Determination of Organochlorine Pesticides in Glycyrrhizae Radix

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The analytical method to determine organochlorine pesticides in natural medicines included in *The Japanese Pharmacopoeia* (JP), 15th edition,[1] is not adequate because the recovery rates of organochlorine pesticides in Glycyrrhizae radix are very low. In this study, we developed a method to analyze organochlorine pesticides in Glycyrrhizae radix with acceptable recovery rates. The method enables analysis of organochlorine pesticides in all natural medicines for which maximum residue levels are set.

Key words —— natural medicine, organochlorine pesticide, Glycyrrhizae radix, gas chromatography/mass spectrometry

INTRODUCTION

Various illnesses are treated with natural medicines. Natural medicines should be confirmed to be as safe as food because patients generally take natural medicines over the long term. In Japan, maximum residue levels (MRL) have been set for eight pesticides [α-benzene hexachloride (BHC), β-BHC, γ-BHC, δ-BHC (BHCs), p,p′-dichlorodiphenyldichloroethylene (DDE), o,p′-dichlorodiphenyltrichloroethane (DDT), p,p′-dichlorodiphenyldichloroethane (DDD), p,p′-DDT] in 13 natural medicines, including Glycyrrhizae radix (Table 1).[1] An analytical method using gas chromatography with electron capture detection (GC/ECD)[1] has been established based on a collaborative study.[2] This method is not adequate because the recovery rates of BHCs and DDTs in Glycyrrhizae radix are very low (between 41% and 69%).[2] Glycyrrhizae radix is an important natural medicine because it is imported in immense quantities (2000–10000 tons/year) and is frequently used in oriental medicines.[1] It is therefore important to ensure the safety of Glycyrrhizae radix. In this study, we developed a method to analyze BHCs and DDTs in Glycyrrhizae radix with acceptable recovery rates and good repeatability. We previously reported that GC/mass spectrometry (MS) with negative chemical ionization (NCI) analyzes organochlorine pesticides more selectively than GC/ECD.[3] GC/MS with NCI permits more sensitive analysis of BHCs and DDTs than GC/MS with electronic ionization mode (EI, data not shown). We therefore analyzed organochlorine pesticides using GC/MS with NCI in this study.

Table 1. 13 Natural Medicines for Which MRL Are Set

GLYCYRRHIZAE RADIX
GINSENG RADIX
SENNAE FOLIUM
ASTRAGALI RADIX
POLYGALAE RADIX
CINNAMONI CORTEX
ASIASARI RADIX
CORNI FRUCTUS
PERILLAE HERBA
ZIZYPHI FRUCTUS
AURANTII NOBILIS PERICARPIUM
ERIOBOTRYAE FOLIUM
MOUTAN CORTEX

MATERIALS AND METHODS

Pesticide Standards —— Pesticide standards were obtained from Wako Pure Chemical Industries (Osaka, Japan) and Riedel de Haën (Hannover, Germany). Each compound was dissolved in acetone to make 0.5 mg/ml of standard stock solution. Spiking solutions were prepared from standard stock solutions at concentrations of 5 μg/ml. Working standard solutions were diluted with extracts of natural medicines to prevent a matrix effect.

Reagents —— Acetone, hexane, toluene, acetonitrile, sodium chloride, and anhydrous sodium sulfate of pesticide analysis grade and activated charcoal were purchased from Wako Pure Chemical Industries. Supelclean ENVI Florisil SPE Tubes (6 ml, 1 g, Florisil) were purchased from Supelco (Bellefonte, PA, U.S.A.). Microcrystalline cellulose was purchased from Funakoshi Co. Ltd. (Tokyo, Japan). Microcrystalline cellulose and activated

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charcoal were mixed in equal amounts and 1 g was weighed into a polypropylene tube (inside diameter, 12 mm) to make an activated charcoal minicolumn.

Sample Preparation —— The sample preparation method was based on the previous reports.[4,5] The proposed method involves acetonitrile extraction,[4] activated charcoal cleanup,[5] and florisil clean-up.[5] Natural medicines were crushed, 10 g was weighed out, and 80, 400, and 2000 μl of spiking solution (each pesticide, 0.4, 2, 10 μg) was added. After 30 min, 40 ml of acetonitrile was added and, following 30-min incubation, the mixture was homogenized for 1 min. Sodium chloride (1 g) and anhydrous sodium sulfate (4 g) were added. The mixture was shaken and centrifuged for 10 min at 3000 rpm. Twenty milliliters of the acetonitrile layer obtained were loaded onto the activated charcoal minicolumn preconditioned with 20 ml of acetonitrile-toluene (3:1). Pesticides were eluted with 20 ml of acetonitrile-toluene (3:1). The eluate and pass-through solutions were mixed and evaporated. The residue was dissolved in 5 ml of toluene and loaded onto a Florisil minicolumn preconditioned with 5 ml of acetone-hexane (3:17) and 5 ml of toluene. Pesticides were eluted with 25 ml of acetone-hexane (3:17). The eluate and pass-through solutions were mixed and evaporated. The residue was dissolved in 5 ml of acetone-hexane (3:17) for GC/MS analysis.

