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New Method for Ethephon ((2-Chloroethyl)phosphonic Acid) Residue Analysis, and Detection of Residual Levels in the Fruit and Vegetables of Western Japan

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A new method for the detection and quantification of ethephon residues in fruit and vegetables was developed. The present study indicates that fruit and vegetables require a rapid and simple cleanup step before using gas chromatograph/mass spectrometry. The recovery and precision of the new method were evaluated by spiking the fruit and vegetable samples with 0.01−0.1 μg/g of ethephon. The amount of ethephon residue can be determined with good accuracy (recovery, 78.6−109%; coefficient variation, 2.65−6.41%), and the detection limit, defined as the amount of ethephon equivalent to three standard deviations (SD) of the noise level in observations at the baseline level of the selected ion (*m*/*z* 110), was 4 pg. The determination limit, defined as the equivalent to 8 SD of the noise level, was 11 pg. The working range was between 10 and 1000 ng/mL, and the correlation coefficient was 0.999 in the five experiments. Ethephon residues were determined between <2 and 97 ng/g in commercial pineapples from Western Japan.

KEYWORDS: Ethephon; fruit and vegetables; analysis; gas chromatograph/mass spectrometry (GC/MS)

INTRODUCTION

Ethephon ((2-chloroethyl)phosphonic acid) is a plant growth regulator used to promote fruit ripening, abscission, flower induction, and other responses. This agricultural chemical is registered for use on a number of food, feed, and nonfood crops, greenhouse nursery stock, and outdoor residential ornamental plants, but is used primarily on cotton. Ethephon is metabolized to ethylene in plants. Since the biological action of ethylene on the development of plants was reported, a number of hormonal responses have been described and reviewed (*1*). The degradation of ethephon at pH values above 3.5 to ethylene, phosphate, and chloride ions offers a convenient possibility for the indirect determination of this growth regulator or for confirmation purposes.

Ethephon results in a considerable inhibition of cholinesterases in blood plasma and erythrocytes in long-term feeding tests on dogs and rats (*2*). Harmful accumulation of this inhibitor in food can be circumvented by well-considered applications based on residue analysis. The Ministry of Health, Labor and Welfare in Japan has set the maximum residue limit (MRL) in crops for 245 pesticides under the Food Sanitation Law. The Ministry of Health, Labor and Welfare intend to set the MRL for ethephon in crops in the near future. Formally, the Ministry of Environment in Japan imposed the tolerance of residual ethephon and the analytical method. However, there are the objections to this analytical method, including the use of toxic ethyl acetate and time consumption for sample preparation.

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The gas chromatographic procedures developed earlier for residue analysis (*3*−*5*) determined the methylated phosphonic acid compound by gas−liquid chromatography with a flame photometric detector or a flame thermionic detector. Even simplified extraction and cleanup methods described previously are rather time-consuming. These analytical methods analyze the ethylene generated under alkaline condition. In addition, the other ethephon analysis methods were also reported (*6*). The method contains the process of extraction with a solvent, such as ethyl acetate, from a sample, derivatization with (trimethylsilyl)diazomethane, or diazomethane, and analysis by FTD or FPD-GC. The indirect quantity method by ethylene is not suitable for residual ethephon analysis in fruit and vegetables with respect to sensitivity. On the analytical methods by GC after the extraction and the derivatization of ethephon, the methylation of ethephon does not fully advance, had the bad reproducibility of data, and many interference peaks appeared on the GC chromatogram. Therefore, a further cleanup procedure was needed. Moreover, there was a problem of ethephon in a sample not being easily extracted with solvents, such as ethyl acetate. The analytical method reported by Hooijschuur et al. (*7*) is not suitable for residual etephon analysis in fruit and vegetables from the point such as the influence of the interference in samples, although it is perfect with respect to the sensitivity. In this paper, a new rapid analysis method is described as most suitable for the routine analysis of ethephon residue in fruit and vegetables and for reporting on ethephon residual levels in fruit and vegetables distributed throughout Western Japan (Fukuoka Prefecture district).

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MATERIALS AND METHODS

Chemicals. (*a) Regents.* Acetone, acetonitrile, methanol, *n*-hexane, and ethyl acetate (pesticide residue analytical grade) were obtained from Wako Pure Chemicals Co. Ind., Tokyo, Japan. Trifluoroacetic acid (TFA), gas chromatographic grade, was also obtained from Wako. Acetone and methanol were pesticide residues analytical quality. Citric acid and acetic acid were purchased from Wako or Kishida Regents Chemicals, Osaka, Japan.

