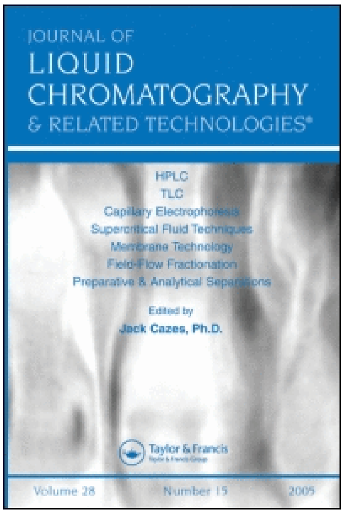

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A SIMPLIFIED METHOD FOR PEROXYOXALATE CHEMILUMINESCENCE DETECTION OF FLUORESCERS BASED ON SOLID STATE REAGENT ADDITION

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ABSTRACT

A peroxyoxalate chemiluminescence detection system which needs no reagent-pumps is described. It is based on solid state addition of bis-(2,4,6,trichlorophenyl)oxalate (TCPO) from a solid state reagent bed. The solid reagent is packed in a column and situated parallel to the analytical column in order to minimize band broadening. If chromatographic separation can be achieved at conditions matching those of the chemiluminescence reaction (>80% acetonitrile) a split-flow system utilizing only the mobile phase pump can be employed. For mobile phase compositions with at least 50% acetonitrile a system with only one reagent pump is developed. The performance of both post-column detection systems are compared to that of a conventional three pump system as described by Sigvardson et al.[5]

INTRODUCTION

The peroxyoxalate chemiluminescence reaction requiring three reagents i.e. hydrogenperoxide, an oxalate and a fluorescer is applicable for the determination of H_2O_2 or fluorescers in flow systems. In our previous work [1,2] the functioning of a solid state reactor for the determination of H_2O_2 by peroxyoxalate chemiluminescence was described. This resulted in a detection system which utilizes only one single flow-line. It was based on immobilized fluorophor (3-aminofluoranthene) and a solid bed reactor from which bis-(2,4,6, trichlorophenyl)oxalate (TCPO) was added. Recently a split flow system for post-column detection of photochemically generated H_2O_2 was developed which needs only one pump[3,4]. One of the main advantages of such a system with a reagent bed used parallel is that no additional band broadening due to the bed-reactor will be encountered and that the reactor can function over prolonged periods of time without the need for repacking.

Peroxyoxalate chemiluminescence is widely known as a selective and sensitive method for the detection of certain polycyclic aromatic hydrocarbons [5] and several derivatized compounds such as dansylated aminoacids [6]. The detection systems reported in the literature are rather complicated, since the three reagents producing chemiluminescence need to be delivered via separate flow-lines. Hence, generally three pumps are used, although recently Imaï and coworkers have examined the possibility to apply a set-up consisting of two pumps by pre-mixing the oxalate and H_2O_2[7].

Poulsen et al. [4] noted that apart from the simplicity of reagent addition from a solid bed it also circumvents the chemical decomposition problems encountered in liquid phase addition of oxalates. In this way a larger range of solvents can be utilized, because TCPO is dissolved very shortly before it is actually used in the chemiluminescence reaction.

The present paper describes the possibility of solid state addition of TCPO for the detection of fluorescers. Both a two pump set-up and a single pump split-flow system are investigated and the results are compared with those of Sigvardson et al. [5], obtained with a conventional three pump system.

EXPERIMENTAL

Chemicals: TCPO was prepared according to Mohan and Turro [8] and recrystallized thrice from benzene (Uvasol®, Merck, Darmstadt, FRG). The product was kept under reduced pressure for several hours to evaporate to dryness and to remove traces of trichlorophenol. All analytes (perylene, 3-aminofluoranthene, 9,10-diphenylanthracene, tetracene, benz(a)pyrene and 1,2-benzanthracene) were analytical grade and purchased from Aldrich (Beerse, Belgium). HPLC-grade acetonitrile (gradient grade, Baker, Deventer, The Netherlands) was purified over an alumina column as described previously[2]. Hydrogen peroxide 30% was obtained from Merck (Darmstadt, FRG); tris buffer from Sigma (St. Louis, USA)

