Decline pattern and risk assessment of cyenopyrafen in different varieties of Asian pear using liquid chromatography and tandem mass spectrometry

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Abstract The dissipation pattern of a commercial cyenopyrafen formulation sprayed at the recommended dose on Asian pears (two different species) grown at two different sites was investigated using liquid chromatography–ultraviolet detection. Samples collected randomly over 14 days were extracted using acetone, partitioned using *n*-hexane/dichloromethane (8/2, v/v), and purified using a Florisil solid-phase extraction cartridge. The residues in field-incurred samples were confirmed via liquid chromatography–tandem mass spectrometry. The method was validated in terms of excellent linearity in the solvent ($R^2=1$); moreover, satisfactory recoveries (89.0–107.3%) were obtained at three fortification levels with a relative standard deviation (RSD) $\leq 5.0\%$ and the limits of detection and quantification of 0.0033 and 0.01 mg/kg, respectively. Although the residual levels at both sites were lower than the maximum residue limit (MRL=1 mg/kg), the dissipation at Site 2 was faster than that at Site 1. Consequently, the half-life ($t_{1/2}$) in Site 2 (5.2 d) was shorter than that in Site 1 (9.8 d). Risk assessment at zero days showed acceptable daily intakes (%) of 27.25% and 24.52% at Sites 1 and 2, respectively, indicating that these fruit species are safe for consumption.

Keywords: cyenopyrafen, Asian pear, decline pattern, liquid chromatography, risk assessment

Introduction

Globally, pesticides are used to protect crops against pests and diseases. The residues of these chemicals occur commonly in food commodities when pesticides are applied improperly (1,2); these residues are noxious for the consumers (3). Cyenopyrafen is a novel beta-ketonitrile acaricide developed and commercialized by Nissan chemicals in 2009 (4). It shows excellent efficacy for preventing fruits, vegetables, and flowers from spider mites (5). In the Republic of Korea, cyenopyrafen is registered as a pesticide for protecting pears from spider mites (6). Pear is one of the most cultivated fruits in the Republic of Korea, with a total cultivation area of 12.664 hectares (7.7% of the total cultivated area for fruits) (7) and an average daily intake of 0.01544 kg per person (8). Thus far, no study has investigated the residual amount and risk assessment of cyenopyrafen in Asian pear. Different fruits and crops need distinctive methods for the detection and quantification of residues belonging to various classes of pesticides because each of them has a different co-extract, e.g., sugar/lipid content, and acidity of the extract (9,10).

Generally, the dissipation pattern of pesticides is only valid for a single analyte on a single crop under specific conditions (11). To explore the dissipation pattern of any pesticide, many factors such as various crop properties (including shape, cuticle features, stage of growth, and growth rate) and application method characteristics (formulation, rate, water volume, pressure, nozzle type, and boom height above the canopy) should be considered under the same environmental condition (12). The maximum residue limit (MRL) regulations require a pre-harvest interval (PHI) to ensure the decline of the residual level below the proposed limit at harvest time (13). The analysis and estimation of the dissipation pattern of a chemical are very important for evaluating the behavior of the residues and for determining the PHI and pre-harvest residue limit (PHRL) (14). Studies employing microextraction and quick, easy, cheap, effective, rugged, and safe “QuEChERS” method are sometimes unable to reflect the real data on dissipation patterns owing to the weight of the samples (15).

Lee *et al.* (16) reported (in Korean) a residue analysis of cyenopyrafen with azoxystrobin in grape using a high-performance liquid chromatography–mass spectrometry method.

graphy with a diode-array detector (HPLC–DAD). In this study, the samples were extracted using acetone and partitioned using dichloromethane (DCM) without further clean-up to assess their decline patterns without establishing the pre-harvest residue limit (PHRL) data. Hwang *et al.* (17) reported an analysis of cyenopyrafen in grapes using ultrafast liquid chromatography coupled with photodiode array detection (UFLC–PDA), in which the samples were extracted using acetone, partitioned using *n*-hexane/DCM (8/2, v/v), and purified using a silica solid-phase extraction (SPE) cartridge. However, no reports were retrieved from the databases for the determination of cyenopyrafen residues in Asian pear. Thus, the objective of the current study was to quantify the cyenopyrafen residue in two different varieties of field-incurred pear samples grown at two different locations in order to propose the decline pattern of the residue, establish the PHRL for safe use, and evaluate the risk assessment from the generated data.

