Relationship between Heptachlor Epoxide Concentration in Pumpkin (Cucurbita maxima) and Soil Concentration Extracted with 50% (v/v) Methanol–Water Solution

Nobuyasu SEIKE1 and Takashi OTANI2

1Institute for Agro-Environmental Sciences, NARO
(3-1-3 Kannondai, Tsukuba, Ibaraki 305-8604 Japan)
2Central Region Agricultural Research Center, NARO
(2-1-18 Kannondai, Tsukuba, Ibaraki 305-8666 Japan)

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Summary

In order to establish a soil diagnosis to avoid the circulation of contaminated pumpkin, the availability of heptachlor exo-epoxide (HEPX) in soil for uptake by pumpkin was evaluated. Namely, the relationship between HEPX concentrations in soils extracted with 50% (v/v) methanol–water solution and HEPX concentrations in pumpkin was investigated. We first tested the early-growth shoots of pumpkin (Cucurbita maxima Dutch. cv. Ebisu) planted in HEPX-contaminated soil in an environmentally controlled chamber. Significant (P<0.01) relationships were found in regression analysis, which indicated that 50% (v/v) methanol–water solution is suitable for evaluating HEPX availability in soil by early-growth shoots of pumpkin. Next, HEPX concentrations in soils collected from the plant bases and extracted with 50% (v/v) methanol–water solution, and in the fruits of three varieties of pumpkin (C. maxima Dutch. cv. Ebisu, C. maxima Dutch. cv. Ajihei, and C. maxima Dutch. cv. TC2A) planted in eight fields, showed significant (P<0.01) relationships. However, the coefficients of determination (R2) of the regression lines for pumpkin fruit were lower than those for the early-growth pumpkin shoots. This implied that the HEPX concentration in soil extracted with 50% (v/v) methanol–water solution, nodal positions of fruits, and dry matter production of fruits contribute to the HEPX concentration in pumpkin fruit. The result of multiple regression analysis using these factors showed that the adjusted R2 values in all varieties increased compared to those determined by single regression analysis. However, from the viewpoint of soil diagnosis, it is better to estimate the HEPX concentration in pumpkin fruits not by using multiple factors, but by using the HEPX concentration in soil extracted with 50% (v/v) methanol–water solution only.

Key words: heptachlor epoxide, persistent organic pollutants, pumpkin, cucurb, soil

INTRODUCTION

Heptachlor (1,4,5,6,7,8-heptachloro-3a,4,7,7a-tetrahydro-1H-4,7-methanoindene), one of the persistent organic pollutants (POPs) adopted in May 2001 and entered into force in May 2004 by the Stockholm Convention1, is a toxic compound ubiquitously found in the environment. More than 20 compounds, including dioxin, polychlorinated biphenyls (PCBs), dichlorodiphenyltrichloroethanes (DDTs), and dieldrin, have been recognized as POPs. Heptachlor was widely used as an insecticide from 1953, and was banned worldwide in the 1970s because of its toxicity. In Japan, heptachlor was not manufactured, but 1500 tons were imported from 1958–19722. Heptachlor exo-epoxide (HEPX, 2,3-epoxy-1,4,5,6,7,8-heptachloro-3a,4,7,7a-tetrahydro-1H-4,7-methanoindene) is one of the major metabolites of heptachlor in soil3. According to the International Program on Chemical Safety (IPCS), WHO4, the acute toxicity of HEPX is higher than that of heptachlor and other metabolites, and the chronic toxicity of HEPX is the same as or higher than that of heptachlor and other metabolites. Because the log Kow values of heptachlor and HEPX are 5.27 and 5.0, respectively5, they strongly adsorb onto soil particles, particularly soil organic carbon (SOC). As HEPX is more stable6 than heptachlor and other metabolites, HEPX has recently been detected in arable soils, although heptachlor has not been used in 40 years.

The aging of pesticides and POPs is the one of the most important processes for understanding the temporal change and behaviors of organic compounds in soil. Even if the total concentration of compounds is constant, the availability of these compounds can decrease over time if they become sequestered in the soil7. This temporarily reduces the availability of compounds that can be volatilized, leached, degraded, and taken up by organisms. Although the availability of POPs in the soil is low, cucurbits can take up these compounds8 and in some cases uptake can exceed the residue limit set by the Japanese Food Sanitation Law9. The residue limit for heptachlor in pumpkin
is set at 0.03 ppm in total, with HEPX as "heptachlors". Therefore, to avoid the production and circulation of POPs contaminating cucurbits, risk reduction methods, such as soil diagnosis, should be established by characterizing the availability of POPs for cucurbits from soil.

