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ORIGINAL ARTICLE

Establishment of Analytical Method for Cyazofamid Residue in Apple, Mandarin, Korean Cabbage, Green Pepper, Potato and Soybean

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Abstract A precise single residue analytical method was developed for fungicide cyazofamid in various crops. Apple, mandarin, Korean cabbage, green pepper, potato, and soybean were selected as representative crops, and clean-up system, partition solvent and extraction solvent were optimized. Limit of quantitation (LOQ) of cyazofamid was 2 ng (S/N>10) and good reproducibility was observed with small coefficient of variation (<4%). Excellent linearity was achieved between 0.05 and 20 mg/kg of cyazofamid standard solutions, with coefficients of determination of 1.000. Method limit of quantitation (MLOQ) was 0.02 mg/kg. For recoveries tests, crop samples were macerated and fortified with cyazofamid standard solution at three fortification levels (MLOQ, 10 MLOQ, and 100 MLOQ). And then those were extracted with acetone, concentrated and partitioned with dichloromethane. Then the extracts were concentrated again and cleaned-up through Florisil® column with ethyl acetate : *n*-hexane (30:70, v/v) before concentration and analysis with HPLC. Good recoveries from 75.3 to 98.5% with coefficients of variation of less than 10% were obtained, regardless of sample type, which satisfies the criteria of KFDA. Those results were reconfirmed with LC-MS/MS. The method established in this study could be applied to most of crops as an official and general method for residue analysis of cyazofamid.

Keywords cyazofamid · high-performance liquid chromatography · limit of quantitation · method limit of quantitation · recovery

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Introduction

Pests are organisms that are competitive to mankind or his interests in some aspect. The world's main source of food is plants, but they are susceptible to 80,000 to 100,000 kinds of diseases, 1,800 weed species, 10,000 insect species, and 1,000 nematode species as pests. Pesticides are any substance or mixture of substances intended for preventing, destroying, repelling or migrating any pest (Ware and Whitacre, 2004) and is applied to protect food crops from the pests at various stages of cultivation and during post-harvest storage, and is essential in agricultural production. However, with their use, the risk of residues remaining on the food is of major concern in food safety issues. Legislations were enacted throughout the world to regulate pesticides in food products (Ahmed, 2001), and maximum residue limits (MRLs) in foodstuffs have been set by government agencies to guarantee consumer safety and to regulate international trade. For this reason, a variety of analytical methods have been developed and applied routinely for the control of pesticide residue in food. Analysis of pesticide residues is extremely difficult, because sample matrix is complex, pesticides consist of many types of compounds, and its residues exist at ppm level or

lower. Therefore, analytical methodologies employed must be capable of residue measurement at very low levels and must also provide unambiguous evidence to confirm both the identity and the magnitude of any residues detected (Taylor et al., 2002).

Cyazofamid [4-chloro-2-cyano-N,N-dimethyl-5-*p*-tolylimidazole-1-sulfonamide; Table 1] is a sulfonamide fungicide (Tomlin, 2006) and registered 2001 in Korea for protection of ginger, potato, tomato, pepper, mandarin, ginseng, grape, Korean cabbage, melon, and green onion from various diseases (Korean Crop Protection Association, 2010).

It has very low mammalian toxicity (LD_{50} for rats: >5000 mg/kg) and ecological effect, indicated by low Log P_{ow} (3.2 at 25°C). Pharmacokinetics and metabolism studies in rats showed rapid absorption and elimination. Both the urine and feces were major routes of excretion, and excreted material was mainly 4-(4-chloro-2-cyanoimidazol-5-yl)benzoic acid (US EPA, 2004). Cyazofamid degraded rapidly in aerobic soil (DT_{50} in soil: 3–5 days) and was covalently bound to organic matter after degradation into the major degradates such as 4-chloro-5-*p*-tolylimidazole-2-carbonitrile and 4-chloro-5-*p*-tolulimidazole-2-carboxylic acid (Evaluation Report Cyazofamid, 2004).

