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ARTICLES

Determination of Three Phenylphenols in Grapefruit Juice by HPLC after Pre-Column Derivatization with 4-Fluoro-7-Nitro-2,1,3-Benzoxadiazole[1]

Yasuhiko Higashi* and Youichi Fujii

Department of Analytical Chemistry, Faculty of Pharmaceutical Sciences, Hokuriku University Ho-3, Kanagawa-machi, Kanazawa, 920-1181 Japan

**e-mail: y-higashi@hokuriku-u.ac.jp*

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Abstract—*o*-Phenylphenol is generally utilized as a disinfectant for citrus fruits. The purpose of this study is to develop a high-performance liquid chromatography coupled with ultraviolet detection (380 nm) method for simultaneous determination of *o*-phenylphenol, and its analogues (*m*-phenylphenol and *p*-phenylphenol) in grapefruit juice after pre-column derivatization with 4-fluoro-7-nitro-2,1,3-benzoxadiazole (**NBD-F**). 2-Hydroxyfluorene was used as an internal standard (**IS**). Standard curves were obtained after derivatization with NBD-F in borate buffer (pH 8.0) at room temperature for 5 min. The three NBD-F derivatives were almost completely separated on a Cholester column (5 μm, 3.0 mm i.d. × 150 mm). Calibration plots were linear in the range of absolute amount of 1.04 ~ 2.08 to 41.6 ng/50 μL injection volume, with r^2 values ≥0.9981, for the three compounds. The lower limits of detection were 0.3 to 0.7 ng/50 μL injection volume (signal-to-noise ratio of 3 : 1). The coefficients of variation were less than 11.1%. After extraction of grapefruit juice (2.0 mL) with *n*-pentane, the level of *o*-phenylphenol in the juice was estimated to be 20.2 ± 2.0 ng/mL ($n = 6$, mean ± SD), while *m*-phenylphenol and *p*-phenylphenol were below the lower limits of quantification. The recovery values of the three phenylphenols from samples spiked with a standard mixture of authentic compounds and IS were satisfactory (99.1 to 118.7%).

Keywords: phenylphenol, 4-fluoro-7-nitro-2,1,3-benzoxadiazole, high-performance liquid chromatography (HPLC), cholester column, grapefruit juice

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o-Phenylphenol (**OPP**) has bactericidal and virucidal activities, and is widely used in households, industry, and hospitals to disinfect surfaces, in addition to being utilized as a preservative in cosmetics, plastics, etc. [1, 2]. OPP exhibits low acute toxicity in animal experiments [3]. The Japanese government approved its use as a food additive only for citrus fruits in 1977 with the permitted maximum residue level of 10 mg/kg in whole fruits [4, 5]. The WHO view on the toxicity of OPP is as follows [6] "A health-based value of 1 mg/L can be calculated for OPP on the basis of an ADI of 0.4 mg/kg of body weight, based on a NOAEL of 39 mg/kg of body weight per day in a 2-year toxicity study for decreased body weight gain and hyperplasia of the urinary bladder and carcinogenicity of the urinary bladder in male rats, using an uncertainty factor of 100. Because of its low toxicity, however, the health-based value derived for OPP is much higher than OPP concentrations likely to be found in drinking-water. Under usual conditions, therefore, the presence of OPP in drinking-water is unlikely to represent a hazard to human health."

To improve the selectivity and sensitivity of methods for determination of various analytes, derivatization with an ultraviolet (**UV**)-absorbing or fluorescent agent is one of the most useful techniques, and may make sample clean-up unnecessary. Yang et al. [5] developed a highly sensitive method of OPP determination by HPLC with electrochemical detection, using a microbore column; this afforded a limit of detection of 3.4 pg. GC–mass spectrometric methods for determination of OPP after derivatization with pentafluorobenzoyl bromide and ferrocenecarboxylic acid chloride were applied to beer and citrus fruit samples, respectively [1, 2]. On the other hand, the WHO does not set guideline values for two positional isomers of OPP, *m*-phenylphenol (**MPP**) and *p*-phenylphenol (**PPP**), and, to our knowledge, only one method has been reported for assay of MPP or PPP [7].