(b) Standard. Ethephon standard (98% pure) was obtained from Wako. The stock solution of the ethephon standard was prepared in acetone at 1 mg/mL and stored in the dark at −20 °C. Working standard solutions (0.01−1.0 μg/mL) were prepared by appropriate dilutions. The diluted standard solutions were stored in the dark at 4 °C. For recovery experiments, the standard solutions were used.

(c) Diazomethane Preparation. For diazomethane preparation, the method described by de Boer and Backer (*8*) was modified, and diazomethane gas was trapped in ice-cold acetone.

Apparatus. *(a) Cartridge Columns.* Isolute NH_2 and SAX solid-phase extraction (SPE) columns (each 500 mg) were obtained from International Sorbent Technology Ltd., Mid-glamorgan, U.K.

(b) Homogenizer. Polytron PT3100 (KINEMATICA, Switzerland).

(c) Rotary Evaporator. R3000 (Shibata Scientific technology Ltd., Tokyo, Japan).

(d) Centrifuge. RX200 (Tomy, Tokyo, Japan).

(e) Shaker. SR2w (TAITEC, Tokyo, Japan).

Samples. According to the annual monitoring plan of the Ministry of Health, Labor and Welfare of Japan, Food Sanitation Inspectors working at 3 health centers established within the Fukuoka Prefecture collected agricultural products at wholesale and retail markets and brought them to my laboratory. A sample whose producer or imported trader is clear and limited to a single producer was chosen, and the information for checking a lot was recorded in detail. The tested samples that were used consisted of cherry, corn, grape, Japanese summer orange, peach, pineapple, tomato, Japanese persimmon, Satsuma mandarin (early ripening type), and Japanese pear and totaled 40 samples (36 domestic and 4 imported) from May 2001 to January 2002. Samples > 1 kg were submitted, and the specimens supplied for the tests were taken from each sample according to the regulatory monitoring method of the Japanese Food Sanitation Law. About 500 g of the specimens were homogenized. When all the samples were homogenized, 1 mL of 1 M citric acid per 10 g of sample was added. Samples were homogenized immediately after their arrival at my laboratory and stored in the dark at −20 °C until analysis.

Extraction. The amount equivalent to 10 g of the sample was weighed in a 500 mL polypropylene centrifuge tube (Nalge Nunc International, NY) from the homogenate, and 100 mL of acetone was added. The mixture was slowly mixed and extracted for 20 s and centrifuged at 4 °C, 7400*g* for 20 min. The operation was performed once again. The supernatant was evaporated at 40 °C on a rotary evaporator until most of the acetone had been removed; then it was diluted with 30 mL of distilled water.

Column Cleanup. Isolute NH_2 SPE column was preconditioned with 5 mL of methanol, 10 mL of 0.5 N acetic acid, and 10 mL of 0.05 N acetic acid, respectively. The extract was charged onto the column, was rinsed with 10 mL of methanol, and was eluted with 15 mL of 1% (v/v) TFA in methanol. The eluate was evaporated at 40 °C to near dryness on a rotary evaporator, and dried up under a nitrogen gas stream.

Gas Chromatograph/Mass Spectrometry Analysis. Apparatus. *(a) GC/MS.* HP 6890 gas chromatograph/HP 5973 mass spectrometer (Hewlett-Packard Co., CA) equipped with electronic flow control and autosampler HP 6890.

(b) Capillary Column. CLPestisides-II (Restek Corp., CA), 0.25 mm i.d. × 30 m, 0.25 μm film thickness.

(c) GC Operating Parameters. ChemStation (Hewlett-Packard) was used for analysis of ethephon. The splitless injection port had a carbofrit inserted in the liner and was purged for 3 min. The injection port temperature was maintained at 250 °C; the initial column temperature was held at 50 °C for 2 min, programmed to 220 °C at 8 °C/min, held at 220 °C for 1 min, programmed to 290 °C at 40 °C/min, held at 290 °C for 1 min, and postrun at 330 °C for 1 min. The average velocity of the carrier gas (helium) was 30 cm/s. The injection volume was 2 μL.