Apparatus: The experimental set-ups used in the present study are depicted schematically in figure 1 (I) and (II). Mobile phase was delivered by a Kratos SF 400 HPLC pump. In the two pump experiments the additional pump was a Kontron 414 HPLC pump equipped with a home-made membrane pulsedampener. The sample was introduced by a Rheodyne injection valve equipped with a 20 µl loop. The analytical column was a home-packed 25 cm, 3 mm i.d. Spherisorb ODS-5. Another home-packed 25 cm Spherisorb ODS-5 in series with the reagent bed reactor served as pressure regulator in the split-flow mode. The reagent bed was hand-packed into a 6 cm, 3 mm i.d. stainless steel column with a mixture of solid finely ground TCPO and 40- 80 µm glass beads. The splitting and later remixing of the mobile phase was performed with Valco low dead volume tee-pieces. The light resulting from the chemiluminescence reaction was measured with a Kratos FS 970 fluorescence detector equipped with a 5 µl cell.

RESULTS AND DISCUSSION

Our earlier work [1,2] showed that for TCPO, the appropriate chemiluminescence conditions are about 80% acetonitrile and a pH of about 8 (achieved with a Tris buffer). One of the crucial points in applying this reaction to HPLC detection is the compatability of the above conditions with the eluent compositions used to achieve chromatographic separation. Furthermore additional band broadening caused by the post-column detection system should be as small as possible.