4-Fluoro-7-nitro-2,1,3-benzoxadiazole has been used as a fluorescent labeling agent for primary and secondary amino groups for HPLC–fluorescence de-

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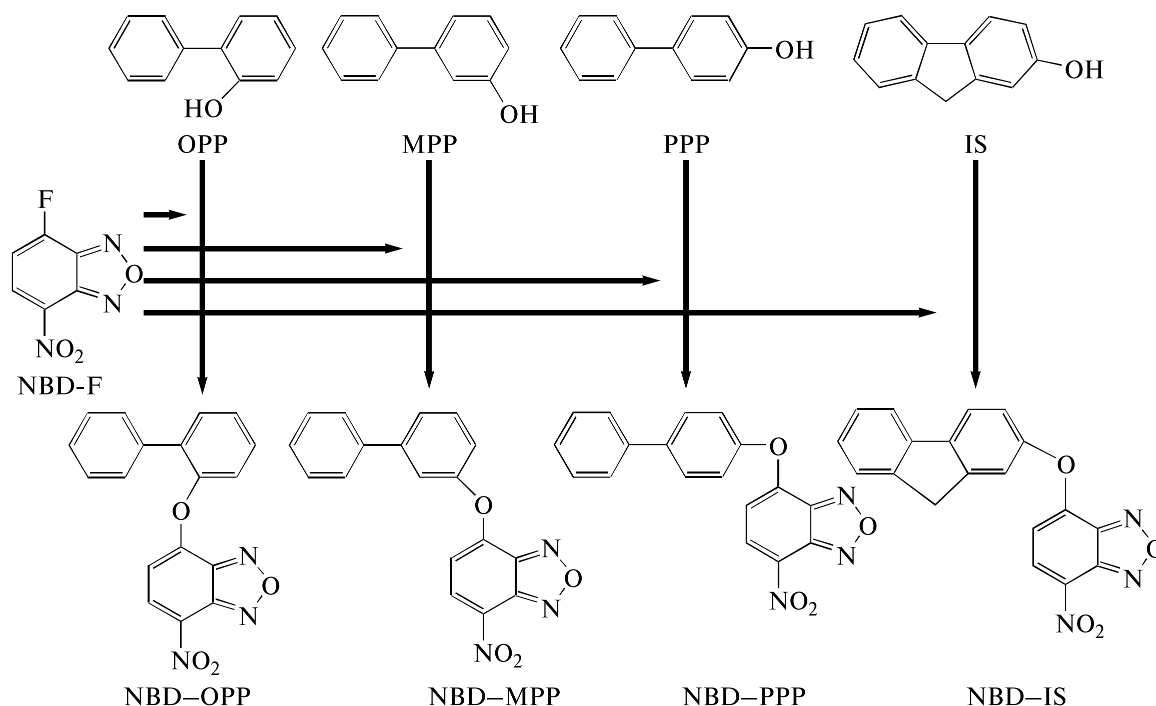

Fig. 1. Scheme illustrating the pre-column NBD-F derivatization of OPP, MPP and PPP in grapefruit juice, as well as the IS.

tection [8–12]. It has also been used as a UV-labeling reagent reactive with the phenolic hydroxyl group of N-acetyltyrosine [13]. In addition, we have previously developed methods employing HPLC–UV after derivatization with NBD-F for the determination of phenolic compounds such as chlorophenols and eugenol [14, 15].

In this paper, we present a simple HPLC–UV method for simultaneous determination of three phenylphenols (**PPs**) (OPP, MPP and PPP) in grapefruit juice after pre-column derivatization with NBD-F. The derivatization scheme is shown in Fig. 1.

EXPERIMENTAL

Chemicals and reagents. OPP and PPP were purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). MPP and NBD-F was obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo). 2-Hydroxyfluorene, used as IS, was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). *n*-Pentane and other general reagents were obtained from Wako Pure Chemical Industries (Osaka, Japan). Grapefruit juice was bought at a market in Kanazawa City, Ishikawa Prefecture, Japan.

Chromatographic system. The HPLC system consisted of a model LC10-AT*vp* pump (Shimadzu, Kyoto, Japan), a Rheodyne injection valve (Cotati, CA, USA) with a 50-μL loop, and a model SPD-10A*vp* UV detector (Shimadzu) operating at 380 nm. The HPLC column was a Cholester column (Nacalai tesque, Kyoto), 150 × 3.0 mm i.d., containing 5 μm particles. Quantification of peaks was performed using a Chromatopac Model C-R8A integrator (Shimadzu). The mobile phase was prepared by the addition of acetonitrile (600 mL) to 400 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.

