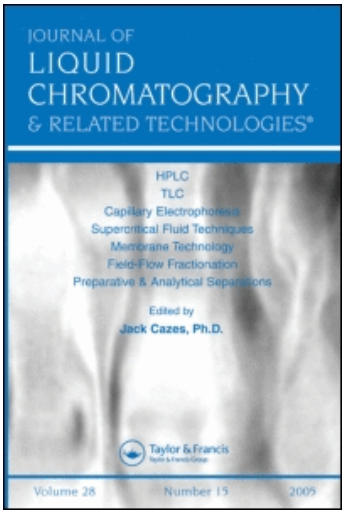

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF FUNGICIDES IN CITRUS FRUITS

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ABSTRACT

The determination of three common citrus fungicides, diphenyl (DP), o-phenylphenol (oPP) and thiabendazole (TBZ), was investigated with reversed-phase high-performance liquid chromatography. DP and oPP were successfully chromatographed and quantitated by utilizing a reversed-phase Unisil Q C_{18} column after extraction with an essential oil distillation apparatus. An acetonitrile-0.1M H_3PO_4 (55:45) mobile phase was used. TBZ was chromatographed by using a Unisil Q C_8 column with a mobile phase of acetonitrile-0.1M H_3PO_4 (80:20) after the extraction with ethyl acetate. The fungicides were detected with fluorescence detection at typical residue levels on citrus. The

practical quantitation limits of DP, oPP and TBZ were 0.1, 0.05, and 0.05 mg/kg, respectively. The recoveries were between 98.0 and 100 %.

INTRODUCTION

DP, oPP and TBZ are used as fungicides in citrus fruits. The prevailing standard analytical method, gas chromatography, is used for determining DP and oPP individually after reflux extraction with an essential oil distillation apparatus and clean-up. To determine TBZ, a fluorescence method or gas chromatography is utilized after its extraction with ethyl acetate and clean-up (1, 2). However, the recovery of DP is reduced when the sample solution needs to be concentrated and interferring peaks of citrus constituents are observed on the gas chromatogram for DP (Fig. 1).

Recently, chromatographic techniques for the determination of DP, oPP and TBZ have been reported by high-performance liquid chromatography (HPLC) (3-7). These solvent extraction-HPLC methods are simple, and paved the way for the simultaneous determination of these three kinds of fungicides. However, these HPLC methods gave lower analytical values than those which were measured by the prevailing reflux extraction-gas chromatography method on DP and oPP in many cases. Thus, another sensitive and no-interfering HPLC method is proposed for the determination of DP, oPP and TBZ.

EXPERIMENTAL

A. Apparatus

A Shimadzu LC-3A liquid chromatograph (Shimadzu Seisakusho Co. Ltd., Kyoto, Japan) with a syringe pump was used. Sample injection was performed by a Rheodyne injector (Model 7125) with a 20 µl loop. The detector was a Hitachi fluorescence

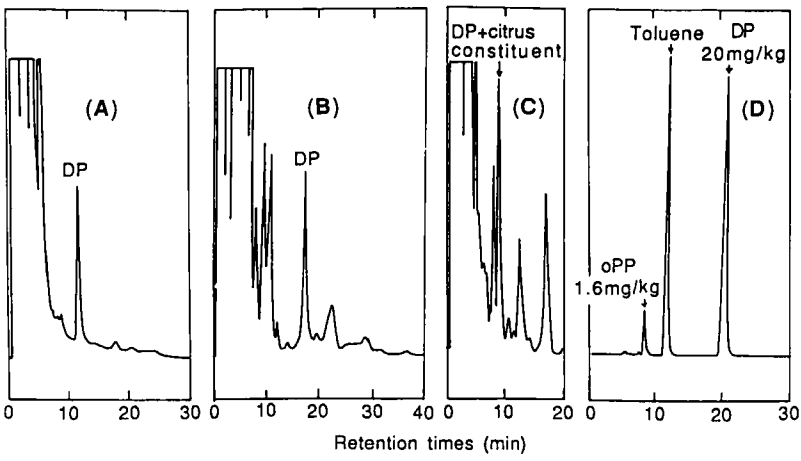

Figure 1. Gas chromatograms (A, B and C) from analysis of 50 g of lemon, using Shimadzu gas chromatograph GC-7AG with FID.