Only few reports were available for the analysis of cyazofamid residues in crop or food including grapes and ginseng (Pesticide Residue Analytical Group, 2006; Choi et al., 2007; González-Rodríguez et al., 2011; González-Álvarez et al., 2012a; 2012b). However, such methods are not suitable for standard analytical method, because the subject crops are limited, not validated in full manner, time-consuming, and labor-intensive due to many steps involved in the clean-up procedure (Pesticide Residue Analytical Group, 2006). In Korea, the analytical method of cyazofamid residues in crop/food is listed in Food Code (Korean Food and Drug Administration, 2010) only as a part of multiresidue method for screening, therefore it cannot be used as a precise and reliable standard analytical method due to lack of method validation data.

The purpose of the present study is the establishment of a standard analytical method of cyazofamid residues in crop/food using HPLC, which is easily accessible and approved by the goverment for many different crop/food samples through full method validation and improvement for more efficient and simpler clean-up procedures than other existing methods using HPLC. Representative crops were selected among five crop groups such as cereal, fruits, vegetables, beans and oil crops, and potatoes.

Materials and Methods

Subject pesticides and crops. Standard material of cyazofamid (98.4%) (Fig. 1) was purchased from Fluka™ (Buchs, Switzerland). Apple, mandarin, Korean cabbage, green pepper, potato, and soybean of "residue-free grade" were purchased from a local market. They were chopped, macerated, and kept in a freezer at a temperature below –20°C in polyethylene bags.

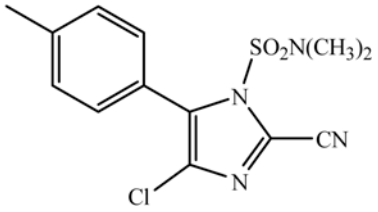

Fig. 1 Structure of cyazofamid.

Chemicals, reagents, and standard solutions. Acetonitile, acetone, *n*-hexane, and ethyl acetate were HPLC grade (Burdick and Jackson®, Ulsan, Korea). Sodium sulfate (GR grade) and sodium chloride (GR grade) were from Junsei Chemical Co. Ltd. (Tokyo, Japan). Florisil® (60–100 mesh) was purchased from Fluka™ (Saint Louis, MO) and activated by drying at 130°C for over 5 h. Filter papers (GF/A) were from Whatman International Ltd. (Maidstone, England). Fat-removing solvent (FR solvent) was prepared by saturating of *n*-hexane in acetonitrile. A stock solution of cyazofamid was prepared in acetonitrile at 1000 mg/L, and the working solutions were prepared by appropriate dilutions of the stock solutions with acetonitrile.

Measurement of instrumental sensitivity, reproducibility, and calibration curve linearity. Cyazofamid standard solutions (1, 0.1, and 0.05 mg/L) were analyzed with HPLC and the signal/noise ratio (S/N) of cyazofamid peak on chromatograms was calculated for LOD (S/N of 3) and LOQ (S/N of 10). For the assessment of reproducibility, a standard solution (0.05 mg/L) was analyzed with HPLC in seven replicates, and variations of retention time (t_r), peak area. and symmetry were examined. The standard solutions at 0.05, 0.1, 0.5, 1, 10, and 20 mg/L were analyzed with HPLC, and calibration curve linearity (R^2) was measured.

Establishment of the HPLC condition for separation of cyazofamid in crop samples. HPLC analysis was performed using an Agilent HPLC 1100 series system (Santa Clara, CA) with Agilent Eclipse XDB-C18 column (250 mm × 4.6 mm i.d., 5 μm particles), and column temperature was maintained at 35°C. Mobile phase was acetonitrile-water and the flow rate was 1 mL/min. For the analysis of apple, Korean cabbage, potato, and soybean, 65:35 (acetonitrile : water) was used as mobile phase, 62:38 for mandarin, and 63:37 for green pepper samples. The injection volume was 20 μL, and the detection wavelength of cyazofamid in crop samples was 280 nm.

For the selection of optimum detection wavelength of cyazofamid, aliquot (20 μL) of a standard solution (1 mg/kg) was analyzed to obtain a full UV spectrum with diode-array detector (DAD; 180–400 nm) under isocratic elution (acetonitrile:water=70:30).

