Simultaneous Determination of Imazalil and Its Major Metabolite in Citrus Fruit by Solid-Phase Extraction and Capillary Gas Chromatography with Electron Capture Detection

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A method was developed for the simultaneous determination of imazalil and its major metabolite, R 14821, in citrus fruit. This method (designated the SPE method) is based on solid-phase extraction (SPE) and capillary gas chromatography (GC) with electron-capture detection (ECD). The SPE method is highly sensitive for both compounds (limit of detection for each: 0.001 µg/g) and is less time consuming than the previously reported method based on repeated liquid–liquid partitioning and GC–ECD. Recoveries for 3 levels of fortification (0.02, 0.2, and 2.5 µg/g) from satsuma mandarins ranged from 94.3 to 96.5% for imazalil and from 93.9 to 96.3% for R 14821, with coefficients of variation (CVs) ranging from 3.1 to 6.3% for imazalil and from 4.5 to 5.6% for R 14821. Average residual levels found in grapefruit, oranges, and lemons by the SPE method and the previously reported method, and the corresponding CVs, were similar.

Imazalil, 1-[2-(2,4-dichlorophenyl)-2-(2-propenloxy) ethyl]-1H-imidazole, is used as a post-harvest fungicide to suppress blue and green molds and sour rot on some raw agricultural commodities and to control their decay during storage (1, 2). It is easily metabolized to relatively stable R 14821, 1-(2,4-dichlorophenyl)-2-(1H-imidazole-1-yl)-1-ethanol, in vivo in rats (3) and in agricultural commodities. However, the toxicities of the metabolites have not been established. Therefore, there is concern about R 14821 contamination of foods, especially citrus fruit imported to Japan.

Although several analytical methods (4–9) have been developed for quantitation of imazalil in citrus fruit and many surveys of its residual levels have been conducted (4–6, 8–10), few attempts to detect R 14821 in foods have been reported, except for a method published in Vol. II of the Pesticide Analytical Manual (11) that allows simultaneous determination of both compounds. However, this method has the drawback of high detection limits and is time consuming because of the requirement for repeated extractions by liquid–liquid partitioning (LLP).

We attempted to lower the detection limit for R 14821, because its level in citrus fruit was thought to be far lower than that of imazalil, which was fully detectable even by previously published methods (4–9). By a combination of acetone extraction and concentration steps, it was possible to detect R 14821 at ng/g levels (12); however, this method (designated the LLP method) was still very time consuming.

In this paper, we describe a method (designated the SPE method) that decreases analysis time, and uses solid-phase extraction (SPE) and capillary gas chromatography (GC) with electron-capture detection (ECD) for simultaneous determination of imazalil and R 14821 residues in citrus fruit.

METHOD

Apparatus

(a) Homogenizer.—Ultra-Turrax, Model T25-S1 (IKA-Werke, Staufen, Germany).

(b) Vortex mixer.—Automatic mixer, Model S-50 (Taiyo Sci., Tokyo, Japan).

(c) Centrifuge.—Model H150C (Kokusan, Tokyo, Japan).

(d) Rotary evaporator.—Model RE-46 (Yamato, Tokyo, Japan).

(e) Hot block bath.—Model TPB-32 (Toyo Seisakusyo, Tokyo, Japan).

(f) Test tubes.—40 mL, glass, with glass cap (Shibata Rika, Osaka, Japan) and 10 mL, glass, with screw cap (Iwaki Glass, Tokyo, Japan).

(g) Flasks.—100 mL, round-bottom, glass (Iwaki Glass) and 100 mL Erlenmeyer, glass (Iwaki Glass).

(h) Filter paper.—No. C (Toyo Roshi, Tokyo, Japan).

(i) Glass filter.—11G1 (Iwaki Glass).

(j) Disposable pipets.—1K-PAS-9P (Iwaki Glass).

(k) SPE cartridge column.—Bond Elut LRC Diol, 500 mg/10 mL (Varian Sample Preparation Products, Harbor City, CA).