(d) MS Conditions. GC/MS Analysis Performed Selected Ion Monitoring (SIM). The mass interface temperature and ion source temperature were maintained at 150 and 200 °C, respectively. EI tuned per factory recommendations.

Quantification. A 10 mL aliquot of diazomethane in acetone was added to the residue and allowed to stand for 30 min at room temperature. The reaction mixture was evaporated at 40 °C to near dryness on a rotary evaporator and then completely dried under a dried nitrogen gas stream. The residue was dissolved in 1 mL of acetone and analyzed by GC/MS. The concentration of ethephon was calculated on the basis of a peak area calibration curve. The calibration curve was constructed with the methyl derivative generated from intact ethephon standard by diazomethane methylation. Each injection was performed 3 times to test reproducibility. The sample solution was automatically injected into the GC/MS for ethephon residue analysis.

Recovery Test. Homogenated samples (10 g) were fortified with 0.1, 0.5, and 1.0 μg/g of ethephon standard. Recovery data represent six replications.

Optimization of SPE Column Chromatography. The optimization of ethephon elution from SPE columns was performed with 0.5 μg/mL of ethephon standard. The ethephon standard solution was charged on two types of SPE columns (NH_2 and SAX columns) preconditioned in a manner similar to the above-mentioned method, respectively. They were rinsed with 10 mL of methanol and eluted with various volumes (5, 10, and 15 mL) of 1% (v/v) TFA in methanol.

Furthermore, ethephon elution from the NH_2 column was investigated by different concentrations (0.1, 0.5, and 1% (v/v)) of TFA in methanol.

In addition, the influence of interfering substances from the sample on the SIM chromatogram of GC/MS was also investigated. The extracts from the samples (each 10 g),which were used in the present study, spiked or not spiked with 0.05 μg/g of ethephon standard were charged on the NH_2 column preconditioned in a manner similar to the above-mentioned method; the column was washed with methanol or acetonitrile and eluted with 15 mL of 1% (v/v) TFA/methanol. The influence of interfering substances from the sample was estimated by GC/MS analysis according to the above-mentioned method.

RESULTS AND DISSCUSSION

Ethephon Extraction. According to the official analytical methods of registered pesticides in Japan, a test sample is homogenized with 1 mL of 1 M citric acid per 20 g of sample, 0.5 mL of hydrochloric acid and 50 g of anhydrous sodium sulfate are added, and the mixture is extracted with 100 mL of ethyl acetate. The ethephon recovery from tomato samples spiked with 0.05 μg/g of ethephon standard was about 70% in the extraction process. In particular, the recovery rate of ethephon was low, because sodium sulfate could not absorb water contained in the samples (*9*). In the subsequent process, the hexane/acetonitrile partition rate was also very low. Finally, the resulting ethephon recoveries were ND to 28.7% for the five experiments (data not shown). Ethephon was difficult to extract with ethyl acetate from the sample. Perhaps, the amount of water in the sample affected the low rate of ethephon extraction. Furthermore, vigorous mixing for the extraction may also have caused the low recovery of ethephon. Therefore, it was necessary to develop a new analytical method for the residual analysis of ethephon.

The extract solvent used was acetone, which dissolves ethephon freely and is usually used in the other pesticides analyses. Samples were homogenized with 1 M citric acid. The process was the same as the official analytical methods used in Japan, but acidic acetone containing 0.05 N acetic acid was used as the extracting solvent. Though acetic acid is a weaker acid

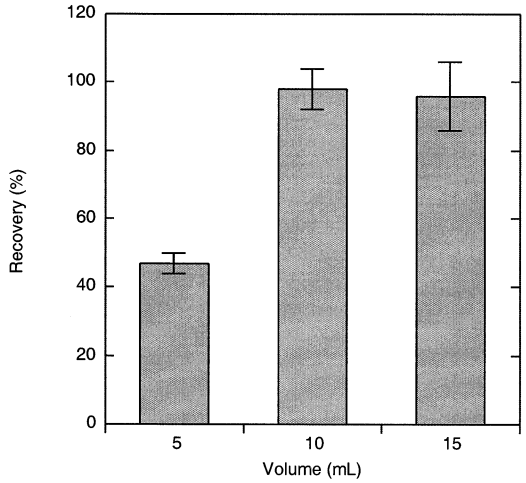

Figure 1. Ethephon elution profiles using a NH_2 solid-phase extraction column. The data represent the mean of ethephon recovery (%) ± standard deviation for three experiments.

