Journal of Chromatography, 83 (1973) 321–330
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CHROM. 6800

FLUORIGENIC LABELLING IN HIGH-SPEED LIQUID CHROMATOGRAPHY

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SUMMARY

Fluorigenic labelling techniques have been used for several years in conjunction with thin-layer chromatography, and are now being used also to good advantage in high-speed liquid chromatography. In this work, reagents such as 4-chloro-7-nitrobenzo-2,1,3-oxadiazole, dansyl chloride and dansyl-hydrazine were used for the fluorigenic labelling of carbamates, ureas, organophosphorous compounds, aliphatic amines, aldehydes, ketones, biphenyls and some compounds of pharmaceutical interest. The labelling reactions and the nature of derivatives are discussed, together with the column liquid chromatographic properties of these derivatives in both liquid–liquid and solid–liquid modes. Detection limits obtained for most compounds with a new fluorimetric detection device range between 1 and 10 ng per 4-μl injection volume. The method is suitable for quantitative analysis, with a reproducibility of *ca.* 3% relative standard deviation. Linear calibration plots for peak area or peak height *versus* concentration were observed up to 600 ng per injection. The method has been applied successfully to the environmental sample analysis of pesticides in water, soil and vegetables without the necessity for a preliminary clean-up. The method is comparable in sensitivity and accuracy to gas chromatography.

The advantages of this method can be summarized as follows: improved detection properties, such as better sensitivity, flow-insensitive detection mode and higher specificity; lowering of polarity of the compounds, hence greater ease of separation; suitable for automation and routine analysis; the range of possible applications is limited only by the available labelling reagents and separation procedures.

INTRODUCTION

The use of fluorigenic labelling procedures in conjunction with thin-layer chro-

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matography (TLC) for the analysis of amino acids has been known for more than a decade[1]. A great variety of compounds of biological interest has been studied by means of similar labelling reactions[2-4]. Recently, the technique has been applied with much success to the TLC analysis of carbamates and urea pesticides using dansyl chloride[5-8] or 4-chloro-7-nitrobenzo-2,1,3-oxadiazole (NBD-Cl)[9,10] as the labelling reagent. The technique has been applied to the residue analysis of these pesticides in water and soil samples, without clean-up procedures. The disadvantages of the method are primarily related to the inherent drawbacks of TLC, such as the lack of automation possibilities and the difficulty of controlling chromatographic parameters. These drawbacks could be partially eliminated by replacing TLC by high-speed liquid chromatography (HSLC). HSLC has experienced a tremendous upsurge in development during recent years[11] and combines many advantages of TLC and gas chromatography (GC). The use of fluorescence detectors has recently been shown to present interesting possibilities[12] and it seemed attractive, in order to overcome the limitations of this detection mode (need for highly fluorescent species), to combine fluorigenic labelling with HSLC. A first study in this direction has been described by Frei *et al.*[13] for some N-methyl carbamates. In the present paper it is intended to give as broad a survey as possible of the various application possibilities of this method.

EXPERIMENTAL

Reagents

The amino acids were obtained from Mann Research Laboratories, New York, U.S.A. Aliphatic amines, ketones and aldehydes were obtained from commercial sources. A list of the pesticides investigated and their manufacturers is given in Table I. The labelling of amino acids and pesticides was carried out with a 0.1% solution of analytical-grade dansyl chloride (Mann Research Laboratories) in redistilled acetone. The dansyl-hydrazine was prepared as follows. A 10-ml volume of a 0.1% solution of dansyl chloride in chloroform was added to a 50-ml flask containing 10 ml of a 0.05% methanolic solution of hydrazine (Fisher Scientific, 65% aqueous hydrazine). The contents were stirred for 2 min and transferred into a 50-ml round-bottomed