For toxic heavy metals, such as arsenic and cadmium\(^\text{10}\), and plant nutrients\(^\text{11}\), crop-available fractions in soil were evaluated by non-exhaustive soil extraction, because parts of them strongly adsorb onto the soil particles. Ibarraki et al. indicated that a content of 0.025 mol/L of HCl-extractable cadmium from soil showed a significant correlation with the cadmium content of wheat grain\(^\text{12}\). Motoki et al.\(^\text{12}\) also tried to evaluate the pesticide availability of plants, and confirmed that concentrations of eight pesticides in Komatsuna (Brassica rapa var. perviridis) shoots showed higher positive correlations with water-extractable concentrations than with exhaustive soil concentrations obtained from shaking the extraction twice with acetone.

Concerning POPs, numerous studies have examined the uptake of POPs and related compounds in the soil by organisms such as bacteria and earthworms, while that in plants has not been widely examined. Tao et al.\(^\text{14}\) found that polycyclic aromatic hydrocarbon accumulation in wheat roots is negatively correlated with the SOC. Sakai et al.\(^\text{14}\) found a negative correlation between the SOC and bioconcentration factor (BCF), which was calculated as the dieldrin concentration in the cucumber shoot divided by the exhaustive soil concentration obtained from Soxhlet extraction with acetone for 16 h. These reports indicated that the availability of POPs for plants cannot be explained by the exhaustive extraction of soil with different SOCs. In our previous studies, it was found that non-exhaustive soil extraction using 50% (v/v) methanol–water solution can be used to determine the availability for dieldrin residue in early-growth cucumber shoots\(^\text{15}\) and fruits\(^\text{16}\). However, compounds differ between dieldrin and HEPX, and dieldrin uptake abilities among 11 cucurbits differed by about 10 times\(^\text{16}\). Therefore, it is necessary to confirm whether soil extraction by 50% (v/v) methanol–water solution can be used to determine the availability for HEPX residue in pumpkin.

In this study, in order to confirm whether the same trend as in the previous study\(^\text{16}\) can be obtained, the relationship between the extractability of HEPX from the soil by 50% (v/v) methanol–water and the BCF of early-growth pumpkin shoots and SOC were examined. Additionally, the relationship between HEPX concentrations in soils extracted with 50% (v/v) methanol–water solution and HEPX concentrations in early-growth pumpkin shoots grown in pots in an environmentally controlled chamber was confirmed. Next, we performed a field test to evaluate the relationship between HEPX concentrations in soils extracted with 50% (v/v) methanol–water solution and HEPX concentrations in pumpkin fruits grown in eight fields. Further, we analyzed the factors for HEPX accumulation in pumpkin fruits, then performed multiple regression analysis with these factors to determine whether the coefficient of determination \(R^2\) improved.

**MATERIAL AND METHODS**

**Pot experiment (early-growth pumpkin shoots)**

Approximately 20 kg of HEPX contaminated soil per field was collected from the upper layers (0–15 cm depth) of eight cultivated fields in Japan. All soils were air-dried, sieved (< 2 mm), used for plant cultivation, and subjected to soil analysis. Soil particle sizes were analyzed using a soil analysis method and classified according to the World Reference Base for Soil Resources 2006\(^\text{17}\). The SOC was determined using the dry combustion method and analyzed with a nitrogen and carbon analyzer (Sumigraph NC analyzer, NC-900; Sumika Chemical Analysis Service, Ltd., Osaka, Japan)\(^\text{18}\). The water content was measured according to the Japanese Industrial Standard specifications\(^\text{19}\). HEPX and heptachlor analysis were performed in triplicate.