Column: (A) 5% Diethylene glycol succinate, 1% phosphoric acid on Chromosorb W (AW), 60-80 mesh, 2 m x 3.0 mm i.d.. (B) 5% Polyethylene glycol 20M on Chromosorb W (AW), 60-80 mesh, 2 m x 3.0 mm i.d.. (C) 10% Silicone SE-30 on Chromosorb W (AW, DMCS), 80-100 mesh, 2 m x 3.0 mm i.d..; Oven temperature: (A) 120°C, (B) 165°C and (C) 150°C.; Flow rate: 50 mL/min (N_2 gas).; Sample size: 5 µL.

(D) High-performance liquid chromatogram of DP and oPP in lemon. Analytical column-Unisil Q C18 (5µ, Gaskuro Kogyo), 250 x 4.0 mm i.d., guard column-Unisil Q C18 (5µ), 50 x 4.0 mm i.d., mobile phase-acetonotrile-0.1M H_3PO_4 (55:45), Flow rate-1.0 mL/min, column temperature-40°C, detection-Fluorescence, Ex-245nm, Em-330nm, injection-10µL.

spectrophotometer (Hitachi Co. Ltd., Model F-3000 with a 150 watt xenon lamp and LC microflow cell unit with a cell volume of 18 µl.) The following additional equipment was also used: an essential oil distillation apparatus as defined in the Pharmacopoeia of Japan of 1986, juicer (Mitsubishi Electric Co. Ltd., Model JM-1200), autohomogenizer with stainless steel cup (EXCEL, Nihon Seiki Ltd., Tokyo, Japan), and rotary vacuum

TABLE 1
HPLC Conditions for Three Fungicides

HPLC conditions	DP and oPP	TBZ
Analytical column	Unisil Q C_{18} (5 μ) 250 x 4 mm	Unisil Q C_8 (5 μ) 250 x 4 mm
Guard column	Unisil Q C_{18} (5 μ) 50 x 4 mm	Unisil Q C_8 (5 μ) 50 x 4 mm
Mobile phase	55:45 Acetonitrile/0.1M H_3PO_4	80:20 Acetonitrile/0.1M H_3PO_4
Column temperature	40°C	40°C
Flow rate	1.0 mL/min	1.0 mL/min
Fluorescent λ's	245 (Ex); 330 (Em)	302 (Ex); 350 (Em)
Sample size	10 μL	10 μL

evaporator (Tokyo Rikakikai Co. Ltd., Model N-2, Tokyo, Japan). HPLC conditions for the three fungicides are shown in Table 1.

A Shimadzu gas chromatograph GC-7AG with a flame ionization detector (FID) was also used.

B. Reagents

All solvents and chemicals were reagent grade. DP, oPP, phosphoric acid, sodium chloride, toluene and anhydrous disodium hydrogen phosphate were from Wako Pure Chemical (Osaka, Japan). Acetonitrile, 2-ethoxyethyl acetate and ethyl acetate were from Kanto Chemical (Tokyo, Japan). TBZ was from Tokyo Kasei Kogyo Co. Ltd..

A standard solution for the determination of DP and oPP is prepared as follows: 0.1000 g each of DP and oPP is weighed in one 100 mL volumetric flask and dissolved with acetonitrile. The primary standard solution is diluted to prepare the solutions containing 0.5, 1.0, 2.0 and 5.0 μg DP and oPP/mL, respectively. A standard solution for the determination of TBZ is also prepared: 0.1000 g TBZ is weighed in one 100 mL volumetric flask and dissolved with methanol. The primary standard solution is diluted to 0.5, 1.0, 2.0 and 5.0 μg TBZ/mL as above.

C. Preparation of sample extracts

A representive sample containing 5-10 individual fruit is obtained. Each fruit is sliced into 16 parts. Randomly, 20 to 40 slices are selected and homogenized with the juicer.