TABLE I

LIST OF PESTICIDES USED

Trade or common name	*Chemical name*	*Manufacturer*
Butacarb	3,5-(*tert.*-Butyl)phenyl-N-methyl carbamate	Chevron Chemical Co.
Carbofuran	2,3-Dihydro-2,2-dimethylbenzofuranyl-7-N-methyl carbamate	Niagara Chemical Co.
Carzol	*m*-{([Dimethylamino]methylene)amino} phenyl-N-methyl carbamate	Morton Chemical Co.
Dioxacarb	4-(1,3-Dioxol-2-yl)phenyl-N-methyl carbamate	Chevron Chemical Co.
Fenthion	O,O-Dimethyl-O-[4-(methylthio)-*m*-tolyl]-phosphorothioate	Chemagro Chemical Co.
Chlorpropham (CIPC)	Isopropyl-N-(*m*-chlorophenyl) carbamate	Pittsburgh Plate Glass Co.
Propham (IPC)	Isopropyl-N-phenyl carbamate	Pittsburgh Plate Glass Co.
Linuron	3(3,4-Dichlorophenyl)-1-methoxy-1-methyl-urea	Dupont Chemical Co.

flask and subsequently evaporated by rotary vacuum evaporation almost to dryness. The residue was streaked along the bottom of a 20 × 20 cm preparative silica gel thin-layer plate (layer thickness *ca.* 1 mm) and developed with chloroform. The dansyl-hydrazine band was eluted with methanol, evaporated to dryness in a rotary vacuum evaporator at 35° and the residue was weighed and dissolved (1.0%) in methanol. NBD-Cl was synthesized according to Ghosh and Whitehouse[14] but is now available from commercial sources (Eastman Chemical Co. Inc. and Aldrich Chemical Co. Inc.). A 1.0% (w/v) solution in methyl isobutyl ketone was prepared for the labelling of aliphatic amines.

Labelling reactions

The dansyl amino acids were prepared by heating the amino acids in a glass-stoppered test tube at 45° for 1 h in 1 ml of acetone containing 2–3 drops of a 0.5 *M* Na_2CO_3 solution. The solvent was then evaporated and the derivative dissolved in benzene for injection into the chromatograph.

The N-methyl carbamates were dansylated according to Frei and Lawrence[6] and the derivatives were extracted into benzene for injection. A two-phase reaction system was used for the labelling of N-phenyl carbamate and urea herbicides. The reaction was carried out in glass-stoppered test tubes. The herbicides were hydrolyzed for 45 min at 75° in 1 ml of 2 *M* NaOH, and to this reaction mixture, containing the resulting anilines, two drops of methyl isobutyl ketone and two drops of the dansyl chloride solution were added. The mixture was shaken well and heated at 65° for 30 min and, after cooling, the mixture was acidified with a 10% HCl solution and 0.3 ml of benzene was added for extraction. The labelling of the organophosphorous insecticide was carried out in a similar manner except that the hydrolysis was carried out with a 0.5 *M* NaOH solution.

The labelling of aliphatic amines with NBD-Cl has been described elsewhere[9], and for injection the derivatives were again dissolved in a benzene solution.

Formation of ketone or aldehyde derivatives with the dansyl-hydrazine was effected by heating the compounds for 15 min at 70° in a water-bath in 1 ml of methanol to which a two-fold molar excess of dansyl-hydrazine and two drops of glacial acetic acid had been added. The reaction mixture was then evaporated to dryness in a rotary vacuum evaporator and the derivatives were dissolved in 0.5 ml of benzene for chromatographic analysis.