Pumpkin (Cucurbita maxima Dutch, cv. Ebisu), which is widely produced in Japan, was planted in a pot containing HEPX-contaminated soils in an environmentally controlled chamber in triplicate. The cultivation conditions have been described previously\(^\text{20}\). Briefly, plastic pots (400 mL) were filled with different soil samples (290–530 g). The soil in each pot was fertilized with a chemical fertilizer (0.12 g N as [NH\(_4\)]\(_2\)SO\(_4\), 0.052 g P as Ca\(_2\)H\(_2\)PO\(_4\)\(_2\)\(_2\)H\(_2\)O, and 0.10 g K as KCl) as basal dressing. Pumpkin seeds were germinated in perlite. After 2 weeks of growth, one seedling was transferred to each of the three pots containing different soil samples. The same method was used to grow pumpkin plants in control soil with no detectable HEPX. The pots were cultivated in an environmentally controlled cultivation room, and the plants were grown with a cycle of 14 h of light and 10 h of dark at 25°C; the same conditions as those in the previous study\(^\text{20}\). Each pot was set in a plastic tray for bottom-up irrigation with tap water and mulched with a silver plastic film sheet to avoid/minimize HEPX contamination of the shoots by soil adhesion. During the growing period, additional fertilizer was applied twice to the soil in each pot as 100 mL of nutrient solution (1–2 times the strength of the Otsuka Chemical A prescription [mg/L]: N, 130; P, 26; K, 168; Ca, 82; Mg, 18; Mn, 0.6; B, 0.25; Fe, 1.35; Cu, 0.015; Zn, 0.045; Mo, 0.015) by bottom-up irrigation. Twenty-one days after transplantation, the shoots, including the leaves and stem, were harvested and weighed and the dry matter production was measured. The shoot samples were finely chopped and divided into two subsamples: one was dried at 70°C to measure the moisture content, and the other (approximately 10–20 g) was used for heptachlor and HEPX analysis.

**Field experiment (pumpkin fruits)**

Soils were collected from eight fields in Japan and used for the pot experiments. Next, five or six plots (approximately 20 m\(^2\) per plot) for one variety were placed in each field for this study. Three varieties of pumpkin (C. maxima Dutch, cv. Ebisu, C. maxima Dutch, cv. Ajhei, and C. maxima Dutch, cv. TC2A) were used in this study, because early-growth pumpkin shoots of these varieties have different HPEX uptake abilities in the following order: ‘Ebisu’ ≥ ‘Ajhei’ ≥ ‘TC2A’\(^\text{21}\). These pumpkins were raised in nursery beds in HEPX-free soil and then transplanted to the test fields in May. The three different varieties of pumpkins were cultivated in each plot. Approximately 3 months after transplantation, the pumpkin fruit samples were harvested from all plots, and the nodal positions of the fruits were obtained at the same time. One to five fruits per plant were collected. After harvest, pumpkin fruits were cured for a few weeks, and the fruit weight and dry matter production were measured. The application of fertilizers and pesticides, as well as irrigation, was performed according to conventional methods in all fields. The fruit samples were frozen and preserved at −20°C until extraction for HEPX analysis. Soil samples from each plot were collected from the base of every plant while...
making a planting hole (diameter, 15 cm; depth, 15 cm) before transplantation. The samples were air-dried, sieved (< 2 mm mesh), and stored in a polyethylene bag in a 4°C chamber in the dark until heptachlor and HEPX analysis.

### Analysis of heptachlor and HEPX

The method used to analyze heptachlor and HEPX has been described previously\(^\text{21}\). Briefly, to determine the total heptachlor and total HEPX residue in the soil, 10 g of each air-dried soil sample was Soxhlet-extracted with acetone (Wako Pure Chemical Industries Ltd., Osaka, Japan) for 16 h. This method was defined as "exhaustive extraction" in this study. These soils were also extracted using 30% (v/v) methanol–water solution. In this method, 8 g of each air-dried soil sample was extracted with 40 mL of 30% (v/v) methanol–water solution in a glass test tube with shaking at 120 rpm in a chamber (GBR-300; TAITEC, Saitama, Japan) at 25°C for 24 h. The solutions were then centrifuged at 1400 × g for 10 min. Pumpkin fruits were individually homogenized using a Polytron® PT3100 (Kinematica, Inc. Bohemia, NY, USA) with 150 mL of aceton at 12,000 rpm for 5 min. The solution was passed through a 0.8-μm glass fiber filter (GF, KIRIYAMA, Tokyo, Japan) and concentrated to 50 mL for heptachlor and HEPX analysis.

Portions of soil (5–10%) and cucumber fruit (10%) extracts were spiked with 0.1 μg of \(^{13}C\_0\)-labeled heptachlor (CLM-4759–1.2; Cambridge Isotope Laboratories, Andover, MA, USA) and HEPX (CLM-4734–1.2; Cambridge Isotope Laboratories) for use as internal surrogate standards. The extract was purified on K-solute (GL Science, Tokyo, Japan) and ENV1-Carb II/PSA (SPELCO, Bellefonte, PA, USA) columns by elution with n-hexane (Wako Pure Chemical Industries Ltd.). The purified samples were spiked with 0.01 μg of \(^{13}C\_0\)-labeled 2,2,4,4,5,5-hexachlorophenyl (MBP-153, Wellington Laboratories, Guelph, Ontario, Canada) as a syringe spike, and then concentrated to 50 μL under a gentle stream of nitrogen.

