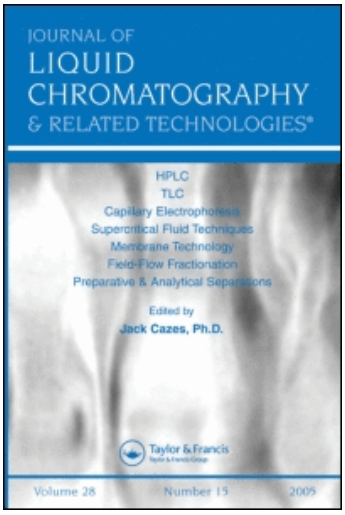

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HPLC-UV ANALYSIS OF EUGENOL IN CLOVE AND CINNAMON OILS AFTER PRE-COLUMN DERIVATIZATION WITH 4-FLUORO-7-NITRO-2,1,3-BENZOXADIAZOLE

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□ *Eugenol is one of the flavor constituents in various medicinally used plant oils, such as clove and cinnamon oils, which are widely used as flavoring agents in foods and beverages. In this study, the levels of eugenol in clove and cinnamon oils were analyzed by HPLC-UV (380 nm) after pre-column derivatization with 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F). A standard curve was obtained after derivatization with NBD-F in borate buffer (pH 8.5) at 40° C for 4 min. The retention time of NBD-eugenol was 12.1 min. The calibration plot was linear in the range of 0.2 to 5 μg/mL with an r^2 value of 0.9976, and the lower limit of detection was 0.04 μg/mL (at a signal-to-noise ratio of 3:1). The coefficient of variation was less than 6.7%. It was found that the content of eugenol in clove oil was 806 ± 29 mg/g (range, 776 to 844 mg/g) and that in cinnamon oil was 39.7 ± 2.0 mg/g (range, 37.9 to 42.2 mg/g). Addition-recovery tests were within the range of 86.9 to 98.8%.*

Keywords 4-fluoro-7-nitro-2,1,3-benzoxadiazole, derivatization, eugenol, UV

INTRODUCTION

Eugenol [4-allyl-2-methoxyphenol], a phenolic constituent in clove (*Syzygium aromaticum*) and cinnamon (*Cinnamomum cassia*),[1,2] is widely used as a flavoring agent for baked foods and beverages as well as in dentistry for its analgesic properties.[3] Eugenol possesses a wide range of activities, including antioxidant,[4] anti-inflammatory,[5] anti-histaminic,[6] anti-anaphylactic,[7] and DNA-protective properties.[8]

Various methods for eugenol determination have been reported based on HPLC and GC with several detectors.[9–16] Beaudry et al. reported a lower limit of detection of eugenol of 0.5 pg injected onto the column

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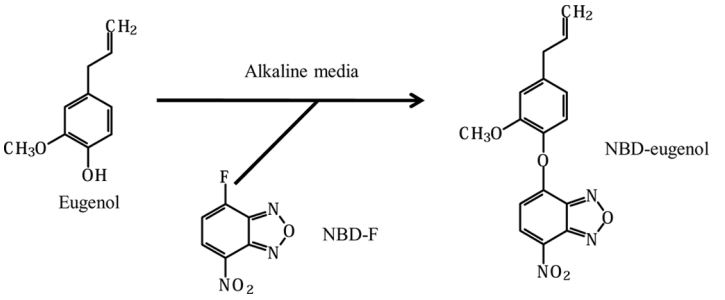

FIGURE 1 Scheme of eugenol derivatization with NBD-F.

(equivalent to 0.44 ng/mL plasma concentration) by using LC-electrospray quadrupole ion trap mass spectrometry after derivatization of eugenol with dansyl chloride.[9] However, this system is expensive and complicated. Derivatization with a UV-absorbing agent generally offers good selectivity and sensitivity, and an HPLC-UV method for assay of eugenol would be desirable for routine quality control, since the equipment is inexpensive, widely available, and easy to use.

4-Fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) has been used as a fluorescent labeling agent of primary and secondary amino groups for HPLC-fluorescence detection,[17–21] and has also been used as a UV-labeling reagent reactive with the phenolic hydroxyl group of *N*-acetyltyrosine and chlorophenols.[22,23] In this paper, we present a simple HPLC-UV method for determination of eugenol in clove and cinnamon oils after pre-column derivatization with NBD-F. The derivatization scheme is illustrated in Figure 1.