Chromatography

The chromatographic apparatus was built in this laboratory. A three-head diaphragm pump (Orlita KG, Giessen, G.F.R., Type S4) capable of operating at pressures up to 350 atm was used. No damping devices were required as the detector used in this work was not sensitive to the flow-rate[12]. The detector is a modified Turner 111 fluorimeter (G. K. Turner, Palo Alto, Calif. 94303, U.S.A.), which has been described elsewhere[12]. The filter combinations used for this study were Turner No. 811 for excitation and No. 817 for emission for the dansyl derivatives and No. 813 ex. 826 em. for the NBD-Cl derivatives. The pressure-relief device and sample introduction valve have been described earlier[15]. All tubing and fittings were made of stainless steel. Sample volumes of 1–10 μl were introduced directly on to the top of the column packing by means of stopped-flow injection. The columns were made from 2.4 mm I.D. seamless

stainless steel (American Instrument Co. Inc., Silver Spring, Md., U.S.A.) and were 1 m long. Before use, the column tubing was washed according to a procedure recommended by Karger *et al.*[16]. All columns were dry-packed by standard procedures[17] and the column packings were washed as described elsewhere[18] prior to being used. The Corasil I (Waters Associates, Framingham, Mass. 01701, U.S.A.) was deactivated with 0.5% of water.

The Zipax®–β,β'-oxydipropionitrile (BOP) packing was prepared by evaporating a known amount of BOP dissolved in chloroform on to Zipax®. A 0.5% (w/w) coating was used, as this has been shown[19] to be the optimum value for obtaining good efficiencies with a dry-packing procedure.

RESULTS AND DISCUSSION

Amino acids

Fig. 1 shows the separation of four amino acid derivatives. Many other solvent systems were investigated in an attempt to improve the efficiency of this separation. The solvent systems that are commonly used for the TLC of these derivatives could not be used in HSLC, either because they were too corrosive or because of an entirely different partitioning pattern of the mobile phase components (3–4 component systems). The use of 0.5% BOP–Zipax® instead of pure Zipax® proved to be unsatisfactory as the compounds were very strongly retained on this polar stationary phase. Attempts are now under way to separate these species by reversed-phase chromatography or possibly ion exchange. With the latter, quenching effects of small amounts of acid or even the presence of water[6,20] in the mobile phase would have to be taken into consideration.

The detection limits of the amino acid derivatives separated by this chromatographic and detection system are of the same order as those reported for TLC[1] (low nanogram range).

N-methyl carbamate insecticides

Good separations of the N-methyl carbamates as dansyl derivatives of the phenyl moiety and the methylamine have been achieved on Corasil® in the adsorption chromatography mode (see Fig. 2) and also on Zipax® loaded with 0.5% of BOP in the partition mode[13]. Column efficiencies above 1000 theoretical plates were observed with these systems. Detection limits between 1 and 10 ng per 4-μl injection have been observed.

Organophosphorous insecticides

Fig. 3 shows a chromatogram following the dansylation of the phenyl moiety of fenthion as compared to a reagent blank reaction. More work is currently being carried out in order to optimize the reaction conditions. Studies are also under way with Ruelene (crufomate), ronnel (fenchlorphos), Proban and parathion. Detection limits may be similar to those observed for N-methyl derivatives.

N-phenyl carbamate and urea herbicides

The labelling of these groups of compounds has been studied in detail in conjunction with TLC, and labelling reactions have been suggested[8]. As these reactions