The purified samples were analyzed using a gas chromatograph mass spectrometer (GC-MS; HP 6890–5973N; Agilent Technologies, Santa Clara, CA, USA) equipped with an ENV-8MS capillary column (30 m × 0.25 mm i.d., 0.25-μm film thickness; Kanto Kagaku, Tokyo, Japan). Details of the GC-MS conditions were described in our previous paper\(^\text{21}\). The concentrations of heptachlor and HEPX in the soils and fruits using \(^{13}C\_0\)-labeled heptachlor and \(^{13}C\_0\)-labeled HEPX as well as the limits of quantification (LOQs) were calculated according to the Japanese Industrial Standard (JIS) K 0312\(^\text{22}\). The LOQs for heptachlor and HEPX in the soils were 0.0010 and 0.0006 mg/kg dry weight (DW) obtained from exhaustive extraction and 0.0016 and 0.0004 mg/kg DW obtained from 50% (v/v) methanol–water extraction, respectively. The LOQ values for heptachlor in pumpkin were determined on 0.0012 and 0.0005 mg/kg FW (fresh weight). The percentage recovery of \(^{13}C\_0\)-labeled heptachlor and HEPX ranged from 54–82% and 52–89%, respectively. The soil reference material (JSAC 0441) for dieldrin, which has the same classification as heptachlor and HEPX as a chlorinated cyclodiene pesticide, was obtained from the Japan Society for Analytical Chemistry and analyzed (n=5) as a quality control. The value obtained was 73 ± 3.7 μg/kg DW (relative standard deviation: 4.4%), which was within the range of the specified values (70 ± 14 μg/g DW). Heptachlor did not exceed the LOQ in any samples. Therefore, heptachlor is excluded from discussion in this paper.

### Statistical analysis

All statistical analyses were performed using SPSS® 22.0 software (SPSS, Inc., Chicago, IL, USA). One-way analysis of variance followed by Tukey’s multiple comparison test with a pair-wise comparison matrix were used to determine significant differences and for regression and multiple regression analyses.

### RESULTS AND DISCUSSION

#### Relationship between HEPX concentrations in soils extracted with 50% (v/v) methanol–water and in early-growth pumpkin shoots (pot experiment)

The soil properties are shown in Table 1. The SOC ranged from 1.04–6.19%, which was within the range of the mean ± standard deviation (3.83% ± 2.88%) in Japan\(^\text{22}\). A high SOC value was identified in soil from field number eight, but the major component was sand, not clay. The fresh weight and dry matter production of the early-growth pumpkin shoots are shown in Table 2. The growth of pumpkin was almost the same in every pot.

The mean concentrations of HEPX in soils collected from the eight fields ranged from < 0.0006–0.031 mg/kg DW when analyzed by exhaustive extraction and from < 0.0004–0.025 mg/kg DW when analyzed by 50% (v/v) methanol–water extraction (Table 3). The extractability of HEPX from the soil extracted with 50% (v/v) methanol–water, calculated as the HEPX concentration in soil obtained by 50% (v/v) methanol–water extraction divided by HEPX concentration in soil and obtained by the exhaustive extraction method, showed a negative correlation with the SOC (Fig. 1). A similar tendency has also been confirmed in dieldrin\(^\text{3}\). The mean HEPX concentrations in early-growth pumpkin shoots ranged from < 0.0005–0.031 mg/kg FW (Table 3). The BCF was calculated as the HEPX concentration...
tion in the shoot divided by the exhaustive soil concentration (Table 3). The significant negative relationship between the BCF and SOC (Fig. 2) indicated that the SOC related to the uptake of HEPX by pumpkin from the soil, as is the case with dieldrin uptake by early-growth cucumber shoots (15). The HEPX concentration in early-growth pumpkin shoots and HEPX concentration in soils extracted with 50% (v/v) methanol–water were significantly correlated (P < 0.01) (Fig. 3). This regression line was assumed to pass through the origin, because if the concentration in the soil is zero, the concentration in the shoots must also be zero. The 95% confidence interval was small, indicating that the relationship between 50% (v/v) methanol–water extractable HEPX concentration in soil and HEPX concentration in early-growth pumpkin shoots is significant. These results agree with the dieldrin uptake by early-growth cucumber shoots (15) and indicate that 50% (v/v) methanol–water solution is optimal for evaluating the POP availability in soil for Cucurbitaceae plants growing in an environmentally controlled chamber.