Materials and Methods

Chemical and reagents Standard cyenopyrafen (98% purity) was acquired from Wako Chemicals USA (Richmond, VA, USA). HPLC-grade acetone, acetonitrile (ACN), dichloromethane (DCM), *n*-hexane, and ethyl acetate (EA) were purchased by Honeywell Burdick and Jackson (Ulsan, Republic of Korea). Analytical reagent (AR)-grade sodium chloride (NaCl), anhydrous sodium sulfate (Na₂SO₄), and Celite 545 were supplied by Junsei Chemical Co. Ltd (Tokyo, Japan). SPE Florisil cartridge (1 g, 6 mL) was purchased from Phenomenex (Torrance, CA, USA). Water was purified using a Milli-Q apparatus (UltiMA Duo 200 (COMBI); Balman Tech, Daegu, Korea) prior to use.

Standard solution 10.20 mg of cyenopyrafen was taken in a 100-mL volumetric flask, and 100 mL of ACN was added to it to prepare a 100-mg/L standard stock solution, which was stored at –20°C before use. Moreover, a 20-mg/L working solution was prepared from this stock solution, and a calibration curve was constructed by diluting the working solution with ACN to obtain concentration levels of 0.05, 0.1, 0.2, 0.5, 1.0, 2.0, and 5.0 mg/L.

Field experiment Two different varieties of pear samples were grown at two different sites (one variety at each site), namely, Site 1 in Naju and Site 2 in Gochang, Republic of Korea, separated by a geographical distance of 80 km. At each site, four trees were selected—three for three replicates and one for the control pear samples. The commercial formulation of cyenopyrafen [25% suspension concentration (SC) obtained from Hankook Samgong Co., Ltd. (Iksan, Korea) was diluted at the dose suggested by the manufacturer (10 mL/20 L) and sprayed (at August 16 and 26, 2015, for Sites 1 and 2, respectively) on the pears using a power sprayer. The spray time and volume of the solution remaining in the sprayer after spraying was measured to calculate the sprayed amount. The samples (seven

pears for each replicate) were harvested randomly at 0 (2 h), 1, 2, 3, 5, 7, 10, and 14 days after cyenopyrafen application. During the sampling process, the temperature, humidity, and fruit size were recorded. The samples were chopped into small slices, and seeds were removed from them. These small slices were then mingled to ensure proper mixing. The mixed sample was placed in a zip-lock bag at –24°C, pending assessment.

Sample preparation A 20 g homogenized pear sample and 100 mL of acetone were added to a 250-mL conical flask, which was shaken for 30 min using a horizontal mechanical shaker (DS-31F; DASOL Scientific Co., Ltd., Hwaseong, Korea). Then, the sample was vacuum-filtered through a Whatman™ 6 filter paper (GE Healthcare UK Limited, Buckinghamshire, UK) and Celite 545. The filtration was completed by washing the filtrate with 30 mL of acetone. The filtrate was then placed in a 1,000-mL separatory funnel, to which 450 mL of distilled water, 50 mL of saturated NaCl solution, and 100 mL of 20% DCM in *n*-hexane were added, and the separatory funnel was shaken for 5 min. The mixture was left undisturbed for completing the partition. The organic layer was collected in a 250-mL round-bottomed flask using anhydrous Na₂SO₄. The samples were re-extracted with 50 mL of 20% DCM in *n*-hexane. The combined extract was then evaporated on a vacuum evaporator (R-114; Büchi, Flawil, Switzerland) at 40°C, and the residue was reconstituted in 6-mL *n*-hexane for purification. A Florisil SPE cartridge (1 g, 6 mL) was conditioned with 6 mL of *n*-hexane; then, the extracted samples were loaded on it. In addition, 6 mL of 5% EA in *n*-hexane was used for washing the cartridge, and 10 mL of 15% EA in *n*-hexane was used to elute the target analyte. The eluent was evaporated and dissolved in 4 mL ACN for liquid chromatography–ultraviolet detection analysis.