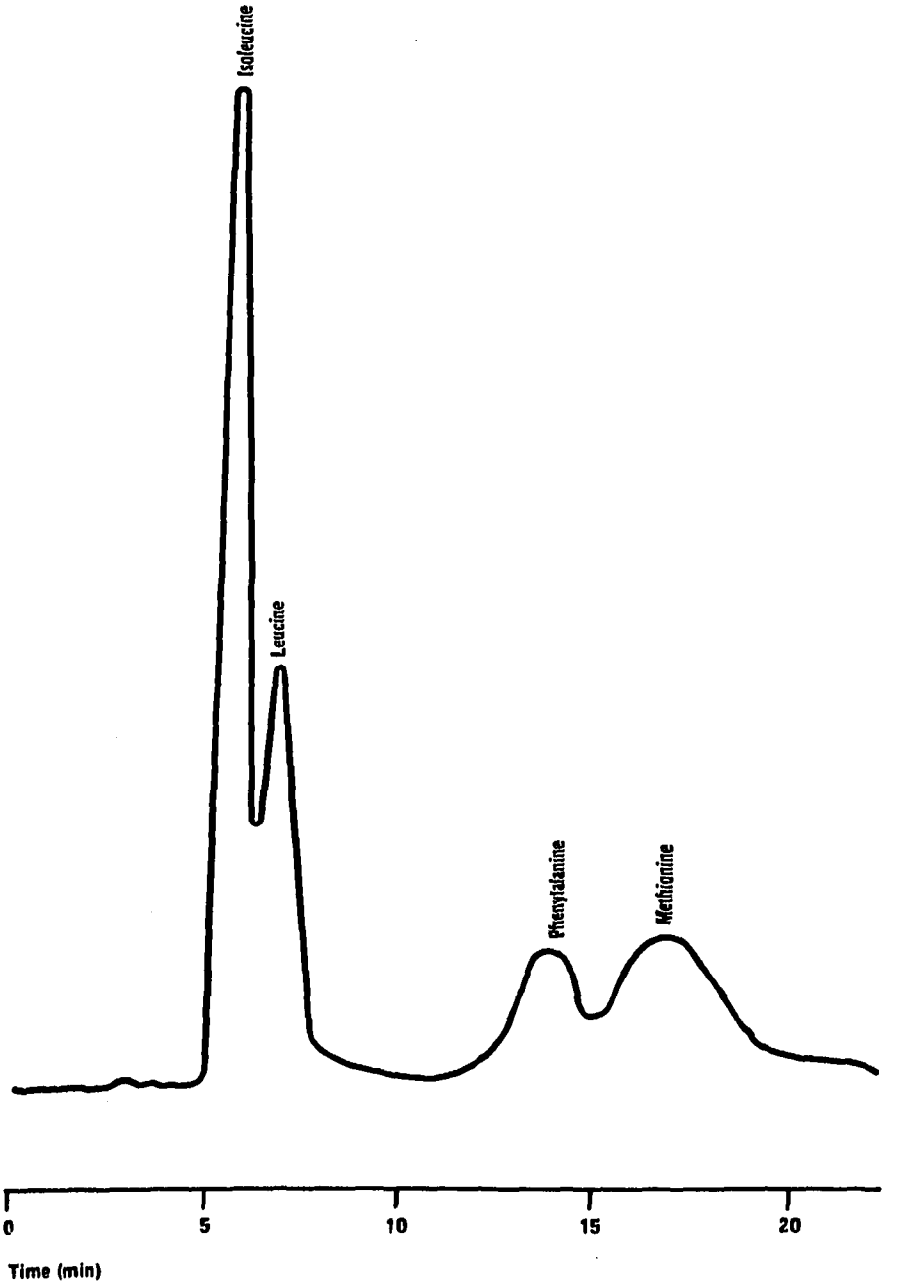


Fig. 1. Separation of dansyl amino acids. Mobile phase, methyl ethyl ketone–light petroleum (5:100, v/v); Zipax ®; flow-rate, 1 ml per 97 sec.

were carried out directly on the plates, some modifications were necessary in order to permit *in vitro* labelling and hence adaptation to HSLC (see Experimental section). The separation of the dansyl derivatives of the aniline moieties is shown in Fig. 4.