Preparation of standard solutions. Ultrapure water was from a Milli-Q water purification system (Simplicity® UV, Millipore Corporation, Bedford, MA, USA). Standard samples of the three PPs were dissolved in methanol to obtain concentrations of 1 mg/mL. Standard mixtures (each 0, 0.0833, 0.167, 0.333, 0.833, 1.67 and 3.33 μg/mL) were prepared by dilution as required with Milli-Q water.

Derivatization. Borate buffer (0.1 M) was adjusted to various pH values by the addition of NaOH. Borate buffer (100 μL) was added to mixtures of diluted standard samples (100 μL) and IS solution (100 μL, 1 μg/mL). Then, NBD-F solution in acetonitrile (2 mg/mL, 100 μL) was added. The mixture was vortexed and allowed to react at room temperature, then an aliquot (50 μL, absolute amount of 0 to 41.6 ng each) was taken and injected into the HPLC system.

Application to grapefruit juice samples. A mixture of grapefruit juice (2.0 mL), a standard mixture of analytes (100 μL, 0, 0.0833, 0.167, 0.333, 0.833, 1.67 and 3.33 μg/mL each), HCl (100 μL, 0.1 M), and IS (100 μL, 1 μg/mL) was extracted with *n*-pentane (4 mL, twice). The organic layers were combined and

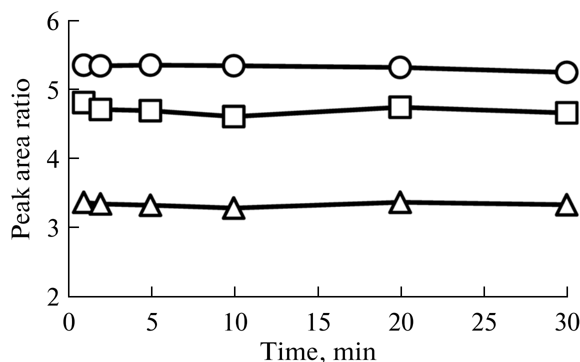

Fig. 2. Time courses of formation of NBD-F derivatives of OPP, MPP and PPP. Standard samples (each 3.33 μg/mL) were reacted with NBD-F in borate buffer at pH 8.0 at room temperature. (○) – OPP, (Δ) – MPP, (□) – PPP.

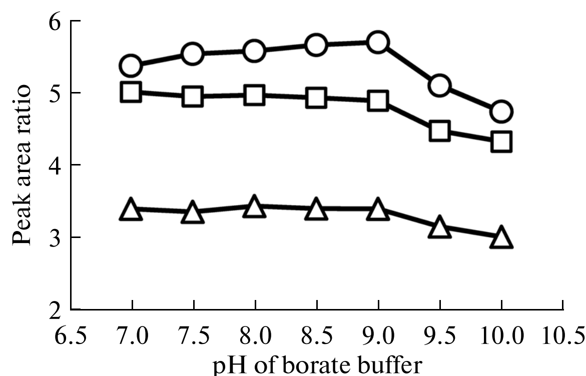

Fig. 3. pH Dependency of the formation of NBD-F derivatives of OPP, MPP and PPP. Standard samples (each 3.33 μg/mL, 41.6 ng/injection) were reacted with NBD-F for 5 min in various borate buffers at room temperature. (○) – OPP, (Δ) – MPP, (□) – PPP.

evaporated. After the addition of water (200 μL) to the residue, derivatization was performed in the same manner as described above and the products were analyzed.

Evaluation of relative recovery. Relative recovery was expressed as the ratio of the slope of the calibration curve prepared from a grapefruit juice sample spiked with the standard sample to that of the standard calibration curve prepared as described above. Relative recovery data were used to assess the accuracy of the method.

RESULTS AND DISCUSSION

Reaction time courses of the three PPs with NBD-F. For the time course study, the reaction time was set at 1, 2, 5, 7, 10 and 20 min (Fig. 2). PP (100 μL, each 3.33 μg/mL), borate buffer (100 μL, pH 8.0), and NBD-F (100 μL, 2 mg/mL) were added to IS solution (100 μL), and each solution was left to stand for the appropriate time. Derivatives of the three PPs and IS reached a maximum at 5 min, while the peak area ratios of the three PPs almost reached a plateau at 1 min (Fig. 2). Thus, the derivatization time of 5 min was selected.

pH dependency of derivatization of the three PPs with NBD-F. pH dependency (pH 7.0 to 10.0) was examined at the derivatization time of 5 min (Fig. 3). The peak area ratio of derivatives showed little variation in the range of pH 7.0 to 9.0. However, the peak area ratios of the three derivatives at pH 9.5 and 10.0 were markedly reduced. Therefore, pH 8.0 was selected for the derivatization buffer.