Establishment of sample preparation procedure for cyazofamid. For establishment of optimum clean-up system, a glass column (35 cm × 1.5 cm i.d.) was filled with activated Florisil® (10 g) and added with anhydrous sodium sulfate (3 g). The column was pre-conditioned with *n*-hexane (100 mL) before

loading the cyazofamid standard solution (5 mL, 1 mg/L). The column was eluted with 50 mL of 10, 20, 30, 40, and 50% ethyl acetate/*n*-hexane mixture sequencially. Each eluate was evaporated under 40°C to dryness; the residue was dissolved with acetonitrile (5 mL) and analyzed with HPLC.

For the optimization of the liquid-liquid partitioning system, an aliquot of cyazofamid solution (1 mL, 5 mg/L) was added to water (25 mL) and left standing about 30 min in a separatory funnel before water (50 mL) and saturated sodium chloride solution (50 mL) were added. The mixture was extracted with each portion of three solvents (dichloromethane, *n*-hexane and ethyl acetate; 100 and 50 mL for each solvent). Organic phases were dried over anhydrous sodium sulfate and evaporated under 40°C to concentration. The residue was dissolved with acetonitrile (5 mL) and analyzed with HPLC.

To establish a fat removal system for soy bean, an aliquot of cyazofamid solution (1 mL, 5 mg/L) was added to 50 mL of *n*-hexane saturated with acetonitrile, and extracted with FR solvent (50, 30 mL). Acetonitrile layer was evaporated under 40°C. The residue was dissolved with acetonitrile (5 mL) and analyzed with HPLC.

To select the proper sample extraction solvent, an aliquot of cyazofamid solution (1 mL, 5 mg/L) was added to green pepper sample (25 g) and left standing fot about 30 min in flask. The flask was shaken at 180 rpm (1 h) for extraction after three solvents (acetone, acetonitrile, and methanol; 100 mL each) were added. The extract was filtrated, evaporated, partitioned, purified, and concentrated as mentioned in recovery procedure. The residue was dissolved with acetonitrile (5 mL) and analyzed with HPLC.

Recovery test of cyazofamid in crop samples. Samples (25 g) of apple, mandarin, Korean cabbage, green pepper, soybean, and potato were macerated and fortified with cyazofamid standard solution at 0.02, 0.2, and 2 mg/kg levels before the samples were extracted with shaking in the reciprocal shaker (SA-2s, Taitec, Japan) at 180 rpm for 1 h with acetone (100 mL). The mixture was filtered under reduced pressure through a Whatman™ GF/A filter paper, and the filter cake was rinsed with acetone (30 mL). The filtrate was concentrated under vacuum at 40°C (R-114, Büchi, Switzerland). The concentrate was dissolved in dichloromethane (100 mL), water (50 mL), and saturated sodium chloride solution (50 mL) for partitioning by shaking. Partitioning was repeated once more with 50 mL of dichloromethane and the combined dichloromethane layer was dried over anhydrous sodium sulfate, concentrated, and dissolved in *n*-hexane (5 mL). After loading the extract on the Florisil® column, which was conditioned with *n*-hexane (100 mL), the column was washed with 100 mL of ethyl acetate/*n*-hexane (10:90, v/v) and eluted with 100 mL of ethyl acetate/*n*-hexane (30:70, v/v). The eluate was concentrated, dissolved with acetonitrile (5 mL) and analyzed with HPLC.

Retention factor of cyazofamid of chromatogram. Retention factor (capacity factor, *k*) was calculated from equation using retention time (t_r) and adjusted retention time (t_r').

$k = t_r'/t_m$

t_r = retention time (min)

t_m = retention time of a non-retained compound (min)

$t_r' = t_r - t_m$ = adjusted retention time (min)

Number of theoretical plate (*N*) and height equivalent to a theoretical plate (*H*). *N* was calculated using t_r and peak width (Korean Food and Drug Administration, 2010). *N* and column length was used for calculation of *H* (Rood, 2007).

$N = 5.545\ (t_r/W_h)^2$

W_h = peak width at half height

H (mm) = column length (mm)/*N*

Optimization of $[M+H]^+$ ion (*m/z* 325) of cyazofamid in LC-MS/MS and analysis of crop samples. Varian 500-MS IT-MASS spectrometer (Walnut Creek, CA) equipped with HP 1100 HPLC was used with Phenomenex Kinetex C18 column (100A 2.1 mm × 50 mm, 2.6 μm particles, Torrance, CA). Elution solvent for HPLC was 0.1% formic acid in acetonitrile + 0.1% formic acid in water (60:40; v/v), and flow rate was 0.2 mL/min. Drying gas temperature, drying gas pressure, and nebulizer gas pressure were 350°C, 40, and 30 psi, respectively.

