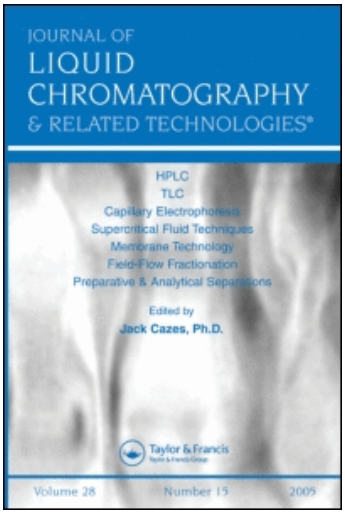

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Fluorogenic Labelling of Carbonylcompounds with 7-Hydrazine-4-nitrobenzo-2-oxa-1,3-diazole (NBD-H)

G. Gübitz[a]; R. Wintersteiger[a]; R. W. Frei[b]
[a] Institute for Pharmaceutical Chemistry University of Graz, Graz, Austria [b] Department of Chemistry, Free University of Amsterdam, HV Amsterdam, The Netherlands

To cite this Article Gübitz, G. , Wintersteiger, R. and Frei, R. W.(1984) 'Fluorogenic Labelling of Carbonylcompounds with 7-Hydrazine-4-nitrobenzo-2-oxa-1,3-diazole (NBD-H)', Journal of Liquid Chromatography & Related Technologies, 7: 4, 839 — 854
To link to this Article: DOI: 10.1080/01483918408074006
URL: http://dx.doi.org/10.1080/01483918408074006

JOURNAL OF LIQUID CHROMATOGRAPHY, 7(4), 839-854 (1984)

FLUOROGENIC LABELLING OF CARBONYLCOMPOUNDS WITH 7-HYDRAZINO-4-NITROBENZO-2-OXA-1,3-DIAZOLE (NBD-H)

G.Gübitz and R.Wintersteiger
Institute for Pharmaceutical Chemistry
University of Graz
A-8010 Graz, Austria

R.W.Frei
Department of Chemistry
Free University of Amsterdam
1081 HV Amsterdam
The Netherlands

ABSTRACT

A method for the prechromatographic fluorescence derivatization of carbonyl compounds with 7-hydrazino-4-nitrobenzo-2-oxa-1,3-diazole (NBD-H) is presented. The separation and quantitation of the hydrazones is carried out by TLC and HPLC on silica gel and RP-materials. Detection limits obtained for benzaldehyde by TLC with fluorodensitometric evaluation are 5 ng/spot and by HPLC with fluorescence detection 200 pg.

INTRODUCTION

Carbonyl compounds are widely occuring in the environment. The determination of traces of carbonyls in air and water is of great importance. Various aldehydes and ketones are also constituents of food aromas. Furthermore, a great number of pharmaceuticals contain carbonyl groups.

 0148-3919/84/0704-0839$3.50/0

The low extinction coefficients of most of the carbonyl compounds however, do not permit a sensitive detection Therefore, several authors applied derivatization methods to enhance the detectability.

2,4-Dinitrophenylhydrazine was described as a UV-derivatization reagent for HPLC-separations of aldehydes by various authors (1-6). Dansyl hydrazine, a fluorescence reagent, was used for TLC- as well as HPLC-separations of various carbonyl compounds (7), sugars (8,9) and ketosteroids (1o-14). Degradation products from the reagent however, interfere often with the quantitation of some compounds.

An alternative to this reagent is 7-hydrazino-4-nitrobenzo-2-oxa-1,3-diazole (NBD-hydrazine, NBD-H), which was proposed by Lawrence and Frei (7) as a potential fluorescence reagent for carbonyl compounds. NBD-hydrazine was prepared by treatment of 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) with hydrazine. NBD-Cl (15-2O) as well as NBD-F (2O-22) have been used as fluorescence labelling reagents for amines and amino acids.

This work deals with the application of NBD-hydrazine to the pre-chromatographic derivatization of various carbonyl compounds for TLC and HPLC with fluorescence detection.