Instrumental analysis For LC analysis, a Shimadzu HPLC (SCL-10A VP; Shimadzu, Tokyo, Japan) system was used, which comprised an auto sampler (SIL-20A), a column oven (CTO-20 A), a dual pump (LC-6 AD), and a ultraviolet (UV)–vis detector (SPD0 10 AVP). The operating system was controlled using the Class VP software. For chromatographic separation, a Gemini-NX 5u C18 110 Å (4.6 mm I.D.×300 mm, Phenomenex) column was used, and the column temperature was maintained at 35°C. The injection volume was 20 µL, and the mobile phase (ACN: deionized water; 80:20, v/v) was in the isocratic mode at a total flow rate of 1 mL/min. The analyte was detected at a wavelength of 297 nm.

A Waters Alliance 2695 LC separation module and a Micromass Quattro Mico triple-quadrupole tandem mass spectrometer (Waters Crop., Milford, MA, USA) was used for mass confirmation, and a C18 column (150×3.0 mm i.d., 3.5 µm particle sizes, Agilent, Santa Clara, CA, USA) was used for separation. An isocratic mobile phase comprising a binary solvent (80% A and 20% B, where A and B comprised 0.1% formic acid+5 mM ammonium formate in methanol and distilled water, respectively) was used at a total flow rate of 0.35 mL/min.

Method validation The parameters of specificity, linearity, limit of detection (LOD), limit of quantification (LOQ), recovery, and precision were used to validate the analytical method. The linearity of the analyte in the solvent was calculated by comparing the proportionality of the instrumental response as peak area at different concentration levels. The determination coefficient (R^2) from the seven-point calibration curve was used to assess the linearity. LOD is the minimum detectable amount at the minimum concentration and produces a signal-to-noise ratio (S/N) of 3; LOQ is the minimum quantifiable amount at the minimum concentration and produces an S/N of 10. The accuracy (expressed as recovery) was determined by fortifying the blank samples (in triplicate) with the standard solution at three levels of LOQ, 10× LOQ, and MRL. All the spiked blank samples were extracted and purified as mentioned earlier for sample preparation

using the same operating system. Precision was expressed as the relative standard deviation (RSD, %).

Results and Discussion

Method optimization Extraction using acetone and partitioning with *n*-hexane/DCM (8/2, v/v) without a clean-up procedure were not possible because of the presence of interference peaks around the retention time of the tested analyte. Washing the Florisil SPE cartridge with *n*-hexane/EA (95/5, v/v) and elution with *n*-hexane/EA (85/15, v/v) afforded a cleaner chromatogram compared with that obtained when *n*-hexane/acetone (95/5, v/v) eluent was used without washing. Therefore *n*-hexane and EA were selected for cleaning the

Fig. 1. Chromatograms of (A) blank pear sample, (B) standard injected at 1.0 mg/L, (C) blank pear sample spiked with 0.1 mg/kg of standard, (D) field-incurred sample collected at 0 days

Table 1. Linear range (mg/L), coefficient of determination (R^2), recovery (%), limit of detection (LOD), and limit of quantification (LOQ) of cyenopyrafen in Asian pear

Pesticide	Linear range (mg/L)	R^2	Recovery % (Mean $\pm$ RSD)			LOD (mg/kg)	LOQ (mg/kg)
			0.01 mg/kg	0.1 mg/kg	1.0 mg/kg		
Cyenopyrafen	0.05–5.0	1.000	107.3 $\pm$ 3.8	89.0 $\pm$ 0.8	101.0 $\pm$ 5.0	0.0033	0.01

Florisil SPE cartridge used in this study.

