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Short communication

Determination of 4-hydroxy-2-nonenal by precolumn derivatization and liquid chromatography with laser fluorescence detection

Yi-Ming Liu[a,1], Jian-Rong Miao[b], Toshimasa Toyo'oka[c,*]

[a]*National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158, Japan*

[b]*Hunan Provincial Institute for the Control of Drugs, Changsha 410001, China*

[c]*Department of Analytical Chemistry, School of Pharmaceutical Sciences, University of Shizuoka, 52-1 Yada, Shizuoka 422, Japan*

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Abstract

The lipid aldehyde 4-hydroxy-2-nonenal (4-HNE) was derivatized with a novel fluorescence labelling reagent, 4-(2-carbazoylpyrrolidin-1-yl)-7-nitro-2,1,3-benzoxadiazole (NBD-ProCZ). The labelling reaction was carried out at 60°C for 10 min in the presence of trichloroacetic acid. The resultant 4-HNE-NBD-ProCZ hydrazone was separated from other aldehyde–NBD-ProCZ derivatives by high-performance liquid chromatography with a ternary eluent consisting of water, methanol and acetonitrile. An argon ion laser at 488 nm, which was very close to the excitation maximum (490 nm) for the labelling fluorophore, was used for excitation and the fluorescence emission was collected at 540 nm. The detection limit (signal-to-noise ratio = 3) was 10 fmol on-column for 4-HNE. Edible oil samples were analysed by this method. 4-HNE contents were found to be at the pmol/g level.

1. Introduction

Lipid peroxidation has been studied extensively because of its involvement in the pathogenesis of certain human diseases, including cancer, atheroscleosis and cerebal apoplexy [1]. Although *n*-alkanals may be quantitatively the major carbonyl products of lipid peroxidation, 4-hydroxyalkenals, particularly 4-hydroxy-2-nonenal (4-HNE), are far more significant products because they are produced in relatively large amounts and are biologically very active. They inhibit, for example, various thiol-dependent enzymes and the synthesis of DNA, RNA and protein [2]. 4-HNE has been found in extracts of various oxidized biological specimens, such as rat hepatic microsome and human low-density lipoprotein. It has also been detected in foodstuffs, e.g. in fried meat [3], and has been identified as a major toxic component of cooking oils associated with a toxic oil syndrome outbreak [4].

A variety of analytical techniques have been developed for the determination of 4-HNE. 4-HNE was selectively determined by high-per-

* Corresponding author.

[1] Present address: Department of Chemistry, University of Illinois at Urbana–Champaign, 600 South Mathews Avenue, Urbana, IL 61801, USA.

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formance liquid chromatography (HPLC) with UV detection [3]. More sensitive gas chromatographic–mass spectrometric (GC–MS) methods based on the formation of volatile 4-HNE oxime derivatives have also been reported [5,6]. To enhance the analytical performance, an HPLC-aided GC–MS method has been proposed for the determination of trace amounts of 4-HNE in biological samples [7].

Fluorescence detection-oriented HPLC techniques, owing to their inherent high sensitivity and selectivity, have received much research interest. However, only one procedure of this kind with 1,3-cyclohexanedione (CHD) as the precolumn derivatizing reagent has so far been reported for 4-HNE determination [8]. The fluorescent aldehyde–CHD derivatives were separated on reversed-phase HPLC columns and detected fluorimetrically. This HPLC method has a determination sensitivity similar to those of GC–MS methods. Its poor selectivity, however, might preclude its use in real sample analysis [9]. Recently, Holley et al. [10] described a modification of this CHD method.

In this paper, we report on the development of an HPLC method for trace 4-HNE determination with the use of a novel fluorescence labelling reagent, 4-(2-carbazoylpyrrolidin-1-yl)-7-nitro-2,1,3-benzoxadiazole (NBD-ProCZ) (Fig. 1) [11]. After separation, 4-HNE-NBD-ProCZ hydrazone was detected by laser-induced fluorescence (LIF) detection. The detection limit was found to be 10 fmol per 4-HNE injection (5 μl). Compared with conventional fluorescence detection, LIF detection was 250 times more sensitive in this particular case. A convenient procedure for the analysis of edible oil samples based on this method is also described.