MATERIALS AND METHODS

Reagents

Eugenol (99%) was purchased from Sigma-Aldrich, Inc., (St. Louis, MO, U.S.A.). Clove oil (Lot No. M5R8724) and cinnamon oil (Lot No. M3B9370) were obtained from Nacalai Tesque (Kyoto, Japan). NBD-F and general reagents were obtained from Wako Pure Chemical Industries (Osaka, Japan).

Equipment

The HPLC system consisted of a model LC-10ATvp pump (Shimadzu, Kyoto, Japan), a Rheodyne injection valve (Cotati, CA, U.S.A.) with a 50-μL loop, and a model SPD-10Avp UV detector (Shimadzu) operating

at 380 nm. The HPLC column (C_{18}-MS-II, Nacalai Tesque) was 150 × 3.0 mm i.d., containing 5 μm particles of C_{18} packing material. Quantification of peaks was performed using a Chromatopac Model C-R8A integrator (Shimadzu). The mobile phase was prepared by the addition of acetonitrile (620 mL) to 380 mL of Milli-Q water containing trifluoroacetic acid (0.1 v/v%). The samples were eluted from the column at room temperature at a flow rate of 0.43 mL/min.

Derivatization

Ultrapure water was from a Milli-Q water purification system (Simplicity® UV, Millipore Corporation, Bedford, MA, U.S.A.). A standard solution of eugenol (100 mg) in methanol (50 mL) was prepared and stocked at 4°C. Working standard solutions (0, 0.2, 0.5, 1, 2, and 5 μg/mL) were prepared by dilution with 10% methanol. Borate buffer (0.1 M) was adjusted to pH 8.5 by the addition of NaOH. Borate buffer (100 μL) was added to a diluted standard sample (100 μL), and then NBD-F solution in acetonitrile (2 mg/mL, 100 μL) was added. The mixture was vortexed and allowed to react for 4 min at 40°C, then an aliquot (50 μL) was injected into the HPLC system.

Application to Clove and Cinnamon Oils, and Recovery Test

Clove and cinnamon oils (each 210 mg) were each dissolved in methanol (100 mL). The methanol solution (200 μL) of clove oil was 1000-fold diluted to 200 mL with 10% methanol to obtain test samples. The methanol solution (400 μL) of cinnamon oil was 500-fold diluted to 200 mL with 10% methanol. Derivatization of the diluted samples was performed, and the derivatives were analyzed as described previously. Addition-recovery tests were carried out to assess the accuracy of the method by spiking each oil solution with eugenol (80.0 and 160 mg of eugenol for clove oil; 4.00 and 8.00 mg of eugenol for cinnamon oil).

$$\text{Recovery value (\%)} = \frac{(\text{Total amount after spiking}) - (\text{Spiked amount})}{(\text{Original amount})} \times 100$$

RESULTS AND DISCUSSION

Derivatization of Eugenol with NBD-F

For the time course study, the reaction time was set at 2, 4, 6, 10, or 15 min at 40°C. Eugenol (100 μL, 2 μg/mL), borate buffer (100 μL, pH

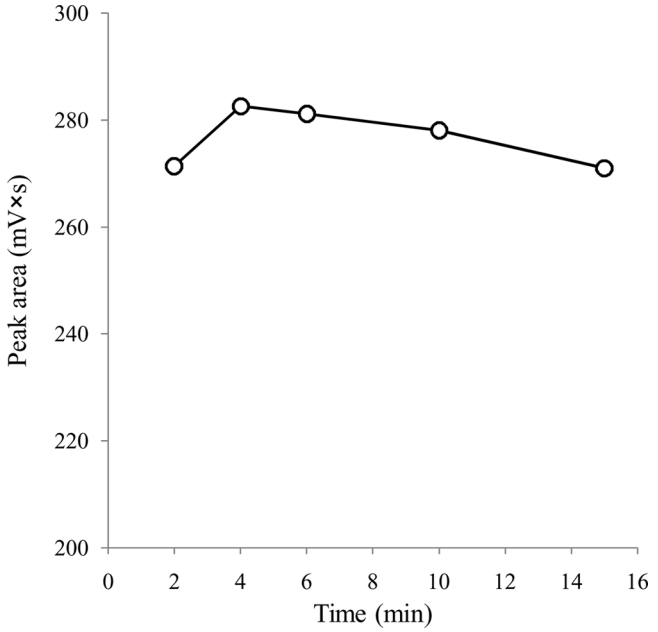

FIGURE 2 Time course of formation of the NBD derivative of eugenol Standard sample (2 μg/mL) was reached with NBD-F in borae buffer, pH 8.5, at 40°C.