For the preparation of sample extracts of DP and oPP, 50 g of homogenized mixture is poured into a 1000 mL Kjeldahl short neck flask containing 200 mL of water. 50 g of sodium chloride, 2 mL of phosphoric acid, 2-3 drops of silicone oil and 5-6 boiling stones are added the flask. The flask is then connected to the essential oil distillation apparatus (extractor). Appropriate amounts of water are added to the extractor and 3 mL of toluene is added slowly, and then a reflux condenser is attached. The sample is refluxed for 2 hours by heating intensely with a gas burner. After cooling, the organic layer in the extractor is separated and made up to 50 mL with acetonitrile. The prepared solution is mixed and filtered through a 0.45 μ filter (Fluoropore FP-45, Sumitomo Electric Ind. Ltd., Osaka Japan).

For the preparation of sample extracts of TBZ, 10 g of homogenated mixture is weighed into a blender cup. 3 g of anhydrous Na_2HPO_4, 10 g of anhydrous Na_2SO_4 and 80 mL of ethyl acetate are added and then extracted by homogenizing for 10 min at 7000 rpm. The supernatant is filtered through filter paper (No. 5B, Toyo Rosshi Kaisha Ltd., Tokyo, Japan) into a 300 mL round bottom flask. The precipitate is homogenized twice with additional 80 mL aliquots of ethyl acetate and then filtered. 5 mL of 2-ethoxyethyl acetate is added to the combined ethyl acetate extracts and the mixture is concentrated to about 5 mL on a rotary vacuum evaporator (40°C water bath). The concentrated solution is transfered quantitatively to a 20 mL volumetric flask and brought to volume with methanol. The solution is mixed and then filtered through a 0.45 μ filter (FM-45. Fuji Photo Film Co. Ltd., Tokyo, Japan).

RESULT AND DISCUSSION

Although the peak of oPP was not completely separated from that of toluene with the Unisil Q C8 column, a Unisil Q C18 column

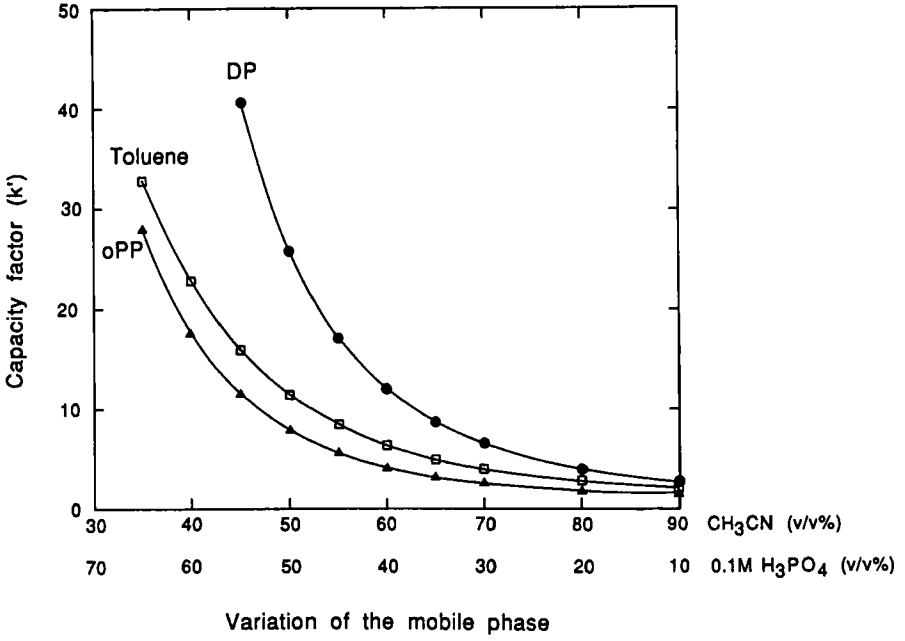

Figure 2. Variation of capacity factor k' for DP, oPP and toluene as a function of the mobile phase.

separated these peaks satisfactorily. Hence, the Unisil Q C18 column was adopted to determine DP and oPP.