EXPERIMENTAL

Apparatus:

Perkin-Elmer Spectrofluorimeter MPF 44 with TLC-attachement and M 56 recorder.
Camag Nanomat (Muttens, Switzerland).
Perkin-Elmer Liquid Chromatograph Series 2, in combination with the Perkin-Elmer Spectrofluorimeter attached with a Hellma flow-through cell, 20 µl, or a Perkin-Elmer UV-detector LC 15.

Chemicals and materials:

7-Chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl)
(Serva, Heidelberg, FRG)
Hydrazine suprapure (Merk, Darmstadt, FRG)
Methanol, chloroform uvasol (Merck, Darmstadt, FRG)
Benzene UVasol for fluorescence spectroscopy (Merck, Darmstadt, FRG)
Precoated silica 60 - F_{254} TLC-plates 20 x 20 cm, washed twice with methanol-chloroform 3:1 prior to use.
HPTLC-RP-8 plates F_{254} 10 x 20 cm, (Merck, Darmstadt, FRG)
2 µl Microcaps, (Drummond Scientific)
200 nl Platin-Iridium capillaries (Antech, Bad Dürkheim, FRG)
Hibar LiChrosorb, 10 µ, 25 x 0.46 cm columns

Hibar LiChrosorb RP 8, 10 µ, 25 x 0.46 cm columns (Merck,Darmstadt, FRG).

Synthesis of NBD-hydrazine:

10 mg NBD-Cl are dissolved in 5 ml chloroform. After addition of 5 ml of a 1% hydrazine solution (0.2 ml 24% hydrazine suprapure solution in 5 ml methanol UVasol), the reaction mixture is allowed to stand in the dark at room temp. for one hour under nitrogen. The precipitated product is washed with benzene and dried at room temp. The product is to be stored in a vessel, which has been gased with nitrogen in the refrigerator.

Derivatization procedure for carbonyl compounds:

10 µl of a methanolic sample solution, containing not more than 100 nmole of carbonyl compounds are treated in conical vials with 10 - 50 µl of a freshly prepared solution of NBD-H in methanol-water (3:1), corresponding to a 5 fold molar excess, for 30 min (ketones for 2 hours) at 50°C.

To minimize the formation of fluorescent byproducts, the reaction is carried out under nitrogen in the dark.

After cooling 100 µl of water are added and the derivatives are extracted with 100 µl benzene. After

centrifugation an aliquot of the benzene layer is used for the TLC or HPLC separation.

TLC:

2 µl of the benzene layer are transfered with microcaps to silica gel plates. For the separation on HPTLC-RP-8 plates, 200 nl are applicated by Pt-Ir-capillaries.

Solvent systems:

I : Benzene-methanol (95:5)	for silica gel plates, migration distance: 10 cm
II : Benzene-ethylacetate (85:15)	
III: Methanol-water (80:20)	for RP-8 plates, migration distance: 4 cm
IV : Methanol-water (70:30)	
V : Acetonitril-water (80:20)	

The quantitation is carried out by fluorodensitometry at an excitation of 470 and an emission between 530 and 570 nm.

HPLC:

20 µl of the benzene layer are injected. As a mobile phase on the silica gel column benzene-chloroform 95:5 was used, for the RP-8 column acetonitril-water 50:50.

Detection is carried out either with a fluorescence detector or an UV-detector at 254 nm.

RESULTS AND DISCUSSION

Reaction:

The scheme of the reaction of carbonyl compounds with NBD-H is given in Fig. 1. Ketones show a considerably lower reactivity in comparison with aldehydes. With benzaldehyde, for example, the reaction is complete in about 10 minutes, acetone requires a reaction time of more than 1 hour. Fig. 2 shows the kinetics of the reaction of benzaldehyde and acetone with NBD-H.

The reaction yield was determined by comparison of the results of the reaction on analytical scale with the isolated and purified derivatives and was found to be 99% for benzaldehyde. To avoid the formation of fluorescent degradation products of the reagent, the reaction temperature should not exceed 50°C. The use of a higher temperature to reduce the reaction time leads to the formation of fluorescent byproducts. The same occurs, when acid catalysts are used. To minimize the reagent blank, it is advantageous to use a freshly prepared reagent.

Spectra

The excitation and emission spectra of benzaldehyde, butyraldehyde and acetone are shown in Fig. 3.

