline and of some amino acids. The detection limits for the two catecholamines were 6 and 16 fmol, respectively, and less than 0.5 fmol for the amino acids. To reach these low levels all reagents were purified or selected for ultra-high purity. The effects of reagent contaminants and of various optical filters have been studied using adrenaline as the test substance.

INTRODUCTION

Introduction of chemiluminiscence in liquid chromatography can be interpreted as an attempt to increase the sensitivity and selectivity of fluorescence methods, and thus extend their applicability to the assay of chemical compounds of various kinds. The effective detection limit in fluorescence analysis using a high-energy light source, e.g. a xenon lamp, and a photomultiplier tube is restricted to about 100 pmol for dansyl derivatives, due to problems with stray light and intensity variations of the light source. Application of UV- and tunable dye lasers have improved the situation. However, UV-lasers are limited to a few

Copyright © 1983 by Marcel Dekker, Inc. 0148-3919/83/0609-1603$3.50/0

wavelengths and tunable lasers are rather expensive, making them available to a small number of laboratories.

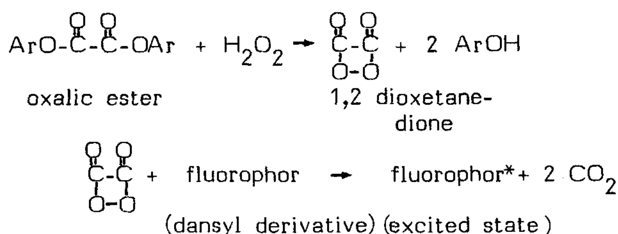
Another problem when trying to decrease the detection limit arises from the light measurement. In conventional fluorescence systems, light is detected with a photomultiplier tube connected to an electric circuitry measuring the photon induced current in a continous manner. Even when no photons strikes the light sensitive surface of the photomultiplier, a small current arises from thermally excited electrons in the dynode chain. This dark current restricts the low limit of detection, unless precautions are taken to reduce it.

Due to the fact that photons propagate as distinct quanta, every photon striking the first dynode gives rise to a very short pulse of electrons, which is amplified in the dynode chain. Pulses generated by thermally excited electrons are amplified to a smaller extent, making them distinguishable from true light pulses. By electronic discrimination and counting, one can register only those pulses having an energy above a preset value. As the background can be kept as low as a few photons per sec., it is possible to count the number of photons interacting with the first dynode. This is the principle of photon counting.

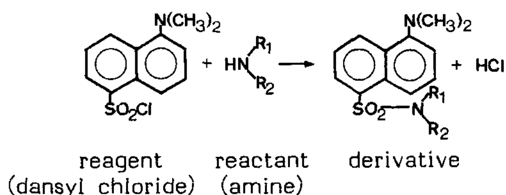
Usually the number of pulses is counted during a preset period of time, e.g. 1 sec., then the result is displayed and a new counting cycle is started. By using this method the instrument background can virtually be kept at zero, making it possible to measure very low sample concentrations. In previous applications of chemiluminiscence to thin-layer chromatography (1) and high performance liquid chromatography (HPLC) (2,3) use has not been made of photon counting for light measurement, whereby the sensitivity has been restricted by dark current fluctuations in the photomultiplier.

In clinical work, bio- and chemiluminiscence have been used for numerous analytical purposes (4) utilizing different methods for chemical excitation. The method used in this work was of the peroxylate type with the proposed mechanism:

Downloaded At: 17:36 24 January 2011



The structure of the oxalate substituents (Ar) has been shown to influence both the reagent stability and the fluorescence intensity (5). The use of bis(2,4,6-trichlorophenyl)oxalate (TCPO) as the oxalic ester gives good reagent stability together with high quantum yields. The reaction of dansyl chloride with amines and phenols occurs according to the general equation:



Downloaded At: 17:36 24 January 2011

EXPERIMENTAL

Apparatus

HPLC was performed with a 5.0 mm I.D. x 200 mm column packed with 5 µm Nucleosil C_{18} particles (Macherey-Nagel & Co., Düren, G.F.R.). The pump used for chromatography was a LDC Constametric III double piston HPLC pump (Laboratory Data Control, Riviera Beach, Fl., U.S.A.) and the injector was a Rheodyne 7120 (Rheodyne, Berkeley, CA., U.S.A.) equipped with a 20 µl sample loop. The photon counter was developed and constructed by Auratronic Electronic Consultant, Stockholm, Sweden. It utilizes a Hamamatsu type R268UH-HA-P photomultiplier tube (Hamamatsu TV. Co. Ltd., Hamamatsu-City, Japan). Filters were manufactured by Carl Zeiss, Oberkochen/Würft, G.F.R. Catecholamine samples were prepared using a Bond Elut C_{18} column (Analytichem International, Harbour City, CA., U.S.A.).