2. Experimental

2.1. Reagents

The fluorescence labelling reagent NBD-ProCZ was synthesized as described previously [11]. 4-HNE, synthesized according to the method of Esterbauer [12], was a gift from Dr. Kurihara (Division of Organic Chemistry, National Institute of Health Sciences, Tokyo, Japan). Standard solutions of 4-HNE were prepared in water and the concentrations were determined spectrophotometrically with $\lambda = 223$ nm and $\varepsilon = 13\,750$ [10]. Other aldehydes including C_1–C_9 *n*-alkanals, benzylaldehyde, malondialdehyde, 2-butenal, 2-hexenal, 2-nonenal, 2-undecenal, 2,4-heptadienal and 2,4-nonadienal were obtained from either Sigma (St. Louis, MO, USA) or Wako (Osaka, Japan). 2,6-Di-*tert.*-butyl-*p*-cresol (BHT) was purchased from Sigma. Acetonitrile (CH_3CN) and methanol (MeOH) were of HPLC grade (Wako). Other

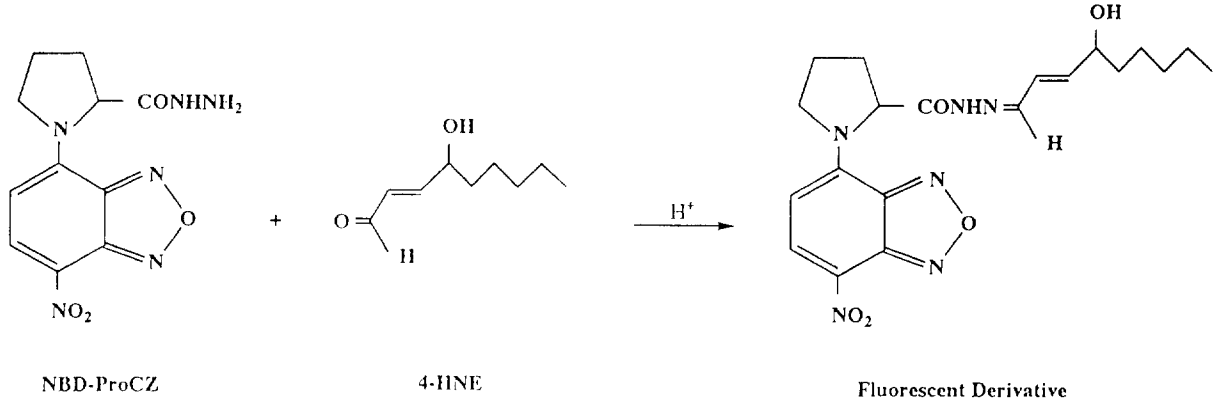

Fig. 1. The novel fluorescence labelling reagent NBD-ProCZ and its reaction with 4-HNE.

chemicals were of analytical-reagent grade. HPLC-grade water from Wako was used throughout.

2.2. Apparatus

The liquid chromatograph (Shimadzu, Kyoto, Japan) consisted of three LC-10AD pumps and an SCL-10A system controller. Sample injections were made with a SIL-10A autosampler equipped with a sample cooler. The analytical column was a 5-μm Inertsil ODS-80A (150 × 4.6 mm I.D.) from GL Sciences (Tokyo, Japan). A CTO-10AC column oven (Shimadzu) was used to maintain the column temperature at 40°C. The effluent was monitored with an LF-8010 laser-induced fluorescence detector equipped with a 5-μl flow cell and interference filter at 540 ± 20 nm (Tosoh, Tokyo, Japan). A 10-mW argon ion laser at 488 nm was used for excitation. For comparison, a Shimadzu RF-10A fluorescence scanning detector equipped with a 12-μl flow cell was used alternatively. The excitation and emission wavelengths were 490 and 540 nm, respectively. An on-line degasser (DGU-3A; Shimadzu) was used for solvent degassing. The mobile phase was a ternary mixture of H_2O, CH_3CN and MeOH. Linear gradient elution with 15% (v/v) (0–10 min), 40% (v/v) (20–30 min) and 15% (v/v) (30.1–40 min) of CH_3CN and a fixed MeOH content of 40% (v/v) in the mixture was applied. The flow-rate of the eluent was 1.0 ml/min in all cases.