NCI Mode GC/MS —— GC/MS conditions were set based on the previous report.[4] A 5973MSD was connected to a GC6890 (Agilent, Wilmington, DE, U.S.A.). GC conditions were: column, DB-1701 capillary column 30 m × 0.25 mm × 0.25 μm (J&W Scientific, Folsam, CA, U.S.A.); helium carrier gas flow, 1.7 ml/min; injection temperature, 200°C; interface temperature, 270°C; ion source temperature, 180°C; ion mode, NCI/selected ion monitoring (SIM) mode; reaction gas, methane; oven temperature program, 50°C for 1 min, 25°C/min to 100°C, and then 5°C/min to 270°C and held for 10 min; injection mode, splitless; and injection volume, 2 μl.

RESULTS AND DISCUSSION

Recovery Test Using *The Japanese Pharmacopoeia* (JP), 15th Edition Method

Glycyrrhizae radix was obtained in Japan (2007, originally from China). The recovery tests were conducted three times for Glycyrrhizae radix according to JP method (Table 2).[1] The recovery rates were between 7.9% and 30.3%, and relative standard deviations (RSDs) were between 9.2% and 45.7%. The recovery rates were very low, and the RSDs of DDTs were unsatisfactory.

Table 2. Recoveries of BHCs and DDTs in Glycyrrhizae Radix by JP method (n = 3)

Compound	Average (%)	RSD (%)
α−BHC	28.0	11.9
γ−BHC	30.3	9.2
β−BHC	25.0	10.5
δ−BHC	25.7	11.1
p,p′-DDE	8.5	45.7
o,p′-DDT	9.3	31.0
p,p′-DDD	9.5	33.8
p,p′-DDT	7.9	37.0

Spiked levels were set 0.2 μg/g.

Analysis

The mass spectrum of pesticides in NCI mode are shown in Fig. 1. The monitoring ions selected for SIM detection are shown in Table 3. Hexane solutions were usually loaded onto the Florisil minicolumn.[2,3] When the evaporated residue of eluate and pass-through solutions from the activated charcoal minicolumn were dissolved in 10 ml of hexane, the recovery rates were very low (Table 4). Furthermore, when the evaporated residue of eluate and pass-through solutions from the activated charcoal minicolumn were dissolved in 10 ml of hexane and sonicated for 10 min, the recovery rates were also very low (Table 5). We therefore dissolved the residue in 5 ml of toluene instead of hexane as a nonpolar solvent.

Many natural medicines are dry matter, and thus extracts of natural medicines are subject to a matrix enhancement effect in GC/MS analysis. When working standard solutions were diluted with acetone-hexane (3:17), the recovery rates were very high from many of the natural medicines (Table 6). To prevent the matrix enhancement effect, Okihashi *et al.* diluted working standard solutions with food extracts.[6] We also diluted working standard solutions with extracts of natural medicines.[3,4] When working standard solutions were diluted with extracts of natural medicines, the recovery rates were satisfactory (Table 7). It is believed that diluting working standard solutions with extracts of natural medicines overcomes the matrix enhancement effect. This procedure was simple and prevented the matrix enhancement effect, indicating that diluting working standard solutions with extracts of natural