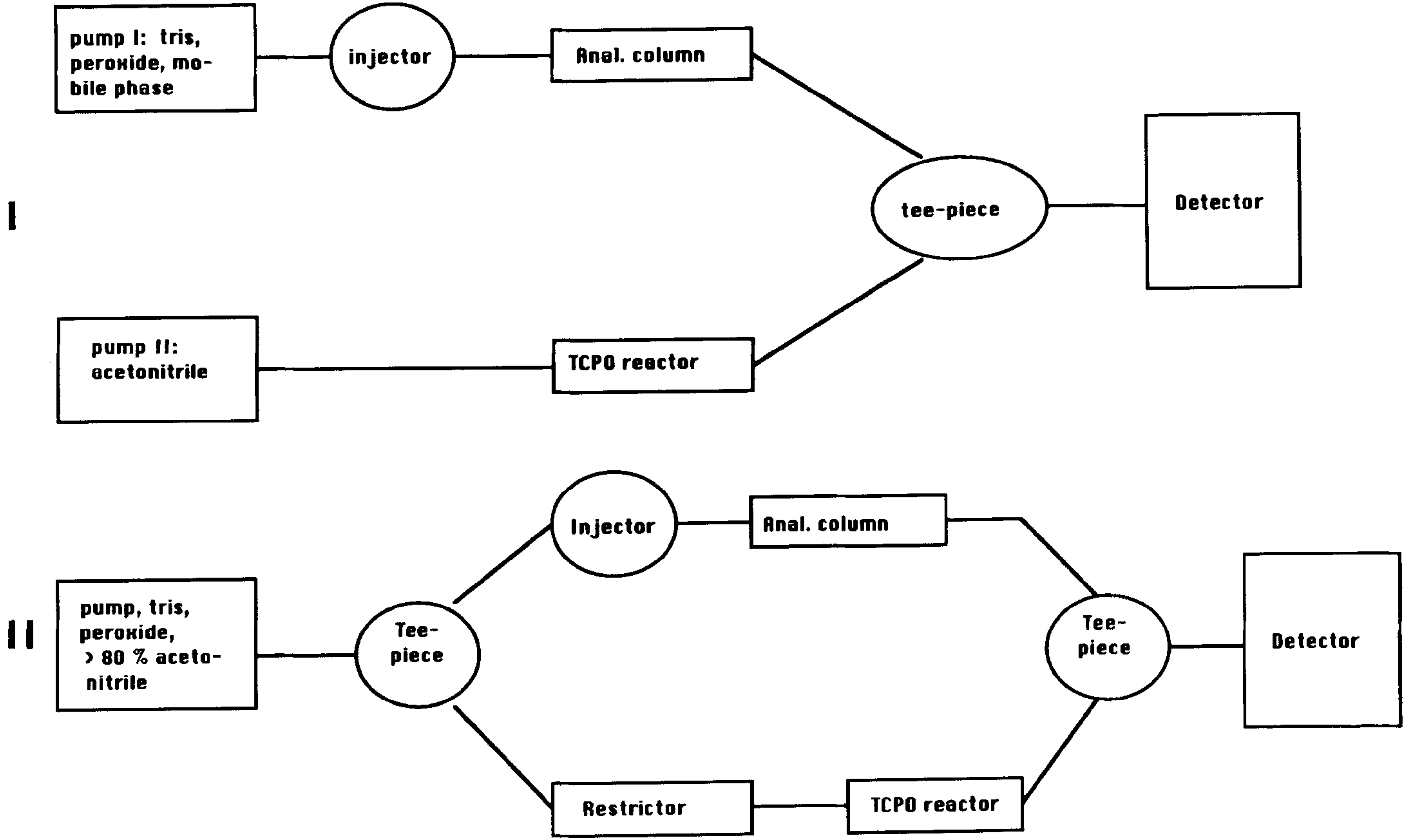

FIGURE 1. Schematical representation of the two pump system I and the single pump system II for the detection of fluorescers based on peroxyoxalate chemiluminescence.

We have found that band broadening problems due to bed-reactors can be circumvented by using these reactors parallel to the analytical column rather than in series. Obviously configuration I (see figure 1) offers considerably more freedom in choice of mobile phases for the separation of analytes because the make-up flow can correct for the deficiency of organic modifier in the ultimate reaction medium. However due to the insolubility of TCPO in more aqueous media it is not possible to have mobile phase compositions with less than 50 % acetonitrile in the set-up described here. In the present study no other solvents were employed, but data from Poulsen et al.[4] indicate that the method can also be used with other solvents such as acetone and methanol.

If more aqueous media are employed TCPO dissolved in the bed-reactor will precipitate in the mixing tee-piece, causing a noisy background signal due to clogging of the flow system. In configuration II (see figure 1) a split-flow mode is applied so that the mobile phase composition is exactly the same as the one passing through the TCPO reactor. For a reasonable signal to noise ratio we have found that the acetonitrile content should be at least 80%. In principle the split ratio can be varied by means of interchanging restriction colums or by the application of a needle valve to regulate the back pressure in either channel. The influence of such a variation in the split-ratio on the signal to noise ratio was examined with the two pump system using identical fluids in both channels. The flow through the analytical column was set at 0.5 ml min^{-1} whereas the flow through the TCPO reactor was varied from 0.1 to 1 ml min^{-1}. The results shown in figure 2 imply that over a large variation of split-ratios the signal to noise ratio hardly varies. Hence the use of a highly sophisticated system to regulate the split ratio will not be necessary. On the other hand the split ratio should not vary too much in time because otherwise the retention times of analytes will not be constant and so a systematic error can be introduced. In the present study the total scattering of retention times was within 2 % (14 chromatograms).

Another point to be considered is the H_2O_2 concentration in the mobile phase. It was varied from 10^{-3}M to 10^{-1}M. The signal is linearly dependent on $[H_2O_2]$ and so the best signal to noise ratio was found at the highest peroxide concentration. In the high concentration region, however, the influence of H_2O_2 on the performance of the system is not very strong; the background noise will

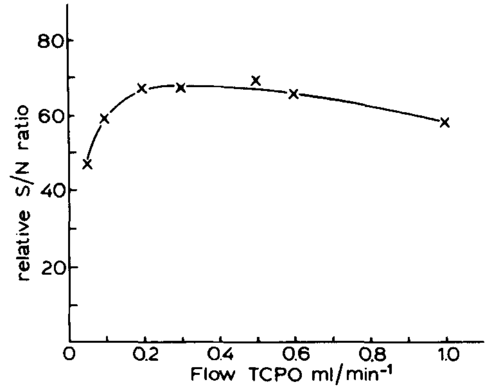

FIGURE 2. dependency of S/N on the split ratio.

also increase upon enlarging the concentration. From 10^{-3} to 10^{-2} an 8-fold increase in signal to noise ratio was observed while from 10^{-2} to 10^{-1} only a 3-fold improvement was observed. For practical reasons such as corrosion of pumps and stainless steel parts of the system we have not utilized concentrations higher than 10^{-1}M of H_2O_2. Under these conditions the performance of the analytical column remains constant for at least a month.