Figure 2 represents the capacity factor (k') of DP, oPP and toluene as a function of the mobile solvent. Optimization of eluent composition within thirty minutes gives a solvent ratio of 55:45 (acetonitrile-0.1M H_3PO_4). The peaks of citrus constituents and that of oPP were thoroughly separated by the eluent composition.

From the above experiments and previous report (7), practical applications were studied for the separation of fungicide mixtures on Hassaku oranges.

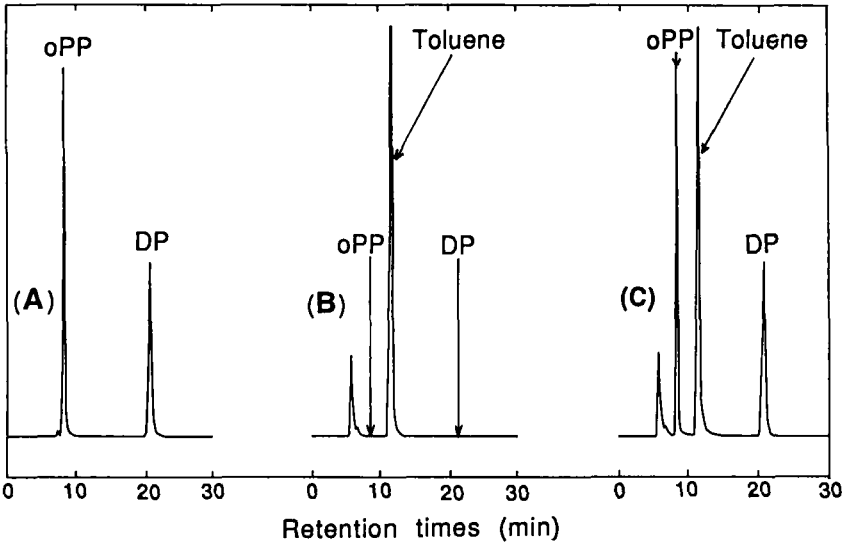

Figure 3. High-performance liquid chromatograms of DP and oPP. (A) Chromatogram of 5 mg/mL each of DP and oPP standard solution. (B) Chromatogram of the extract of Hassaku orange (fungicide-free). (C) Chromatogram of the extract of Hassaku orange in which 5 mg/kg DP and oPP was added. RP-HPLC conditions: Analytical column-Unisil Q C18 (5 μ, Gasukuro Kogyo), 250 x 4.0 mm i.d. Guard column-Unisil Q C18 (5 μ), 50 x 4.0 mm i.d., Mobile phase-acetonitrile-0.1M H_3PO_4 (55:45). Flow rate-1.0 mL/min. Column temperature-40°C. Detection-Fluorescence, Ex-245 nm, Em-330 nm. Injection-10 μl.

Figure 3(A) is a chromatogram of 5 μg/mL each of DP and oPP. Figure 3(B) is a chromatogram of extracted fungicide-free Hassaku oranges. Other untreated fruit (i.e. lemon, satsuma mandarin, kumquat, ponkan, Navel orange and lime) were analyzed to check the background interference from natural substances. No interfering peaks were noted for the determination of DP and oPP. Figure 3(C) is a chromatogram of 5 mg/kg of DP and oPP added to Hassaku oranges.

