

Analysis of Cyazofamid and its Metabolite in the Environmental and Crop Samples Using LC–MS/MS

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Received: 3 March 2014 / Accepted: 23 August 2014 / Published online: 31 August 2014
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Abstract A rapid and robust LC–MS/MS method for the analysis of cyazofamid and its metabolite, 4-chloro-5-*p*-tolylimidazole-2-carbonitrile (CCIM), in environmental samples (soil and water) and a variety of crops (apple, mandarin, Kimchi cabbage, green pepper, potato and soybean) was established in this study. Those compounds were analyzed by selected reaction monitoring with electrospray ionization (positive mode) on LC–MS/MS. Method limit of quantitations were 2 ng g⁻¹ (cyazofamid) and 5 ng g⁻¹ (CCIM) for soil/crop samples, while 0.02 ng mL⁻¹ (cyazofamid) and 0.05 ng mL⁻¹ (CCIM) were achieved for water samples. Matrix effect (%) was different depending on sample matrices. For recovery tests, soil/crop samples were treated with QuEChERS method and water samples were extracted with dichloromethane. The recoveries of target analytes in the environmental and crop samples were 80.2 %–105.1 % for cyazofamid and 75.1 %–99.1 % for CCIM (coefficients of variation; ≤16.4 %).

Keywords Cyazofamid · LC–MS/MS · CCIM · QuEChERS · Recovery

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Cyazofamid (4-chloro-2-cyano-*N,N*-dimethyl-5-*p*-tolylimidazole-1-sulfonamide, Fig. 1a) is a sulfonamide fungicide (Tomlin and British Crop Protection Council 2009) which has been used for protection of several vegetables and fruits from various diseases (Pesticide Handbook 2011).

It has very low mammalian toxicity (LD₅₀ for rats: >5,000 mg kg⁻¹) and ecological effect [LC₅₀ (96 h) for carps: >0.14 mg L⁻¹] (Tomlin and British Crop Protection Council 2009). In aerobic soil, cyazofamid degraded rapidly (DT₅₀ in soil: 3–5 days) into the major degradates such as CCIM (Fig. 1b), 4-chloro-5-*p*-tolylimidazole-2-carboxamide (CCIM–AM), and 4-chloro-5-*p*-tolylimidazole-2-carboxylic acid (CTCA) which were covalently bound to organic matter (Evaluation Report Cyazofamid 2004). When cyazofamid was treated on field for crop residue trial, CCIM was found as a major metabolite (Evaluation Report Cyazofamid 2004).

Only few reports were available for the analysis of cyazofamid residues in the limited environmental or crop/food samples including grapes, ginseng, soil and water (Choi et al. 2007; González-Álvarez et al. 2012; González-Rodríguez et al. 2009, 2011; Lee et al. 2012; Tandon and Singh 2012). Such methods utilized HPLC–UVD (Choi et al. 2007; Lee et al. 2012; Tandon and Singh 2012) or GC–ITMS (González-Álvarez et al. 2012; González-Rodríguez et al. 2009, 2011) after conventional sample treatment, and only cyazofamid was analyzed without metabolites.

The objective of this study is to establish a rapid and effective analytical method of cyazofamid and its metabolite, CCIM, using LC–MS/MS after QuEChERS sample treatment. Upland soil and deionized water were used for environmental samples, while apple, mandarin, Kimchi cabbage, green pepper, potato, and soybean were selected for the representative crops.

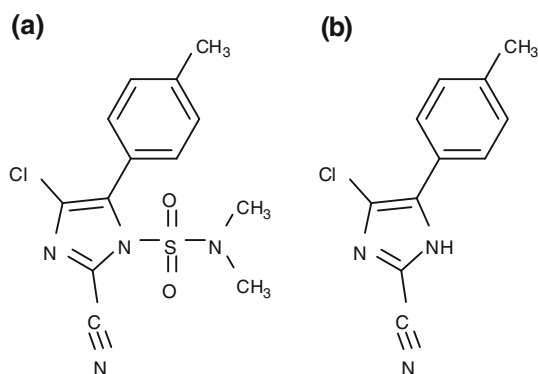


Fig. 1 Structure of cyazofamid (a) and CCIM (b)