HEPX residue in soil and pumpkin fruits (field experiment)

The nodal position, fresh weight, and dry matter production of pumpkin fruits are shown on Table 4. In our previous study, cucumber fruit samples were collected from the same nodal position (6th to 10th node on the 1st lateral branch). However, pumpkin fruits were not collected from the same nodal position because we could not control the pollination of pumpkin in field. The mean dry matter production of pumpkin fruits ranged from 21.5–31.7%, indicating that the sample contained immature pumpkins.

The mean concentrations of HEPX in soils collected from the eight fields ranged from < 0.0006–0.030 mg/kg DW when analyzed by exhaustive extraction and from < 0.0004–0.021 mg/kg DW when analyzed by 50% (v/v) methanol–water extraction (Table 5). The mean HEPX concentrations in pumpkins fruits ranged from < 0.0005–0.052 mg/kg FW in ‘Ebisu’, < 0.0005–0.072 mg/kg FW in

### Table 3  HEPX concentrations in soils (mg/kg DW) and the early-growth pumpkin shoots (mg/kg FW)

| Field No. | Exhaustive Extraction (mg/kg DW) | 50% (v/v) methanol–water extraction (mg/kg DW) | Early-growth pumpkin shoots (mg/kg FW) |
|-----------|---------------------------------|-----------------------------------------------|----------------------------------------|
|           | Mean SD                          | Mean SD                                        | Mean SD                                |
| 1         | 0.015 0.001                      | 0.013 0.0009                                  | 0.021 0.0003                           |
| 2         | 0.030 0.001                      | 0.025 0.0014                                  | 0.031 0.0014                           |
| 3         | < 0.0006 -                       | < 0.0004 -                                    | < 0.0005 -                             |
| 4         | 0.014 0.000                      | 0.008 0.0005                                  | 0.009 0.0004                           |
| 5         | 0.031 0.001                      | 0.021 0.0003                                  | 0.012 0.0026                           |
| 6         | 0.019 0.001                      | 0.015 0.0008                                  | 0.015 0.0027                           |
| 7         | 0.022 0.002                      | 0.015 0.0004                                  | 0.017 0.0024                           |
| 8         | 0.024 0.001                      | 0.010 0.0009                                  | 0.008 0.0006                           |

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**Fig. 1** Relationship between soil organic carbon (SOC) and HEPX extractability by 50% (v/v) methanol–water solution.

**Fig. 2** Relationship between the SOC and bioconcentration factor (BCF) calculated as the HEPX concentration in the pumpkin shoot divided by the exhaustive soil concentration.

**Fig. 3** Relationship between the HEPX concentration in soil extracted with 50% (v/v) methanol–water and HEPX concentration in the pumpkin shoot. The solid line shows the regression line, and the dashed lines show the 95% confidence interval.
Ajihei', and \(< 0.0005-0.033\) mg/kg FW in ‘TC2A’. The differential HEPX concentration in soil extracted with 50% (v/v) methanol–water in individual plots from the eight fields (Fig. 4) and differential HEPX uptake from soil with different SOC’s between the eight fields may have affected the HEPX concentrations in the three pumpkins. Accordingly, the fruit concentration factor (FCF) was calculated as the HEPX concentration in the fruits divided by the soil concentration obtained from 50% (v/v) methanol-water solution extraction (Fig. 5). The mean values of FCF were in the order of ‘Ebisu’, ‘Ajihei’, and ‘TC2A’, which was consistent with the trend in HPEX uptake ability in early-growth pumpkin shoots\(^{20}\). This indicates that the differential HEPX uptake ability of pumpkin in the early growth stage was maintained until the harvest stage.

### Relationship between HEPX concentrations in soils extracted with 50% (v/v) methanol–water and in pumpkin fruits (field experiment)