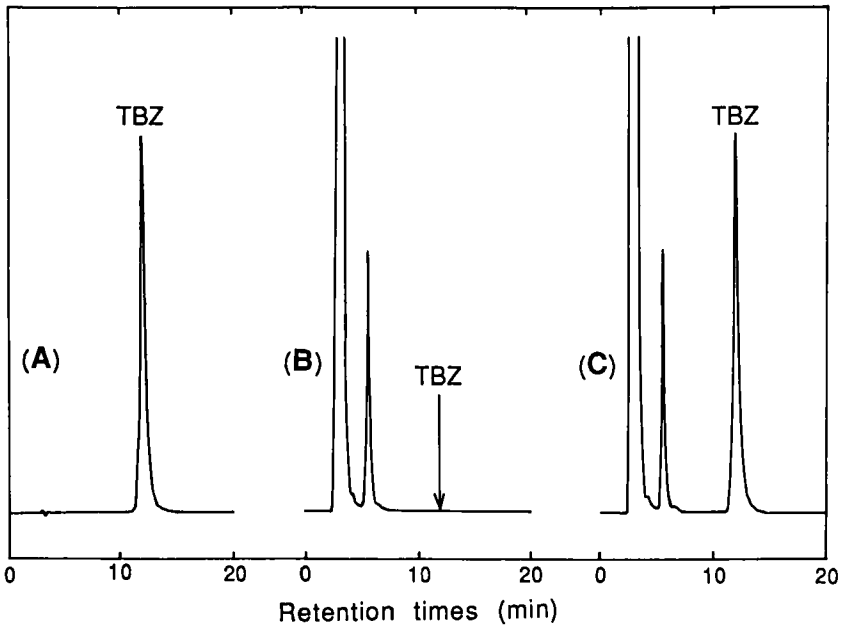

Figure 4. High-performance liquid chromatograms of TBZ.

(A) Chromatogram of 0.5 mg/mL TBZ standard solution. (B) Chromatogram of the extract of Hassaku orange (fungiside-free). (C) Chromatogram of the extract of Hassaku orange in which 1.0 mg/kg.TBZ was added. RP-HPLC conditions: Analytical column-Unisil Q C8 (5 μ Gasukuro Kogyo), 250 x 4.0 mm I. d. Guard column-Unisil Q C8 (5 μ), 50 x 4.0 mm i.d., Mobile phase-acetonitrile-0.1M H_3PO_4 (80:20). Flow rate-1.0 mL/min. Column temperature-40°C. Detection-Fluorescence, Ex-302 nm, Em-350 nm. Injection-10 μl.

Figure 4 (A) is a chromatogram of 0.5 μg/mL of TBZ. Figure 4 (B) is a chromatogram of fungicide-free Hassaku oranges. No peaks were noted to interfere with the determination of TBZ. Figure 4 (C) is a chromatogram of 1 mg/kg of TBZ added to Hassaku oranges.

Percent recoveries were determined when various concentrations of fungicides were added to Hassaku oranges (All Hassaku oranges used as a control were initially free from these

TABLE 2
Recovery and Coefficient of Variation (c.v.) of DP, oPP and TBZ from Hassaku Orange.

Spiked (mg/kg)	DP		oPP		TBZ	
	Recovery[a] (%)	C.V. (%)	Recovery[a] (%)	C.V. (%)	Recovery[a] (%)	C.V. (%)
0.5					99.3	1.9
1.0	99.1	0.7	99.9	0.6	98.3	1.4
2.0	100	0.8	99.1	0.5	99.1	1.9
5.0	100	0.7	99.2	0.7	98.5	1.4
10.0	100	0.4	99.1	0.9	98.6	1.5
20.0	100	0.7	99.0	0.7		
50.0	100	0.5	98.7	0.4		
100	100	0.3	98.0	0.6		

a) Average of five trials.

fungicides). Spiked levels of DP and oPP were 1.0, 2.0, 5.0, 10.0, 20.0, 50.0 and 100 mg/kg respectively. The levels of TBZ were 0.5, 1.0, 2.0, 5.0 and 10.0 mg/kg.

Table 2 represents recovery data. The recoveries were 98.0 to 100 % of added fungicides.

Figure 5. shows a high-performance liquid chromatogram of extracted oranges which were on the market.

Table 3 represents a comparison of fungicide concentration in citrus fruits determined by ordinary fluorescence or a gas chromatographic method and the proposed method. This table demonstrates that the proposed method gave almost the same analytical results compared with the results which were determined with the prevailing standard analytical method.

The proposed method is simple and sensitive for determining DP, oPP and TBZ in citrus fruits and showed excellent recoveries for these three fungicides.