(l) Gas chromatograph.—Varian GC 3300 (Varian Associates, Inc., Sunnyvale, CA), equipped with 63Ni ECD and a DB-1701 fused-silica capillary column (0.25 mm id × 30 m, 0.25 µm film thickness; J&W Scientific, Folsom, CA).
Table 1. Elution patterns of imazalil and R 14821 on the Diol column

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Imazalil (CV, %)</th>
<th>R 14821 (CV, %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>78.4 (4.3)</td>
<td>82.9 (5.0)</td>
</tr>
<tr>
<td>2</td>
<td>11.9 (6.2)</td>
<td>8.0 (5.7)</td>
</tr>
<tr>
<td>3</td>
<td>3.5 (4.5)</td>
<td>3.2 (6.1)</td>
</tr>
<tr>
<td>4</td>
<td>1.7 (6.9)</td>
<td>2.3 (5.8)</td>
</tr>
<tr>
<td>5</td>
<td>0.6 (7.2)</td>
<td>0.7 (6.7)</td>
</tr>
</tbody>
</table>

(a) Each fraction consisted of 2 mL eluate.
(b) Average of 3 determinations.
(c) CV = coefficient of variation.

Materials and Reagents

(a) Citrus fruit.—Grapefruit, oranges, lemons, and satsuma mandarins were purchased from fruit stores in Osaka, Japan.
(b) Imazalil.—Purity, 99% (Riedel-de Haën, Hannover, Germany).
(c) R 14821.—Purity, 98% (Janssen Chimica, Beerse, Belgium).
(d) Acetone, n-hexane, ethyl acetate, dichloromethane, methanol (MeOH), toluene, and anhydrous sodium sulfate.—For pesticide analysis (Wako Pure Chemicals, Osaka, Japan).
(e) Sodium hydroxide (NaOH).—Special grade (Wako Pure Chemicals).
(f) N,O-bis(trimethylsilyl)acetamide (BSA) and pyridine.—GC grade (Wako Pure Chemicals).
(g) Water.—Tap water previously extracted with n-hexane to remove interfering substances.

(h) pH indicator.—Alkalit®, pH 7.5–14 (Merck, Darmstadt, Germany).

Standard Solutions

(a) Imazalil stock solution (1 mg/mL).—Dissolve 50 mg imazalil in 50 mL MeOH. Store at 4°C.
(b) R 14821 stock solution (1 mg/mL).—Dissolve 50 mg R 14821 in 50 mL MeOH. Store at 4°C.
(c) Imazalil and R 14821 intermediate solution (each analyte: 5 µg/mL).—Transfer 2 mL imazalil stock solution (1 mg/mL) and 2 mL R 14821 stock solution (1 mg/mL) to a 100 mL volumetric flask, and dilute to 100 mL with toluene (each analyte: 20 µg/mL). Dilute 25 mL solution to 100 mL with toluene. Store at 4°C.
(d) Imazalil and R 14821 working standards (each analyte: 0.1, 0.2, and 0.4 µg/mL).—Transfer 2, 4, and 8 mL aliquots of imazalil and R 14821 intermediate solution (each analyte: 5 µg/mL) to individual 100 mL volumetric flasks, and dilute to 100 mL with toluene. Store at 4°C.
(e) Imazalil and R 14821 standards for fortification (each analyte: 0.4, 4, and 50 µg/mL).—Transfer 5 mL imazalil stock solution (1 mg/mL) and 5 mL R 14821 stock solution (1 mg/mL) to a 100 mL volumetric flask, and dilute to 100 mL with MeOH (each analyte: 50 µg/mL). Dilute 8 mL solution to 100 mL with MeOH (each analyte: 4 µg/mL), and then dilute 10 mL solution to 100 mL with MeOH (each analyte: 0.4 µg/mL). Store at 4°C.

Sample Preparation

Cut 50 g whole citrus fruit into small pieces and chop well with a kitchen knife.

Sample Extraction and Cleanup by the SPE Method

Weigh 10 g finely chopped citrus fruit into a 100 mL Erlenmeyer glass flask. Add 50 mL acetone to the flask. Homogenize the mixture with an Ultra-Turrax homogenizer for 2 min, and let stand at room temperature for 30 s. Add 5 mL water to the flask and homogenize again if the sample becomes sticky and difficult to separate into 2 phases during this

Table 2. Recovery of imazalil and R 14821 from satsuma mandarins

<table>
<thead>
<tr>
<th>Compound</th>
<th>Added, µg</th>
<th>Mean recovery, %</th>
<th>CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Imazalil</td>
<td>0.2</td>
<td>95.8</td>
<td>6.3</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>96.5</td>
<td>3.1</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>94.3</td>
<td>5.8</td>
</tr>
<tr>
<td>R 14821</td>
<td>0.2</td>
<td>96.3</td>
<td>5.6</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>95.2</td>
<td>4.5</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>93.9</td>
<td>5.1</td>
</tr>
</tbody>
</table>

(a) Each compound was added to 10 g satsuma mandarins in which no residue was detected in advance.
(b) Average of 3 determinations.
(c) CV = coefficient of variation.
step. Filter the upper layer through 30 g anhydrous sodium sulfate on a filter paper (No. C). Add 50 mL acetone to the flask. Repeat homogenization and filtration. Rinse the solid residue on the filter with 10 mL acetone. Collect acetone extracts/rinses in the same filtering flask, and concentrate, using a rotary evaporator (water bath, 40°C).