A standard solution (1 mg/kg) of cyazofamid (2 μL) was analyzed via LC-MS/MS in ESI (Elecrospray) positive mode (mass range: *m/z* 100–400), and the instrumental conditions such as capillary voltage, RF loading storage, and needle voltage were optimized for the best formation of its protonated molecular ion ($[M+H]^+$; *m/z* 325). A full scan spectrum of cyazofamid was obtained with the optimized conditions and then *m/z* 325 was used as a selective ion monitoring (SIM) ion in analysis of crop samples (0.02 mg/kg).

Results and Discussion

Analyses of representative crops and pesticide residue. MRLs of cyazofamid were established for the crops (Table 1). Apple and mandarin from fruits, Korean cabbage, green pepper, potato, and soybean, and oily crops were selected as representative crops, in consideration of their popularity and matrix characteristics in analytical aspects for application of developed method to the crops.

Analytical methods for pesticide residues involve several discrete steps, such as 1) Matrix modification (homogenizing of samples), 2) Extraction (extracting pesticide residues from samples), 3) Liquid-liquid partitioning (removing polar impurities), 4) Solvent evaporation (concentration of analytes in solution, 5) Clean-up (removing the co-extractives such as lipids and pigments, 7) Resolution (resolving analyte from remaining co-extractives by refined chromatography), 8) Detection (obtaining a response of analyte as a signal several on chromatography), and 9) Determination (calculating the amount of analyte present in sample) (Fong et al., 1999). However, in establishing of residue

Table 1 MRLs of cyazofamid in various crops (Korea Food and Drug Administration, 2010)

Crop	MRL	Crop	MRL	Crop	MRL
Potato	0.1	Peach	1.0	Cucumber	0.5
Green pepper	2.0	Watermelon	1.0	Tomato	0.5
Other crops	0.05	Ginger	0.5	Sesame	0.1
Pear	0.2	Spinach	3.0	Grape (Wild grapes)	2.0
Korean cabbage	2.0	Onion	1.0	Sweet pepper	2.0
Mandarin	0.5	Melon	0.5	Oriental melon	0.5
Welch onion	1.0	Leaf beet	10.0		

analytical method of a pesticide the procedure goes in reverse: detection, clean-up, liquid-liquid partitioning, extraction, and resolution.

Establishment of analytical method of cyazofamid from various crops. Optimum detection wavelength of cyazofamid in HPLC was investigated for sensitive detection. When a full UV spectrum of cyazofamid was recorded using DAD, λ_{max} was observed at 280 nm, therefore 280 nm was used as a detection wavelength in the present study. Other reports also engaged 280 nm (Pesticide Residue Analytical Group, 2006) and 290 nm (Suciu et al., 2011) as a detection wavelength.

Clean-up method with Florisil® column chromatography. In pesticide residue analysis, adsorption chromatography is generally used for clean-up of the interfering coextractives (e.g. lipids and pigments), which were not removed by liquid-liquid partitioning. Florisil®, silica gel, and alumina were used traditionally as column chromatography sorbents. On the other hand, Choi et al. (2007) used NH_2 cartridge. In the present study, Florisil®, the most popular material for clean-up in pesticide analysis, was chosen for absorption column chromatography. Various combinations of ethyl acetate/*n*-hexane mixture were used in sequence after loading of cyazofamid on Florisil® column. As a result, a mixture of 30:70 (ethyl acetate/*n*-hexane, v/v) gave a good recovery, indicating a mixture of such combination of 100 mL could be used as an elution solvent, whereas a mixture of 10:90 (ethyl acetate/*n*-hexane, v/v) be used for washing of the column to remove early eluting impurities without losing cyazofamid during clean-up procedure (Table 2).