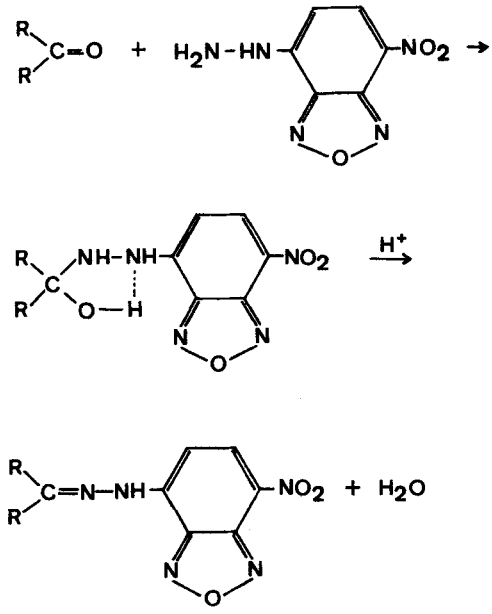

FIGURE 1: Reaction of carbonyl compounds with NBD-H.

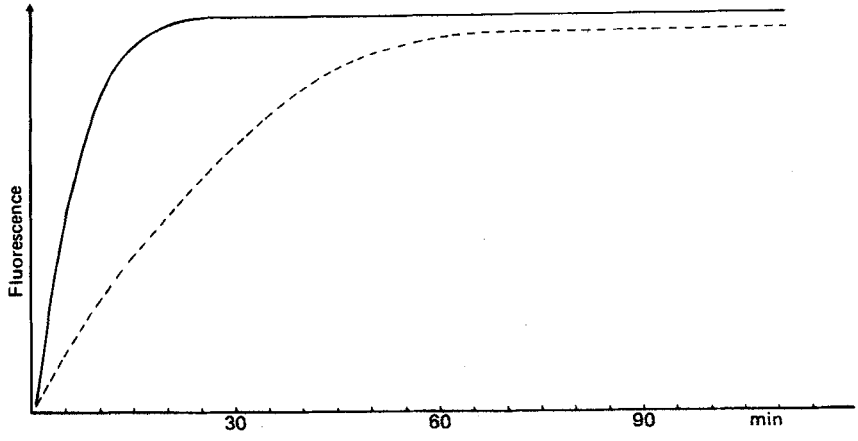

FIGURE 2: Kinetics of the reaction of benzaldehyde ——— and acetone ---- with NBD-H.

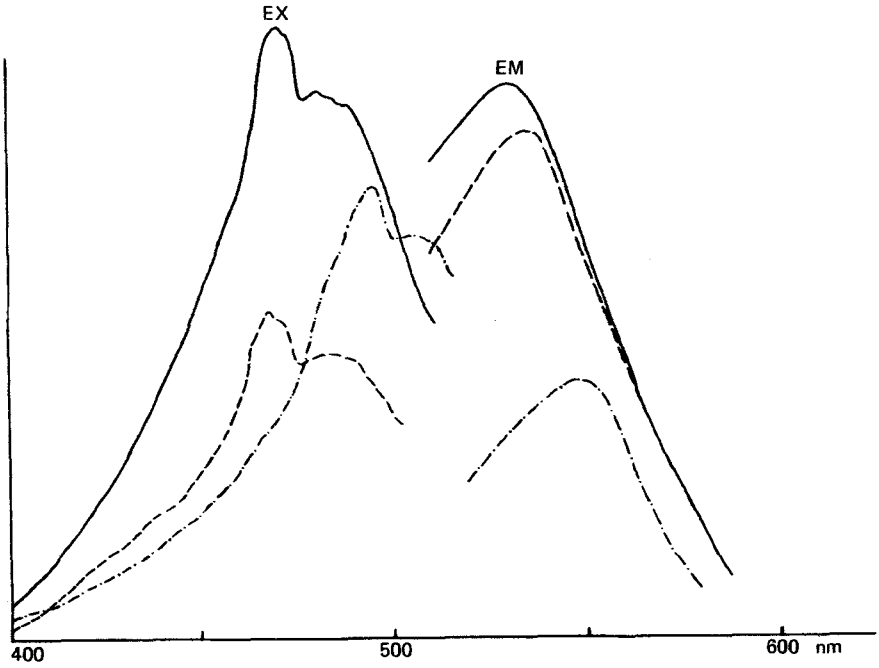

FIGURE 3: Excitation and emission spectra of the NBD-hydrazones of benzaldehyde -·-·-·, butyraldehyde ----- and acetone ———, recorded on a TLC-plate.