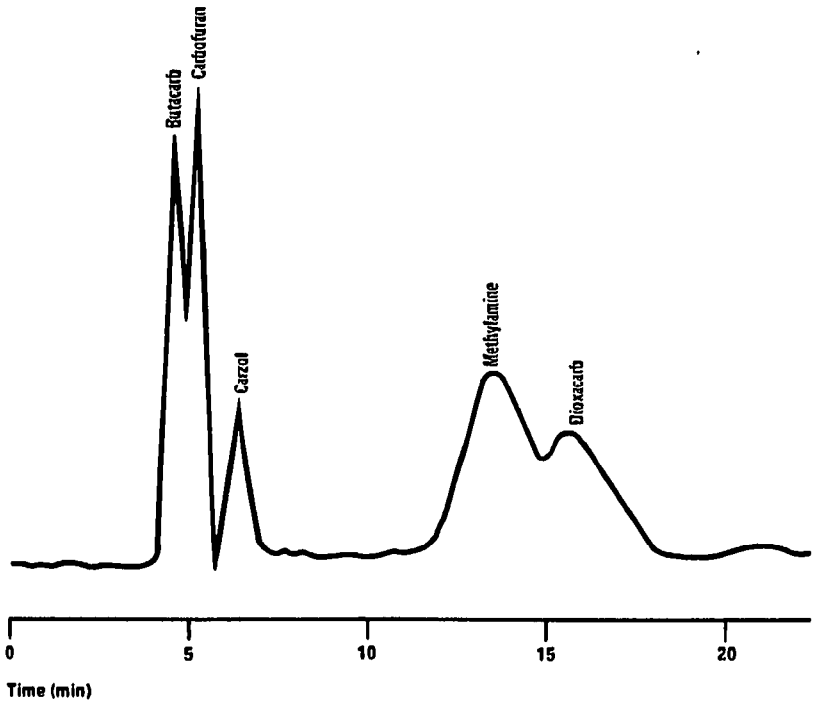


Fig. 2. Separation of dansyl derivatives of N-methyl carbamates. Mobile phase, 2% (v/v) acetone in hexane; Corasil I ®; flow-rate, 1 ml per 81 sec.

The same chromatographic system has been used for other carbamate and urea herbicides with good efficiency. The same technique is also applicable to anilide herbicides. Detection limits are usually below 5 ng per injection.

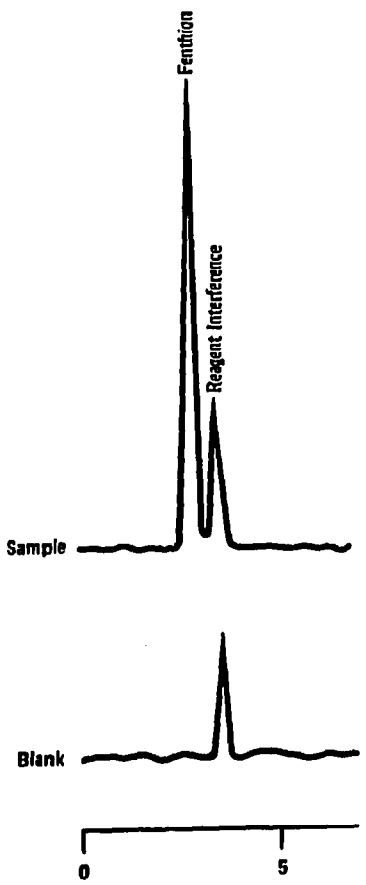
Aliphatic amines

Labelling reactions of NBD-Cl with methyl- and dimethylamines that occurred as hydrolysis products of certain pesticides have been discussed in detail elsewhere[9]. The advantages of NBD-Cl over dansyl chloride are its high specificity toward aliphatic amines, the absence of fluorescence prior to labelling and excitation and emission maxima in the visible region. This permits the use of simpler optics, and interferences from naturally fluorescing impurities in complex matrices are minimized.

Chromatograms of several aliphatic amines are demonstrated in Fig. 5. Satisfactory separations are possible on both Zipax® and Corasil®. The method can be applied directly to the analysis of aliphatic amines or to any compounds that yield aliphatic amines upon hydrolysis. Detection limits are below 1 ng per injection in some instances.