Selectivity and specificity The selectivity of the method was validated by obtaining identical retention times for both the injected standard and spiked samples. The interference with the analyte was examined by comparing the chromatogram of the standard sample with those of the blank and fortified samples. The chromatogram for the blank sample (Fig. 1) shows no other peak at the retention time for the standard sample.

Linearity The linearity of the analyte was assessed by injecting samples at seven concentration levels ranging from 0.05 to 5.0 mg/L; a good calibration curve was obtained with a linearity equation of $y=2396.8199x-474.1615$ and $R^2=1.0000$.

Limit of detection (LOD) and limit of quantification (LOQ) The LOD and LOQ for cyenopyrafen in the pears were 0.0033 and 0.01 mg/kg, respectively (Table 1). The LOQ was considerably lower than the MRL (1 mg/kg) established by the Korean Ministry of Food and Drug Safety (18) for pears.

Recovery The percentage recoveries of the analyte at LOQ, 10 $\times$ LOQ, and MRL were 103.3–107.4%, 88.4–89.9%, and 96.9–106.6%, respectively, with RSD $\leq$ 5.0% (Table 1). To assess the storage stability of the same analyte, the percentage recovery was checked at the LOQ and 10 $\times$ LOQ levels (extraction was conducted 30 days after spiking the blank sample); the observed recoveries were 106.9–

109.8% and 78.6–88.5%, respectively, with RSD $\leq$ 6.0%. In summary, this suggests that the developed method is accurate and that the analyte was stable under storage conditions.

Method application The method was successfully applied to treated pear samples collected from two different sites. Initial depositions at Sites 1 and 2 were 0.90 and 0.81 mg/kg, respectively, and the residual amounts 14 days after the treatment were 0.28 and 0.11 mg/kg at Sites 1 and 2, respectively (Fig. 2). As noted, the residual amounts were slightly higher at Site 1 compared to Site 2, this variation is related to the sprayed amount (19.5 L for Site 1 and 12.5 L for Site 2). We were unable to attribute the variation in the initial deposits to rainfall because it was recorded on days (8, 9, 11, and 12 post-application) other than the sampling dates at Site 2. The decline rate on Day 1 at Sites 1 and 2 were 24.44 and 34.57%, respectively, and those on Day 14 were 68.89 and 86.42%, respectively (Table 2). In addition, the half-lives of 9.8 and 5.2 days for Sites 1 and 2, respectively, were different. This means that the species cultivated at Site 1 slowly degraded the pesticide in comparison to the species cultivated at Site 2. The average temperature and humidity at Sites 1 and 2 were 22.5°C and 85.0% and 24.7°C and 83.3%, respectively (Fig. 3). Slightly higher temperatures, light intensity during summer (Site 2, located to the south of Site 1), and fruit species could be among the factors contributing to the shorter half-life of the analyte at Site 2. There are many factors that affect the pesticides in plants, including environmental conditions [temperature, humidity, light intensity, and rainfall (19)], crop species (20–24), physicochemical

Fig. 2. (A) Dissipation pattern and (B) PHRL curve of cyenopyrafen at Sites 1 (Naju) and 2 (Gochang)

Table 2. Residue amount, percentage decline, and risk assessment of cyenopyrafen at Sites 1 and 2