2.3. Procedures

Derivatization of 4-HNE with NBD-ProCZ

In a 0.5-ml reaction vial (GL Sciences), 5 μl of 4-HNE standard solution were mixed with 45 μl of trichloroacetic acid (TCA) methanol solution (0.5%, w/v) and 50 μl of 1 m*M* NBD-ProCZ solution in MeOH. The reaction vial was tightly capped, votex mixed for several seconds and then heated with a dry heating block at 60°C for 10 min. The derivatization solution was kept in the autosampler at 5°C and 5 μl of the solution were injected on to column without further purification.

Analysis of edible oil samples

To 1.0 g of soybean oil samples or 0.25 g of sesame oil samples in a centrifuge tube was added 1.0 ml of the freshly prepared reagent solution, which contained 0.25% (w/v) TCA, 150 μg/ml BHT and 0.5 m*M* NBD-ProCZ in MeOH–H_2O (8:2). BHT was used to prevent the potential formation of additional 4-HNE by oxidation during analysis [7]. The reaction solution was vortex mixed for 1 min and then heated at 60°C for 10 min. During heating the tubes were sealed with Teflon-lined caps. The solution was then cooled on ice and centrifuged at 3000 *g* for 10 min at 5°C. Aliquots of 5 μl of the supernatant were injected on to column.

3. Results and discussion

3.1. Fluorescence labelling

NBD-ProCZ reacted readily with 4-HNE in the presence of TCA as catalyst. The resulting fluorescent derivative showed much higher stability than the *n*-alkanal–NBD-ProCZ derivatives. The 4-HNE derivative solution was stable for at least 12 h when kept at 5°C in the dark. This was probably due to the presence of the conjugated C = C double bond in the 4-HNE molecules (Fig. 1). The experimental conditions for derivatizing 4-HNE in various reaction solvents including MeOH, CH_3CN, H_2O and their mixtures were optimized. To obtain a reproducible and optimum labelling yield for 4-HNE, the reaction solution was heated at 60°C for 10 min. The presence of the acidic catalyst, TCA, was found to be essential for the derivatization. No major difference in labelling yield was observed when the TCA concentration was varied over the range 0.05–0.4% (w/v). Excess NBD-ProcCZ (more than 25 times the molar amount of 4-HNE) was used. It is worth noting, however, that too much unreacted reagent in the deriva-

tive solution would deteriorate the chromatographic quantification of 4-HNE owing to the appearance of a too large and tailing reagent peak in the chromatogram.

3.2. HPLC separation

All aldehydes tested reacted with NBD-ProCZ forming fluorescent hydrazones. These aldehydres included C_1–C_9 *n*-alkanals, benzylaldehyde, 2-butenal, 2-hexenal, 2-nonenal, malondialdehyde, 2-undecenal, 2,4-heptadienal and 2,4-nonadienal. Most of these hydrazones could be easily separated from 4-HNE–NBD-ProCZ derivative by reversed-phase HPLC. Pentanal–NBD-ProCZ, however, was found to elute with a retention time very close to that of 4-HNE–NBD-ProCZ under many of the HPLC conditions tested. Various CH_3CN–H_2O and MeOH–H_2O combinations were tried as mobile phases but none of them was found to be satisfactory. Fortunately, a complete separation of 4-HNE–NBD-ProCZ from all the aforementioned aldehyde–NBD-ProCZ derivatives was achieved with a MeOH–CH_3CN–H_2O ternary mixture as the mobile phase using linear gradient elution. A typical chromatogram of a derivative solution containing 4-HNE and C_4–C_9 *n*-alkanal–NBD-ProCZ hydrazones is shown in Fig. 2. The gradient elution conditions are indicated in the figure. It should be pointed out that although in our work three HPLC pumps were used for executing the gradient elution, two pumps in fact