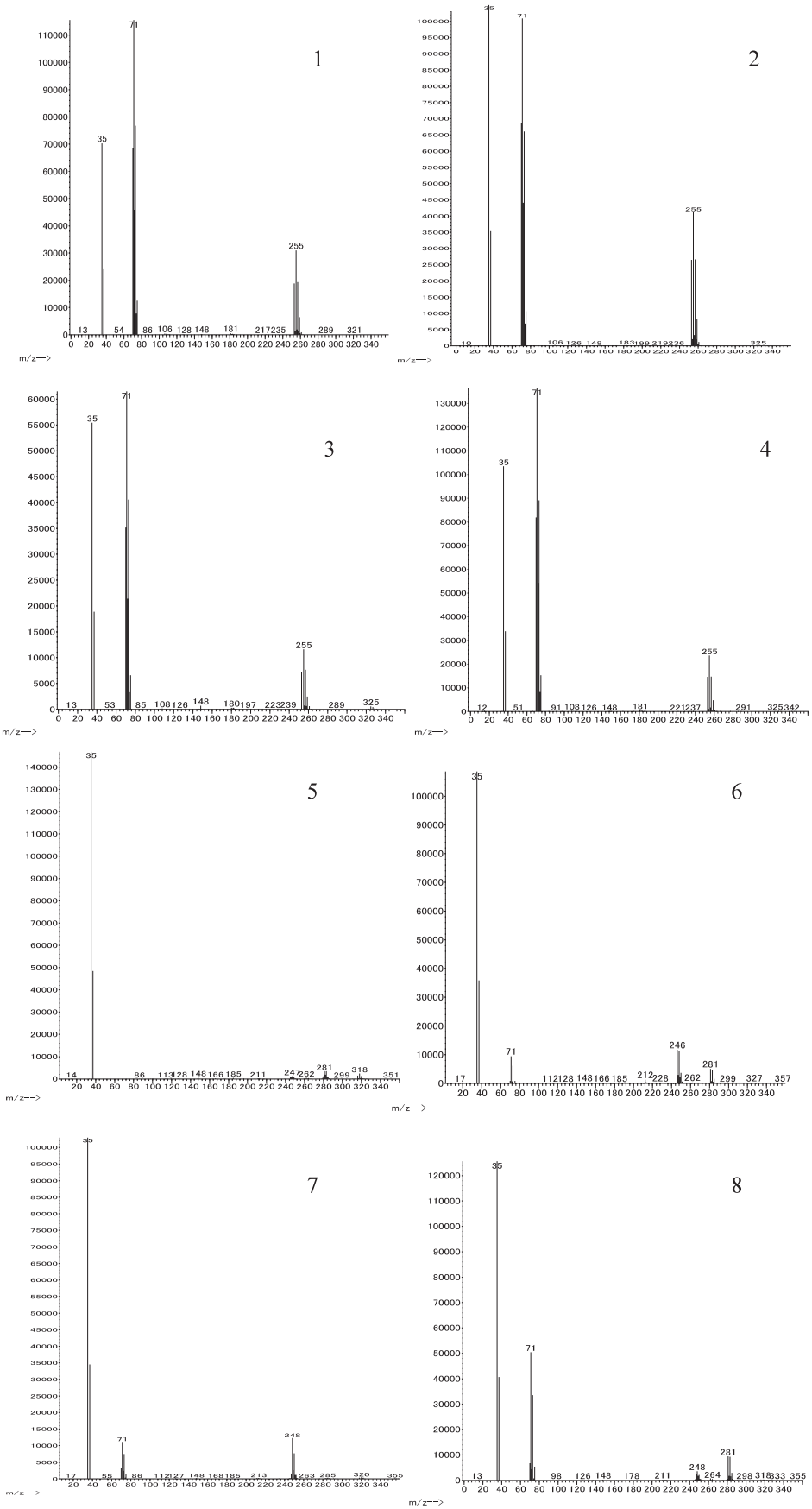

Fig. 1. Mass Spectra of Pesticides in the NCI Mode
1: α-BHC, 2: γ-BHC, 3: β-BHC, 4: δ-BHC, 5: p,p′-DDE, 6: o,p′-DDT, 7: p,p′-DDD, 8: p,p′-DDT.

medicines is useful for analyzing BHCs and DDTs in them.

SIM chromatograms of the Glycyrrhizae radix extract fortified with pesticides are shown in Fig. 2. Pesticide peaks were clearly detectable. The recovery tests were conducted three times for Glycyrrhizae radix. The intraday recovery rates of BHCs and DDTs were between 71.6% and 92.2%, and RSDs were between 0.6% and 10.2% (Table 7). The interday recovery rates of BHCs and DDTs were between 81.5% and 87.4%, and RSDs were between 3.1% and 17.5% (Table 7). The recovery rates and RSDs were satisfactory for residue analysis. The recovery rates and RSDs using the proposed method are much better than the JP method; therefore the proposed method appears useful for analyzing BHCs and DDTs in Glycyrrhizae radix. The correlation coefficients of linearity can be seen in Table 8, which vary from 0.9991 to 0.9996. Standard solution diluted with extracts of Glycyrrhizae radix showed good linearity. The instrument detection limit (IDL) of BHCs and DDTs is shown in Table 9. The proposed method had sufficient sensitivity. The IDL was estimated using the Japanese Ministry of Environment Approach.[7)]