The emission maxima of the diverse carbonyl compounds range between 530 and 570 nm. The colors of the spots are yellow to purple. (Table 1)

The fluorescence intensity in solution depends greatly on the polarity of the solvent. In apolar solvents a high fluorescence is observed, in polar solvents like water or methanol an intensification of the color and a bathochromic shift occurs and the fluorescence is reduced such that visible detection becomes more sensitive than fluorescence detection.

TABLE 1. Excitation and emission maxima and visual appearance of the spots of some NBD-hydrazones.

Compound	Ex	Em	vis	Color
Butyraldehyde	467	535	450	yellow
Benzaldehyde	470,495	555	490	orange
Anisaldehyde	471	570	550	purple
Vanilline	471	570	550	purple
Acetone	470	530	450	yellow
Acetophenone	470	535	480	orange
Propiophenone	470	535	480	orange
Cyclopentanone	470	535	480	yellow

Separation of the NBD-hydrazones by TLC and HPLC:

The separation of the NBD-hydrazones of aldehydes were carried out on silica gel sheets as well as on HPTLC-RP-8 plates. A better separation and a higher sensitivity was obtained with the reversed phase systems. In Table 2 and 3 the Rf-values of the derivatives of various aldehydes and ketones, respectively, are given.

The quantitative evaluation was carried out by fluorodensitometry. Fig. 4 shows a scan of mixtures of the hydrazones of some aldehydes and ketones.

TABLE 2: Rf-values of NBD-hydrazones of aldehydes:

Compound	Rf x 100	
	Solv.I (silica gel)	Solv. IV (RP-8)
Formaldehyde	26	18
Butyraldehyde	40	24
Crotonaldehyde	37	26
Valeraldehyde	44	16
Benzaldehyde	44	15
Cinnamylaldehyde	44	13
Anisaldehyde	39	18
Aminobenzaldehyde	17	36
4-Dimethylaminobenzaldehyde	39	5
4-Hydroxybenzaldehyde	9	33
Salicylaldehyde	6	35
Vanilline	14	31
3-Nitrobenzaldehyde	27	25
Pyridin-2-aldehyde	26	25

As preliminary experiments have shown, this derivatization method is also applicable to the HPLC-separation of carbonyl compounds. The separation of some aromatic aldehydes was carried out on a silica gel column. (Fig. 5) For aliphatic aldehydes a RP-8 column

TABLE 3: Rf-values of NBD-hydrazones of various ketones:

Compound	Rf x 100		
	Solv.II (silica gel)	Solv.III (RP-8)	Solv.V (RP-8)
Acetone	21	66	81
Acetophenone	49	44	65
Propiophenone	-	47	65
Methylphenylketone	4	74	92
Isopropylmethylketone	-	56	65
Isobutylmethylketone	-	51	59
Diisopropylketone	-	47	65
Benzalacetone	39	43	52
Cyclohexanone	33	50	65
Cyclopentanone	38	56	71
Cycloheptanone	26	56	48
Indan-1,3-dione	7	63	67
1,4-Napthoquinone	11	64	57
Vitamine K_1	6	63	47
Prednisolone	-	34	46
Haloperidole	-	0	77
Methadone	-	60	72
Ketobemidone	-	58	70
Hydrocodone	-	55	68