Transfer the concentrate (ca 8 mL) to a 40 mL glass tube. Add 5N NaOH, and dip a pH indicator strip into the solution to confirm that the pH is 13–14. Add 10 mL ethyl acetate, mix on vortex mixer for 20 s and centrifuge at 1800 rpm for 10 min. Draw up the ethyl acetate extract, using a disposable pipet connected to a silicone rubber bulb, and transfer extract to a 100 mL Erlenmeyer glass flask. Add 10 mL ethyl acetate to the residual solution, and repeat extraction and centrifugation.

Transfer the ethyl acetate extract to the flask. Add 30 g anhydrous sodium sulfate, and shake flask for dehydration. Pass the extract through a glass filter (11G1) and concentrate using a rotary evaporator at 40°C. Transfer the concentrate to a 10 mL test tube. Remove the solvent by evaporation to 1 mL with a stream of nitrogen gas, while warming the tube by heating with a hair dryer to prevent formation of water droplets.

Transfer the solution to a Bond Elut LRC Diol column pre-conditioned with 8 mL dichloromethane and 8 mL ethyl acetate. Rinse the tube with an additional 1 mL ethyl acetate, and add the rinses to the column. Wash the column with 7 mL ethyl acetate, and elute imazalil and R 14821 from the column into a 50 mL round-bottom glass flask with 8 mL ethyl acetate–MeOH (1 + 1, v/v).

Silylation of R 14821 with BSA

Add 20 μL BSA to 0.5 mL imazalil and R 14821 working standards (each analyte: 0.1, 0.2, and 0.4 μg/mL). Mix on vortex mixer for 20 s and heat to 90°C for 15 min. After cooling, adjust the volume to 5 mL with n-hexane–pyridine (20 + 1, v/v) to prepare the standard solutions for GC (each analyte: 0.01, 0.02, and 0.04 μg/mL).

After sample extraction and cleanup, concentrate the eluate from the SPE column, using a rotary evaporator at 40°C. 

Figure 1. Gas chromatograms of (A) control satsuma mandarins (10 g), (B) control satsuma mandarins (10 g) fortified with imazalil (0.2 μg) and R 14821 (0.2 μg), and (C) standard solution of imazalil (0.02 μg/mL) and silylated derivative of R 14821 (0.02 μg/mL). IZ = imazalil; R1-TMS = silylated derivative of R 14821.
Transfer the concentrate to a 10 mL test tube. Evaporate the solution to dryness with a stream of nitrogen gas, while warming the tube by heating with a hair dryer as described in the sample extraction and cleanup section. Add 0.5 mL toluene and 20 mL BSA to the tube, mix the contents of the tube, and heat. After cooling, adjust the volume to 5 mL with n-hexane–pyridine (20 + 1, v/v).

**Determination**

Measure the level of each compound in the final solution with a Varian gas chromatograph with electron-capture detector by comparison of GC peak responses (area or height) obtained for the working standard and the sample. Determine the linear regression coefficients for the standard calibration graph from the plot of imazalil or R 14821 chromatographic peak response versus the corresponding imazalil or R 14821 concentration in the standard solution (each analyte: 0.01, 0.02, and 0.04 mg/mL). The graphs should be linear with a coefficient of determination >0.98. Calculate the concentrations of imazalil and R 14821 in citrus fruit samples with the following equation:

\[
\text{Imazalil or R 14821, } \mu\text{g/g} = C \times \frac{\text{Hsp}}{\text{Hst}} \times \frac{V}{W} \times F
\]

where \(C\) = concentration of the standard solution (\(\mu\text{g/mL}\)), \(\text{Hsp}\) = peak height (mm) or area count for sample extract, \(\text{Hst}\) = peak height (mm) or area count for the standard solution, \(V\) = sample volume for GC (5 mL), \(W\) = sample weight (10 g), and \(F\) = dilution factor, if needed.