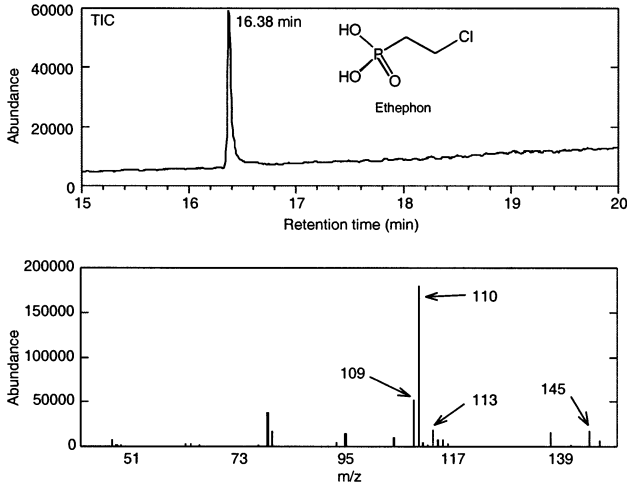

Figure 2. Mass spectrum and total ion chromatogram of ethephon methyl derivative (intact ethephon, 100 pg). The upper figure shows the total ion chromatogram of a derivertized ethephon standard, and the lower figure shows the mass spectrum of the ethephon methyl derivative.

than hydrochloric acid, producing a sample of pH ~3.5, ethephon was not degraded using the above extraction conditions.

Column-Cleanup Condition. The extract containing ethephon was purified using anion-exchange chromatography. The SPE columns used were NH_2 and SAX columns. The SAX column clogged when the sample extract was charged on the column; therefore, the SAX column could not be estimated. The NH_2 column did not clog significantly, the ion exchange capacity is bigger than that of the SAX column, and this column could be used to create the optimum conditions to obtain accurate results. The solid phase of the NH_2 column has a weak anion exchange phase at less than pH 7.8 in water conditions, and anion molecules could be retained. The preconditioned NH_2 column maintained a pH of under 3.5. After acetone extracts from the sample were charged in the column, the column was rinsed with 10 mL of methanol. Attempts were made to rinse the column with other solvents (acetonitrile and acetone), and it became clear that methanol thoroughly eluted any interferences in order to obtain an accurate result. In the case of rinsing the column with acetone, the rate of ethephon recovery was very low, because ethephon bound to the column eluted with acetone.