To confirm the correlation between HEPX concentrations in soils extracted with 50% (v/v) methanol–water and in pumpkin fruits, single linear regression analysis was performed. The regression line was assumed to pass through the origin because if the concentration in the soil is zero, the concentration in the fruit should also be zero. A significant \((P < 0.01)\) relationship was observed between the HEPX concentration in pumpkin fruits and 50% (v/v) methanol–water extractable HEPX concentration in the soil (Fig. 6). The 95% confidence interval was small in all three varieties (Fig. 6), indicating that the relationship between the 50% (v/v) methanol–water extractable HEPX concentration in soil and HEPX concentration in pumpkin fruits was significant. The residual sum of squares obtained from the estimated value and the observed value was 0.0193, 0.0263, 0.0051, for each of varieties ‘Ebisu’, ‘Ajihei’ and ‘TC2A’, respectively. However, \(R^2\) of the regression lines for the three pumpkins fruits were lower than those for the early-growth pumpkin shoots (Fig. 3 and Fig. 6). A similar trend was confirmed in the case of dieldrin in the early-growth cucumber shoots \((R^2 = 0.966)\) and fruits \((R^2 = 0.286 to 0.460)\), and it was considered that the difference in the cultivation season influ-

### Table 4 Nodal position, fresh weight (g FW) and dry matter production (%) of pumpkin fruits

| Field No. | Variety | N\(^{oo}\) Nodal positions of fruits | Fresh weight (g FW) | Dry matter production (%) |
|-----------|---------|------------------------------------|---------------------|--------------------------|
|           |         | Mean SD                            |                     | Mean SD                  |
| 1         | ‘Ebisu’ | 10 4–7                             | 1280.2 280.9        | 26.2 2.5                 |
|           | ‘Ajihei’| 7 2–14                             | 1598.1 687.8        | 23.5 6.6                 |
|           | ‘TC2A’  | 5 6–10                             | 944.8 264.6         | 27.0 4.2                 |
| 2         | ‘Ebisu’ | 6 5–7                              | 1507.3 181.2        | 26.0 1.3                 |
|           | ‘Ajihei’| 5 5–8                              | 1191.2 291.3        | 27.8 1.3                 |
|           | ‘TC2A’  | 7 6–14                             | 1228.0 375.4        | 23.2 7.2                 |
| 3         | ‘Ebisu’ | 12 4–8                             | 1824.9 356.2        | 27.4 5.5                 |
|           | ‘Ajihei’| 6 5–8                              | 1901.7 226.2        | 28.4 2.2                 |
|           | ‘TC2A’  | 10 8–12                            | 2293.2 486.6        | 29.5 5.7                 |
| 4         | ‘Ebisu’ | 9 6–10                             | 1800.1 475.9        | 21.5 1.5                 |
|           | ‘Ajihei’| 8 6–13                             | 1713.6 498.6        | 25.8 6.9                 |
|           | ‘TC2A’  | 7 9–12                             | 2377.6 465.1        | 25.5 8.2                 |
| 5         | ‘Ebisu’ | 7 4–7                              | 1275.6 518.6        | 24.8 3.6                 |
|           | ‘Ajihei’| 5 7–12                             | 1368.4 453.9        | 26.3 3.9                 |
|           | ‘TC2A’  | 5 6–10                             | 1699.0 438.0        | 26.3 4.7                 |
| 6         | ‘Ebisu’ | 12 4–41                            | 1507.8 451.1        | 22.8 4.6                 |
|           | ‘Ajihei’| 12 2–44                            | 1188.3 325.1        | 24.0 5.6                 |
|           | ‘TC2A’  | 10 6–32                            | 1577.7 762.1        | 26.4 3.6                 |
| 7         | ‘Ebisu’ | 16 3–25                            | 2048.1 465.4        | 25.5 4.0                 |
|           | ‘Ajihei’| 12 2–17                            | 1720.3 618.4        | 29.5 3.0                 |
|           | ‘TC2A’  | 14 2–13                            | 2563.7 547.8        | 31.7 1.9                 |
| 8         | ‘Ebisu’ | 20 4–37                            | 2062.9 652.6        | 20.9 3.0                 |
|           | ‘Ajihei’| 16 5–43                            | 1700.8 505.6        | 25.0 5.4                 |
|           | ‘TC2A’  | 10 4–33                            | 2037.4 557.7        | 24.4 3.4                 |
Table 5  HEPX concentrations in three varieties of pumpkin fruits (mg/kg FW) and soils (mg/kg DW)