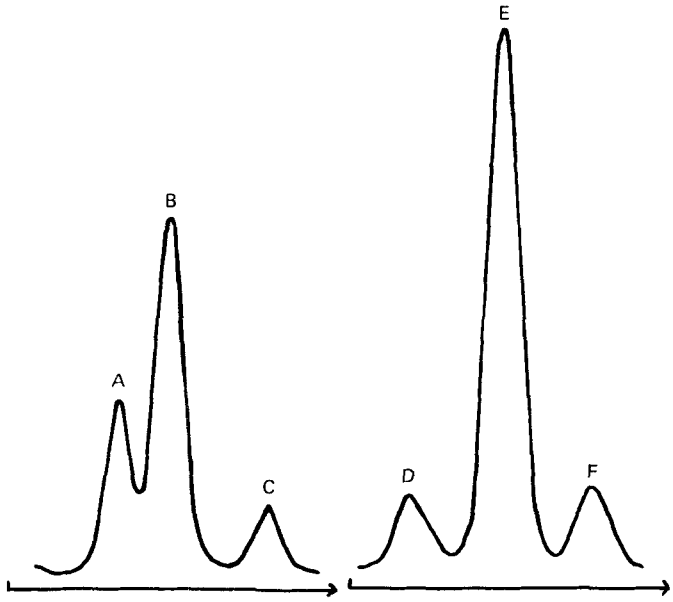

FIGURE 4: Scan of a TLC of the NBD-hydrazones of A: cinnamylaldehyde, B: nitrobenzaldehyde, C: vanilline, D: acetophenone, E: cyclopentanone, F: acetone. HPTLC-RP-8 plates, Solvent: methanol-water (70:30) Ex: 470 nm/Em: 530 nm.

showed a better selectivity, however, the high polarity of the usual eluents quenches the fluorescence considerably. Therefore an UV-detection was used in this case, with a resulting loss of sensitivity. Fig. 6 shows a separation of 4 aliphatic aldehydes on a RP-8 column.

Quantitation:

Calibration curves were prepared for various carbonyl compounds both for TLC and HPLC. The curves

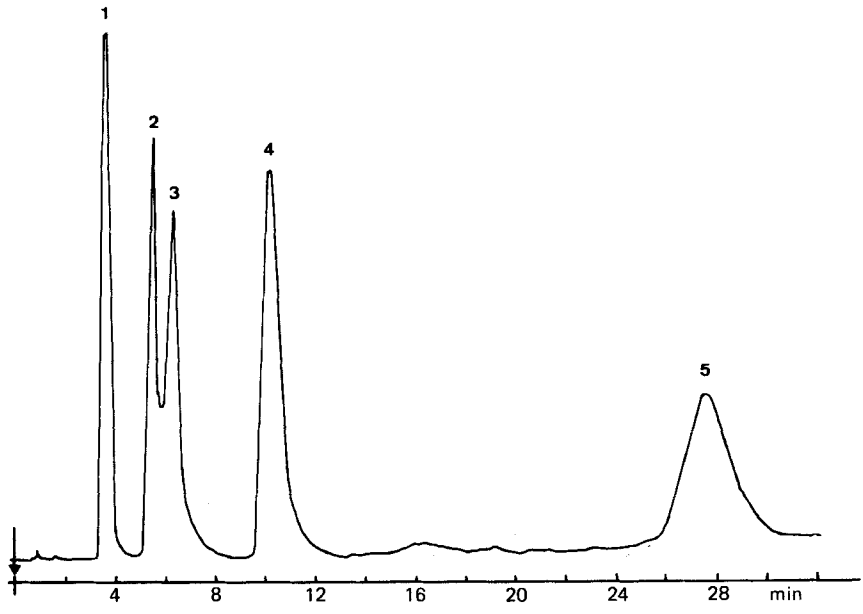

FIGURE 5: Separation of the NBD-hydrazones of aromatic aldehydes by HPLC. 1: Benzaldehyde, 2: cinnamylaldehyde, 3: anisaldehyde, 4: vanilline. Column: Silica gel, 25 x 0.46 cm. Mobile phase: Benzene-chloroform (95:5). Flow: 2 ml/min. Detection: Fluorescence, Ex: 470/Em: 560 nm.

are linear over a range of more than one decade. The correlation coefficients were between 0 .996 and 0.999.

The relative standard deviation determined for benzaldehyde (50 ng/spot) by the TLC-method was 2.5% (n=8) and 3.8% for 5 ng, determined by HPLC.

The fluorescence detection limits obtained by TLC at a signal to noise ratio of 3:1 are 2 ng/spot for benzaldehyde and 10 ng for acetone. For vitamine K_1 the detection limit is 2 ng.