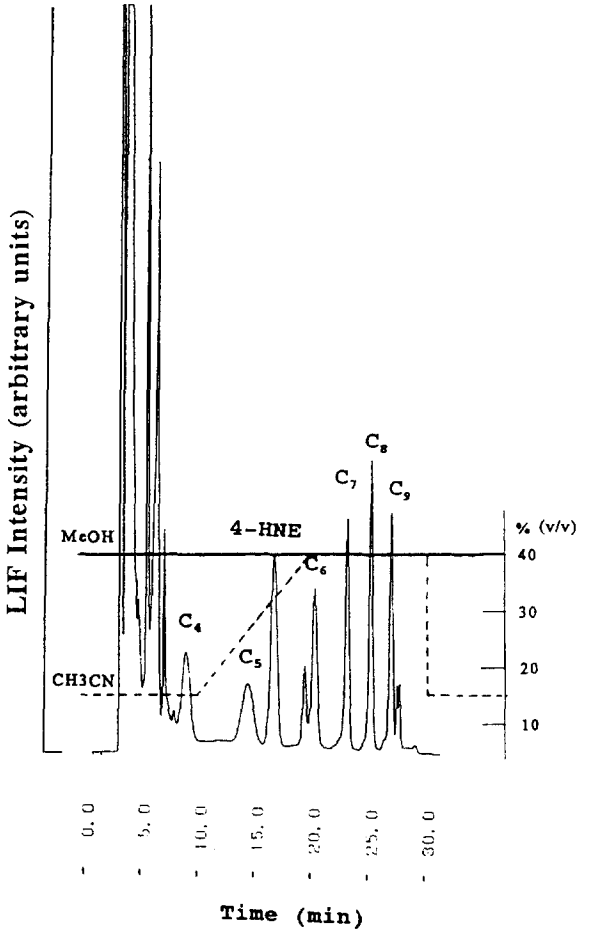

Fig. 2. HPLC of a mixture of 4-HNE and C_4–C_9 *n*-alkanal–NBD-ProCZ derivatives. The volume of 5 μl injected contained 1 pmol each of the aldehydes. LIF detection: λ_{ex} = 488 nm, λ_{em} = 540 nm. Elution conditions as indicated in the figure.

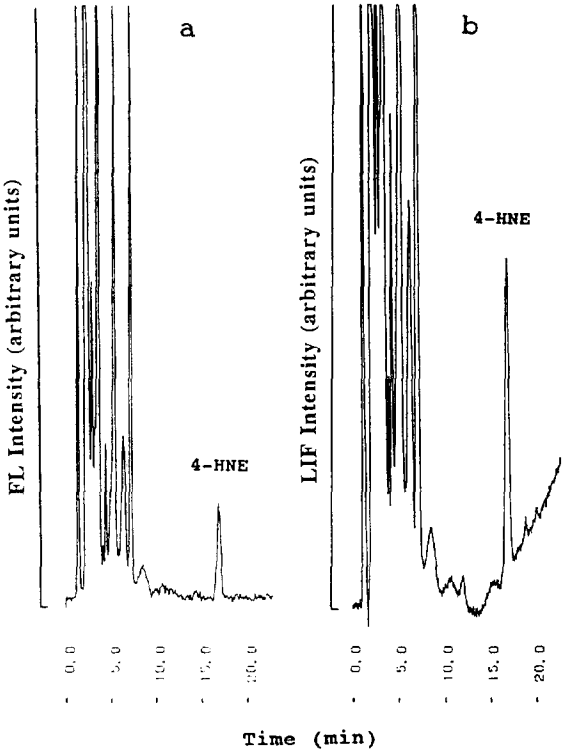

Fig. 3. Comparison between (a) conventional fluorescence detection and (b) LIF detection of 4-HNE labelled with NBD-ProCZ. 4-HNE injected: (a) 5 pmol and (b) 25 fmol. HPLC conditions as in Fig. 2.

are sufficient to do so if MeOH–CH_3CN (4:6) and MeOH–H_2O (4:6) mixtures are prepared in advance.