The photomultiplier tube was mounted as shown in Fig. 1. Immediately in front of the tube is a filter holder with a filter followed by a spiral-type flow cell. To avoid signal deterioration the pre-amplifier is placed as close to the photomultiplier tube as possible, inside the light-proof box. A μ-metal magnetic shield prevents disturbance from laboratory equipment. On top of the box is a carefully sealed cover, giving a background count of only 50-150 photons per sec. The design of the flow cell is shown in Fig. 2. It was constructed from borosilicate glass and on the revers side it was covered with aluminium foil to direct all light towards the photon counter.

Chemicals

l-Adrenaline, l-noradrenaline, dansyl chloride and cyklohexylamine salts of dansyl-l-glutamic acid, dansyl-l-methionine, dansyl-d,l-norleucin and dansyl-l-α-alanine were obtained from Sigma Chemical Company, St. Louis, MO., U.S.A. Ethyl acetate, acetone, hydrogen peroxide, tris(hydroxymethyl)aminomethane (TRIS) and hydrochloric acid, all of analytical grade, and ethyl acetate, acetone and acetonitrile of spectroscopic grade were obtained from E. Merck, Darmstadt, G.F.R. Toluene of HPLC-grade was supplied by the same source. Acetonitrile for HPLC was from Rathburn Chemicals Ltd., Walkerburn, Gt. Britain and from BDH Chemicals Ltd., Parkstone, Gt. Britain. Hydrogen peroxide "Aristar" was also supplied by BDH. TCPO was prepared by the method of Mohan and Turro (6). All water was doubly distilled.

Purification of chemicals

Analytical grade ethyl acetate and acetone were distilled using a 1-m filled column. The TCPO was recrystallized from HPLC-grade toluene, the solution being treated with charcoal, and TRIS was recrystallized from aqueous ethanol (7).

Analytical conditions

In the determination of catecholamines the mobile phase consisted of 85% (v/v) acetonitrile and 15% (v/v) 0.05 mol l^{-1} TRIS-HCl-buffer, pH 7.7. The flow-rate was 1 ml min^{-1} . TCPO was dissolved

Downloaded At: 17:36 24 January 2011

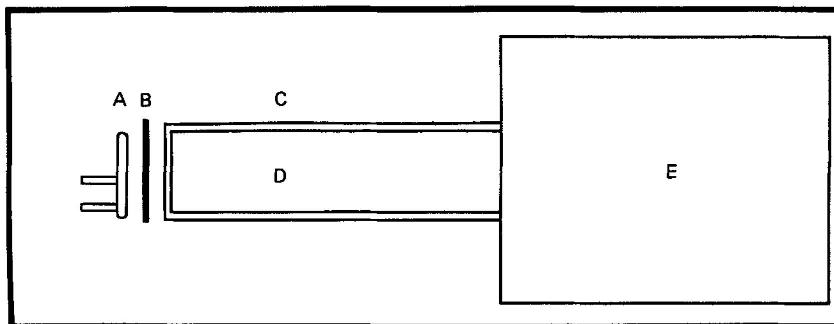


FIGURE 1

Light measurement compartment with A) Flow cell, B) Filter, C) μ-metal shield, D) Photomultiplier and E) Pre-amplifier.

Downloaded At: 17:36 24 January 2011

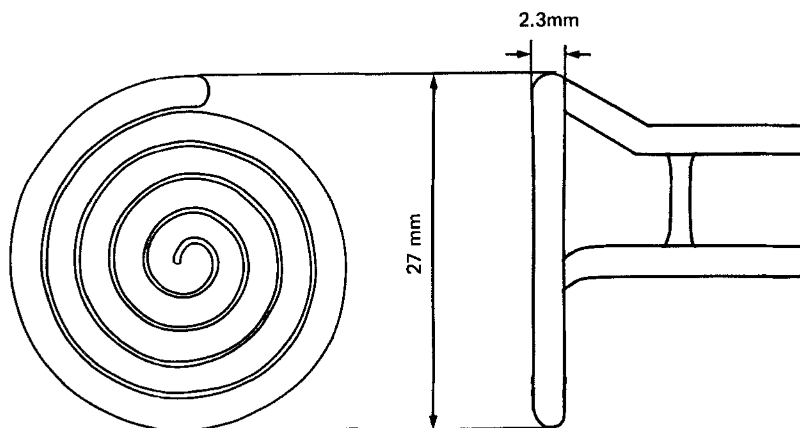


FIGURE 2

Design of flow cell.