Materials and Methods

Cyazofamid (98.4 %) was purchased from FlukaTM (Buchs, Switzerland) and its metabolite, CCIM was kindly donated from the manufacturer. All of solvents (HPLC grade) were obtained from Burdick and Jackson[®] (Korea) and other reagents were purchased from Sigma Aldrich (USA). Water was distilled and purified with a LaboStarTM TWF UV7 Ultra-pure water system (Siemens Water Technologies LLC, USA). Upland soil sample was collected from a local province. Apple, mandarin, Kimchi cabbage, green pepper, potato, and soybean of ‘Residue free grade’ were purchased from a local market. The QuEChERS materials were obtained from commercial suppliers. For extraction of crops, ‘Ultra QuECh extract kit’ (Ultra Scientific, USA), which is containing 4 g of MgSO₄, 1 g of NaCl, 1 g of Na₃Citrate·2H₂O and 0.5 g of Na₂HCitrate·1.5H₂O was used. For the dispersive SPE (d-SPE) cleanup of crop extracts from apple, mandarin Kimchi cabbage, green pepper, and potato samples, ‘Ultra QuECh dSPE-General’ (2 mL centrifuge tubes containing 150 mg of MgSO₄ and 25 mg of PSA, Ultra Scientific, USA) was used. For soybean samples, ‘Agilent Dispersive SPE 2 mL Fatty Samples’ (2 mL centrifuge tubes containing 150 mg of MgSO₄, 25 mg of PSA and 25 mg of C18, Agilent Technologies, USA) was chosen.

Standard stock solutions of cyazofamid and CCIM were prepared at the concentrations of 1,000 and 100 mg L⁻¹ with acetonitrile, respectively. Those two stock solutions were combined to make standard mixture solution, and then it was diluted with acetonitrile to prepare the standard working solutions of various concentrations. The calibration curves were constructed in solvent (acetonitrile), or in matrix extract for matrix matched calibration at 5, 10, 20, 50, 100, and 200 ng mL⁻¹ levels. Matrix effect (ME, %) was calculated by the equation of $ME (\%) = [(S_m/S_s) - 1] \times 100$ (Lozano et al. 2012). S_s is the slope of solvent calibration curve, while S_m is the slope of matrix matched calibration curve.

A 10 g of homogenized crop sample was placed in a 50 mL conical tube. And the sample was fortified with the standard mixture solution at 10 and 100 $\mu\text{g kg}^{-1}$ levels before the crop was extracted with acetonitrile (10 mL) by shaking with the reciprocal shaker (SA-2 s, Taitec Corporation, Japan) at 250 rpm for 10 min. Then, ‘Ultra QuECh extract kits’ were added in the conical tubes, shaken for 2 min, and centrifuged for 5 min at 3,500 rpm (Combi 408, Hanil Science Industrial Co., Ltd., Korea). In the case of soybean sample, it was soaked in water (10 mL) for 20 min before extraction. An aliquot (1 mL) of extract was transferred in 2 mL d-SPE tube, before centrifuged for 2 min at 15,000 rpm (Micro 17TR, Hanil Science Industrial Co., Ltd., Korea). The aliquots (400 μL) of crop extracts from d-SPE cleanup were also mixed with 50 μL acetonitrile with 1 % formic acid and 50 μL of acetonitrile for matrix matching before analyzed with LC–MS/MS. For crop matrix matched calibration, an aliquot (400 μL) of control crop extract was mixed with 50 μL acetonitrile which contains 1 % formic acid and 50 μL of standard working solution. For the true sample applications, grapes were purchased from local market and 10 g of homogenized grape sample were processed and analyzed according to the crop method.

A dried and sieved (2 mm) soil (10 g, sandy loam, pH 6.5) was placed into a 50 mL conical tube, and was fortified with the standard mixture solution at 10 and 100 $\mu\text{g kg}^{-1}$ levels. And then it was extracted with 10 mL acetonitrile containing 1 % acetic acid by shaking at 250 rpm for 5 min with the reciprocal shaker. Four grams of MgSO₄ and 1 g of NaCl were added into extraction mixture, and the mixture was shaken with the reciprocal shaker for 5 min, before centrifuged at 3,500 rpm for 5 min. The aliquots (800 μL) were mixed with 200 μL of acetonitrile, and analyzed with LC–MS/MS. For soil matrix matched calibration, an aliquot (800 μL) of control soil extract was mixed with 200 μL of standard working solution. For the field sample applications, orchard soil was collected and the dried and sieved (2 mm) soil (10 g, sandy loam, pH 6.5) was processed and analyzed according to the soil method.