Liquid-liquid partitioning of cyazofamid. After successful establishment of the clean-up procedure, liquid-liquid partitioning system was examined. Through this procedure, the polar interfering coextractives (e.g. carbohydrates) could be removed from sample extract (Fong et al., 1999). Sodium chloride was added in partitioning system, because as more 'salt' dissolves in the aqueous phase, more of the pesticide is partitioned into the organic phase (Fong et al., 1999). In the present study, three organic solvents such as dichloromethane, ethyl acetate, and *n*-hexane, were used with water (Table 3). Cyazofamid was well partitioned with dichloromethane, giving a total recovery of 105.4%. Therefore, dichloromethane (100, 50 mL) was selected as the organic solvent for liquid-liquid partitioning system.

Selection of extraction solvent. Three common solvents such as acetone, acetonitrile, and methanol were used to extract cyazofamid from crop samples. All three solvents gave reasonable recoveries (94.2–97.8%); however, acetone (94.9%) was chosen as the extraction solvent, because it is cheaper and more volatile than other solvents.

Method validation. Method validation is a set of procedures to evaluate the performance characteristics such as recovery, reproducibility, linearity and range of calibration, limits of detection, and quantitation of a method for specific analyte and sample types (Codex Alimentarius Commission, 2003).

Instrumental LOD and LOQ express the sensitivity of analytical instruments (Fong et al., 1999; Miller, 2005). Based on the analysis of several concentrations, 0.4 and 2 ng was determined as LOD and LOQ, respectively, which are satisfactory for sensitive analysis of cyazofamid residue. Choi et al. (2007)

Table 2 Recovery rate by sequential elution of ethyl acetate/*n*-hexane

Ethyl acetate/*n*-hexane		Recovery (%)
v/v	Volume	Florisil®
10 : 90	50 mL	-
20 : 80	50 mL	-
30 : 70	50 mL	91.7
40 : 60	50 mL	4.3
50 : 50	50 mL	-
Total		96.0

Table 3 Efficiency of liquid-liquid partitioning with three different solvents

Solvents	Recovery (%)		
	100 mL	50 mL	Total
n-Hexane	98.8	3.1	101.8
Ethyl acetate	92.2	5.6	97.8
Dichloromethane	100.3	5.1	105.4

Table 4 LOD, LOQ and reproducibility of analysis of cyazofamid

LOD	LOQ	Reproducibility		
0.4ng	2 ng	Factors	Average	C.V(%)
		t_r (min)	8.78	0.08
		Area	5.03	1.81
		Peak symmetry	1.08	3.20

Table 5 Recovery and MLOQ for cyazofamid in crops

Fortified level (mg/kg)	Recovery (%)[a]/C.V (%)[b]						MLOQ (mg/kg)
	Apple	Mandarin	Korean cabbage	Green pepper	Potato	Soybean	
0.02	79.1/5.2	75.3/6.0	92.0/2.6	90.1/1.3	87.0/3.4	98.5/4.4	0.02
0.2	95.2/0.8	78.5/4.5	89.9/1.3	86.1/3.7	83.0/4.7	89.8/2.7	
2	91.7/0.5	86.1/0.5	88.0/2.7	88.6/3.4	78.9/9.2	82.6/5.9	

[a]Average of triplicate experiments
[b]Coefficient of variation, standard deviation / mean × 100

reported that LOQ was 1.6 ng using HPLC.

For reproducibility study, LOQ level of cyazofamid solution (2 ng) was analyzed seven times (Table 4). Good reproducibility was observed with small coefficient of variation (<4%) for retention time (t_r), peak area, and peak symmetry, providing a good stability of instrument for reliable analysis. Good peak shape was also observed within values of 0.9–1.1 (Rood, 2007).

Excellent linearity was achieved between 0.05 and 20 mg/kg of cyazofamid standard solutions, with coefficient of determination (R^2) of 1.000. The regression equations were $y = 50.7810x - 0.3448$ for apple, Korean cabbage, potato, and soybean, $y = 51.8090x + 0.2325$ for mandarin, and $y = 51.1591x - 0.9011$ for green pepper.