| Field No. | Number of plots | Variety | Pumpkin Fruits (mg/kg FW) | Soil 50% (v/v) methanol-water extraction (mg/kg DW) |
|-----------|----------------|---------|---------------------------|-----------------------------------------------|
|           |                |         | Mean  | SD     | Mean  | SD     | Mean  | SD     |
| 1         | 5              | ‘Ebisu’ | 0.052 | 0.014  | 0.016 | 0.0021 | 0.014 | 0.0018 |
| 2         | 5              | ‘Ajihei’| 0.072 | 0.024  | 0.018 | 0.0022 | 0.014 | 0.0012 |
| 3         | 5              | ‘TC2A’ | 0.033 | 0.0069 | 0.017 | 0.0014 | 0.014 | 0.0019 |
| 4         | 5              | ‘Ebisu’ | < 0.0005 | -     | < 0.0006 | -     | < 0.0004 | -     |
| 5         | 5              | ‘Ajihei’| < 0.0005 | -     | < 0.0006 | -     | < 0.0004 | -     |
| 6         | 5              | ‘TC2A’ | < 0.0005 | -     | < 0.0006 | -     | < 0.0004 | -     |
| 7         | 5              | ‘Ebisu’ | 0.0120 | 0.0019 | 0.012 | 0.0010 | 0.0085 | 0.0006 |
| 8         | 5              | ‘Ajihei’| 0.0130 | 0.0034 | 0.012 | 0.0011 | 0.0088 | 0.0002 |
| 9         | 5              | ‘TC2A’ | 0.0084 | 0.0027 | 0.012 | 0.0015 | 0.0080 | 0.0004 |
| 10        | 5              | ‘Ebisu’ | 0.0130 | 0.0020 | 0.016 | 0.0005 | 0.013 | 0.0008 |
| 11        | 5              | ‘Ajihei’| 0.0190 | 0.0048 | 0.017 | 0.0008 | 0.013 | 0.0008 |
| 12        | 5              | ‘TC2A’ | 0.0091 | 0.0043 | 0.017 | 0.0011 | 0.013 | 0.0010 |
| 13        | 6              | ‘Ebisu’ | 0.0370 | 0.011  | 0.017 | 0.0022 | 0.014 | 0.0013 |
| 14        | 6              | ‘Ajihei’| 0.0490 | 0.021  | 0.021 | 0.0051 | 0.017 | 0.0041 |
| 15        | 6              | ‘TC2A’ | 0.0270 | 0.010  | 0.022 | 0.0038 | 0.017 | 0.0029 |
| 16        | 6              | ‘Ebisu’ | 0.0360 | 0.015  | 0.012 | 0.0015 | 0.0086 | 0.0010 |
| 17        | 6              | ‘Ajihei’| 0.0290 | 0.0056 | 0.011 | 0.0019 | 0.0087 | 0.0019 |
| 18        | 6              | ‘TC2A’ | 0.0210 | 0.0039 | 0.013 | 0.0026 | 0.0094 | 0.0022 |

Fig. 4  HEPX concentration in the soil in individual plots extracted by 50% (v/v) methanol–water

Fig. 5  Fruit concentration factor (FCF) of HEPX in three varieties of pumpkins. Error bars indicate standard deviation. Data were analyzed by one-way analysis of variance followed by Tukey’s multiple comparison test (P<0.01). The same letters indicate that the values are not significantly different.
enced the uptake of dieldrin by cucumber\(^{16}\). However, the pumpkin cultivation season is the same in this study. Thus, other factors may contribute to HEPX accumulation in pumpkin fruits. The 95% confidence interval was small, indicating that the relationship between the 50% (v/v) methanol–water extractable HEPX concentration in soil and HEPX concentration in early-growth pumpkin shoots is significant.

To identify the factors reducing \( R^2 \), regression analysis between the FCF and HEPX concentrations in soils extracted with 50% (v/v) methanol–water, weight of fruits, nodal positions of fruits, and dry matter production of fruits was performed (Table 6). Because the HEPX concentration in the fruit is influenced by the concentration in the soil (Fig. 6), FCF was used, as it ignores the influence of the soil concentration. No correlation was observed between the FCF and HEPX concentrations in soils extracted with 50% (v/v) methanol–water and fruit weight. Thus, the concentration-dependence of HEPX uptake from soil by pumpkin and fruit size-dependent HEPX accumulation did not occur. However, a negative relationship was observed between the FCF and nodal positions of fruits, while a positive relationship was observed between the FCF and dry matter production of fruits. In general, pumpkin fruit is cured to improve qualities such as flavor by promoting the conversion of starch to sugar and prevent rotting by exposure to 30°C for a few weeks. The positive relationship between the FCF and dry matter production of fruits may be because HEPX in pumpkin fruits becomes concentrated when the moisture is evaporated during curing. In contrast, the negative relationship between the FCF and the nodal positions of fruits indicates that the concentrations of HEPX in pumpkin fruits collected near the root were higher than those collected near the tip of the plant. The same trend was found for PCBs in pumpkin\(^{24}\). Murano et al.\(^{25}\) suggested that the protein-like materials in the xylem sap delivered dieldrin from the roots to the above-ground tissue. When HEPX is not completely dissolved in the xylem sap, HEPX may not be uniformly distributed throughout the whole Cucurbitaceae plant body.