Sampling (Days)	Site 1 (Naju)				Site 2 (Gochang)			
	Residue (average±RSD, mg/kg)	Decline (%)	EDI (mg)	%ADI	Residue (average±RSD, mg/kg)	Decline (%)	EDI (mg)	%ADI
0	0.90±0.16	--	0.0139	27.25	0.81±0.07	--	0.0125	24.52
1	0.68±0.12	24.44	0.0105	20.59	0.53±0.07	34.57	0.0082	16.00
2	0.65±0.06	27.78	0.0100	19.68	0.53±0.11	34.57	0.0082	16.00
3	0.61±0.11	32.22	0.0094	18.47	0.35±0.06	56.79	0.0054	10.60
5	0.55±0.03	38.89	0.0085	16.65	0.23±0.01	71.60	0.0035	6.96
7	0.46±0.09	48.89	0.0071	13.92	0.24±0.03	70.37	0.0037	7.27
10	0.44±0.06	51.11	0.0068	13.32	0.16±0.03	80.25	0.0025	4.84
14	0.28±0.03	68.89	0.0043	8.48	0.11±0.02	86.42	0.0017	3.33
Half-life	9.8				5.2			
FDI ¹⁾	0.01544 kg							
ADI	0.051 mg							

¹⁾FDI=Fruits (pear) average daily intake per Korean

properties of the chemicals, formulations, and application methods (25,26).

The PHRL curve shows that if the residue level is less than 1.71 and 2.61 mg/kg 10 days before harvest for Sites 1 and Site 2, respectively, (Fig. 2), the pear is safe for consumption.

Liquid chromatography–tandem mass spectrometry (LC–MS/MS) confirmation In cases where false-positive results may be obtained from a conventional LC detector, mass spectrometry (MS) has proved to be a valuable analytical technique for the unambiguous identification of chemical contaminants in a variety of environmental samples because it provides information on the molecular structure of a compound (27). The cyenopyrafen standard (5 mg/L) and the field-incurred sample were confirmed via LC–MS/MS in the positive electrospray ionization (ESI⁺) mode using multiple reaction monitoring (MRM) with two mass transitions of m/z 310.0 and 254.0, attributed

to the quantifier and qualifier ions, respectively (Fig. 4). This LC–MS/MS result authenticated the LC–UVD results.

Risk assessment The data obtained from the quantification of cyenopyrafen on pear was used for safety evaluation. In the Republic of Korea, the cyenopyrafen MRL in pear was set by MFDS at 1 mg/kg (18). The acceptable daily intake (ADI) of cyenopyrafen is 0.051 mg (Table 2), and the average body weight of a Korean is 55 kg (8). The estimated daily intake can be calculated using the following equation:

$$\text{Estimated daily intake (EDI)} = \text{Residue amount} \times \text{average uptake. (28)}$$

The ADI% was calculated as $\text{EDI}/\text{ADI} \times 100$.

The exposure level of the Korean population to cyenopyrafen (applied at the recommended dose) was lower than 30% in both species cultivar cultivated at both Site 1 and 2 (Table 2); this value is much lower than the level suggested (100%) to produce health risk (29).

Fig. 3. (A) Average weight of the pears and (B) temperature and humidity at Sites 1 and 2

Fig. 4. LC-MS/MS chromatograms (A1) of the cyenopyrafen standard injected at 5.0 mg/L, (A2) relative mass spectrum of the cyenopyrafen standard, (B1) field-incurred sample collected at 0 days, and (B2) relative mass spectrum of the field-incurred sample at 0 days.

In conclusion, the dissipation pattern of cyenopyrafen on Asian pears cultivated at two locations was observed. From the dissipation pattern, the half-lives of the pears at Sites 1 and 2 were obtained as 9.8 and 5.2 days, respectively. The PHRL curves suggest that if the residue 10 days before harvest is less than 1.71 or 2.61 mg/kg, the residue would be below the MRL at the time of harvest. The decline rate was found to depend on temperature, light intensity, and species cultivar from the two different locations. The data generated from risk assessment indicates that the fruits were fit for consumption when the pesticide was applied according to the manufacturer's recommendations.

Disclosure The authors declare no conflict of interest.

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