The water sample (500 mL) was placed into a 1,000 mL separatory funnel and fortified with the standard mixture solution at 0.1 and 1 $\mu\text{g L}^{-1}$ levels. Then the sample was added with 20 g of NaCl before extracting with 100 and 50 mL of dichloromethane successively, by hand shaking. Two dichloromethane extracts were combined, dried over anhydrous Na₂SO₄, and concentrated with rotary evaporator (Rotavapor R-114, Büchi, Switzerland) under reduced pressure. The residue was dissolved with 5 mL of acetonitrile and an aliquot (400 μL) was mixed with 50 μL of acetonitrile which contains 1 % formic acid and 50 μL of acetonitrile before analyzed with LC–MS/MS. For water

Table 1 SRM conditions for cyazofamid and CCIM on LC–MS/MS

Name	Transition (<i>m/z</i>)	Dwell time (sec)	Q1 pre bias (v)	CE (v)	Q3 pre bias (v)
Cyazofamid	325.0 > 108.1	100	−12	−14	−18
CCIM	218.0 > 139.1	100	−10	−23	−24

matrix matched calibration, an aliquot (400 μL) of control water sample extract was by mixed with 50 μL of acetonitrile which contains 1 % formic acid and 50 μL of standard working solution.

LC–MS/MS analysis was performed on LCMS-8030 (Shimadzu, Japan) coupled to Nexera UHPLC (Shimadzu, Japan) with electrospray (ESI, positive mode). The analytical column was a Kinetex C18 (100 \times 2.1 mm i.d., 2.6 μm , Phenomenex, USA) and the column oven temperature was 40°C. The injection volume was 4 μL and the mobile phases were eluted at a 0.2 mL min^{-1} . Mobile phases were 0.1 % formic acid in water (A) and 0.1 % formic acid in acetonitrile (B). For gradient elution, the initial combination was 40:60 (A:B, v/v) and the B solution was increased to 100 % in duration of 2 min, holding for

1 min. To establish the selected reaction monitoring (SRM) condition on LCMS-8030, precursor ions, product ions, Q1 and Q3 pre bias voltage, and collision voltage were optimized through the flow injection of cyazofamid and CCIM standard solutions (1 $\mu\text{g mL}^{-1}$).

Results and Discussion

On LC–MS/MS, the protonated molecular ion $[\text{M} + \text{H}]^+$ at $m/z = 325.0$ for cyazofamid, and $m/z = 218.0$ for CCIM were obtained in full scan spectra at 4,500 v of needle voltage at the positive ESI mode. And product ions of cyazofamid and CCIM were selected in product scan during the SRM optimization of Q1 pre bias, Q3 pre bias and collision voltages (Table 1). The other conditions such as DL temperature, nebulizing gas flow, heat block temperature were set at the recommended values of the instrument. Good and clear separation was observed on SRM for cyazofamid and CCIM in a variety of samples, showing better sensitivity for cyazofamid than CCIM (Fig. 2).

Method limit of quantitations (MLOQs) were 2 ng g^{-1} (cyazofamid) and 5 ng g^{-1} (CCIM) for soil/crop samples,

Fig. 2 SRM chromatograms of cyazofamid and CCIM in soil (a, fortified at 100 $\mu\text{g kg}^{-1}$), water (b, fortified at 1 $\mu\text{g kg}^{-1}$), and potato (c, fortified at 100 $\mu\text{g kg}^{-1}$) extracts