in ethyl acetate at a concentration of 5 mmol l^{-1} and 30% (v/v) H_2O_2 was diluted with acetone to a final concentration of 0.5 mol l^{-1}. The flows were 0.3 ml min^{-1} for the TCPO solution and 0.5 ml min^{-1} for the H_2O_2 reagent. In order to analyse the amino acids the buffer content of the mobile phase was increased to 65% (v/v) and acetonitrile made up the final 35% of the mobile phase. The flow-rate was maintained at 1 ml min^{-1}. To avoid phase separation due to the low solubility of TCPO in aqueous systems, the TCPO reagent was pumped at 0.5 ml min^{-1} and the H_2O_2 reagent at 1.3 ml min^{-1}.

Sample preparation

Dansyl amino acid samples were freshly prepared by dissolving the derivatives in doubly distilled water. Dansyl derivatives of catecholamines were prepared as follows: Adrenaline and noradrenaline were dissolved in 0.1 mol l^{-1} HCl. A 100-μl volume of this solution was mixed with 330 μl of $NaHCO_3$ buffer (pH 8.5) and 2 ml of 0.1% (w/v) dansyl chloride in acetone. After vigorous mixing the samples were allowed to react at 55°C for 10 min. To remove the excess reagent, 2 ml of 0.5% (w/v) l-α-alanine was added and the sample mixture was once again placed at 55°C for 10 min. After application on a Bond Elut C_{18} column and rinsing with 5 ml of 50% ethanol, the sample was eluted with 500 μl of acetone and diluted to 10 ml with mobile phase. Care was taken to prevent UV-irradiation during sample preparation.

Procedure

The entire system outline is shown in Fig. 3. The eluent was kept in a glass container and was pumped through the injector and the column at a pressure of 1500 psi. The TCPO and H_2O_2 reagents, dissolved in ethyl acetate and acetone, were placed in two 500-ml stainless steel containers pressurized with helium and forced through a 1-m x 0.15 mm I.D. stainless steel or teflon capillary tubing giving a flow of 2 ml min^{-1} or less. The exact flow rates were adjusted by carefully regulating the helium pressure. This method gives a constant and absolutely pulse free delivery, which is important to achieve a low background noise.