3.3. LIF detection

This work takes advantage of the favourable spectral characteristics of the nitrobenzoxadiazole (NBD) fluorophore. Its maximum excitation wavelength of 490 nm is very close to the emission wavelength of the popular argon ion laser (488 nm). Further, the sufficient photostability of 4-HNE–NBD-ProCZ hydrazone also permitted the use of the sensitive argon laser-induced fluorescence detection. Fig. 3 shows a comparison between a conventional fluorescence detector and an LIF detector as used for the detection of NBD-ProCZ labelled 4-HNE. As little as 10 fmol per 4-HNE injection can be detected with LIF detection. This LIF detection limit is about 250 times lower than that with conventional fluorescence detection and is comparable to those obtained by GC–MS methods [6,7].

A typical calibration graph prepared with a standard solution of 4-HNE over the range 0–250 n*M* using LIF detection is described by the equation

$$y = 8.59x + 0.39, \quad r = 0.994$$

where y is the peak area, x the 4-HNE concentration in n*M* and r the correlation coefficient.

3.4. Analysis of edible oil samples

Fig. 4 shows typical HPLC traces obtained from soybean oil samples. To identify the chromatographic peak which corresponds to 4-HNE, the standard addition method was used. An oil

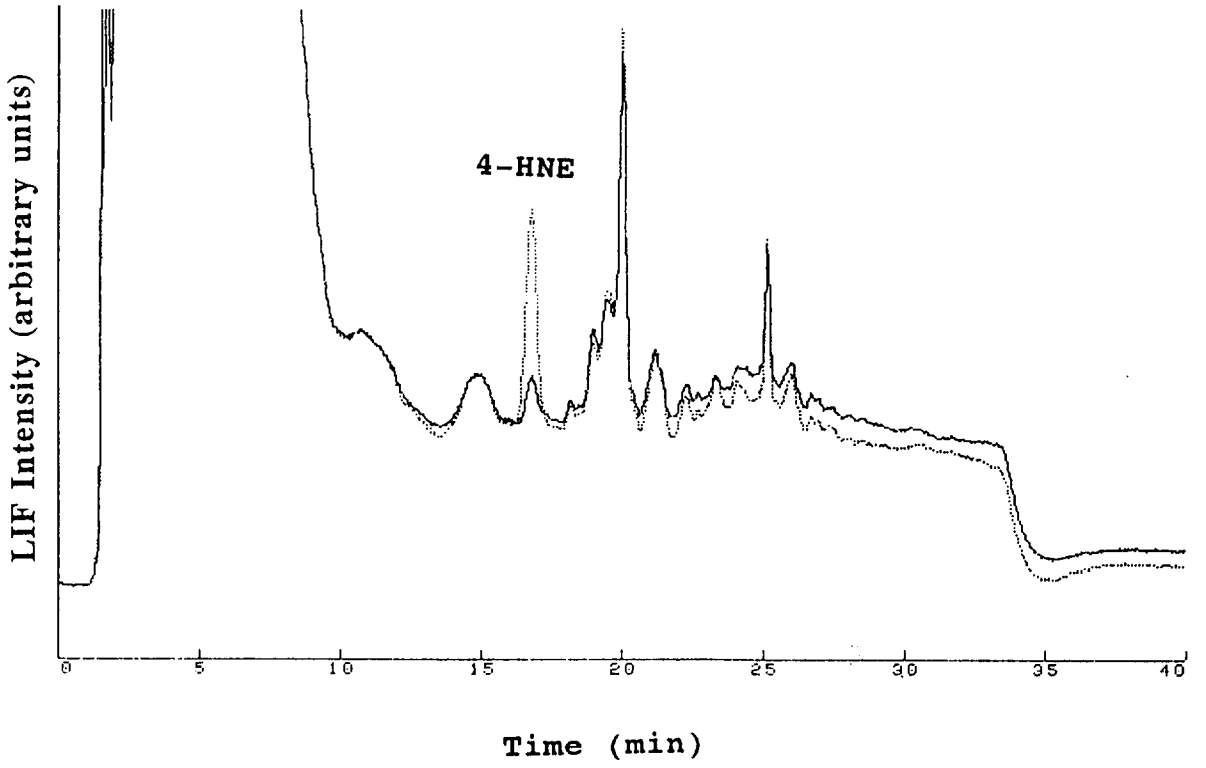

Fig. 4. Overlaid HPLC traces obtained from a soybean oil sample (solid line) and a sample spiked with 80 pmol/g of 4-HNE (dotted line). HPLC and detection conditions as in Fig. 2.