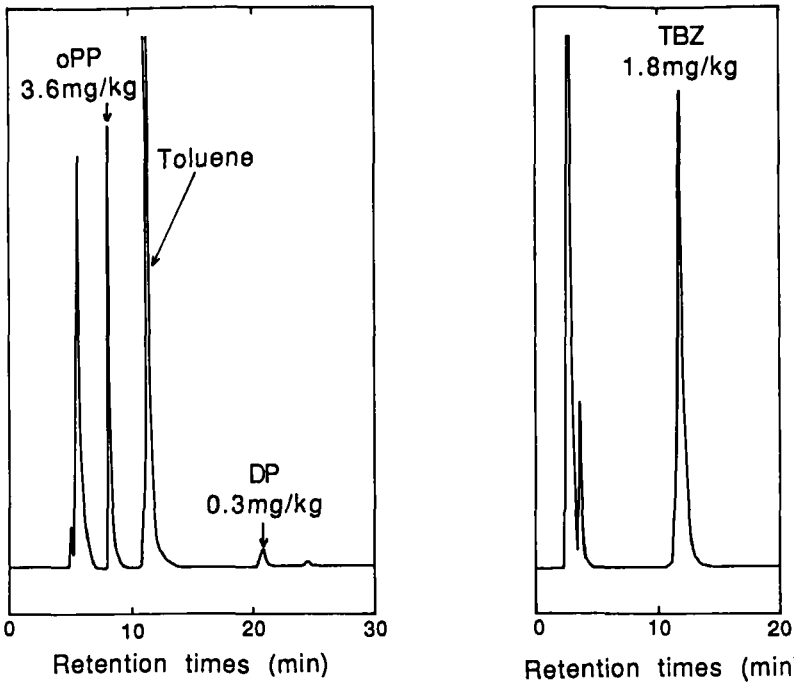

Figure 5. High-performance liquid chromatograms of extracted market oranges. (A) Analytical column-Unisil Q C18 (5 μ), 250 x 4.0 mm i.d., Guard column-Unisil Q C18 (5 μ), 50 x 4.0 mm i.d., Mobile phase-CH_3CN-0.1M H_3PO_4 (55:45). Flow rate-1.0mL/min. Column temperature-40°C. Detection-Fluorescence, Ex-245, Em-330 nm. Injection-10μL. (B) Column-Unisil Q C8 (5 μ), 250 x 4.0 mm i.d., Guard column-Unisil Q C8 (5 μ), 50 x 4.0 mm i.d., Mobile phase-CH_3CN-0.1M H_3PO_4 (80:20). Flow rate-I.0 mL/min. Column temperature-40°C. Detection-Fluorescence, Ex-302, Em-350 nm. Injection-10 μl.

TABLE 3
Comparison of Fungicide Concentration in Citrus Fruits Determined by Ordinary Fluorescence or Gas Chromatographic (GLC) Method and the Proposed Methods.

Sample	No.	DP		oPP		TBZ	
		GLC[a] (mg/kg)	HPLC[a] (mg/kg)	GLC[a] (mg/kg)	HPLC[a] (mg/kg)	Fluorescence[a] (mg/kg)	HPLC[a] (mg/kg)
Lemon	1	2.1	2.3	-[b]	-	-	-
	2	19[c]	20	1.5	1.6	0.5	0.5
	3	10[c]	10	2.0	2.1	0.3	0.3
	4	2.6	3.0	1.6	1.7	-	-
Orange	1	1.8	2.8	0.9	0.9	2.4	2.5
	2	18[c]	20	1.4	1.5	5.1	5.2
	3	41[c]	42	1.0	1.1	0.6	0.8
Grapefruit	1	0.2	0.6	-	-	1.1	1.2
	2	0.6	1.0	-	-	-	-
	3	trace	0.2	1.1	1.2	0.3	0.4
	4	12[c]	12	0.7	0.8	1.6	1.8

a) Average of three trials.
b) -; Less than 0.1mg/kg.
c) The sample solutions of DP were prepared without evaporation of the solvent.

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