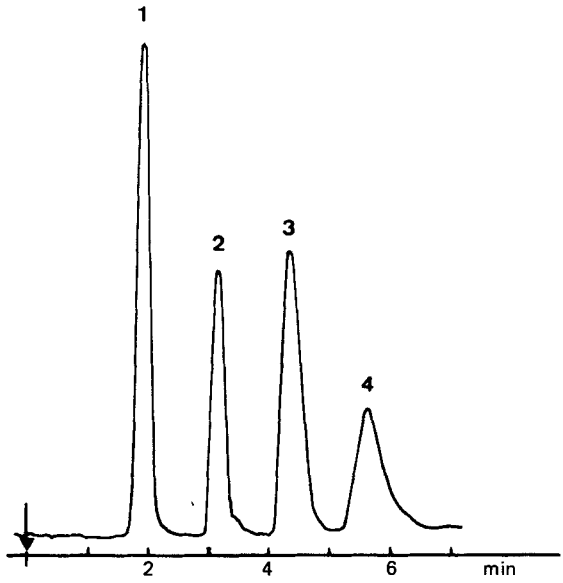

FIGURE 6: Separation of the NBD-hydrazones of aliphatic aldehydes by HPLC. 1: Propionaldehyde, 2: butyraldehyde, 3: valeraldehyde, 4: capronaldehyde. Column: RP-8, 25 x 0.46 cm. Mobile phase: acetonitril-water (50:50). Flow: 1 ml/min. Detection: UV 254 nm.

With HPLC the fluorescence detection limit for benzaldehyde was 200 pg. This could be improved by further optimization of the experimental conditions. The detection limits with UV-detection at 254 nm were 5-10 ng. It should be noted, that the sensitivity depends greatly on the purity of the reagent and the used solvents.

The optimization of the method with the goal of a sensitive determination of drugs and other compounds of interest containing carbonylgroups by HPLC is under way.

REFERENCES

1. Kikta, E.J. and Grushka, E., Anal.Chem., 48, 1098, 1976.
2. Selim, S., J.Chromatogr., 136, 271, 1977.
3. Demko, P., J.Chromatogr., 179, 361, 1979.
4. Vigh, G., Varga-Puchony, Z., Hlavay, J., Petro-Turcza, M. and Szarföldi-Szalma, I., J.Chromatogr., 193, 432, 1980.
5. Fung, K. and Grosjean, D., Anal.Chem., 53, 168, 1981.
6. Reindl, B. and Stan, H.J., J.Chromatogr., 235, 481, 1982.
7. Frei, R.W. und Lawrence, J.F., J.Chromatogr., 83, 321, 1973.
8. Avigad, G., J.Chromatogr., 139, 343, 1977.
9. Takeda, M., Maeda, M. und Tsuji, A., J.Chromatogr., 244, 347, 1982.
10. Chayen, R., Dvir, R., Gould, S. und Harrel, A., Anal.Biochem., 42, 283, 1971.
11. Graef, V., Z.Klin.Chem.u.Klin.Biochem., 8, 320, 1970.
12. Kawasaki, T., Maeda, M. und Tsuji, A., J. Chromatogr., 163, 143, 1979.
13. Kawasaki, T., Maeda, M. und Tsuji, A., J. Chromatogr., 226, 1, 1981.
14. Wintersteiger, R. und Gamse, E., Proc.of the Symp. on the Analysis of Steroids, Eger, Hungary, 453, 1981.
15. Lawrence, J.F. and Frei, R.W., Anal.Chem., 44, 2046, 1972.
16. Klimesch, H.-J. and Stadler, J.Chromatogr., 90, 141, 1974.
17. Wolfram, J.H., Feinberg, J.I., Doerr, R.C. and Fiddler, W., J.Chromatogr., 132, 37, 1977.

18. Ahnoff, M., Grundavik, I., Arfwidsson, A., Fonselius, J. and Persson, B., Anal.Chem., 53, 484, 1981.

19. Roth, M., Clin.Chim.Acta, 83, 273, 1978.

20. Imai, K. and Watanabe, Y., Anal.Chim.Acta, 130, 377, 1981.

21. Watanabe, Y. and Imai, K., Anal.Biochem., 116, 471, 1981.

22. Watanabe, Y. and Imai, K., J.Chromatogr., 239, 723, 1982.