MLOQ (Method Limit of Quantitation) is not an instrumental LOQ, but instead is a practical LOQ for the total analytical method. It is calculated using LOQ, injection volume, final extract volume, and sample weight in analytical method (Lee et al., 2008).

$$\text{MLOQ (mg/kg)} = (2\ \text{ng} \times 5\ \text{mL})/(20\ \mu\text{L} \times 25\ \text{g}) = 0.02\ \text{mg/kg} \quad (1)$$

MLOQ value for cyazofamid calculated using the equation 1 was 0.02 mg/kg. This value satisfied the criteria of KFDA (Korea Food and Drug Administration) which are below 0.05 mg/kg or half of MRL (Lee, 2011). Compared to other works with grapes (MLOQ = 0.72 mg/kg) (González-Álvarez, 2012b), our result is relatively more sensitive.

Recoveries of cyazofamid from crop samples. Recovery test can provide accuracy and precision of sample preparation method by recovered rate (accuracy, %) and C.V (precision, %) (Fong et al., 1999). Untreated samples were spiked with MLOQ (0.02 mg/kg), 10MLOQ (0.2 mg/kg) and MRL (2 mg/kg) levels of cyazofamid standard solutions, and the analysis was performed using the established method of extraction, partitioning and clean-up to give reasonable recoveries (75.3–98.5%) and low C.V (0.5–9.2%) (Table 5, Fig. 2).

Other studies also reported reasonable recoveries; González-Rodríguez et al. (2011) reported that recoveries were 85–103% in grapes and wines by HPLC-DAD and GC-ITMS, and Choi et al. (2007) reported that recovery was 80.6% in ginseng by HPLC-UVD.

Retention factor (*k*) and chromatographic efficiency in resolution of cyazofamid on HPLC. Retention factor (*k*) measures the extent to which a solute (cyazofamid) is retained and

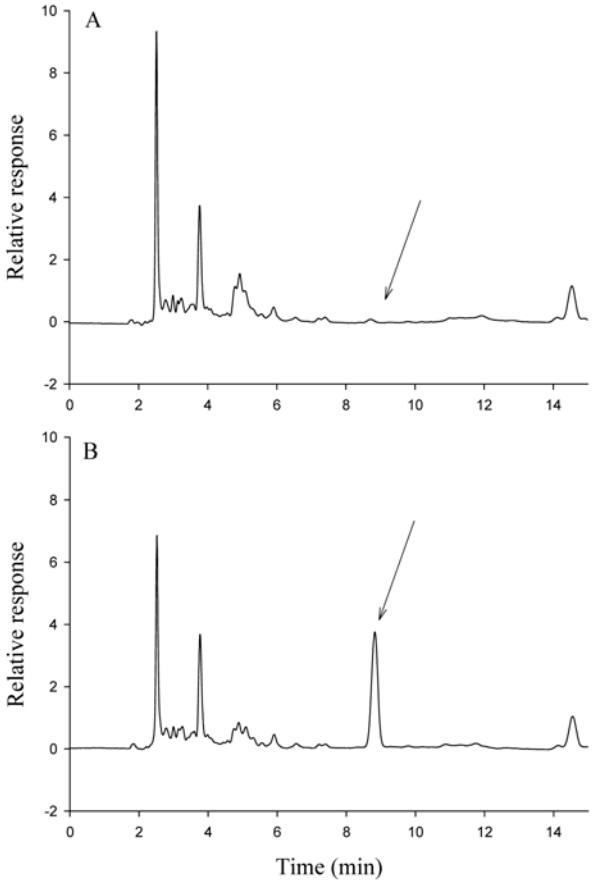

Fig. 2 Representative chromatograms of control (A) and recovery (B) of cyazofamid in apple extracts (0.2 mg/kg level).

is commonly called the partition ratio or capacity factor (McNair and Miller, 1998; Miller, 2005; Rood, 2007). It is proportional to the time a compound spends in the stationary phase (t_r') relative to the time it spends in the mobile phase (t_m) (Korean Food and Drug Administration, 2010). *k* value was 4.165–4.880 for cyazofamid (Table 6), indicating enough retention for good separation.