Downloaded At: 17:36 24 January 2011

Downloaded At: 17:36 24 January 2011

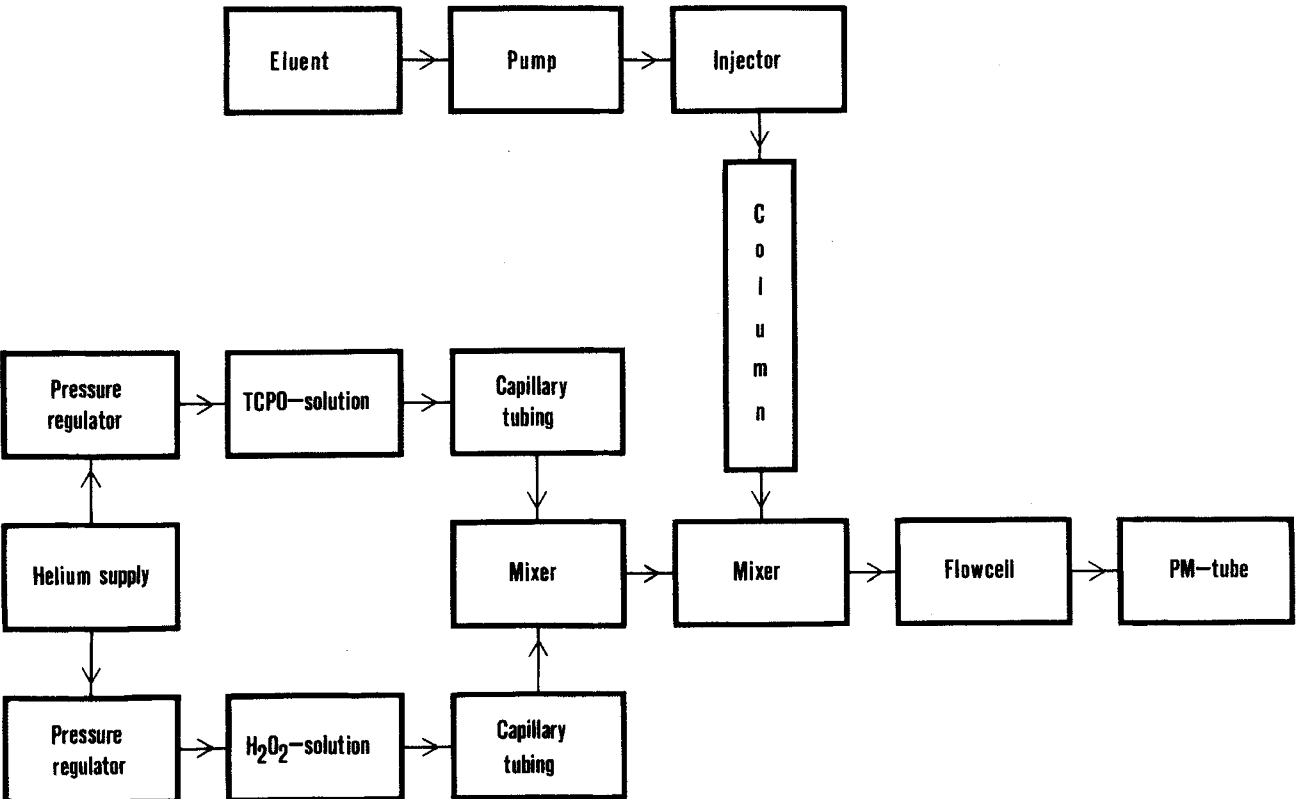


FIGURE 3

Block diagram of the complete analytical system.

The TCPO and H_2O_2 solutions were mixed in a static mixer according to Kobayashi and Imai (3), and then immediately brought in contact with the column effluent using an identical mixer. Upon mixing, 1,2-dioxetanedione is formed which excites fluorescent molecules when encountered in the column effluent. Since the chemiluminiscent radiation declines very rapidly, the flow was conducted directly to the flow cell where the light intensity was measured by photon counting. The number of photons striking the photomultiplier tube during 1 sec. was counted, the digital reading was converted automatically to an analog result and the chromatogram registered on a recorder in conventional manner. When starting up the system, it was allowed to operate for 10 to 15 min. so that the base line became stable. If the system has been turned off less than a couple of hours, it can be used within a few min.

Downloaded At: 17:36 24 January 2011

RESULTS AND DISCUSSION

Effect of reagent purity

Table 1 lists the effect of mobile phase purity on signal and background readings. It can be seen that acetonitrile may contain relatively large amounts of fluorescent contaminants and still fulfil the UV-absorbance criterion of a HPLC-grade solvent. Therefore a careful choice of acetonitrile supplier is essential. The purification of TRIS is of smaller importance but still affects the results. During the remainder of this work BDH HPLC acetonitrile has been used in the mobile phase together with recrystallized TRIS.

In Table 2 the effects of purity of TCPO and H_2O_2 reagents are summarized. It is evident that the make of H_2O_2 has little influence on the peak height to background ratio, while distillation of the reagent solvents is essential.

Test of optical filters

In an attempt to increase the sensitivity of the detector system, 25 different filters were tested using the dansyl derivative of adrenaline as the test substance. A filter may be used to reduce the background level since the sample and the contaminants emit light at different

TABLE I

Effect of Mobile Phase Purity on the Peak Height to Background Ratio*

TRIS	Aceto-nitrile	HCl	Background (cps)	Peak height/background x1000
P.A. recryst.	BDH HPLC	P.A.	15600	1.67
P.A.	BDH HPLC	P.A.	18000	1.50
P.A. recryst.	Rathburn HPLC	P.A.	36700	0.75
P.A. recryst.	Merck Uvasol	P.A.	15200	1.71

*Constant amounts of approximately 0.9 pmol of tridansyl-adrenaline were injected.

TABLE II

Effect of Purity of TCPO and H_2O_2 Reagents on the Peak Height to Background Ratio*

Acetone	Ethyl acetate	H_2O_2	Background (cps)	Peak height/background x1000
P.A. dest.	P.A. dest.	Merck P.A.	84100	1.080
P.A. dest.	P.A.	Merck P.A.	190000	0.500
P.A.	P.A. dest.	Merck P.A.	235000	0.404
P.A. dest.	P.A. dest.	BDH Aristar	95000	0.989

* Constant amounts of approximately 0.9 pmol of tridansyl-adrenaline were injected.