Chromatograms. Figure 4 shows typical chromatograms obtained from blank (A), a standard mixture (each 20.8 ng) (B), and a grapefruit juice sample (C) after derivatization with NBD-F. The retention times of NBD–OPP, NBD–MPP, NBD–PPP, and NBD–IS were 14.1, 17.4, 19.1 and 23.2 min, respectively. The resolution value between the NBD–MPP and NBD–PPP peaks was 1.32 using the Cholester column. Our preliminary tests showed that the value using the Cholester column was higher than those obtained with C_{18}-MS-II (0.91) and C_{22}-AR-II (0.99) columns (Nakalai tesque, 150 × 3.0 mm i.d., containing 5 μm particles) under the same HPLC conditions. On the other hand, the values were more than 1.5 between NBD–OPP and NBD–MPP and between NBD–PPP and NBD–IS using all three tested columns (i.e., complete separation). Thus, the Cholester column was found to be more useful for simultaneous determination of the analytes. As shown in Fig. 4c, the peaks of NBD–OPP and NBD–IS was clearly detected, and the OPP level in grapefruit juice was estimated as described later (see Determination of the three PPs in grapefruit juice), whereas NBD–MPP and NBD–PPP were below the lower limit of quantification.

Standard curves of the three PPs. The standard curves of the three PPs were constructed by plotting integrated peak area ratios to IS area *vs.* absolute amount (ng/50 μL injection volume). The calibration data are summarized in Table 1. All the plots were linear in the range of 1.04 ~ 2.08 to 41.6 ng, with r^2 values E0.9981. The values of the lower limit of quantification were taken as the lowest concentration on the standard curve, and the lower limits of detection were 0.3, 0.7, and 0.5 ng/50 μL injection volume for OPP, MPP and PPP, respectively (signal-to-noise ratio of 3 : 1). The limit of detection of OPP (0.3 ng) represents only moderate sensitivity compared with previous reports (range of 3.4 to 350 pg) [4, 5, 16, 17]. The sensitivity of MPP (0.7 ng) is reported for the first time in the present study, while that of PPP (0.5 ng) was improved over the previous value [7].

Precision and accuracy. Precision and accuracy for intra-day and inter-day assays of these derivatives are shown in Table 2. In the intra-day assay, the range of standard deviation was within 1.5 to 7.4% of the mean.