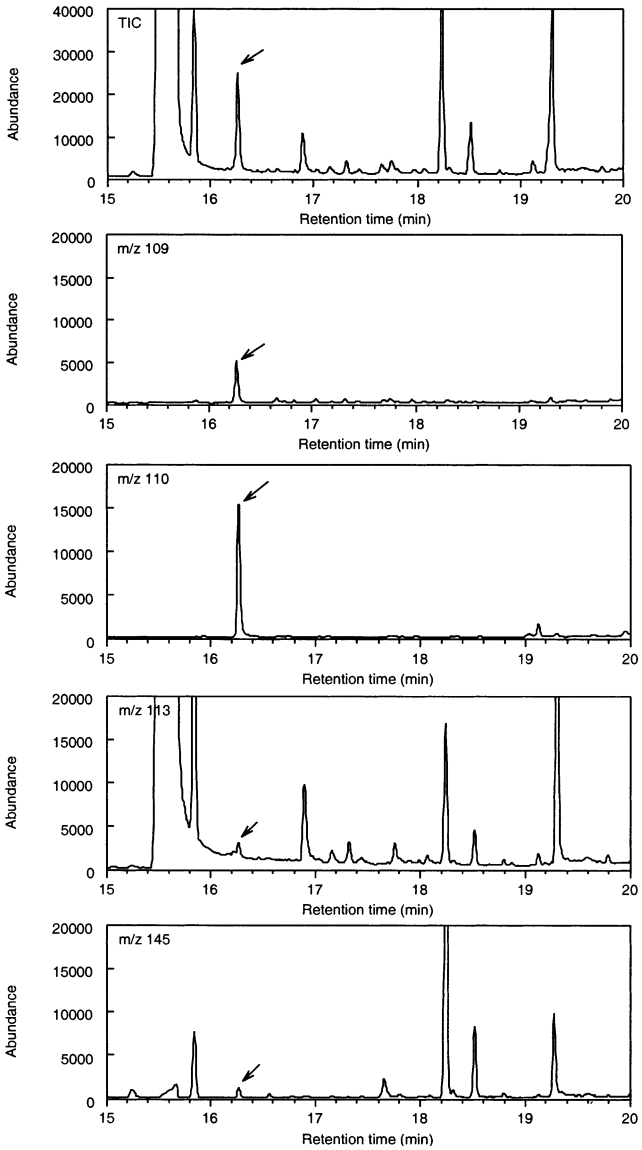

Figure 3. Selected ion chromatograms of ethephon methyl derivative from a tomato sample spiked with the ethephon standard. The arrows show the ethephon's signals.

TFA in methanol was used for the ethephon elution from the column. When 15 mL of 0.1 (v/v) and 0.5% (v/v) TFA in methanol were used for the column elution of ethephon, ethephon was not absolutely eluted from the NH_2 column, 1% (v/v) TFA in methanol, however, eluted ethephon from the column completely. Figure 1 shows the effects of the volume of 1% (v/v) TFA/methanol on the ethephon recovery from the NH_2 column. When the NH_2 columns were eluted with 10 and 15 mL of 1% (v/v) TFA/methanol, the recovery rates were not different. Therefore, ethephon elution from the column was performed with 15 mL of 1% (v/ v) TFA/methanol.

Analysis of Ethephon by GC/MS. Figure 2 shows a mass spectrum and total ion chromatogram of the ethephon standard esterified with diazomethane. Four fragment ions (*m*/*z* 109, 110, 113, and 145) were chosen for the detection of ethephon. Figure 3 shows the selected ion chromatograms of ethephon added to the tomato sample. The fragment ion chosen for the ethephon determination was *m*/*z* 110, and the qualified ion chosen was *m*/*z* 109. The other chosen ions were not detected in a low concentration of ethephon because many disturbances appeared around the ethephon fragment ion peak at *m*/*z* 113, and *m*/*z*

Table 1. Recovery of Ethephon in Fruit and Vegetable Samples

samples	amt of ethephon spiked (ng/g)	recovery (%)	SD	coeff of variation (%)
tomato	100	104.	2.76	2.65
	50	97.6	3.81	3.90
	10	84.2	4.01	4.76
natsumikan (Japanese summar orange)	100	100	3.21	3.21
	50	97.3	3.93	4.04
	10	78.6	5.04	6.41
corn	100	109	3.81	3.50
	50	100	4.51	4.51
	10	98	5.25	5.36
peach	100	107	3.97	3.71
	50	98.1	4.23	4.31
	10	89.5	4.74	5.30
cherry	100	93.2	3.64	3.91
	50	96.6	3.97	4.11
	10	92.7	4.11	4.43
grape	100	103	2.87	2.79
	50	101	3.01	2.98
	10	99.7	3.76	3.77

[a] Data represent the mean ± SD for six experiments.

145 of the ethephon fragment ion was a low intensity. On the other hand, the other fragment ion peaks (*m*/*z* 109 and *m*/*z* 110) had no distinct interference peaks. In the other samples, the fragment ion peaks of *m*/*z* 109 and 110 observed were the same as those of the tomato sample. Finally, the fragment ions of *m*/*z* 109 and 110 for the ethephon determination were chosen.

When the fragment peaks for the two selected *m*/*z*'s signals (109 and 110) were present, the retention time matched that of the ethephon standard (±5 s) and the ratio of integrated areas of the two selected signals were within ±15% of the ratio for the ethephon standard, the peak was identified as the signal for ethephon. In addition, the standard ratio for ethephon was 3.19 ± 0.243 in the five experiments. The detection limit, defined as the amount of ethephon equivalent to three standard deviations (SDs) of the noise level in observations at the baseline level of the selected ion (*m*/*z* 110), was 4 pg. The determination limit, defined as those equivalent to 8 SDs of the noise level, was 11 pg. The working range was actually between 10 and 1000 ng/mL, and the correlation coefficient was 0.999. The lowest amount for ethephon detection was 4 pg, and the lowest amount for ethephon determination was 10 pg.