Aldehydes and ketones

The separation of two aldehyde derivatives of the dansyl-hydrazine is shown



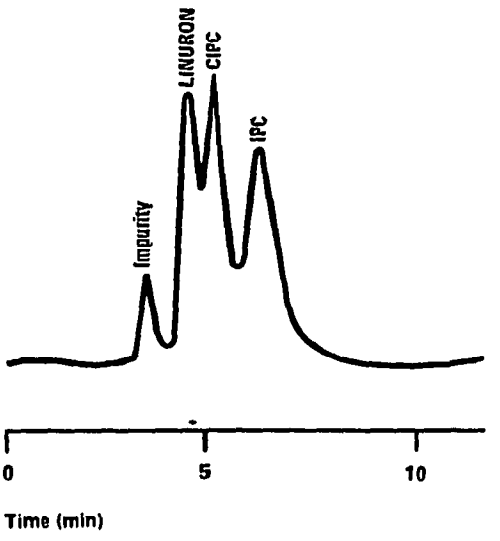
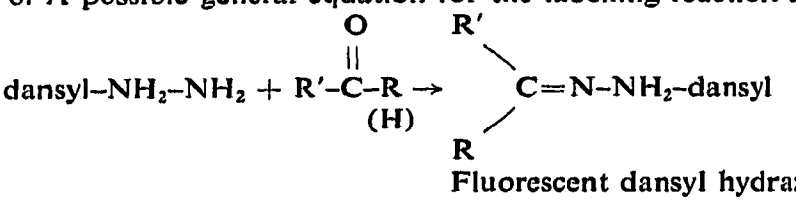


Fig. 3. Chromatogram of a fenthion standard analysis (200 ng). Mobile phase, 5% methanol in hexane; 0.5% of BOP on Zipax ®; flow-rate, 1 ml per 77 sec.

Fig. 4. Separation of herbicides after dansylation of their aniline moieties. Column conditions are identical with those in Fig. 3.

in Fig. 6. A possible general equation for the labelling reaction is



Fluorescent dansyl hydrazone

The derivatives were found to fluoresce with an emission maximum at *ca.* 530 nm and an excitation maximum at *ca.* 365 nm. A large number of organic compounds with suitable keto or aldehyde groups can be labelled with this reagent. It has been used for keto-steroids, for example, with enhanced sensitivity in comparison with colorimetric techniques based on the formation of coloured hydrazones[20,21]. The