Downloaded At: 17:36 24 January 2011

TABLE III

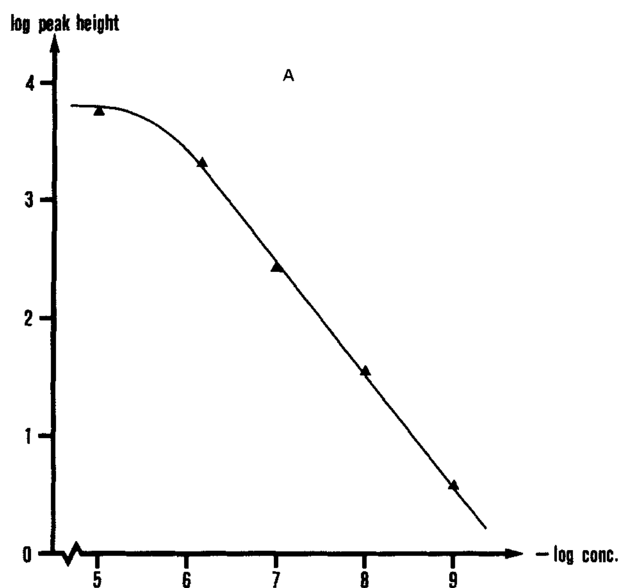
Effect of Filters in the Optical Path on the Peak Height to Background Ratio

Filter	Background (cps)	Peak height/background x1000	Characteristics
None	142900	0.308	
GG16	13600	0.750	
GG14	33900	0.697	
GG8	43000	0.588	
OG3	810	0.543	
Inter-ference	1905	0.522	
GG15	94700	0.394	
GG17	108200	0.333	
BG23	77000	0.252	

Wave-length
400nm
700nm
100%
0%
Transmittance

wavelengths. The filters were placed next to the photomultiplier tube immediately in front of the flow cell. As can be seen from Table 3, the best choice (GG16) increases the signal to background ratio more than twice compared to measurements without a filter. As the chemi-luminiscence from the dansyl derivative of adrenaline gives a wide wavelength distribution with a maximum at 512 nm, a 90 nm bandwith interference filter with its peak transmittance at 519.5 nm was expected to increase the signal to background ratio significantly. The

Downloaded At: 17:36 24 January 2011



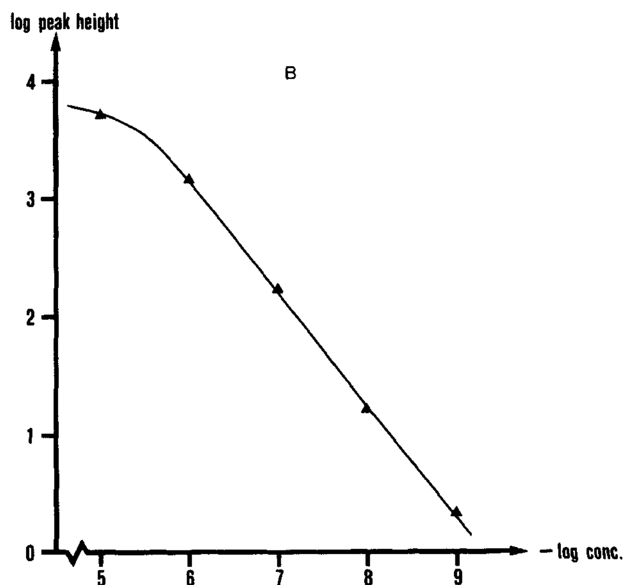


FIGURE 4

Correlation between sample concentration (mol l^{-1}) and peak height for A) adrenaline and B) noradrenaline.

Downloaded At: 17:36 24 January 2011

gain was, however, not as large as accomplished with some of the other filters.

Linear detection range and detection limit for catecholamines

A plot of peak height versus concentration for adrenaline and noradrenaline displays a linear correlation over more than three orders of magnitude (Figs. 4A and B). At the high concentration end the peaks were flattened at the top probably due to reagent shortage. The peak intensities were in the order of 3 million cps. Extrapolating to the noise level gives a detection limit of 6 fmol for adrenaline and 16 fmol for noradrenaline. This is to be compared with the value of approximately 12 pmol obtained for the same derivatives by Frei, Thomas and Frei using a conventional fluorescence detector (8).