Ethephon Residues in Fruits and Vegetables Distributed in Western Japan. Table 1 shows the results of ethephon recovery from fruit and vegetables. As the results indicate, ethephon recovery was obtained in the range from 93.2 to 109% for fruit and vegetables. The recovery values from the official Japanese method ranged from ND to 28.7%. Figure 4 shows selected ion chromatograms of ethephon detected in pineapples, and Table 2 shows the concentrations of ethephon residue remaining in commercial fruit and vegetables. The residual levels were ND to 97 ng/g in pineapples. Ethephon residue was not detected in the other fruit and vegetables.

Ethephon had been shipped to Japan in the amount of 5844 kg/year in 1999 (*10*). However, the current status is unclear. Recently, several pesticides are known to be endocrine disrupting chemicals (EDC). Straube et al. (*11*) reported that testosterone and especially estoradiol in the blood decreased in professional pesticides applicators after acute pesticide exposure, including ethephon, and also reported that CD4 and CD8 lymphocytes slightly increased. In their study, maximal residues on the applicators bodies surfaces exposed to ethephon were

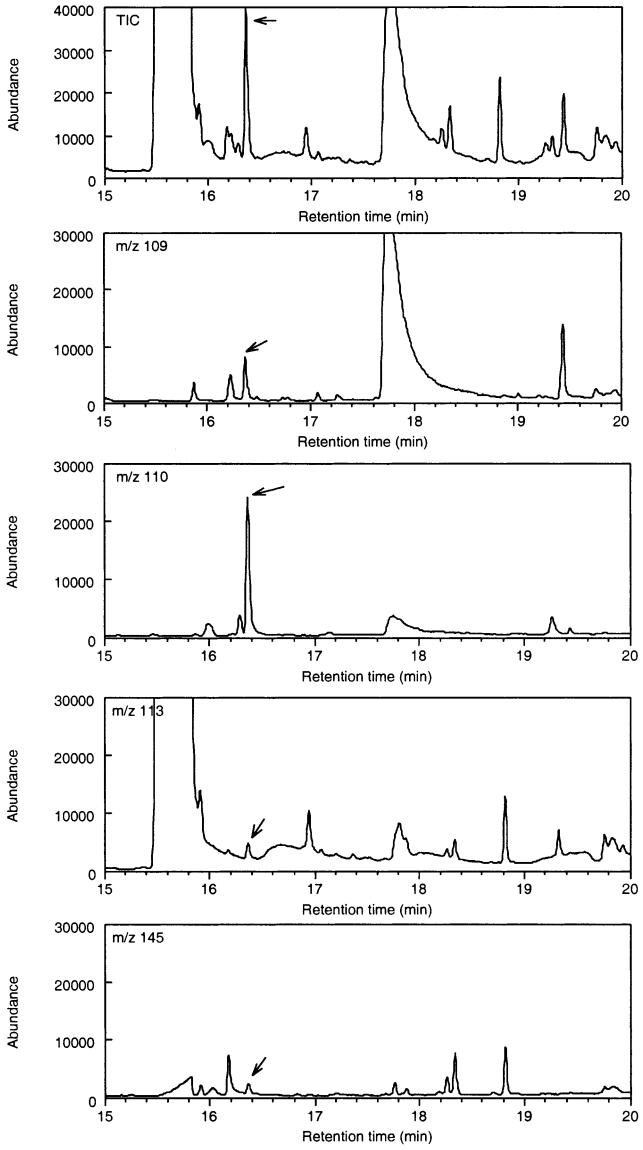

Figure 4. Selected ion chromatograms of ethephon methyl derivative detected in commercial pineapple. The arrows show the ethephon's signals.

Table 2. Results of the Ethephon Residue Analysis in Japanese Fruit and Vegetables

sample	no.	results[a] (μg/g)
cherry	4	ND
corn	4	ND
grape	4	ND
Japanese summer orange	4	ND
peach	4	ND
pineapple	4	ND – 0.097
tomato	4	ND
Japanese persimmon	4	ND
Satsuma mandarin (early ripening type)	4	ND
Japanese pear	4	ND

[a] ND, value is less than 2 ng/g.

from 0.52 to 1.39 mg. The ethephon residual levels in pineapples, however, are of a high value in comparison with other pesticide residual levels in Japan. If we ingest [100 g/(60 kg of body weight)]/day of pineapple, the intake of ethephon residue is about [8 μg/(60 kg of body weight)]/day. Whether this value is as dangerous as other EDC, it will be hereafter necessary to investigate.

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