sample was divided into two portions and to one of them a suitable amount of the standard solution was added. They were then derivatized and chromatographed identically. The peak retention times and peak shapes were compared for the two chromatograms to see if the targeted peak given by the compound was identical with that of the standard added. Two such chromatograms obtained from a soybean sample and a sample spiked with 4-HNE at 80 pmol/g are overlaid in Fig. 4. As can be seen, the proposed method is highly sensitive. 4-HNE at n*M* concentrations in the soybean oil samples can be accurately determined. Chromatographic peak areas were used to determine 4-HNE concentrations. Analytical results for some soybean oil and sesame oil samples are summarized in Table 1. Calibration graphs prepared with water, soybean oil or sesame oil as the matrix showed no significant difference in their slopes. With the proposed procedure, the recovery of 4-HNE from these oil samples was quantitative. The precision of the method was evaluated by replicate analyses of oil samples and relative standard deviations (R.S.D.) were found to be less than 10% in all cases (Table 1).

A typical chromatogram for the determination of 4-HNE in sesame oil samples is shown in Fig. 5. As can be seen, this chromatogram is much more complicated than those obtained from soybean oil samples (Fig. 4). The 4-HNE peak was identified using the standard addition method. As there is a possibility that unknown peak(s) are overlapped with the 4-HNE peak in different samples, a better separation is obviously desirable for the accurate determination of trace amounts of 4-HNE in these samples. It is hoped that this can be achieved by using different HPLC columns and/or mobile phases. The numerous peaks here suggest that many kinds of carbonyl compounds exist in this cooking oil. Some of them must be responsible for the special smell and flavour of sesame oil, which make it so popular in Asian dishes.

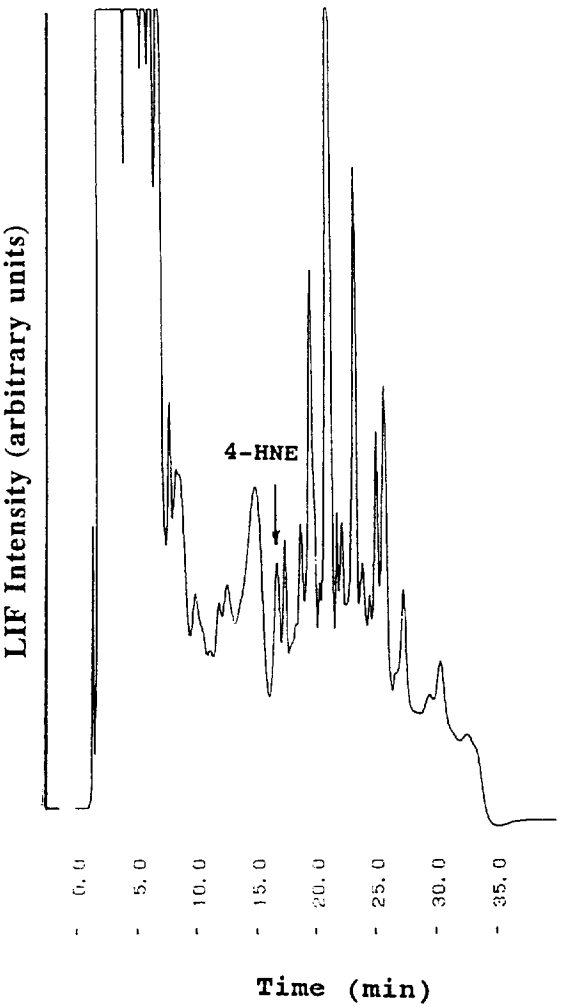

Fig. 5. Typical chromatogram for determination of 4-HNE in sesame oil. HPLC and detection conditions as in Fig. 2.

Table 1
Determination of 4-HNE in edible oil samples

Sample[a]	4-HNE found (pmol/g)[b]	R.S.D. (%) ($n = 5$)
Soybean oil I	26	8.1
Soybean oil II	36	3.5
Sesame oil I	597	3.0
Sesame oil II	786	5.4

[a] Samples were purchased in a local supermarket.
[b] Means of five determinations.

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