Table 6  Correlation coefficient between FCF and four factors

| Factors                        | ‘Ebisu’ | ‘Ajihai’ | ‘TC2A’  |
|--------------------------------|---------|----------|---------|
| HEPX concentrations in soils   |         |          |         |
| extracted with 50% (v/v) methanol-water | 0.012   | -0.012   | 0.144   |
| Weight of fruits               | -0.018  | 0.133    | -0.074  |
| Nodal positions of fruits      | -0.307**| -0.373** | -0.317* |
| Dry matter production of fruits| 0.488** | 0.202*   | 0.309*  |

\(^*P < 0.05, **P < 0.01\)

![Graph 1](image1.png)

![Graph 2](image2.png)

![Graph 3](image3.png)

![Graph 4](image4.png)

**Fig. 6** Relationship between the HEPX concentration in soil extracted with 50% (v/v) methanol–water and the HEPX concentration in pumpkin fruits. The solid line shows the regression line, and the dashed lines show the 95% confidence interval.
Our findings indicate that the nodal positions of fruits and dry matter production of fruits may contribute to variations in the HEPX concentrations in pumpkin fruits. Therefore, to improve the $R^2$ value of the regression line, multiple regression analysis was performed using these factors and HEPX concentrations in the soil samples extracted with 50% (v/v) methanol–water solution as a regressor. The results showed that the adjusted $R^2$ values in all varieties increased (Table 7) compared to those determined by single regression analysis (Fig. 6). The residual sum of squares obtained from the estimated value and the observed value was reduced to 0.0148, 0.0099, 0.0041, for ‘Ebisu’, ‘Ajihei’, and ‘TC2A’, respectively.

In order to establish soil diagnosis based on these results, it is necessary to analyze and evaluate the accuracy and uncertainty of the equation for the estimation of HEPX concentration in pumpkin by statistical analysis, such as regression analysis and/or discriminant analysis, with a large number of samples (soil and pumpkin). At that time, the estimation of pumpkin concentration can be performed using a single factor (soil concentration), or multiple factors including the fruit of pumpkin and dry matter content. In general, the shipping of crops by farmers and (or) entire production areas are stopped when they exceed the residue limit of compounds. In the case of estimation based on multiple factors, the HEPX concentration in pumpkin is estimated after harvesting, and the economic loss is great when it is determined that shipment should be stopped. Therefore, it seems that a method of estimating HEPX concentration in pumpkin from a single factor (soil concentration) obtained before cultivation is preferable.

However, we found another important problem for establishing a soil diagnosis method. In some cases, the HEPX concentrations in soils collected from the plant base exhibited large variations in individual fields. (Fig. 4 and Table 5). Pumpkins may be cultivated in a large area exceeding 1 ha. Namely, it is necessary to propose a minimum number of soil samples for evaluating the horizontal distribution of HEPX concentration in the field based on statistical analysis. Therefore, it is necessary to create the management flow for soil diagnosis based on soil concentration after making the equation for estimating the HEPX concentration in pumpkins, and proposing suitable numbers of soil samples.

**CONCLUSION**

This research confirmed that soil extraction by 50% (v/v) methanol–water solution is suitable for evaluating HEPX availability by pumpkin, which was confirmed to be useful for dieldrin in cucumber. The HEPX concentration in early-growth pumpkin shoots grown in pots in an environmentally controlled chamber and HEPX concentration in soils extracted with 50% (v/v) methanol–water was significantly correlated, as is the case with dieldrin uptake by cucumber. A significant relationship was observed between the HEPX concentration in pumpkin fruits collected from eight fields and 50% (v/v) methanol–water extractable HEPX concentration in the soil. These results indicated that soil extraction by 50% (v/v) methanol–water solution can be used to determine the availability for HEPX residue in pumpkin. However, the $R^2$ values of the regression lines for pumpkin fruits were lower than those for the early-growth pumpkin shoots. It was thought that the nodal positions of fruits and dry matter production of fruits contributed to the variations in HPEX concentration in pumpkin fruit. Additionally, in order to establish a soil diagnosis method and prevent the potential distribution of contaminated pumpkins, it is necessary to create a management flow for soil diagnosis to estimate the HEPX concentration in pumpkin and propose a suitable number of soil samples.

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