The most common measure of the efficiency of a chromatographic system is plate number (*N*) and a related parameter, which expresses the efficiency of a column as the plate height (*H*) (McNair and Miller, 1998; Miller, 2005; Rood, 2007). The greater the number of total theoretical plate a unit length (mm) of column,

Table 6 Retention times (t_r), retention factor (k), number of plates (N), and height of theoretical plate (H) of cyazofamid (each analytical condition)

Crops	t_r (min)	t_m (min)	t_r'	k	N	H (mm)
Apple, Korean cabbage, potato and soybean	8.78	1.7	7.08	4.165	22413	0.0112
Mandarin	10.82	1.84	8.98	4.880	10110	0.0247
Green pepper	9.96	1.86	8.10	4.355	10600	0.0236

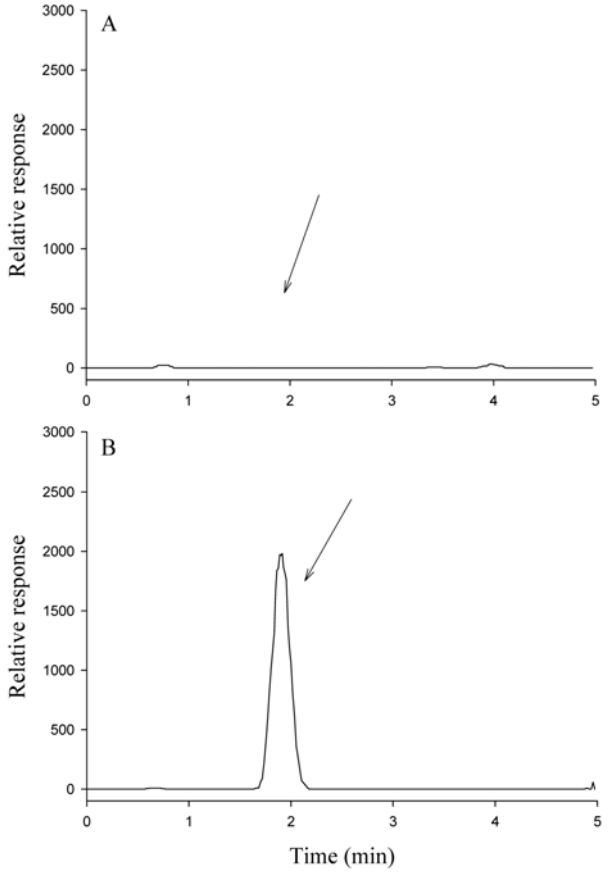

Fig. 3 LC/MS/MS-SIM chromatogram of the representative crop extracts (A: Apple control sample, B: apple recovery sample fortified at 0.02 mg/kg)

the shorter each theoretical plate. Therefore, high efficiency columns have large N and small H values. For cyazofamid, N was 10110–22413 and H was 0.0112–0.0247 mm in each crop sample (Table 6), suggesting high column efficiency.

Confirmation of cyazofamid in crop matrices by LC-MS/MS. LC-MS/MS was used for the confirmation of the fortified cyazofamid residues in crop samples. Before sample analysis, three parameters (capillary voltage, RF loading, and needle voltage) were tuned to reach highest sensitivity of cyazofamid. Capillary voltage effects the focusing of ions into the capillary as well as the energy imparted to them as they exit the capillary and enter the skimmer. RF Loading (%) optimizes the amount of energy that an ion acquires after injection into the ion trap. The needle voltage is the voltage applied to the tip of the spray needle to make charged fine droplets with the aid of nebulizer gas. As the optimized volt, 5000 volts was set for optimal condition, because voltage higher than 5000 results in overloading of the instrument and thus is not practical for analysis. By using all three combinations, full scan spectrum of cyazofamid afforded *m/z* 325 and the protonated molecular ion $[M+H]^+$ as the most intensive peak. Analysis by LC-(ESI)MS/MS with SIM mode at *m/z* 325 reconfirmed that the corresponding peak in recovery samples (0.02 mg/kg level) is a true cyazofamid residue (Fig. 3).

Furthermore, LC-MS/MS result proved that it is better than HPLC for performing pesticide analysis, because Fig. 3, which gave a clean and clear peak of shorter retention time without other coextractive peaks present in Fig. 2, even though the fortified level in Fig. 3 was 10 times lower than that in Fig. 2. Therefore LC-MS/MS also could be used in cyazofamid residue analysis.

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