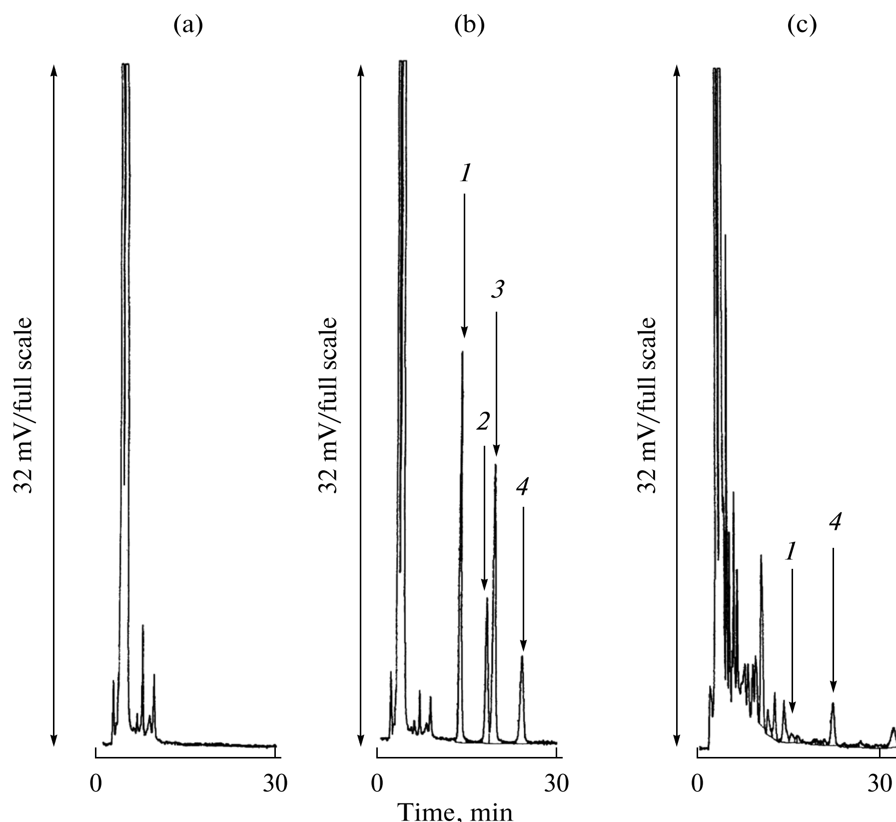

Fig. 4. Typical chromatograms of blank (a), standard sample (b) and grapefruit juice sample (c) after derivatization with NBD-F. Standard samples were reacted with NBD-F for 5 min at pH 8.0 at room temperature. Compounds: three PPs, each 20.8 ng/50 μL injection volume; IS, 12.5 ng/50 μL injection volume. Retention time, min: 14.1, NBD–OPP (*1*); 17.4, NBD–MPP (*2*); 19.1, NBD–PPP (*3*); 23.2, NBD–IS (*4*).

Recoveries were within the range of 90.1 to 98.6%. In the inter-day assay, the range of standard deviation was within 3.8 to 11.1% of the mean. Recoveries were within the range of 88.0 to 98.6%.

Determination of the three PPs in grapefruit juice. The described method was used to determine the levels of the three PPs in grapefruit juice spiked or not spiked with authentic standards. The level of OPP in grapefruit juice was found to be 20.2 ± 2.0 ng/mL (mean ± SD, $n = 6$, range from 16.4 to 22.3 ng/mL), while the levels of MPP and PPP in grapefruit juice were both below the lower limit of quantification. It was found that the OPP level in the juice was much less than 10 mg/kg, the maximum permitted level in Japan.

Calibration curves prepared from grapefruit juice samples spiked with three PPs showed linear relationships between absolute amount and peak area ratio, with r^2 0.9932, and the relative recovery values of OPP, MPP, and PPP were 118.7 ± 10.6% (range, 108.7 to 120.8%), 99.1 ± 5.3% (range, 91.6 to 104.4%), and 102.1 ± 5.6% (range, 96.8 to 108.2%). These results indicate that our method is capable of monitoring grape-

Table 1. Linear correlation parameters

Compound	Slope	Intercept	Range*	r^2	Lower limit of detection*, **
OPP	0.133	+0.0118	1.04 to 41.6	0.9982	0.3
MPP	0.0771	+0.0265	2.08 to 41.6	0.9981	0.7
PPP	0.119	+0.0282	2.08 to 41.6	0.9985	0.5

* ng/50 μL injection volume.
** Signal-to noise ratio of 3.

Table 2. Intra- and inter-day assay reproducibility for determination of three PPs

Compound, ng		Measured, ng (mean ± SD, $n = 5$)	CV, %	Recovery, %
Intra-day				
OPP	4.16	3.75 ± 0.20	5.3	90.1
	41.6	40.1 ± 1.1	2.7	96.4
MPP	4.16	3.80 ± 0.28	7.4	91.3
	41.6	41.0 ± 0.6	1.5	98.6
PPP	4.16	3.86 ± 0.15	3.9	92.8
	41.6	40.6 ± 0.7	1.7	97.6
Inter-day				
OPP	4.16	3.69 ± 0.41	11.1	88.7
	41.6	40.3 ± 2.5	6.2	96.9
MPP	4.16	3.66 ± 0.38	10.4	88.0
	41.6	41.0 ± 3.2	7.8	98.6
PPP	4.16	3.74 ± 0.26	7.0	89.9
	41.6	40.0 ± 1.5	3.8	96.2

fruit juice for contamination with OPP, MPP, and PPP.

CONCLUSION

HPLC–UV method was developed using a Cholester column for simultaneous determination of OPP, MPP and PPP, with NBD-F as a pre-column UV-labeling reagent. The OPP level in grapefruit juice was determined by means of this assay after *n*-pentane extraction; on the other hand, MPP and PPP were below the lower limit of quantification. The presented system is simple and suitable for routine testing of grapefruit juice for contamination with the three PPs.

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