8.5), and NBD-F (100 μL, 2 mg/mL) were mixed as described in Materials and Methods. The derivatization of eugenol reached a plateau at 4 min (Figure 2).

Next, pH dependency (pH 7.5 to 9.5) was examined at the derivatization time of 4 min at 40°C. Peak area of NBD-eugenol was maximal at pH 8.5 (Figure 3). Thus, the derivatization time of 4 min at pH 8.5 was selected.

Chromatogram

Figure 4 shows typical chromatograms obtained from (A) blank, (B) standard sample (2 μg/mL), (C) test sample of clove oil (2.1 μg/mL), and (D) test sample of cinnamon oil (4.2 μg/mL). The retention time of NBD-eugenol was 12.1 min. A peak of NBD-eugenol was observed in the two test samples.

Standard Curve of Eugenol

A standard curve was constructed by plotting integrated peak area vs. concentration of eugenol. The plot was linear ($y = 142.6x + 1.222$) in the range of 0.2 to 5 μg/mL with an r^2 value of 0.9976. The values of the lower

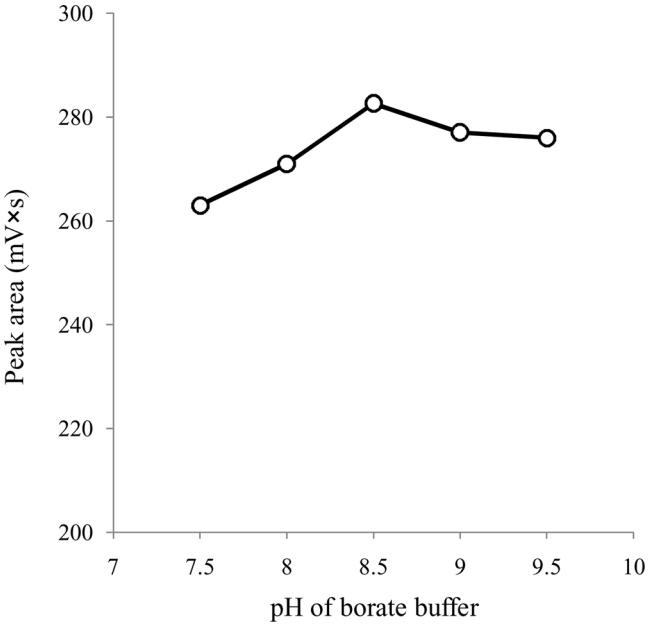

FIGURE 3 pH dependency of formation of the NBD derivative of eugenol Standard sample (2 μg/mL) was reacted with NBD-F for 4 min at 40°C in borae buffer a various values of pH.

limits of quantification and detection were 0.13 (signal-to-noise ratio of 10:1) and 0.04 μg/mL (signal-to-noise ratio of 3:1), respectively. While the sensitivity is about 3-fold greater than that of a previous HPLC method with a diode array detector,[10] it is 12.5- to about 90-fold inferior to other reported methods.[9,13]

Precision and Accuracy

Precision and accuracy for intra-day and inter-day assays of eugenol are shown in Table 1. In the intra-day assay, the range of standard deviation was within 2.9 to 4.9% of the mean, and recoveries were within the range of 91.5 to 102.8%. In the inter-day assay, the range of standard deviation was within 3.6 to 6.7% of the mean, and recoveries were within the range of 89.5 to 104.2%.