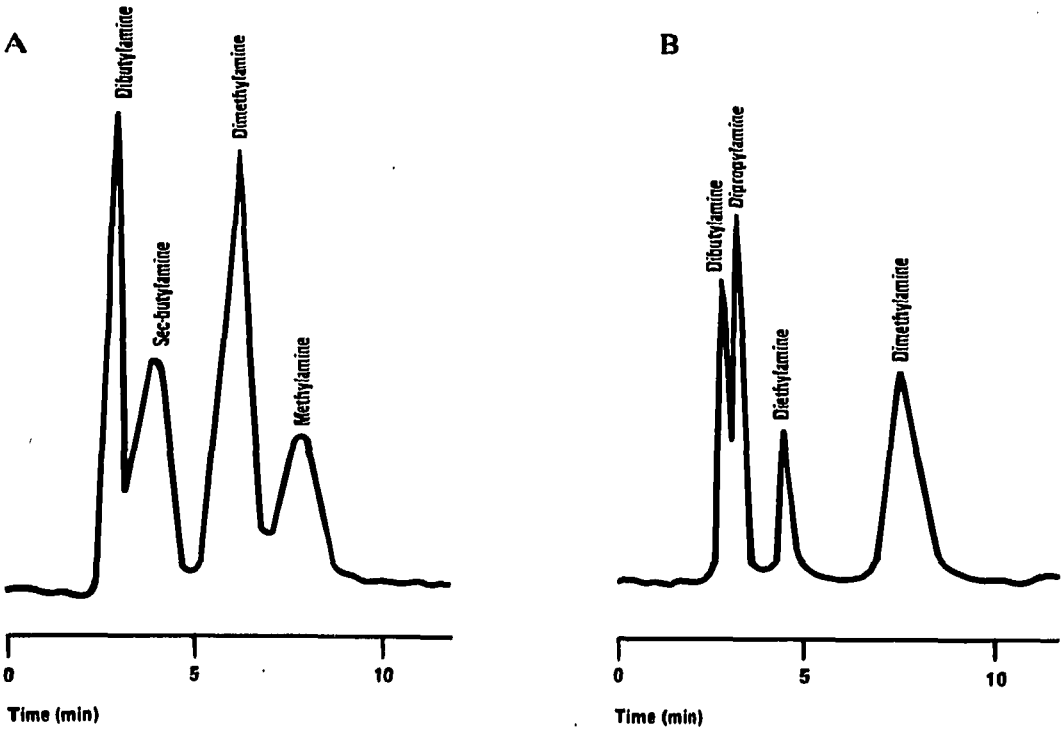


Fig. 5. Separation of NBD-amines. A: mobile phase, 1% of tetrahydrofuran in hexane; Corasil I®; flow-rate 1 ml per 90 sec. B: same mobile phase; Zipax ®; flow-rate, 1 ml per 87 sec.

Fig. 6. Separation of dansyl-hydrazones of hexanal and 3-hydroxy-2-butanone. Mobile phase, diisopropyl ether; 0.5% of BOP on Zipax ®; flow-rate, 1 ml per 66 sec.

filter combination on the fluorescence detector has been chosen to be the same as for dansyl-amines or -phenols. Detection limits were between 1 and 10 ng per injection.

NBD-hydrazine, which is prepared in the same manner as dansyl-hydrazine (see Experimental section), reacts also with aldehydes and ketones but forms fluorescent derivatives only with aldehydes[22].

Application of labelling to residue analysis

The same advantages that have been observed for the labelling methods in residue analysis in connection with TLC[6–10] can be found in connection with HSLC[13]. The labelling procedure itself already serves as a pre-clean-up step, and hence a smaller amount of interfering co-extractives is encountered. It is therefore not surprising that with the higher separation efficiency of HSLC over TLC, a residue analysis can be carried out without clean-up even in a complex biological matrix. Fig. 7 shows the results obtained for a crop sample, extracted only with acetonitrile, followed by the labelling process and subsequent injection. Fluorescent co-extractives are essentially not retained and do not interfere with the pesticide peak.

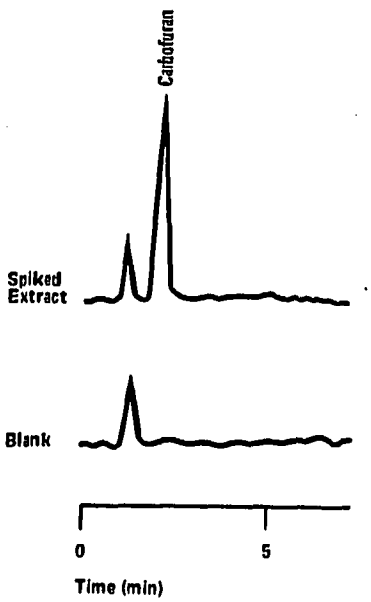


Fig. 7. Chromatogram of lettuce extract spiked with 100 ng of carbofuran. An equivalent of 1 g of lettuce was injected. Mobile phase, 2% of acetone in hexane; Zipax ®; flow-rate, 1 ml per 36 sec.

CONCLUSIONS

Even though the chromatographic conditions or the detection device used in this study are far from optimum, it can be shown that fluorigenic labelling in conjunction with HSLC can be a very useful analytical tool. The favourable performance of the fluorescence detector[12] would also permit the use of low-cost chromatographs. Sensitivity and specificity are comparable with, and in some instances superior to,

GC and the technique should have good potential in toxicological, pharmacological or environmental problem-solving.

ACKNOWLEDGEMENTS

This work was supported by funds provided by the Public Health Research Grant (Project No. 602-7-141) of the National Health Grants Program. Additional assistance was provided by the Canada Department of Agriculture and the National Research Council of Canada.

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