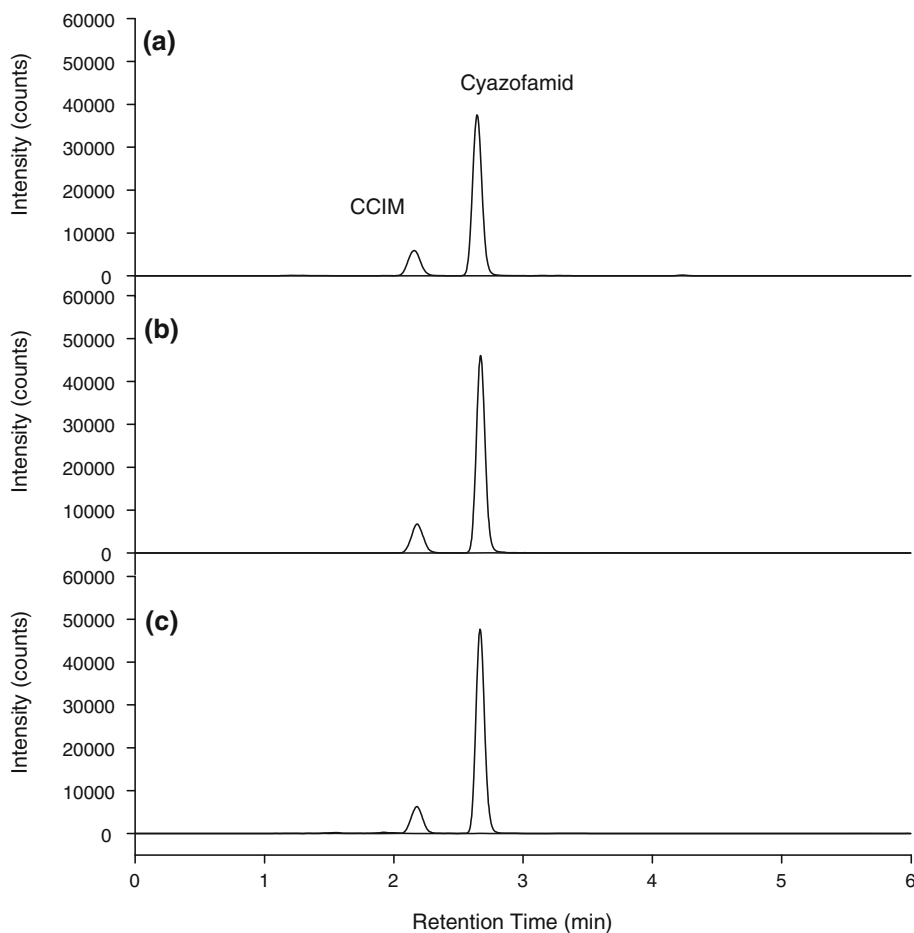


Table 2 Regression equation and matrix effect (ME) for cyazofamid and CCIM

Crops	Cyazofamid		CCIM	
	Regression equation	ME (%)	Regression equation	ME (%)
Solvent	$y = 2,598.3x + 810.6$	–	$y = 560.8x + 143.4$	–
Apple	$y = 2,662.9x + 90.2$	2.5	$y = 548.0x - 201.5$	–2.3
Mandarin	$y = 2,294.1x + 2,554.1$	–11.7	$y = 412.8x + 211.1$	–26.4
Kimchi cabbage	$y = 2,714.2x - 577.1$	4.5	$y = 512.6x - 146.1$	–8.6
Green pepper	$y = 2,485.7x + 2,219.8$	–4.3	$y = 446.2x + 293.6$	–20.4
Potato	$y = 2,849.1x + 873.9$	9.7	$y = 548.4x - 225.5$	–2.2
Soybean	$y = 2,337.4x + 1,669.0$	–10.0	$y = 503.4x + 179.4$	–10.2
Soil	$y = 2,446.7x + 406.0$	–5.8	$y = 531.4x - 177.9$	–5.2
Water	$y = 2,976.1x - 2,093.3$	14.5	$y = 565.4x - 533.3$	0.8

Table 3 Recoveries of the cyazofamid and CCIM residues in soil, water, and crop samples

Crops	Cyazofamid		CCIM	
	Recovery (%) / C.V. (%)		Recovery (%) / C.V. (%)	
	10 $\mu\text{g kg}^{-1}$	100 $\mu\text{g kg}^{-1}$	10 $\mu\text{g kg}^{-1}$	100 $\mu\text{g kg}^{-1}$
Apple	105.1/2.6	103.6/1.1	86.0/5.8	88.1/3.8
Mandarin	88.1/3.6	101.8/3.4	90.3/4.1	98.2/2.4
Kimchi cabbage	101.0/1.4	95.7/16.4	92.3/0.8	91.0/15.1
Green pepper	80.2/1.2	90.5/1.8	75.1/4.1	85.5/2.1
Potato	96.4/1.9	99.0/0.5	82.4/10.7	91.1/2.6
Soybean	98.1/15.7	96.9/2.6	99.1/8.9	89.9/2.5
Soil	95.3/2.1	96.5/1.0	91.1/9.5	91.6/3.7
Water ^a	99.0/2.3	91.3/2.2	92.1/4.7	94.9/1.7

^a The water samples were fortified at 0.1 and 1.0 $\mu\text{g L}^{-1}$ instead fortified at 10 and 100 $\mu\text{g kg}^{-1}$, respectively

while 0.02 ng mL^{-1} (cyazofamid) and 0.05 ng mL^{-1} (CCIM) for water samples. Linearity of matrix matched standard solutions (5–200 ng mL^{-1}) of cyazofamid and CCIM was good, showing higher than 0.999 of coefficients of linear determination (Table 2). In water samples ME (%) was generally enhanced, while those of upland soil, mandarin, green pepper, and soybean samples were suppressed for the both of cyazofamid and CCIM. On the other hand in apple, Kimchi cabbage, and potato samples, enhancement for cyazofamid, and suppression for CCIM were observed.