Analysis of Plant Oils

The described method was used to determine eugenol in clove and cinnamon oils and in samples spiked with standards. As shown in Table 2,

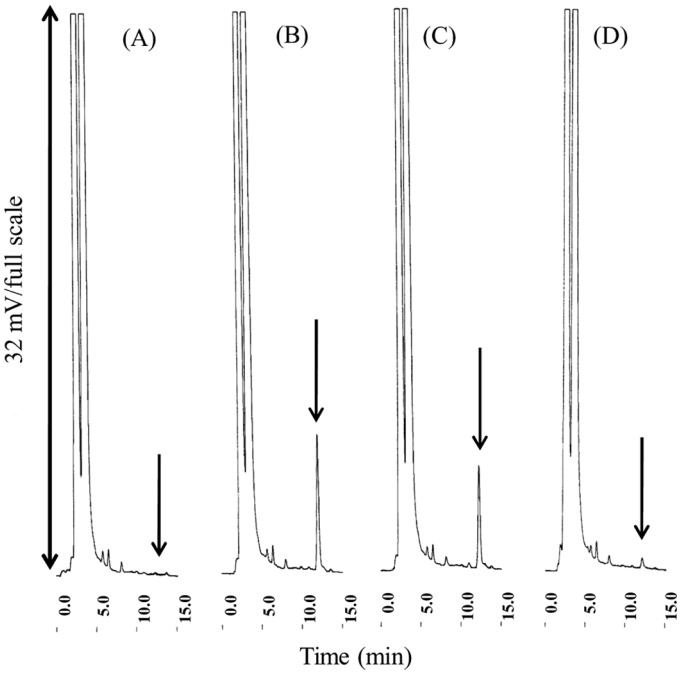

FIGURE 4 Typical chromatograms of blank (A), standard sample (B, 2 μg/mL), clove oil sample (C, 2.1 μg/mL), and cinnamon oil sample (D, 4.2 μg/mL) after derivatization with NBD-F. Samples were reacted with NBD-F for 4 min in borate buffer, pH 8.5, a 40°C. Retention time of NBD-eugenol: 12.1 min (arrowed peak).

the contents (w/w%) of eugenol in clove and cinnamon oils were $80.6 \pm 2.9\%$ (range, 77.6 to 84.4%) and $3.97 \pm 0.20\%$ (range, 3.79 to 4.22%), respectively. Recovery values of clove and cinnamon oil samples spiked with eugenol were $94.7 \pm 3.0\%$ (range, 89.8 to 98.8%) and $91.4 \pm 2.9\%$ (range, 86.9 to 94.5%), respectively.

TABLE 1 Intra- and Inter-day Assay Reproducibility for Determination of Eugenol

Eugenol (μg/mL)	Measured (μg/mL, Mean ± S.D., $n = 5$)	C.V. (%)	Recovery (%)
Intra-day assay			
0.2	0.183 ± 0.009	4.9	91.5
1	1.02 ± 0.04	3.9	102.0
5	5.14 ± 0.15	2.9	102.8
Inter-day assay			
0.2	0.179 ± 0.012	6.7	89.5
1	1.04 ± 0.06	5.8	104.0
5	5.21 ± 0.19	3.6	104.2

TABLE 2 Level of Eugenol in Clove and Cinnamon Oils and the Addition-Recovery

Compound	Content (w/w%)	Recovery (%)	
Clove oil		Added (80.0 mg)	Added (160 mg)
Day 1	77.6%	94.4	98.8
Day 2	79.5%	93.2	97.6
Day 3	80.9%	92.2	95.2
Day 4	84.4%	89.8	96.4
Ave. ± S.D. RSD (%)	80.6 ± 2.9% (3.6%, $n = 4$)		94.7 ± 3.0% (3.2%, $n = 8$)
Cinnamon oil		Added (4.00 mg)	Added (8.00 mg)
Day 1	4.22%	92.0	92.6
Day 2	4.06%	93.4	94.5
Day 3	3.79%	87.2	93.3
Day 4	3.82%	86.9	90.9
Ave. ± S.D. RSD (%)	3.97 ± 0.20% (5.0%, $n = 4$)		91.4 ± 2.9% (3.2%, $n = 8$)

CONCLUSION

We have developed a simple HPLC-UV method for determination of eugenol in clove and cinnamon oils by using NBD-F as a labeling reagent. Clove and cinnamon oils were found to contain eugenol (mean 806 mg/g and 39.7 mg/g, respectively) using the present system. This system is simple and convenient, and should be suitable for routine quality assessment of clove and cinnamon oils.

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