**Fortification**

Ensure that no imazalil or R 14821 is present in the materials to be fortified. Homogenize 150 g satsuma mandarins, and fortify 10 g portions of the homogenate with 0.5 mL imazalil and R 14821 standard solution (each analyte: 4, 4, and 50 \(\mu\text{g/mL}\)) to bring the content of each analyte in the sample to 0.02, 0.2, and 2.5 \(\mu\text{g/g}\), respectively.

**Elution Pattern on Diol Column**

Transfer 0.5 mL aliquots of imazalil and R 14821 standards for fortification (each analyte: 4 \(\mu\text{g/mL}\)) to a 10 mL test tube. Evaporate the standard solution to dryness with a stream of nitrogen gas, while warming the tube by heating with a hair dryer. Dissolve the residue in 1 mL ethyl acetate, and transfer the resulting solution to a Bond Elut LRC Diol column as described in the sample extraction and cleanup section. Collect fractions of 2 mL eluate in 10 mL test tubes, and evaporate to dryness with a stream of nitrogen gas, while warming the tubes. Derivatize the residues in each fraction with BSA. Measure the content of each compound in each fraction by GC–ECD, as described above, to determine the elution profile of the compounds.

**Confirmation of Identity**

Inject 2 \(\mu\text{L}\) aliquots of the final solution for each sample and the appropriate standard solution, containing imazalil and the silylated derivative of R 14821, into the GC column. Confirm the presence of both compounds in the sample solution by GC with mass selective detection (GC–MSD) by comparison of retention times and relative abundances of 3 ions (\(m/z\) 173, 215, and 217 for imazalil; \(m/z\) 154, 247, and 249 for the silylated derivative of R 14821) obtained for the sample and the standard (8, 12).

**Results and Discussion**

A method using SPE column cleanup and capillary GC–ECD was developed for the simultaneous determination of imazalil and R 14821 residues in citrus fruit. Compared with previous methods (11, 12), this procedure decreased the analysis time expended.

Table 1 shows the elution patterns of imazalil and R 14821 on the Diol column. Majority portions of both compounds were eluted in the first fraction (each: about 80%), and moderate amounts of both compounds were detected in the second fraction. Smaller quantities of both compounds were detected...
in the third and fourth fractions; <1% of each compound was eluted in the fifth fraction. Total recoveries of both compounds in fractions 1–4 were >95%. Based on this result, the elution volume of ethyl acetate–MeOH (1 + 1, v/v) was set at 8 mL.

A recovery test was conducted to validate the SPE method (Table 2). Both compounds were detected in almost all the imported citrus fruit examined (12). Therefore, domestic satsuma mandarins were used for the recovery test because they contained no residual imazalil or R 14821. At fortification levels of 0.02, 0.2, and 2.5 μg/g, average recoveries were 94.3–96.5% for imazalil and 93.9–96.3% for R 14821. The accuracy of the SPE method was confirmed by the low coefficient of variation (CVs) and high recoveries obtained.

Figure 1 shows gas chromatograms obtained for standard compounds, control satsuma mandarins, and control satsuma mandarins fortified with both compounds. No distinct peaks appeared at the retention times of both compounds in the control chromatogram. Relatively large, unidentified peaks appeared at retention times of 10–15 min in the chromatograms obtained for the control and the fortified sample, but they did not interfere with the determination of either compound. Furthermore, no peaks influenced the determination of either compound in samples of other citrus fruit such as grapefruit, oranges, and lemons (data not shown).

In the chromatograms of control satsuma mandarins (n = 3), the values of the background noise at the retention time of imazalil were <0.5 mm. Also, those at the retention time of R 14821 were <0.5 mm. Because the heights of the imazalil and R 14821 peaks (each: 0.02 ppm) were >40 mm in Figure 1, the detection limits were calculated as 7.5 x 10⁻⁵ ppm (3 × 0.5 mm [maximum value]/40 mm × 0.02 ppm) at a signal-to-noise ratio of 3:1. Therefore, both detection limits were estimated as 0.001 ppm.

Table 3 shows the levels of imazalil and R 14821 in imported citrus fruit as determined by the SPE and LLP methods (12). Each compound was detectable at ng/g levels by both methods. The average values obtained by the 2 methods were compared and found to be in a ratio of 1.00 to 1.08. Moreover, the CVs of these methods were similar and satisfactory (3.7–6.2 for the SPE method; 4.8–6.5 for the LLP method). However, the SPE method was less time consuming than the LLP method and demonstrated sensitivity equivalent to that of the LLP method.

References