The recoveries of crop samples were from 80.2 % to 105.1 % for cyazofamid (C.V; 0.5 %–16.4 %) and from 75.1 % to 99.1 % for CCIM (C.V; 0.8 %–15.1 %). For soil recovery tests, good recoveries from 91.1 % to 96.5 % (C.V; 1.0 %–9.5 %) were obtained, while the recoveries were from 91.3 % to 99.0 % (C.V; 1.7 %–4.7 %) for water samples (Table 3; Fig. 2).

Most of the previous analytical studies dealt with only cyazofamid (Choi et al. 2007; González-Álvarez et al. 2012; González-Rodríguez et al. 2009, 2011; Lee et al. 2012; Tandon and Singh 2012) however, in this study, metabolite CCIM was also analyzed together with cyazofamid because it has been known as the major and common metabolite in soil and crop (Pesticide Fact Sheet 2004; Evaluation Report Cyazofamid 2004) and in the processed food (e.g. wine) (European Food Safety Authority 2013).

Generally, LC–MS/MS offers very sensitive, selective and rapid analysis compared to the conventional HPLC and GC. When SRM mode was used in this study few ppb levels for soil and crop samples, and sub-ppb levels for water samples were observed as MLOQs.

For the rapid and efficient sample treatment, QuE-ChERS method was used because the conventional extraction, liquid–liquid partitioning, column chromatography purification and concentration procedures are tedious and time/labor consuming. The popular QuE-ChERS method was originally introduced in 2003 by Dr. Steven Lehotay's team of USDA ARS (Anastassiades et al. 2003) for the multiresidue analysis of pesticides in food and crops, and was modified into the acetate-buffering version (Lehotay et al. 2005) and the citrate-buffering versions (Anastassiades et al. 2007). This method applied successfully in not only crops but also environmental samples (Kvíčalová et al. 2012; Liu et al. 2014) for pesticide residue analysis.

In this study for crop samples, citrate-buffering QuE-ChERS version was used successfully, giving a reasonable recoveries (75.1 %–105.1 %) and precisions for two target analytes. The other studies (Lee et al. 2012; Choi et al. 2007) with the conventional sample treatment and HPLC analysis of crops reported that the recoveries were 75.3 %–98.5 % for several crops and 80.2 %–80.6 % for ginseng. As expected, the analytical time, cost, reagents and labor were saved greatly compared to conventional procedures,

still keeping reasonable recovery rates. When grape sample from market was analyzed, crop method was applied successfully and no cyazofamid and CCIM was observed.

In soil recovery tests, the modified QuEChERS method (Caldas et al. 2011) which utilizes $MgSO_4$ and NaCl in extraction process was used without d-SPE procedure since the extract was less complicated than crop extracts. By omitting d-SPE step, the analytical time and cost were reduced again, maintaining of good recoveries (91.1 %–96.5 %). In case of field soil analysis, the established soil method was applied nicely and no cyazofamid and CCIM was observed. Tandon and Singh (2012) also obtained the reasonable recoveries (86.4 %) of cyazofamid from soil samples after the conventional extraction and SPE cartridge purification.

However for water samples, the traditional liquid–liquid partitioning of large volumes of sample (500 mL) and concentration step were carried out in order to reach the sub-ppb level of MLOQ because the analytes concentration in field water samples could be significantly low by dilution. Recoveries from 91.3 % to 99.0 % were obtained with MLOQs of 0.02–0.05 ng mL⁻¹ in this study, while 95.3 % of recovery of cyazofamid was reported by Tandon and Singh (2012), which used the partitioning with chloroform of water samples. However, MLOQ of this study was significantly lower than that of their work (Tandon and Singh 2012).

As a conclusion, a rapid and effective LC–MS/MS method for the analysis of cyazofamid and its metabolite CCIM in environmental samples (soil and water) and crops (apple, mandarin, Kimchi cabbage, green pepper, potato and soybean) was established in this study. By employing QuEChERS process for the crop and soil sample treatment, and SRM on positive mode ESI for MS/MS analysis, the method was very sensitive, quick and selective. Matrix matched calibration was used for the proper quantitation and the recoveries of analytes from matrix samples satisfied the criteria of CODEX guideline (2010). Applicability and robustness of established method was proved through true samples (grapes and soil) analysis.

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