In figure 3 a typical chromatogram of a standard mixture of PAH's including 3-aminofluoranthene recorded with the split-flow system (II) is shown. A similar chromatogram can also be obtained with the two pump system. In table I the detection limits obtained from these chromatograms are compared with those reported by Sigvardson et al. with the more complex three pump liquid addition system. It can be seen that the present simplified system provides no substantial loss of sensitivity is. L.O.D.'s generally are in the order of magnitude as those obtained by Sigvardson et al. It should be noted that the L.O.D.s of the latter are defined at a signal to noise ratio of 2 whereas our data are calculated at a S/N of 3.

The reproducibility of the method was tested by an 8-fold injection of 300 pg of 9,10-diphenylanthracene. Relative standard deviations were calculated to be 2.8%(N=8) for the split-flow system and 2.4% for the two pump system. The method was shown to be linear for at least three orders of magnitude (for diphenylanthracene N=7, r=0.9996).

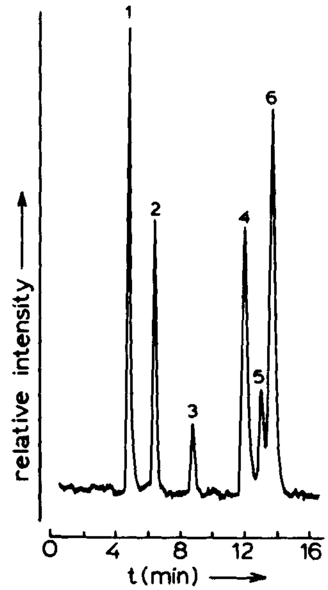

FIGURE 3. Chromatogram obtained by the split-flow method. Mobile phase composition: 90 % acetonitrile, 10 % aqueous Tris (5 mM, pH=8.0), 10^{-1}M H_2O_2. Injection volume: 20 µl.
1. 25 pg 3-aminofluoranthene
2. 2 ng anthracene
3.300 pg 1,2-benzanthracene
4. 30 pg perylene
5.149 pg benz(a)pyrene
6.350 pg of 9,10-diphenylanthracene.

Results obtained for the split system (II) are somewhat less favourable than those for the two pump system (I). This can be attributed to the fact that in the former (II) the starting point for the chemiluminescence reaction is not well defined because the hydrogenperoxide is already in contact with TCPO in the bed reactor thus providing a higher base-line noise level due to pump pulsations. Nevertheless for chromatographic conditions as used in the present example system II is very appropriate. It presents the most simple system for peroxyoxalate chemiluminescence detection and its sensitivity and selectivity are comparable to other peroxyoxalate based LC detection systems.

Table I

Comparision of detection limits (pg) obtained by Sigvardson et al., our two pump system and our split-flow system.

Analyte	Sigvardson et al. S/N = 2	2-pump system S/N = 3	split system S/N = 3
perylene	0.77	1.6	2.0
3-aminofluoranthene	0.30*	0.6	0.7
9,10-diphenylanthracene	20	30	35
anthracene	130	150	200
tetracene	735	860	980
benz(a)pyrene	45	80	120
1,2-benzanthracene	20.5	53	65

* calculated from text

Finally it is emphasized that the solid TCPO reactor can be manipulated quite simply. It can be employed continuously for over 8 hr without any drift. TCPO can directly be added to the reactor. After about two weeks of continuous use the reactor should be repacked completely since after such a prolonged time large amounts of impurities can accumulate in the reactor.

CONCLUSION

The peroxyoxalate chemiluminescence detection system can be simplified by adding TCPO from a solid state reagent bed. This has been shown for polyaromatic hydrocarbons but should be more generally applicable for fluorescers in a other interesting chromatographic separations. Basic requirement is that the liquid chromatographic separation can be achieved with aqueous acetonitrile containing at least 50 % acetonitrile. With acetronitrile contents higher than 80 % even a single pump system can be applied.

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