Table 3. Monitoring Ions Selected for SIM

Compound	Monitoring ion (m/z)	
α−BHC	71	35
γ−BHC	71	35
β−BHC	71	35
δ−BHC	71	35
p,p′-DDE	35	282
o,p′-DDT	35	71
p,p′-DDD	35	71
p,p′-DDT	35	71

Actual Investigation

Five samples of Glycyrrhizae radix were ana-

Table 4. Recovery Rates of BHCs and DDTs from Glycyrrhizae Radix

Compound	Average (%)	RSD (%)
α−BHC	40.0	22.5
γ−BHC	37.2	20.0
β−BHC	33.8	11.9
δ−BHC	35.8	18.0
p,p′-DDE	40.8	28.0
o,p′-DDT	35.3	29.4
p,p′-DDD	34.2	25.1
p,p′-DDT	30.6	32.1

The evaporated residue of eluate and pass-through solutions from the activated charcoal minicolumn was dissolved in 10 ml of hexane ($n = 3$).

Table 5. Recovery Rates of BHCs and DDTs from Glycyrrhizae Radix

Compound	Average (%)	RSD (%)
α−BHC	47.1	23.9
γ−BHC	44.0	22.4
β−BHC	40.6	15.1
δ−BHC	43.3	17.2
p,p′-DDE	48.7	23.1
o,p′-DDT	41.8	27.2
p,p′-DDD	43.5	21.1
p,p′-DDT	39.4	28.9

The evaporated residue of eluate and pass-through solutions from the activated charcoal minicolumn were dissolved in 10 ml of hexane and sonicated for 10 min ($n = 3$).

Table 6. Recoveries of BHCs and DDTs in 13 Natural Medicines

Compound	Average (%)	RSD (%)
GLYCYRRHIZAE RADIX	83.5–100.3	4.1–10.2
GINSENG RADIX	90.5–130.7	1.7– 7.2
SENNAE FOLIUM	83.0–156.6	0.6– 3.3
ASTRAGALI RADIX	90.5–206.2	5.4– 7.2
POLYGALAE RADIX	58.9–184.7	2.5– 6.4
CINNAMONI CORTEX	90.4–140.8	0.8– 8.4
ASIASARI RADIX	84.8–150.2	2.1–14.9
CORNI FRUCTUS	91.7–113.4	2.8– 9.5
PERILLAE HERBA	79.3–184.4	1.8– 3.6
ZIZYPHI FRUCTUS	95.0–135.5	2.0– 5.1
AURANTII NOBILIS PERICARPIUM	85.0–138.3	1.1– 7.4
ERIOBOTRYAE FOLIUM	80.1–148.8	1.5–10.0
MOUTAN CORTEX	99.9–119.1	1.0– 8.2

The working standard solutions were diluted with acetone-hexane (3:17, $n = 3$).

Table 7. Recovery Rates of BHCs and DDTs from Glycyrrhizae Radix

spiked level (μg/g)	Intraday						Interday[a)]	
	0.04		0.2		1		0.2	
Compound	Average (%)	RSD (%)	Average (%)	RSD (%)	Average (%)	RSD (%)	Average (%)	RSD (%)
α-BHC	74.9	1.0	85.8	4.1	77.9	0.7	84.3	3.8
γ-BHC	72.8	1.4	86.2	4.6	76.7	0.6	84.6	3.1
β-BHC	71.9	1.1	88.0	6.5	75.9	1.5	84.7	5.3
δ-BHC	73.5	1.3	88.2	5.9	76.1	1.8	85.4	4.7
p,p′-DDE	74.2	2.5	87.7	8.1	75.8	1.8	84.5	5.5
o,p′-DDT	71.6	4.7	89.1	8.3	75.7	3.0	82.1	11.4
p,p′-DDD	72.8	2.8	92.2	9.7	76.3	3.1	87.4	6.4
p,p′-DDT	72.5	9.9	91.0	10.2	74.0	4.7	81.5	17.5

a) The interday recovery study was carried out over 3 working days. The evaporated residue of eluate and pass-through solutions from the activated charcoal minicolumn were dissolved in 5 ml of toluene (n = 3).