Assay of amino acids

In order to make a further comparison between this detector and a conventional fluorescence detector (2), some amino acids were analysed under almost identical conditions to those in ref. (2). Dansyl derivatives of glutamic acid, alanine, methionine and norleucine were dissolved in water and analysed using the same system as for the catecholamines. A VG10 filter turned out to be the best choice in order to supress the effect of background radiation. The sensitivity varied somewhat for different derivatives but the methionine derivative, being the least sensitive, had a detection limit of less than 0.5 fmol (Fig. 5). Using a fluorescence detector with the lamp turned off, Kobayashi and Imai (2) reached a detection limit of 10 fmol for the same dansyl amino acids, that is, the present method is more than 20 times as sensitive. This brings chemiluminiscence combined with photon counting up to and possibly beyond the limits of laser induced fluorescence, making it the most sensitive optical method of detection available at present.

CONCLUSIONS AND FURTHER WORK

Chemiluminiscence in combination with photon counting affords a high-sensitive analytical method for the assay of many substances, especially in the biochemical field. The considerable decrease in de-

Downloaded At: 17:36 24 January 2011

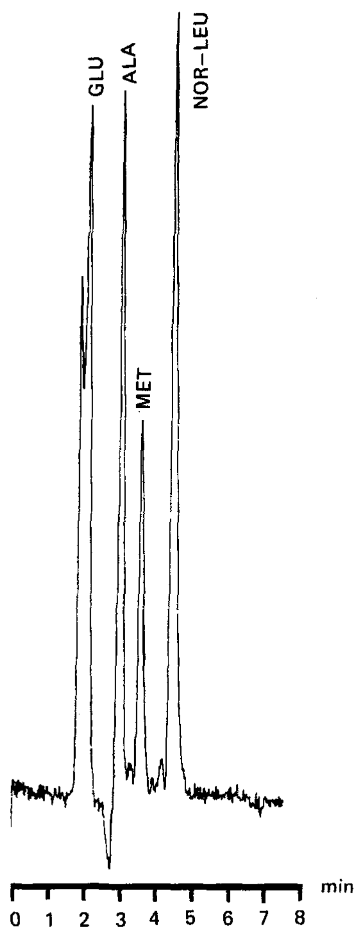


FIGURE 5

Chromatogram of amino acid dansyl derivatives. Injected volume: 20 μl . Sample concentrations: dansyl-l-methionine (MET) 2.07 $\cdot 10^{-10}$ mol l^{-1}, dansyl-l-glutamic acid (GLU) 1.73 $\cdot 10^{-10}$ mol l^{-1}, dansyl-l-α-alanine (ALA) 2.37 $\cdot 10^{-10}$ mol l^{-1} and dansyl-d,l-norleucin (NOR-LEU) 2.15 $\cdot 10^{-10}$ mol l^{-1}. The chromatographic conditions are given in the text.

Downloaded At: 17:36 24 January 2011

tection limits for dansyl derivatives of catecholamines and amino acids in comparison with those attained with conventional fluorescence methods, should be possible to realize for many other compound classes. On that account it is our intention to apply the present method to other fields of interest e.g. to the assay of amines as o-phtaldialdehyde derivatives.

ACKNOVLEDGEMENTS

The author is indebted to Professor Bengt Smith, Head of the Department of Technical Analytical Chemistry, for valuable discussions during the course of this work.

REFERENCES

1. Curtis, T.G. and Seitz, W.R., J. Chromatogr., 134, 343 (1977).
2. Kobayashi, S. and Imai, K., Anal. Chem., 52, 424 (1980).
3. Kobayashi, S., Sekino, J., Honda, K. and Imai, K.,Anal. Biochem., 112, 99 (1981).
4. Gorus, F. and Schram, E., Clin. Chem., 25, 512 (1979).
5. Rauhut, M.M., Bollyky, L.J., Roberts, B.G., Loy, M., Whitman, R.H., Ianotta, A.V., Semsel, A.M. and Clarke, R.A., J. Amer. Chem. Soc., 89, 6515 (1978).
6. Mohan, A.G., and Turro, N.J., J. Chem. Educ., 51, 528 (1974).
7. Perrin, D.D., Armarego, W.L.F. and Perrin, D.R., "Purification of Laboratory Chemicals", Pergamon Press, Oxford, 1966.
8. Frei, R.W. Thomas, M. and Frei, I., J. Liq. Chromatogr., 1, 443 (1978).

Downloaded At: 17:36 24 January 2011