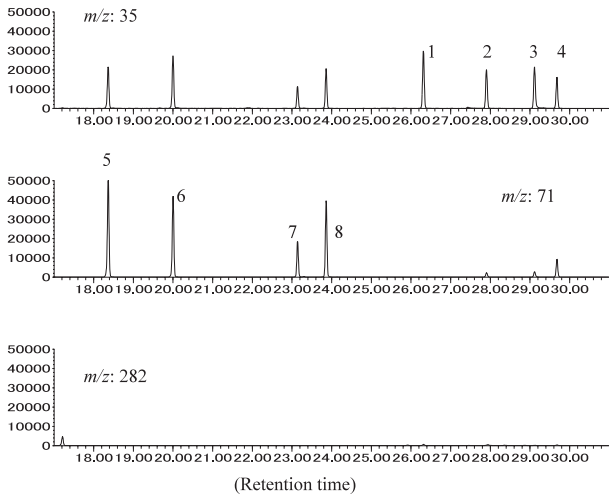

Fig. 2. SIM Chromatograms of Glycyrrhizae Radix Fortified with Pesticides at 0.2 ppm

1: p,p′-DDE, 2: o,p′-DDT, 3: p,p′-DDD, 4: p,p′-DDT, 5: α-BHC, 6: γ-BHC, 7: β-BHC, 8: δ-BHC.

lyzed using to the proposed method. Glycyrrhizae radix was obtained in Japan (2007, originally from China). Five samples contained BHCs with levels between 0.89 and 13.42 ppb (as total BHC). Two samples contained DDTs at 0.29 and 2.37 ppb (total DDT). These values were much lower than the MRL (200 ppb). This result suggests that Glycyrrhizae radix currently distributed in Japan does not contain high levels of BHCs and DDTs.

Recovery Test Using 12 Other Natural Medicines

We attempted to apply the proposed method to 12 other natural medicines for which MRL values are set. The recovery tests were conducted three times for 12 natural medicines. The recovery rates of BHCs and DDTs were between 75.3% and 103.1%, and most RSDs were less than 10% (Table 10). The recovery rates and RSDs were satisfactory. The proposed method can therefore be used to analyze BHCs and DDTs in all 13 natural medicines for which an MRL is set with good recovery rates and RSDs.

In conclusion, a method for determining BHCs and DDTs in Glycyrrhizae radix was developed

Table 8. Linear Ranges and Correlation Coefficients of Standard Solutions Diluted with Extract of Glycyrrhizae Radix

Compound	Range (ng/ml)	Correlation coefficient (γ)
α-BHC	5–1000	0.9996
γ-BHC	5–1000	0.9995
β-BHC	5–1000	0.9992
δ-BHC	5–1000	0.9995
p,p′-DDE	5–1000	0.9993
o,p′-DDT	5–1000	0.9995
p,p′-DDD	5–1000	0.9991
p,p′-DDT	5–1000	0.9996

Table 9. IDLs of Standard Solutions Diluted with Extract of Glycyrrhizae Radix

Compound	MDL (ppb)
α-BHC	0.05
γ-BHC	0.06
β-BHC	0.06
δ-BHC	0.03
p,p′-DDE	0.27
o,p′-DDT	0.60
p,p′-DDD	0.38
p,p′-DDT	1.08

IDLs = $t\,(n-1, 0.01) \times$ SD, where $t\,(n-1, 0.01)$ is Student's t-value at the 99% confidence level and is 3.143 when 7 replicates are used. SD is standard deviation of n replicates.

Table 10. Recoveries of BHCs and DDTs in 12 Natural Medicines (n = 3)

Compound	Average (%)	RSD (%)
GINSENG RADIX	93.9–101.7	1.7– 7.2
SENNAE FOLIUM	91.7– 94.8	0.6– 3.3
ASTRAGALI RADIX	91.3– 94.7	5.4– 7.2
POLYGALAE RADIX	75.3– 87.5	2.5– 6.4
CINNAMONI CORTEX	84.6– 88.9	0.8– 8.4
ASIASARI RADIX	83.9– 91.2	2.1–14.9
CORNI FRUCTUS	90.7– 97.9	2.8– 9.5
PERILLAE HERBA	88.6– 90.9	1.8– 3.6
ZIZYPHI FRUCTUS	87.2– 90.6	2.0– 5.1
AURANTII NOBILIS PERICARPIUM	89.3– 95.0	1.1– 7.4
ERIOBOTRYAE FOLIUM	88.1– 94.3	1.5–10.0
MOUTAN CORTEX	92.4–103.1	1.0– 8.2

and gave satisfactory recovery rates and RSD values for residue analysis. Five samples of Glycyrrhizae radix were analyzed following the proposed method, and no samples contained high levels of BHCs and DDTs. The proposed method can be used to analyze BHCs and DDTs in all 13 natural medicines for which MRLs are set with acceptable recovery rates. Diluting working standard solutions with extracts of natural medicines is simple and overcomes the matrix enhancement effect. This procedure is useful to analyze BHCs and DDTs in natural medicines. The proposed method can be routinely applied because BHCs and DDTs in all 13 natural medicines for which MRLs are set can be analyzed using a common analytical method.

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